Marine biotoxins

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Foreword

FAO is publishing this Food and Nutrition Paper on Marine Biotoxins in an effort to support the exchange of scientific information on an important subject of concern for food safety worldwide. Marine biotoxins represent a significant and expanding threat to human health in many parts of the world. The impact is visible in terms of human poisoning or even death ollowing the consumption of contaminated shellfish or fish, as well as mass killings of fish and shellfish, and the death of marine animals and birds.

This paper provides an extensive review of different aspects of five shellfish poisoning syndromes (paralytic shellfish poisoning, diarrhoeic shellfish poisoning, amnesic shellfish poisoning, neurologic shellfish poisoning, azaspiracid shellfish poisoning), as well as one fish poisoning syndrome (ciguatera fish poisoning). Various aspects of these poisoning syndromes are discussed in detail including the causative toxins produced by marine organisms, chemical structures and analytical methods of the toxins, habitat and occurrence of the toxin producing organisms, case studies and existing regulations. Based on this analysis, risk assessments are carried out for each of these different toxins, and recommendations elaborated to better manage these risks in order to reduce the harmful effect of these toxins on public health.

Work undertaken during this study has underlined the difficulties of performing a scientific-based risk assessment given the lack of data on the toxicology and exposure of diverse marine toxins. The allowance levels currently valid for phycotoxins are generally based on data derived from poisoning incidents in people. However, these data are seldom accurate and complete, and usually restricted to acute toxicity. Therefore, increased attention must be paid to expanding and improving initiatives to monitor, detect and share information on marine biotoxins in the future in order to reduce the public health risks associated with the consumption of contaminated shellfish and fish.

Abbreviations

AOAC	Association of Official Analytical Chemists
ASP	Amnesic shellfish poisoning
AZA	Azaspiracid
AZP	Azaspiracid shellfish poisoning
BRC	Bureau Communautaire de Référence
BTX	Brevetoxin analogues, metabolites formed in fish
bw	Body weight
CE	Capillary electrophoresis
CEN	European Committee for Standardization
CFP	Ciguatera fish poisoning
CID (= CAD)	Collision induced dissociation (= collision activated decomposition)
CRL	Community Reference Laboratory
CTX	Ciguatoxin
C-CTX	Caribbean ciguatoxin
P-CTX	Pacific ciguatoxin
CZE	Capillary zone electrophoresis
DA	Domoic acid
DAP	Domoic acid poisoning
DSP	Diarrhoeic shellfish poisoning
DTX	Dinophysistoxin
EC	European Commission
EIA	Enzyme immuno assay
ELISA	Enzyme-linked immunosorbent assay
ENSO	El Niño-Southern Oscillation
ESI	Electrospray ionisation
EU	European Union
FAB	Fast atom bombardment
FIA	Flow injection analysis
GC	Gas chromatography

GNTX	Gonyautoxin
GTX	Gambiertoxin
HILIC	Hydrophylic interaction liquid chromatography
HP	Hepatopancreas
IAC	Immuno affinity columns
ICES	International Council for the Exploration of the Sea
KB cells	A human cell line derived from epidermoid carcinoma
LC	Liquid chromatography
LC-FD	Liquid chromatography with fluorescence detection
LC-ISP-MS	Ion Spray LC-MS
LC-MS	Liquid chromatography with mass spectrometric detection
LC-UV	Liquid chromatography with ultra violet detection
LOAEL	Lowest observed adverse effect level
LOD	Limit of detection
MAB	Monoclonal antibodies
MEKC	Micellar electrokinetic capillary chromatography
MIA	Membrane immunobead assay
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MS^n	Multiple tandem MS
MTX	Maitotoxin
MU	Mouse units
NMR	Nuclear magnetic resonance
NOAEL	No observed adverse effect level
N : P ratio	Nitrogen : phosphorus ratio
NRL	National Reference Laboratories
NSP	Neurologic shellfish poisoning
OA	Okadaic acid
PbTx	Brevetoxin
PSP	Paralytic shellfish poisoning

PTX	Pectenotoxin
PTX2SA	Pectenotoxin-2 seco acid
RIA	Radioimmunoassay
SAX-SPE	Strong anion exchange-solid phase extraction
SEC	Size exclusion chromatography
SIM (= SIR)	Selected ion monitoring
SIR (= SIM)	Selected ion recording
SMT	Standards, Measurements and Testing Programme
SPIA	Solid-phase immunobead assay
SRM	Selected reaction monitoring (an MS/MS technique that is similar to selected ion monitoring (SIM))
STX	Saxitoxin
STXOL	Saxitoxinol
TDI	Tolerable daily intake
TLC	Thin layer chromatography
TTX	Tetrodotoxin
UV	Ultraviolet (Detection)
Wideband activation	A type of resonance excitation (in ion trap MS) in which the RF voltage is applied to a mass window substantially wider than the parent ion window.
YTX	Yessotoxin

1. Introduction

Microscopic planktonic algae of the world's oceans are critical food for filter-feeding bivalve shellfish (oysters, mussels, scallops, clams) as well as for the larvae of commercially important crustaceans and finfish. Among the 5 000 existing marine algal species, approximately 300 can sometimes occur in such high numbers (blooming) that they obviously discolour the surface of the sea, the so-called "red tides" (Hallegraeff *et al.*, 1995; Lindahl, 1998). The word "bloom" is used to indicate the explosive growth of any of these organisms, which may vary in colour from the commonly cited red (so called "red tides") to different shades of yellow, green, brown or blue depending on the type of protista and their depth and concentration. The commonly used term "red tide" comes from the fact that a massive number of organisms often appear as red streaks across the surface of the water (Bower *et al*., 1981). The conditions for an algal bloom are not yet fully elucidated but the phenomenon is probably influenced by climatic and hydrographic circumstances (Van Egmond and Speijers, 1999). The explosive growths sometimes appear during changes in weather conditions but important contributing causes may be variations in upwellings, temperature, transparency, turbulence or salinity of the water, the concentration of dissolved nutrients, wind or surface illumination (Bower *et al*., 1981).

There are no reasons to assume that shellfish intoxication can be predicted by the properties of the regional area. In general, red tides often occur when heating or freshwater runoff creates a stratified surface layer above colder, nutrient-rich waters. Fast-growing algae quickly strip away nutrients in the upper layer, leaving nitrogen and phosphorus only below the interface of the layers, called the pycnocline. Non-motile algae cannot easily get to this layer whereas motile algae, such as the dinoflagellates, can thrive. Many swim at speeds in excess of 10 metres a day, and some undergo daily vertical migration; they reside in surface water like sunbathers and then swim down to the pycnocline to take up nutrients at night. As a result, blooms can suddenly appear in surface waters that are devoid of nutrients and seem incapable of supporting such prolific growth (Anderson, 1994).

Evidence is increasing from diverse areas (such as the Hong Kong Harbour, the Seto Inland Sea in Japan and North European coastal waters) that "cultural eutrophication" from domestic, industrial and agricultural wastes can stimulate harmful algal blooms. It is even possible that algal species which are normally not toxic may be rendered toxic when exposed to atypical nutrient regimes (e.g. phosphate deficiencies) resulting from cultural eutrophication. Changed patterns of land use, such as deforestation, can also cause shifts in phytoplankton species composition by increasing the concentrations of humic substances in land runoff. Acid precipitation can further increase the mobility of humic substances and trace metals in soils (Hallegraeff, 1993).

Some species produce basically harmless water discolorations. On the other hand, some species can bloom so densely, under exceptional conditions in sheltered bays, that they indiscriminately kill fish and invertebrates due to oxygen depletion. Other algal species can be harmful to fish and invertebrates (especially in intensive aquaculture systems) by damaging or clogging their gills. Furthermore, there are micro-algal species (about 75) which have the capacity to produce potent toxins (called phycotoxins) that can find their way through levels of the food chain (e.g. molluscs, crustaceans and finfish) and are ultimately consumed by humans causing a variety of gastrointestinal and neurological illnesses. Some algal species already produce toxins at low abundances of some hundreds of cells per litre, while other algal species must occur in some millions of cells per litre in order to cause any harm. Most of the harmful species have a restricted distribution pattern but some harmful species have a worldwide distribution (Hallegraeff *et al.*, 1995; Lindahl, 1998).

It is not clear why some micro-algal species produce toxins. These toxins are secondary metabolites with no explicit role in the internal economy of the organisms that produce them and with very specific activities in mammals. They are probably used by their producers as a way to compete for space, fight predation or as a defence against the overgrowth of other organisms (Botana *et al*., 1996).

During the past two decades, the frequency, intensity and geographic distribution of harmful algal blooms has increased, along with the number of toxic compounds found in the marine food chain. Different explanations for this trend have been given such as increased scientific awareness of toxic algal species, increased utilization of coastal waters for aquaculture, transfer of shellfish stocks from one area to another, cultural eutrophication from domestic, industrial and agricultural wastes, increased mobility of humic substances and trace metals from soil due to deforestation and/or by acid precipitation (acid rain), and unusual climatic conditions (Hallegraeff *et al.*, 1995). In addition, monitoring for toxic algae and/or (shell)fish is now carried out in several coastal areas of the world. Figures 1.1 and 1.2 illustrate monitoring in coastal waters of European and North American countries in the International Council for the Exploration of the Sea (ICES).[1] The transportation of dinoflagellate resting cysts, especially from paralytic shellfish poisoning toxin producers (McMinn *et al*., 1997), either in a ship's ballast water or through the movement of shellfish stocks from one area to another provides another possible explanation for the increasing trend of harmful algal blooms (Hallegraeff *et a*l., 1995).

The resting cyst or hypnozygote is the immobile form of some dinoflagellates. These cysts sink to the bottom of the sea and accumulate at the borderline of water and sediment where they over-winter. When favourable growth conditions return, the cysts may germinate and reinoculate the water with swimming cells that can subsequently bloom. In this way the survival of certain dinoflagellates from one season to the other season is assured (Mons *et al*., 1998).

Exchanges in mid-ocean of a ship's ballast water that is derived from the open harbour, with ballast water from the open ocean can be partly effective in controlling not only cysts but also the harmful dinoflagellates and diatoms themselves. Incomplete elimination of harmful organisms is caused by the incomplete discharge of water and sediments in the ballast tank during reballasting (Zhang and Dickman, 1999). However, mid-water exchange within regional seas (for example the North Sea, Irish Sea or English Channel) is less efficient than within oceanic waters. Mid-water exchange in regional seas may reduce the risk from polluted European harbour waters but may result in the transportation of potentially harmful phytoplankton species from the regional seas (Macdonald and Davidson, 1998)

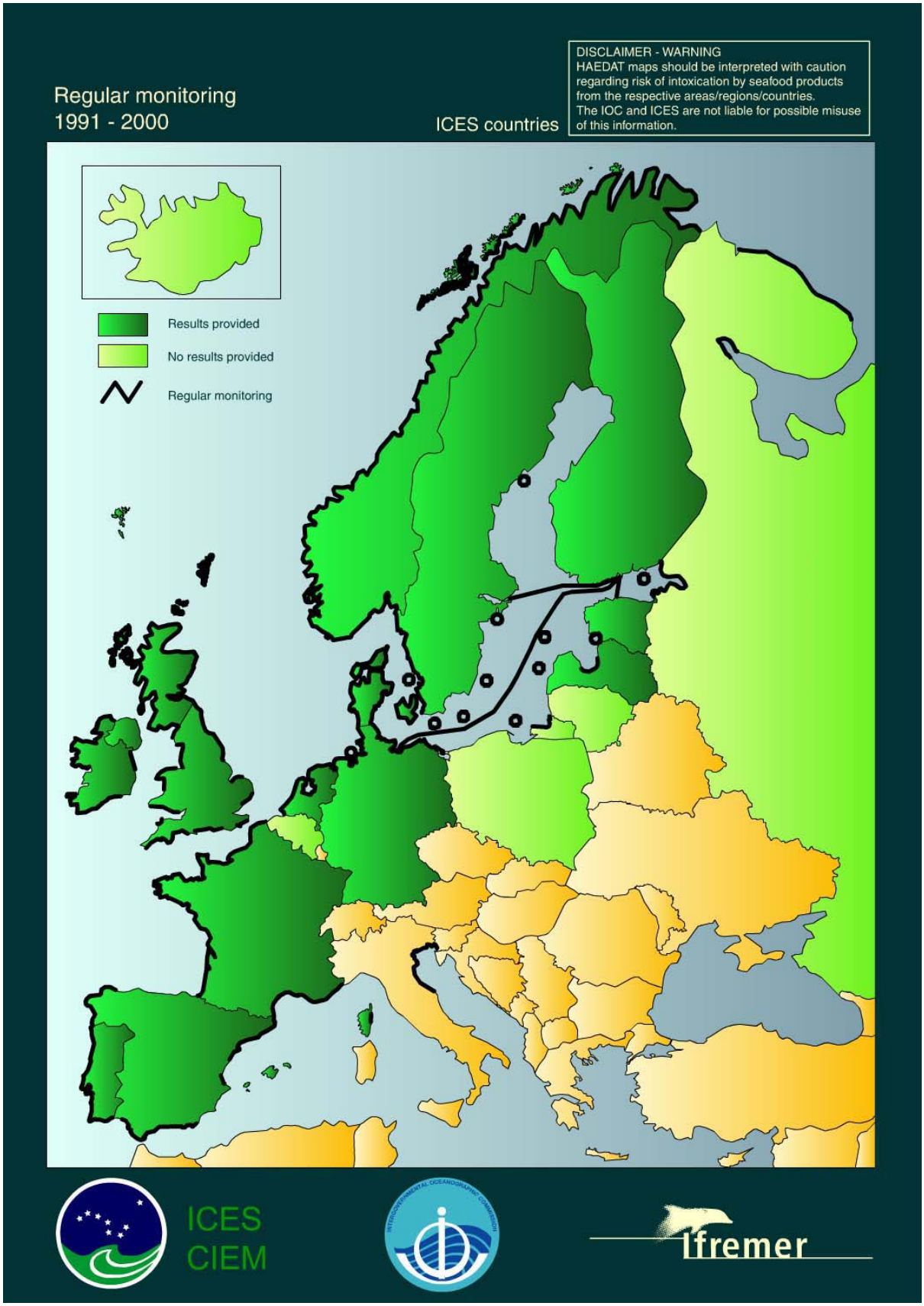
The most important marine phycotoxins are shellfish toxins and ciguatoxins. Until now, five groups of shellfish toxins have been distinguished, namely:

i. paralytic shellfish toxins causing paralytic shellfish poisoning (PSP);
ii. diarrhoeic shellfish toxins causing diarrhoeic shellfish poisoning (DSP);
iii. amnesic shellfish toxins causing amnesic shellfish poisoning (ASP);
iv. neurotoxic shellfish toxins causing neurotoxic shellfish poisoning (NSP); and
v. azaspiracid shellfish toxins causing azaspiracid shellfish poisoning (AZP) (Hallegraeff *et al.*, 1995; Lindahl, 1998).

Ciguatoxins cause ciguatera fish poisoning (CFP). PSP, DSP, ASP, NSP and AZP are caused by human consumption of contaminated shellfish products whereas CFP is caused by the consumption of subtropical and tropical marine carnivorous fish that have accumulated ciguatera toxins through the marine food chain. Various aspects of these toxins will be reviewed in this publication.

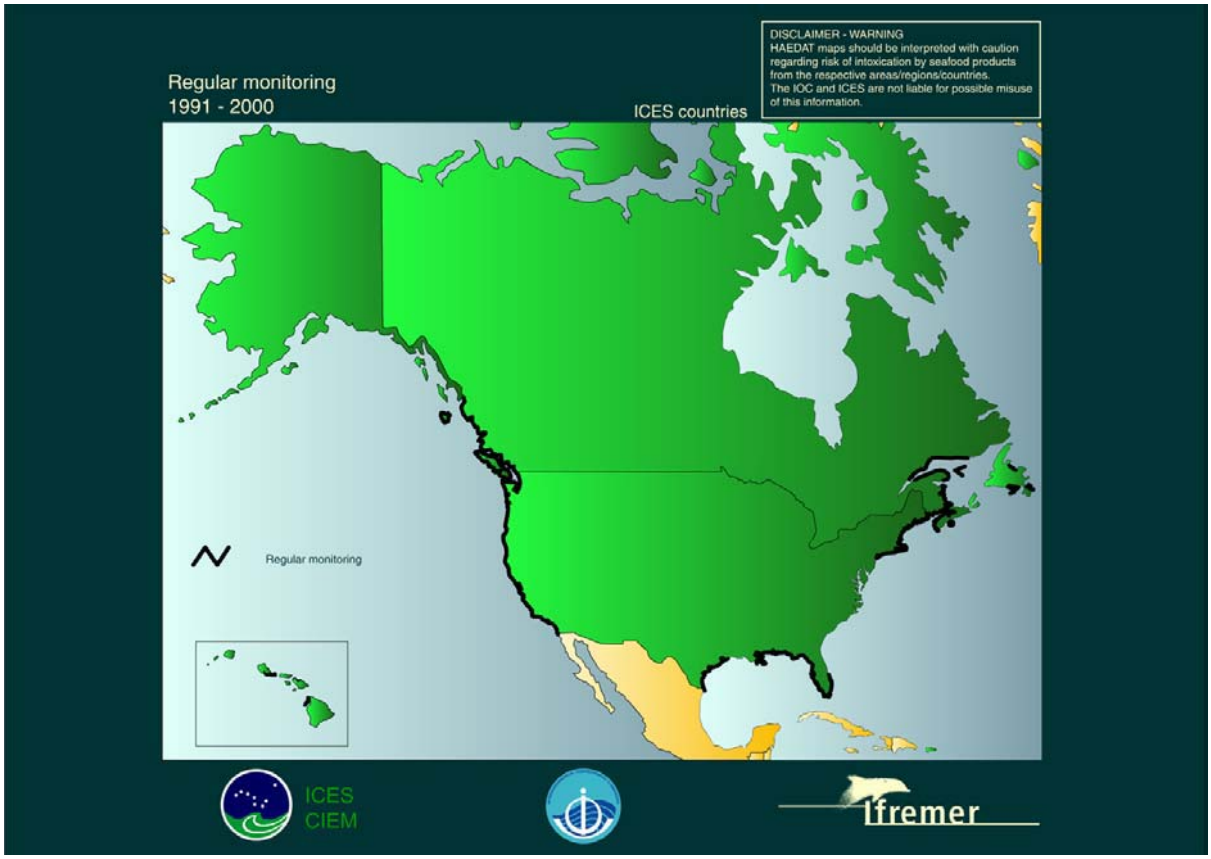
[1] Source: www.ifremer.fr/envlit/documentation/dossiers/ciem/aindex.htm

Figure 1.1 Monitoring of coastal waters in European ICES countries for toxic algae and/or shellfish from 1991 to 2000



Source: www.ifremer.fr/envlit/documentation/dossiers/ciem/aindex.htm

Figure 1.2 Monitoring of coastal waters in North American ICES countries for toxic algae and/or shellfish from 1991 to 2000



Source: www.ifremer.fr/envlit/documentation/dossiers/ciem/aindex.htm

2. Paralytic Shellfish Poisoning (PSP)

Paralytic shellfish poisoning (PSP) in humans is caused by ingestion of shellfish containing PSP toxins. These PSP toxins are accumulated by shellfish grazing on algae producing these toxins. Symptoms of human PSP intoxication vary from a slight tingling or numbness to complete respiratory paralysis. In fatal cases, respiratory paralysis occurs within 2 to 12 hours of consumption of the PSP contaminated food.

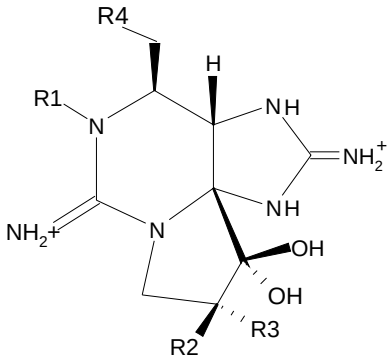
The PSP toxins are a group of 21 closely related tetrahydropurines (see Figure 2.1). The first PSP toxin chemically characterized was saxitoxin (STX). The various PSP toxins significantly differ in toxicity with STX being the most toxic. The PSP toxins are produced mainly by dinoflagellates belonging to the genus *Alexandrium*, which may occur both in the tropical and moderate climate zones. Shellfish grazing on these algae can accumulate the toxins but the shellfish itself is rather resistant to the harmful effects of these toxins. During the last 20 years, there seems to have been an increase in intoxications caused by PSP. However, as yet it is unclear whether the increase is real, whether it could be a consequence of improved identification, detection and medical registration, or whether it is due to expanded shellfish culture and consumption. A few dozen countries have regulations for PSP toxins. Most regulations are set for PSP toxins as a group.

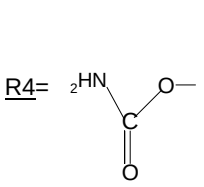
2.1 Chemical structures and properties

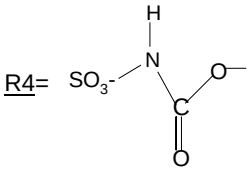
The PSP toxins form a group of closely related tetrahydropurine compounds that make up four subgroups: i) carbamate (STX, neoSTX and gonyautoxins (GNTX1-4); ii) N-sulfo-carbamoyl (GNTX5-6, C1-4); iii) decarbamoyl (dc-) (dcSTX, dcneoSTX, dcGNTX1-4); and iv) deoxydecarbamoyl (do-) (doSTX, doneoSTX and doGNTX1) components. At least 21 PSP toxins (see Figure 2.1), mainly from marine dinoflagellates and shellfish that feed on toxic algae, have been identified. Attempts to isolate PSP toxins began more than one hundred years ago but their occurrence as mixtures of compounds with different ionizable functionalities complicated isolation procedures and early progress was slow. The development of ion-exchange chromatography, guided by mouse bioassays, eventually led to the isolation of a water-soluble basic toxin from the Alaska butterclam *(Saxidomus giganteus).* This compound was later given the trivial name saxitoxin (STX).

In 1975, the first crystalline derivative of STX was synthesized and the structure was studied (Bower *et al.,* 1981). By means of x-ray crystallographic and nuclear magnetic resonance (NMR) spectroscopic studies the structure of STX was elucidated (see Figure 2.1 for the chemical structures of STX and other PSP toxins). The dihydroxy or hydrated ketone group on the five ring is essential for its poisonous activity. Catalytic reduction of this group with hydrogen to a monohydroxy group eliminates the activity. Removal of the carbamoyl group side-chain on the six-membered ring, leaving a hydroxyl group in its place, produces a molecule with about 60 percent of the original toxic activity. The presence of this active hydroxyl group establishes a means for the preparation of various derivatives of STX (Mons *et al.,* 1998). The PSP toxins are heat stable at acidic pH (with the exception of the N-sulfo-carbamoyl components) but unstable and easily oxidized under alkaline conditions (Mons *et al.,* 1998).

Figure 2.1 Chemical structures of PSP toxins







R4= OH

R4=H

R_1	R_2	R_3	carbamate toxins	N-sulfo-carbamoyl toxins	decarbamoyl toxins	deoxy-decarbamoyl toxins
H	H	H	STX	GNTX5(B1)	dcSTX	doSTX
H	H	OSO_3^-	GNTX2	C1	dcGNTX2	
H	OSO_3^-	H	GNTX3	C2	dcGNTX3	
OH	H	H	neoSTX	GNTX6(B2)	dcneoSTX	doneoSTX
OH	H	OSO_3^-	GNTX1	C3	dcGNTX1	doGNTX1
OH	OSO_3^-	H	GNTX4	C4	dcGNTX4	

Source: Mons *et al*., 1998; Quilliam *et al*., 2001

2.2 Methods of analysis

2.2.1 In general

Because of the potential hazard to humans and animals, a quick, sensitive and specific method is needed to determine the presence of the PSP toxins in shellfish. Traditionally, the presence of PSP toxins has been determined using the mouse bioassay. However, the controversial issue of using mammals for testing in addition to the inherent problems and limitations of mammalian bioassays encourages the development of alternative assays such as pharmacological assays, immunoassays, chemical or separation assays and alternative bioassays to detect marine toxins in seafood (Mons *et al*., 1998).

2.2.2 Bioassays

in vivo assays

mouse bioassay

Presently the mouse bioassay still forms the basis of most shellfish toxicity monitoring programmes. The procedure was developed more than half a century ago and has been refined and standardized by the Association of Official Analytical Chemists (AOAC) to produce a rapid and reasonable accurate measurement of total PSP toxins (Hollingworth and Wekell, 1990). Twenty gram mice are injected with 1 ml of an acid extract of the shellfish and the time taken for the animal to die is recorded. Highly toxic extracts are diluted to ensure that mortality occurs within 5 to 15 minutes. The toxicity of the sample is then calculated with reference to dose response curves

established with STX standards and expressed in mouse units (MU). In most countries the action level for closure of the fishery is 400 MU/100 g shellfish (1 MU is the amount injected toxin which would kill a 20 g mouse in 15 minutes and is equivalent to 0.18 σg of STX). The limit of detection of the assay is approximately 40 µg STX/100g of shellfish tissue with a precision of ∂ 15-20 percent. A known interference is a high salt content of samples which suppresses toxic effects (Schantz *et al.*, 1958), whereas zinc accumulation in oysters has been reported to lead to lethal effects in mice at levels that present no health threat to humans (Aune *et al.*, 1998). Highly toxic extracts may give extremely variable results (Park et *al.*, 1986). The practical drawbacks to using the method are:

- a colony of mice between 19 g and 22 g in weight must be maintained, however, at times of increased monitoring the supply of mice may fail;
- the detection limit of the assay is strain dependent;
- the death time versus toxin level is non-linear;
- it is very labour-intensive to determine accurately the death time;
- the sacrifice of a large number of animals is involved.

In spite of these difficulties, the assay has been employed on a wide range of molluscs and crustaceans and is still the official method in most countries that regulate PSP toxins in seafood.

In France, a proficiency study was conducted in which eight laboratories applied the mouse assay for the analysis of oyster samples, contaminated with PSP toxins at levels non-detectable and at levels of 153 and 335 σg STX/100 g meat. The authors concluded that on the basis of overall performance all eight participating laboratories were proficient in their use of the AOAC mouse assay. Within-laboratory variations and between-laboratory variations ranged from 5 to 10 and from 8 to 40 percent respectively. Low recoveries were reported for the spiked samples, which pointed at underestimation because of “salt effects”. This inaccuracy would require an adequate safety margin to protect consumers (LeDoux and Hall, 2000).

The mouse assay was also used in a pilot study on PSP toxins in freeze-dried mussels, organized by the Food Analysis Performance Assessment Scheme (FAPAS®) in 2003 (Earnshaw, 2003). Fifteen laboratories took part in this exercise, nine of which applied the mouse bioassay. The materials used in the study were prepared from certified reference materials (Van Egmond *et al.*, 1998), in such a way that stability and homogeneity were satisfactory. The results of this pilot exercise were not impressive. Analysis results ranged from 1 to 383 σg/100 g (expressed as total PSP toxins on fresh weight basis) with a median value of 137 σg/100 g. Statistical evaluation of the results was not undertaken due to the variable nature of the results received.

in vitro assays

<u>in vitro hippocampal slice assay</u>

Kerr *et al.* (1999) investigated *in vitro* rat hippocampal slice preparations as a means of rapidly and specifically detecting the marine algal toxins STX, brevetoxin and domoic acid (DA) in shellfish tissue or finfish and identified toxin-specific electro-physiological signatures for each. It was concluded that hippocampal slice preparations are useful in detection and analysis of marine biotoxins in contaminated shellfish tissue.

<u>sodium channel blocking assay</u>

The mechanism by which the PSP neurotoxins disrupt cell function has been suggested as an alternative method of assay. The toxins bind to sodium channels in nerve cell membranes disrupting normal depolarization. The amount of binding is proportional to toxicity. Davio and Fontelo (1984) described an assay in which the amount of radiolabeled STX displaced from a rat brain preparation is measured. An alternative approach has been developed in mouse

neuroblastoma cells by Kogure *et al.* (1988) and Gallacher and Birkbeck (personal communication; Van Egmond *et al.,* 1993). Mouse neuroblastoma cells swell and eventually lyse upon exposure to veratridine, which, when added together with ouabain, enhances sodium ion influx. In the presence of STX, which blocks the sodium channels, the action of the other two compounds is inhibited and the cells remain morphologically normal. In this bioassay the fraction of the cells protected from the actions of ouabain and veratridine is in direct proportion to the concentration of STX and its analogues.

Jellett *et al.* (1992) have modified this bioassay to improve its speed and convenience by eliminating the need to count individual cells to determine the STX equivalents. Instead, they have employed a microplate reader for automated determinations of absorption of crystal violet from stained neuroblastoma cells. When these changes and other minor technical modifications were tested in this tissue culture bioassay systematically, the lower detection limit was found to be around 10 ng STX equivalents per ml of extract (= 2.0 σg STX eq/100 g shellfish tissue). This version of the tissue culture bioassay was compared with the standard mouse bioassay using 10 acid extracts of dinoflagellates *(Alexandrium excavata* and *Alexandrium fundyense)* and 47 extracts of shellfish tissues, prepared according to the AOAC procedure. The tissue culture bioassay provided results virtually identical to those obtained with the mouse bioassay ($r > 0.96$), and moreover, was considerably more sensitive. The results obtained from liquid chromatography (LC) analysis of a subset of 12 extracts were less consistent when compared with the results from both bioassay methods (Jellett *et al.,* 1992). Truman and Lake (1996) also compared results of the neuroblastoma cell culture assay with results of the mouse bioassay. Twenty-nine extracts of shellfish gave negative results in both assays. Fifty-seven extracts gave positive results in at least one assay. In spiking studies with shellfish extracts the neuroblastoma assay showed a good response to added STX. The correlation between the assays for STX eq in shellfish was 0.876. The authors concluded that, although the results supported the use of the neuroblastoma assay as a screening procedure, results close to the regulatory limits should be confirmed by the mouse bioassay.

In principle the neuroblastoma cell assay could be a good alternative to the mouse bioassay for testing shellfish for PSP toxins. However, the procedure developed by Jellett *et al.* (1992) did not yield satisfactory results when it was tested in an AOAC International collaborative study in 1999. Disappointing performance in practice, also due to problems in the shipment of study materials, led to discontinuation of the studied method in the evaluation procedure of the Methods Committee on Natural Toxins of AOAC International (Personal information).

Another recent development to detect sodium channel-specific marine toxins like saxitoxin is the hemolysis assay developed by Shimojo and Iwaoka (2000). It is based on the principles of the mouse neuroblastoma tissue culture assay for sodium channel specific biotoxins using red blood cells from the red tilapia (*Sarotherodon mossambicus*). Veratridine and ouabain both react with red blood cells from tilapia by affecting the permeability of the cell's membrane. Saxitoxin can inhibit this action (leaving the cell morphologically normal). By sequencing the addition of veratridine and ouabain, with either the extracted samples or saxitoxin to the red blood cells, PSP toxins can be detected. The authors reported that the test was able to detect saxitoxin in concentrations at 0.3 σg/ml, which is slightly above the limit of detection of the mouse bioassay. No information was provided about its value in screening shellfish in practice.

Both the mouse bioassay and the tissue culture bioassay measure total toxicity but not the content of the individual toxins.

Cheun *et al.* (1998) developed a tissue biosensor system consisting of a Na^+ electrode covered with a frog bladder membrane integrated within a flow cell. The direction of Na^+ transfer,

investigated in the absence of Na^+ channel blockers, established that active Na^+ transport occurs across the frogs bladder membrane from the internal to the external side of the membrane. The tissue sensor response to each of a number of PSP toxins was recorded (GNTX1, 2, 3 and 4). Sensor output was inhibited in the rank order GNTX4 > GNTX3 > GNTX1 > GNTX2. Comparing these results with those obtained from the standard mouse bioassay showed good agreement except for GNTX2.

Comparison of results for neoSTX and dcSTX in the tissue biosensor system with the results in the standard mouse bioassay again showed good agreement (within 5 σg toxin/g wet tissue).

Lee *et al*. (2000) used the method above to examine the toxicity in cultured *Alexandrium tamarensis* strains under various environmental conditions. It appeared that the tissue biosensor system was able to measure very small quantities of PSP toxin within an individual plankton cell (5 fg). However, measurement of at least 100 cells is more desirable for increasing the sensitivity of the system. For comparison: at least 6 000 individual cells must be harvested to measure toxin production using the LC method.

2.2.3 Alternative bioassays

There is growing concern about the continued use of mammals for bioassay and one alternative may be to develop similar assays based on the use of invertebrates such as oyster embryos or fish larvae. One method employed to reduce the number of mouse tests in several European countries is to use enumeration of presumptive toxic algal cells in seawater for monitoring purposes (Hald *et al.*, 1991). This technique could also be described as a qualitative assay but cannot be used for quality control of shellfish for commercial sale.

2.2.4 Biochemical assays

immunoassays

ELISAs

Indirect enzyme-linked immunosorbent assays (ELISA) that exploit antibody-antigen binding are increasingly used as "dip-stick" assays for a variety of compounds. One method for production of PSP assay systems has been described by Chu and Fan (1985). A STX antigen is prepared using bovine serum albumin and injected into rabbits. Antibodies raised by the rabbits are then collected and lyophilized. In the test system, antigens are coated to microtitre plates, STX standards or mussel extracts and appropriate dilutions of antibodies are added, and the amount of bound antibody is determined using goat antirabbit IgG peroxidase conjugate, with measurement by a colorimetric substrate assay. STX present in the mussel extract competes for binding with the STX antigen coated to the microtitre plates. Until recently, commercial ELISA test kits have only been developed for STX. However, these are not totally specific for STX and some reaction is induced to decarbamoyl-STX (dcSTX) and neoSTX. Cembella and Lamoreux (1991) described a polyclonal test kit which measures STX, neoSTX, GNTX1 and GNTX3. Although the kit has not yet been fully evaluated, it appears to be more sensitive than LC and more specific than the mouse bioassay.

Chu *et al.* (1996) compared three different direct competitive ELISAs for the analysis of a large number of contaminated shellfish and concluded that there was excellent agreement between the ELISA data and mouse assay results. Usleber *et al.* (1997) also concluded that ELISA results correlated well with mouse bioassay results when analysing scallops. Kasuga *et al.* (1996) concluded however that the mouse assay cannot be replaced with ELISA for the purpose of screening inshore shellfish samples, as unpredictable cross-reactions occurred, as well as

underestimations of toxicity of some naturally contaminated shellfish samples, harvested in the sea near Japan.

Garthwaite *et al.* (2001) developed an integrated ELISA screening system for ASP, NSP, PSP and DSP toxins (including yessotoxin). The system detects suspected shellfish samples. Thereafter, the suspected samples have to be analysed by methods approved by international regulatory authorities. Alcohol extraction gave good recovery of all toxin groups.

Kawatsu *et al.* (2002) developed a direct competitive enzyme immunoassay based on a gonyautoxin 2/3 (GNTX2/3)-specific monoclonal antibody and a saxitoxin-horseradish peroxidase conjugate. GNTX2/3, dc-GNTX2/3, C1/2, GNTX1/4, STX and neoSTX were detectable at concentrations lower than the regulatory limit of 80 µg/100 of shellfish tissue.

Several more publications have appeared recently about the application of ELISA to the analysis of shellfish for PSP toxins. In view of the presence of cross-reactions with lower binding specificity and the potential lack of response to other toxins than STX within the PSP group, the practical application of these assays probably will remain limited, unless acceptable performance characteristics can be demonstrated in formal collaborative studies according to AOAC International or ISO accepted procedures. Such studies have not yet been published.

2.2.5 Chemical assays

fluorometric and colorimetric techniques

The alkaline oxidation of PSP toxins yields fluorescent products, allowing simple determination using fluorometric techniques (Bates and Rapoport, 1975; Bates *et al.,* 1978). However, such techniques are subject to several sources of variability. The adjustment of pH during extraction and before oxidation is critical, ion exchange column clean-up is necessary to remove interfering co-extractants, and the presence of a variety of metals can affect oxidation and subsequent fluorescent yield. Moreover, the toxins do not fluoresce equally, and for several of the carbamate toxins fluorescence is very weak. One way of circumventing the latter problem is to apply multiple fluorescence and colorimetric assays on the same samples. The fluorescence assay was reported to be an order of magnitude more sensitive, and the colorimetric assay slightly more sensitive, than the mouse assay (Mosley *et al.,* 1985).

Hungerford *et al.* (1991) have automated a fluorescence method by using flow injection analysis. The method allows automatic correction for background fluorescence and rapid screening of shellfish samples for the presence of PSP toxins.

chromatographic techniques

Techniques based on liquid chromatography (LC) are the most widely used non-bioassay methods for determination of PSP compounds. During the last decade considerable effort has been applied to the development of an automated LC method for routine analysis of PSP toxins. The assays are generally based on separation of the toxins by ion-interaction chromatography and use of a post-column reactor that oxidizes the column effluent to produce readily detectable derivatives. The methodology developed by the United States Food and Drug Administration was reported to be capable of resolving 12 carbamate and sulfocarbamoyl PSP toxins (Sullivan, 1988). The methodology has been validated against the mouse bioassay and the correlations between the techniques is generally good ($r > 0.9$) (Sullivan, 1988). Detection limits are generally an order of magnitude lower than with the mouse assay. In practice, the method of Sullivan (1988) has shown difficulties in separating STX from dcSTX (Van Egmond *et al.,* 1994) and has therefore gone out of use in most European laboratories involved in PSP analysis.

Although the LC approach is an interesting development, the system requires a considerable amount of skill and dedicated time to make it operate routinely. Furthermore, the LC technique is not free of problems. Thielert *et al*. (1991) has shown that the 6-decarbamoyl toxins are not resolved by the method of Sullivan (1988). Improved resolution was achieved by sequential analysis of the samples using different buffer and ion-pair reagent systems. Ledoux *et al*. (1991) described problems with discrimination of the C-group toxins from fluorescent material present in non-toxic mussels. Waldock *et al.* (1991) also reported that the LC technique was not sufficiently rapid or robust to cope with the large number of samples generated during bloom events.

Peak spreading is also a problem due to the large volume of post-column reaction tubing. One method of circumventing this problem is to prepare fluorescent derivatives before LC separation (Lawrence and Menard, 1991; Lawrence *et al.,* 1991a), but as yet not all of the known PSP toxins have been separated by this method because some of the known toxins (e.g. GNTX2 and GNTX3) lead to the same oxidation products.

Furthermore, for accurate quantitation, it is essential to calibrate the system continually using PSP toxins standards. This is because of differences in the chemistry of each PSP toxin that result in different oxidation rates for each compound in the post-column reactor. Until recently only a STX standard was commercially available and accurate estimation of the amounts of the other PSP toxins in the mixture was impossible. In 2003, certified standards of STX, neoSTX, GNTX 1-4, GNTX 2/3 and GNTX 5 were commercially available (Laycock *et al.,* 1994; NRC, 2003), and their availability significantly improves the quality of the data that is obtained by the LC method (Wright, 1995). Researchers should be careful when changing from one standard to another as a discontinuity of data may occur. Concentration differences up to 20 percent have been noticed between STX concentrations of three different suppliers (Quilliam *et al.*, 1999).

LC methods were used (in addition to the mouse bioassay, see Section 2.2.2.) in a pilot study on PSP toxins in freeze-dried mussels organized by the Food Analysis Performance Assessment Scheme (FAPAS®) in 2003 (Earnshaw, 2003). Fifteen laboratories took part in this exercise and seven of them applied LC. Practically all laboratories analysed the test materials for STX and dcSTX, some also determined the amounts of neoSTX; GNTX1/4; GNTX2/3; GNTX5, GNTX6, C1/2 and C3/4. The results obtained for STX ranged from non-detectable to 83 σg/100 g (on fresh weight basis), those for dcSTX ranged from 25 to 130 σg/100 g. The test material actually contained < 3.5 σg/100 g for STX and ~ 80 σg/100 g for dcSTX. An analysis of the analytical procedures used showed that those laboratories that found positive values for STX all used HCl, with boiling in the extraction step (as in the mouse assay according to the AOAC-procedure (Hollingworth and Wekell, 1990). In contrast, laboratories that applied acetic acid without boiling in the extraction step found hardly or no saxitoxin. The reason for this is that HCl extraction with boiling leads to partial hydrolysis of certain PSP toxins, leading to conversion of some PSP toxins into more toxic analogues (e.g. GNTX5 is converted into STX). Acetic acid without boiling is a milder extraction procedure, which leaves the toxin profile of the sample practically intact. The sample used in the FAPAS study did not contain STX but it did contain GNTX5. Awareness of this phenomenon and standardization of methodology may largely solve this problem, and may lead to better agreement in analytical results, as demonstrated in a Dutch proficiency study (Van Egmond *et al.*, 2004)

A project was carried out from 1993 to 1997 within the framework of the European Commission's Standards, Measurements and Testing Programme (SMT) (previously called and also known as the Bureau Communautaire de Référence or BCR) to develop shellfish reference materials with certified mass fractions of some PSP toxins. The work was carried out by a consortium of 13 public laboratories and six universities, representing the five main shellfish producing countries in the European Union (EU) and some other EU member countries that had an interest in the area of

PSP-determinations. A preliminary inter-laboratory study in the EU had already shown that there was a basis for the development of reference materials (Van Egmond *et al*., 1994).

The research programme involved:
- studies on the improvement and evaluation of the chemical methodology;
- identification and determination of purity of PSP standards, and their stability in solution;
- two inter-comparison studies of analytical methods;
- preparation of reference materials, including homogeneity and stability studies;
- a certification exercise.

Initially the laboratories were asked to analyse solutions of STX and PSP-containing shellfish extracts with a method of their choice but in the final certification study design only LC-methods involving precolumn or postcolumn derivatization were included. The project was finalised with a report describing the certification of the mass fractions of STX and dcSTX in two mussel reference materials (BCR-CRMs 542 & 543) including the identification of several other PSP-toxins, and a spiking procedure based on an enrichment solution (CRM 663) with a certified mass concentration of STX (Van Egmond *et al.,* 1998; Van den Top *et al.,* 2000, 2001).

Two of the methods used in the SMT project that showed good performance characteristics in the SMT project (Lawrence and Menard, 1991; Franco and Fernandez, 1993) were selected for standardization by the European Committee for Standardization (CEN). At the time of writing, the Franco method had appeared as European Prestandard (CEN, 2002a), and the method of Lawrence as Draft European Standard (CEN, 2002b). The latter method was successfully applied in a proficiency study on PSP in shellfish, carried out in the Netherlands in 2001 at national level (Van Egmond *et al.,* 2004). The method was also further modified by Lawrence and the modification was evaluated in 2002 in an international collaborative study (Lawrence *et al.*, 2003).

electrophoretic techniques

slab electrophoresis

Various methods for separation of PSP toxins have been developed using gel and paper electrophoresis (Boyer *et al.,* 1979; Onoue *et al.,* 1983; Ikawa *et al.,* 1985; Thibault *et al.,* 1991). Used in batch mode and in a single dimension, the technique could allow rapid screening of a number of samples. However, quantitation appears to be a major stumbling block, and most methods employ a peroxide spray and a UV lamp to visualise the toxins on the electrophoretic plate. Perhaps one way forward in this area would be the use of scanning fluorescence detectors (Van Egmond *et al.,* 1993).

capillary electrophoresis

Capillary electrophoresis (CE) is a relatively new technique and to date there have been few applications in the field of toxin analysis, however the flexibility of CE systems suggests that it is a promising area for research. In essence, the technique employs a narrow (~100 σm id) fused silica capillary in place of the electrophoretic gel, and nanolitre amounts of the sample are introduced to the end of the column before it is used to bridge two buffer reservoirs. The toxins migrate through the column when high voltage is applied and may be detected as they pass through a UV or fluorescence cell. The technique is applicable to broad classes of compounds with electrophoretic mobility and even where no net charge occurs it is possible to trap compounds in micelles which will then migrate.

Wright *et al.* (1989) applied a CE system coupled to a laser fluorescence detector for the determination of STX standards. The technique allowed detection of STX at the 1 σg/kg level.

Even though the injection volume is necessarily small (1 to 10 nl), the theoretical detection limits for samples are in the σg/kg range. The present drawbacks to the technique are that the same separation has not been demonstrated for biota with mixed toxins, the equipment is not commercially available and is expensive, and the methodology suffers from the same problems as LC in that a fluorescent derivative must be prepared before separation or detection.

Thibault *et al.* (1991) have applied a CE method to samples of marine biota. Separations of neoSTX and STX were achieved and using UV spectrometry a detection limit of 5 σM (approximately 1.5 σg/ml) was demonstrated. The authors suggest that the CE-UV technique holds considerable promise for the routine screening of these toxins in natural extracts, but presently detection limits appear to be too high to be of use in monitoring programmes.

mass spectrometry

The application of mass spectrometry in the field of marine biotoxins was promoted during the last two decades not only by the development of LC-MS interfacing but also by integration of separation, detection and computer technologies. The key to the growth and success of LC-MS (including LC-tandem MS) is (and will be) in the informing power, reliability, affordability and availability of commercial systems (Willoughby *et al*., 1998).

Already in the late 1980s, Quilliam *et al*. (1989) reported the determination of STX by LC-MS applying ion-spray ™ as ionization technique, being a trademarked name to describe pneumatically assisted electrospray (Sparkman, 2000). In single ion recording (SIM mode) and focussing on positive ions, a concentration detection limit of 0.1 µM (1 µL injection) was estimated from flow injection analysis, which is about five times more sensitive than the AOAC mouse bioassay. Full scan spectra were recorded of 100 ng of STX, as well as product ion (daughter ion) spectra of the single protonated molecule ($[M+H]^+$), providing information useful for confirmation of identity and for development of an SRM method.

Pleasance *et al*. (1992a) reported on analysis of PSP toxins applying LC-MS and CE-MS. LC-MS (SIM and full scan-MS^1 mode) was used to monitor purification of saxitoxin isolated from dinoflagellate cell extracts. Additionally, tandem mass spectrometry (MS^2) has been used to provide structural information. It appeared possible to detect 10 pg injected, that is equivalent to a concentration of 0.03 µM. The improvement was obtained by change of the mobile phase in combination with a reduced flow rate. A calibration curve was shown for standard solutions (external calibration) having a concentration range with ratio 55 (highest/lowest conc.) Although the picture looks fine, values are missing for the linearity and reproducibility indicators (r^2 and s.d. respectively). The applicability of Flow Injection Analysis (FIA) to the determination of PSP toxins in more complex marine extracts was also clearly discussed. It was judged to have serious limitations.

Quilliam *et al.* (1993) reported on an LC-MS study with qualitative aspects. The study focused on the characterization of periodate oxidation products of PSP toxins. Mass spectra (mostly MS^1-spectra) were acquired of the various oxidation products, however sensitivity (relative response) was greatly reduced over that for the parent toxins, and the authors concluded that "The overall sensitivity is such that pre-column oxidation combined with LC/MS will not be a competitive method for the trace level analysis of PSP toxins."

Jaime *et al* (2001) mentioned a PSP quantification method using a linkage of ion exchange chromatography with electrospray ionization (ESI)-mass spectrometry. The chromatographic separation was achieved by gradient elution. Measurements were carried out in SIM mode. Descriptions for automated systems were depicted. The focus was on limits of detection (LOD) and linearity; "the LODs obtained for the individual PSP toxins were comparable to those

obtained by other methods based on ion-pair chromatography with chemical oxidation and fluorescence detection", and well suited for determination of PSP toxins in biological materials (regulatory limit mentioned for mussels and shellfish: 800 µg PSP/kg). Linearity was demonstrated by good correlation coefficients (> 0.99). These were obtained notwithstanding the limited calibration concentration range (approximately one decade on average).

Quilliam *et al*. (2001; 2002) presented various LC-MS methods for the determination of PSP toxins, especially the method where they used hydrophylic interaction liquid chromatography coupled with electrospray ionization tandem mass spectrometry detection (HILIC-ESI-MS/MS). The authors claim to have a method that detects all PSP toxins in a single analysis run. So far, the methods have been presented but not published.

Oikawa *et al*. (2002) have used LC-MS to confirm the accumulation of PSP toxins (GNTXs and C-toxins) in edible crab. A description of quantification with LC-MS was not reported. Partial purification was conducted for ESI-MS analyses, that is successive treatment with activated charcoal and a Bio-Gel P2 column.

To summarise, in the field of PSP toxin analysis, LC-MS articles mainly concern qualitative aspects and reflect conventional use of MS^1 mode, although tandem instruments are used. The application of LC-tandem MS for PSP toxin analysis has recently been presented but has not yet been published.

2.3 Source organism(s) and habitat

2.3.1 Source organism(s)

The PSP toxins are present in some genera of dinoflagellates and one species of blue-green algae. Several species of the genus *Alexandrium* (formerly named *Gonyaulax* or *Protogonyaulax)* are identified as contaminators in shellfish. These are *Alexandrium tamarensis*, *A. minutum* (*syn. A. excavata), A. catenella, A. fraterculus, A. fundyense* and *A. cohorticula.* Other clearly distinct dinoflagellates have also been recognised as sources of the STXs. These are *Pyrodinium bahamense* and *Gymnodinium catenatum* (Mons *et al.*, 1998). The toxicity of the dinoflagellates is due to a mixture of STX derivatives of which the composition differs per producing species and/or per region of occurrence.

In Marlborough, New Zealand, the toxin profile of *A. minutum* consisted predominantly of various proportions of GNTX1, GNTX2, GNTX4, neoSTX and STX (see Figure 2.1). These profiles were similar to those observed in other New Zealand isolates of *A. minutum*. They were, however, rather different from those observed in this species elsewhere in the world (MacKenzie and Berkett, 1997).

There is also an immobile form of some dinoflagellates, the resting cyst or the hypnozygote. The cysts sink to the bottom of the sea and accumulate at the borderline of water and sediment where they over-winter (Mons *et al.,* 1998). When favourable growth conditions return, the cysts may germinate and reinoculate the water with swimming cells that can then bloom. In this way the survival of certain dinoflagellates from one season to the other season is assured. The cysts themselves are also toxic, however their exact toxicity is not clear. Some investigators claim a toxicity of the same order as the dinoflagellate itself but others mention a ten to thousand fold higher concentration of PSP toxins in the cysts than in the mobile cells (Mons *et al.,* 1998). In Jakarta Bay, Indonesia, motile forms of *Pyrodinium bahamense* were recorded just after finding cysts of this species in surface sediments. Probably *P. bahamense* undergoes a complete life cycle in Jakarta Bay (Matsuoka *et al*., 1998). Cysts from three main groups of toxic or potentially toxic dinoflagellates were found along the coasts of Portugal: i) cysts of *G. catenatum* were present

along the whole coast, dominated assemblages by up to 68 percent in the southwest coast; ii) cysts of *P. bahamense* were present on the eastern side of the Atlantic Ocean; and iii) cysts of the genus *Alexandrium* were present along the whole coast and accounted for 8 to 31 percent on the south coast (Amorim and Dale, 1998).

Apart from the protista, the freshwater cyanophyte *Aphanazomenon flos-aquae* has also been shown to contain STX and neosaxitoxin (neoSTX) (Mons *et al*., 1998). Other investigators indicated the presence of PSP components in shellfish and crabs without any sign of the appearance of toxic protista. These species were e.g. *Spondylus butler* and *Zosimous acnus* (Mons *et al*., 1998). It is not clear to what content the consumption of coral reef algae was responsible for this effect. During a recent investigation on dinoflagellate cyst production in the Gulf of Naples spherical smooth-walled cysts, which germinated into *A. andersonii*, were observed in the summer months. Although this species was reported in the past as non-toxic, Ciminiello *et al.* (2000c) found a clonal culture of this species positive for PSP in the mouse bioassay.

2.3.2 Predisposing conditions

It is not predictable when a bloom of dinoflagellates will develop; neither is the population density a predictable factor. A bloom begins as a small population of toxic dinoflagellate cells in the lag phase or in the form of resting cysts residing in the bottom sediment and the timing and location of a bloom depends on when the cysts germinate and where they were deposited. Climatic and environmental conditions such as changes in salinity, rising water temperature, and increased nutrients and sunlight trigger cyst germination to a vegetative stage that enables rapid reproduction. Once the dinoflagellate bloom begins, an exponential growth phase causes a tremendous increase in their population. In time, the depletion of nutrients and carbon dioxide in the water, and degraded environmental conditions caused by the bloom, decrease population growth. A stationary phase ensures levelling off the population. At this high level of the bloom, the water can have a fluorescent reddish colour referred to as red tide. Continued environmental degradation increases cell death and ultimately leads to a population crash. At this phase of the bloom, many dinoflagellate species form resting cysts that sink to the bottom, ready for the next bloom. Within this bloom cycle, the most toxic cells generally occur during the middle of the exponential growth phase (Mons *et al*., 1998).

Cyst beds of *A. catenella* are widespread in coastal and estuarine waters (13 to 25 ∀C) in New South Wales, Australia. Cysts from cultured isolates in Australia exhibited dormancy periods at 17 ∀C as short as 28 to 55 days. This contrasts with the usually longer dormancy requirements of temperate populations of *A. catenella* from Japan (97 days at 23 ∀C) and of *A. tamarensis* from Cape Cod (United States) or British Columbia (Canada). Sometimes a one hour temperature increase from 17 to 25 ∀C improved the germination process of some cultured Australian *A. catenella* cysts up to 100 percent (achieved after 98 days), but cold-dark storage did not produce the lengthened dormancy requirements reported overseas for over-wintering temperate cyst populations. This indicates that different geographic isolates of the same dinoflagellate taxon can have different cyst dormancy requirements (Hallegraeff *et al*., 1998).

In cultures of *A. fundyense* toxin production was discontinuous, induced by light, and always occurred during a defined time frame (approximately 8 to 10 hours) within the early G1 phase of the cell cycle and dropped to zero for the remainder of the interphase and mitosis (Taroncher-Oldenburg *et al*., 1997).

Dinoflagellates develop at relative high temperatures and abundant sunlight. In Europe and South Africa cases of intoxications and mortality thus occurred mainly between May and November,

whereas in North America the intoxications were reported between July and September (Mons *et al*., 1998).

The type of habitat in which PSP intoxications have been observed varies considerably. Hydrographic conditions probably play an important role; in particular, the presence of a thermocline is very important (an upper layer of seawater which does not mix with the underlying water). Indirectly windforce and turbulence in the water may influence the existence of this thermocline (Mons *et al.*, 1998).

The growth characteristics of *A. tamarensis* were studied by artificial culture in the laboratory. The results demonstrated that the optimum situation is temperature 22 to 26 ∀C; salinity 28 to 31 ‰; light intensity 1500-2500 lux and light/dark period 16/8 hours. The average doubling time is 85 hours (Hao, 2001).

There was evidence for a coincidence between *Pyrodinium* blooms and El Niño-Southern Oscillation (ENSO) climatic events. El Niño is caused by an imbalance in atmospheric pressure and sea temperature between the eastern and western parts of the Pacific Ocean and results in a shoaling of the thermocline (Mons *et al.*, 1998).

The amount of nutrients in the seawater has to be adequate to fulfil the needs of the organisms, especially the concentration of trace elements, chelators, vitamins and organic material in general. However, there are many uncertainties in the determination of the exact role of nutrients in the development of red tides. For example, the development of red tides is sometimes stimulated by low salt concentrations, whereas in other cases high concentrations of salt seem to induce the bloom (Mons *et al*., 1998).

Irradiance also has an effect on the growth of, for example, *A. minutum*. Growth of *A. minutum* cultured from a case outbreak in New Zealand (Bay of Plenty), was studied using 54 combinations of irradiance and different N sources (NO_3^-, NH_4^+, urea) and concentrations. Irradiance had more effect on growth in cultures enriched with NO_3^-, than with NH_4^+ or urea. Growth appeared to saturate at relatively low irradiance suggesting that *A. minutum* is able to sustain reasonably good growth rates, even in poorly illuminated depths within the water column (Chang and McLean, 1997). The optimal environmental conditions for cell growth and toxin production of *A. minutum* T1 isolated from southern Taiwan Province of China were temperature 25 ∀C, pH 7.5, light strength 120 σEm^{-2} s^{-1}, and salinity 15 ‰. The optimal levels of nutrients supplemented in the 50 percent natural seawater medium were phosphate 0.02 percent, nitrate 0.01 percent, cupric ion 5.0 ng/g, ferric ion 270 ng/g and humic acid free. Both cell toxicity and total toxicity reached the maximum level at the post-stationary growth phase and decreased quickly (Hwang and Lu, 2000).

For *A. catenella* (in laboratory culture isolated from the waters of the Hong Kong Special Administrative Region, China) the highest amount of toxin/L of medium was recorded at 20 ∀C at the beginning of the stationary phase (four hours after the onset of darkness and lasting four to five hours), when cell density was highest and the amount of toxin/cell was still relatively high. At 10 ∀C the cell density was low while the amount of toxin/cell was high. At 30 ∀C, the population at full capacity was low and the amount of toxin per cell was also low (Siu *et al.*, 1997).

The N:P ratio is expected to have a marked influence on the production of toxin during a bloom. Several studies are reported in the literature which describe the effect of N:P ratios on the growth of *Alexandrium* spp. and also the effect on their toxin content (Mons *et al*., 1998; Béchemin *et al*., 1999; John and Flynn, 2000). Nitrogen restriction reduced population growth and toxin production, while phosphorus restriction reduced only population growth but enhanced toxin

production. When nutrients are non-limiting, the main limiting factors for *A. catenella* are temperature (20-25 ∀C), salinity (30-35 ‰) and pH (8.0-8.5) (Siu *et al*., 1997).

Involvement of eubacteria other than cyanobacteria in the production of PSP toxins has proven to be a controversial subject. It is suggested that bacteria play a role in this area although the precise mechanisms are unclear. It is feasible that the production of PSP toxins is an inherent function of some marine bacteria required for their physiological processes and is incidental in relation to dinoflagellate and shellfish toxicity. Additionally, increasing evidence that bacteria are capable of metabolising PSP toxins may prove to be of practical importance in terms of both dinoflagellate and shellfish toxicity. It may be pertinent to conduct more detailed studies on bacterial and dinoflagellate interactions in marine environments (Gallacher and Smith, 1999).

2.3.3 Habitat

Dinoflagellates and their cysts have mainly occurred in the waters near North America, Europe and Japan but occurrences in Asia are increasingly reported (Mons *et al*., 1998). In northeastern Canada, PSP was reported more than 100 years ago. In the northeast of the USA, particularly in the New England region, where toxicity was restricted to the far eastern sections of Maine near the Canadian border, the first documented PSP case dates from 1958 (Anderson, 1997).

A. catenella has been observed particularly along the coast of North America, southern Japan and Venezuela, whereas *A. tamarensis* is found in North America, northern Japan, southern Europe, Turkey and Australia (Mons *et al*., 1998). Cyst beds of *A. catenella* are widespread in coastal and estuarine waters (13-25 ∀C) in New South Wales, Australia. Cysts from cultured isolates in Australia exhibited dormancy periods at 17 ∀C as short as 28 to 55 days. This contrasts with the usually longer dormancy requirements of temperate populations of *A. catenella* from Japan (97 days at 23 ∀C) and of *A. tamarensis* from Cape Cod or British Columbia (Hallegraeff *et al*., 1998).

A. fundyense occurs in the coastal waters of northeastern North America (Taroncher-Oldenburg *et al*., 1997) and blooms of *Protogonyaulax tamarensis* are a common, seasonal occurrence in the Gulf of Maine (Shumway *et al*., 1988).

A. excavata (syn. *A. minutum)* has been reported from the northeast coast of North America, Egypt, Australia, the North Sea (Denmark, Germany, the Netherlands, Norway and Great Britain), the Mediterranean coast (Mons *et al*., 1998) and New Zealand (Chang *et al*., 1997a). Since 1990, *A. minutum* has been reported from a lagoon in Sicily (Italy) where both an exploitation of natural settlements of clams (*Ruditapes decussata* and *Cardium* spp.) and small-scale farming of blue mussels (*Mytilus galloprovincialis*) are practised. No cases of human intoxication were reported. Cell densities are maximal in May (Giacobbe *et al*., 1996). Along the northwest of the Adriatic coast of Italy, *A. minutum* was found at the Emilia Romagna sampling stations in 1994, 1995 and 1996 from April to July (Poletti *et al*., 1998). In the Mediterranean Sea, only the potentially toxic *A. minutum* and *A. tamarensis* have been reported to be present until now. However, an investigation of Ciminiello *et al*. (2000c), performed on cultured material, namely cysts from the Gulf of Naples germinating to *A. andersoni*, showed positive effects for PSP in the mouse bioassay. The toxicity profile *A. andersoni* consisted mainly of toxins in the STX class, in particular STX and neoSTX.

Outbreaks of PSP in Japan, the northwest coast of North America, southern Ireland, Spain, Mexico, Argentina and Tasmania (Australia) have been caused by blooms of *Gymnodinium catenatum.* The present day distribution of *G. catenatum* includes the Gulf of California, Gulf of Mexico, Argentina, Venezuela, Japan, the Philippines, Palau, Tasmania, the Mediterranean, and the Atlantic coast of Spain and Portugal (Mons *et al*., 1998). *G. catenatum* is not endemic to

Tasmania but was introduced some decades ago. The first bloom was seen in 1980 with major blooms in 1986, 1991 and 1993. Several lines of evidence suggest that ballast water discharge from cargo vessels originating from Japan and the Republic of Korea, or less likely Europe, was the most probable mechanism of introduction (McMinn *et al*., 1997).

The first harmful implications of *Pyrodinium* bloom became evident in 1972 in Papua New Guinea. Since then toxic *Pyrodinium* blooms have apparently spread to Brunei Darussalam, Sabah (Malaysia), and the central and northern Philippines. During a *Pyrodinium* bloom in 1987 in Champerico on the Pacific coast of Guatemala, 187 people had to be hospitalised and 26 people died (Rodrigue *et al.*, 1990). In 1989 another bloom swept northward along the Pacific coast of Central America, again causing illness and death (Mons *et al*., 1998).

Direct measurement of the specific toxicity of cultured isolates of *A. ostenfeldii* suggested a low risk of PSP associated with this dinoflagellate species. *A. ostenfeldii* has been described from numerous locations on the west coast of Europe such as Iceland, the Faeroe Islands (Denmark), Norway and Spain, as well as Egypt, the west coast of the USA, the Gulf of St. Lawrence, Canada and East Asiatic region of the Russian Federation. Cysts of *A. ostenfeldii* were stated to be common in sediments around the New Zealand coast (Levasseur *et al*., 1998; Mackenzie *et al*., 1996).

2.4 Occurrence and accumulation in seafood

2.4.1 Uptake and elimination of PSP toxins in aquatic organisms

During the process of filtration the dinoflagellate cells and cysts are transported to the oesophagus and the stomach of the bivalve molluscs. The digestion takes place in the stomach and the diverticulae whereby the PSP toxins are released and enter the digestive organs. The particular toxin mixture retained in soft tissues of the shellfish varies in concentration and over time, and is determined by the species and strains of the dinoflagellates and shellfish as well as by other factors like environmental conditions. In mussels, it was found that the viscera, which constitute only 30 percent of the total tissue weight, contribute 96 percent of total toxicity. In clams the toxins rapidly concentrate in the viscera and gradually decrease afterwards. After a lag period of four or more weeks, the toxins are mainly detected in the siphon. The composition is not consistent but varies with the time and location in the animals (Mons *et al*., 1998).

Various authors have reported on the toxicity of various scallop tissues and a number of generalities have emerged (Shumway *et al*., 1988):

- The adductor muscle does not accumulate toxins and has in fact been shown to inactivate the toxins when present. One exception was the purple-hinged scallop, *Hinnites giganteus*, where toxin levels reached 2000 σg/100 g of tissue.
- Digestive gland, mantle, gonad and gill tissues all retain the toxins although the levels vary between tissues and between species.
- There are seasonal variations in toxicity level of the various tissues.

After uptake and distribution, the toxins may undergo transformation. In feeding experiments non-toxic butter clams were fed *A. catenella* containing GNTX 1-4 and neoSTX but no STX. After a period of 83 days STX was also detected, leading the authors to conclude that some type of synthesis or biotransformation of GNTX 1-4 and/or neoSTX to STX occurs *in vivo*. Similar findings were reported by other authors (Mons *et al.*, 1998).

One common transformation, termed epimerization, occurs when a portion of the original STX molecule rearranges. Scallop and mussel, for example, can perform epimerization of STX they

receive from the toxic algae when the H and OSO_3^- switch locations on the number 11 position of the STX molecule. Such a transformation can decrease toxicity eleven-fold. On the contrary, there are also transformations that increase toxicity. For example, a six-fold increase in toxicity occurs when the SO_3^- group is separated from position 21 on the STX molecule by acid hydrolysis (Mons *et al*., 1998).

The butter clam has a distinctive ability to chemically bind the highly toxic STX in its siphon tissue and can retain PSP toxins for up to two years after initial ingestion. The littleneck clam, *Prothotaca staminea*, can also become toxic but less so than the butter clam. The lower toxicity of the littleneck clam is partially due to its ability to perform transformations that change highly toxic STXs to the moderately toxic forms. The combined effect of the littleneck clam's capability to transform STXs to less toxic forms, and the ability of butter clams to concentrate and retain highly toxic forms, can result in a wide difference in toxicity between these two species. This toxicity difference is particularly significant since butter clams and littleneck clams can coexist on the same beach and are, to the unskilled harvester, similar in appearance (Mons *et al*., 1998). MacKenzie *et al*. (1996) noted the changes in PSP-toxin profiles in the surfclam tuatua (*Paphies subtriangulata*) inhabiting the beaches in the Bay of Plenty, New Zealand, during the contamination phase (peak levels Ω412 µg STX eq/100 g) in January 1993 and over a six-month period one year later when low toxin levels (40 µg/100 g) persisted. Toxin profiles during peak contamination consisted of various levels of carbamate derivatives GNTX 1-4, neoSTX and STX with some traces of the decarbamoyl derivative dc-STX. These profiles resembled those produced by the dinoflagellate *A. minutum*, which caused the PSP incident. One year later, only traces of derivatives other than STX remained and almost all of this toxin was sequestered within the siphon.

Andrinolo *et al*. (1999a) demonstrated that natural depuration from PSP toxins by *Aulacomya ater*, a native South American filter-feeder bivalve, occurs in the form of an exponential decay of the first order (one-compartment model). Depending on their detoxification kinetics, bivalves have been classified into two major groups: slow detoxifiers (e.g. *Saxidomus giganteus*, *Spisula solidissima*, *Placopecten magellanicus*, *Patinopecten yessoensis*) and rapid-to-moderate detoxifiers (e.g. *Mytilus edulis* and *Mya arenaria*) (Androlino *et al*., 1999a). A biphasic, two-compartment model best describes detoxification kinetics in some species. During toxification, the viscera typically attain toxicities two to five times higher than whole tissues, whereas locomotor tissues (foot and adductor muscle) are least toxic. However, the viscera detoxify faster than other tissues, leading to a steady decline in their contribution to total toxin burden during detoxification. Biotransformation of toxins in tissues is most pronounced in a few clam species capable of enzymatic decarbamoylation (e.g. *Protothaca staminea*), and more limited in others such as *Mya arenaria* and *Mytilus edulis*. Overall, changes in toxin profile are greatest when ingested dinoflagellates are rich in low potency, N-sulfocarbamoyl toxins (Bricelj and Shumway, 1998).

Some bivalves can avoid ingesting toxic dinoflagellates such as the northern quahaug (*Mercenaria mercenaria*) which retracts its siphon and closes its valves in the presence of *Alexandrium* sp. (Mons *et al*., 1998).

Blanco *et al*. (1997) studied detoxification kinetics in the mussel *Mytilus galloprovincialis* previously exposed to a bloom of the PSP producing dinoflagellate *G. catenatum*. The toxin profile observed in the mussels was very similar to that of *G. catenatum*, showing that biotransformation had little or no importance in this case. Detoxification took place in two phases:

i. a fast one, which took place during the early detoxification period (only a small amount of the toxin, relative to the initial amount, remains in the bivalves after the first few days of detoxification); and

ii. a slow one, lasting from the end of the first phase to the end of detoxification. Environmental conditions (salinity, temperature and light transmission) and body weight affected detoxification especially during the fast first phase.

When Pacific oysters (*Crassostrea gigas*) were fed toxic or non-toxic *A. tamarensis* and *A. fundyense*, a stop/start clearance behaviour (filter pump switched off/on) of the oysters was observed suggesting that PSP toxins were not directly involved in inhibiting the initial feeding response. When control oysters were fed a reference microalga, *Isochrysis* sp., known to support their growth, this behaviour was not seen. When Pacific oysters, which were acclimated to *Isochrysis* sp., were fed mixtures of *Alexandrium/Isochrysis*, further evidence of stop/start clearance behaviour was seen (Wildish *et al*., 1998).

Adult Pacific oysters (*Crassostrea gigas*) experimentally contaminated with PSP toxins (by exposure to *A. minutum*) up to concentrations of 150-300 σg STX eq/100 g, were fed diets based on non-toxic dinoflagellates or diatoms in order to study detoxification. Despite the large individual variations in toxin levels, a detoxification time of three to four days was measured for reaching the safety threshold of 80 σg/100 g in the oysters. Detoxification rates did not differ significantly when oysters were fed *Isochrysis galbana, Tetraselmis suesica, Thalassiosira weissflogii* or *Skeletonema costatum.* GNTX2/GNTX3 were the major compounds found in the oysters during depuration, whereas C toxins were quite low and STX and neoSTX undetectable. The toxin profile was the same as in *A. minutum* suggesting no biotransformation in the oyster (Lassus *et al*., 2000).

The Chinese scallop, *Chlamys farreri*, has a high ability to accumulate PSP toxins. After exposure for 48 hours to toxic *A. minutum* 5000 σg STX eq/100 g were found in the viscera of these scallops and the rate of detoxification was slow. The viscera accounted for 97 percent of the total toxin content. The ratio of different PSP toxins has changed during the experimental period, for example, the ratio of GNTX1 and GNTX4 to total toxins decreased while that of GNTX2 and GNTX3 increased. The toxin profile in the scallops was different from that in the algae. Toxin profile in the scallop faeces matched well with that in the early stage of *A. minutum* in batch culture (Zou *et al*., 2001).

In large containers (20 litres), the adult pelagic harpacticoid copepod, *Euterpina acutifrons*, was incubated with a high toxic strain of *A. minutum* (1 000 or 10 000 cells/ml) for up to five days. Only trace levels of PSP-toxins were found in the extracts analysed by LC. With a low and a high toxic strain of *A. minutum* (1 000 and 10 000 cells/ml), 10 to 15 percent of copepods were inactive after one to two days. It is suggested that *E. acutifrons* avoids feeding on the dinoflagellates after tasting a few cells (Bagøien *et al*., 1996).

Purple clams (*Hiatula diphos*) were contaminated with PSP toxins by feeding them with cells of *A. minutum* and then fed to maculated ivory shells (*Babylonia areolata*), which are carnivorous gastropods. The toxin composition in the clams, gastropods and dinoflagellates were similar but the profile differed in the gastropods. There was a notable degradation of GNTX1 in the gastropod compared to the clam and the dinoflagellate that resulted in a decrease in toxicity while the total amount of toxins was accumulatively increasing. The transmitted GNTX1-4 of *A. minutum* could only be found in the viscera of these shellfish species (Chen and Chou, 1998). In a later study, Chou and Chen (2001a) studied accumulation, distribution and elimination of PSP toxins in purple clams (*Hialuta rostrata*) after feeding a toxic strain of *A. minutum*. The high toxicity of the digestive gland was confirmed. Depuration efficiency between toxic clams fed non-toxic algae and those put in starvation was similar. Toxin profile of the clams was similar to that of *A. minutum* at the end of the feeding period (GNTX4 and GNTX1 were dominant). However, at the end of the elimination period GNTX3 and GNTX2 were dominant indicating inconsistent removal

rates of different toxins or transformation of toxins. No PSP toxins other than GNTX1-4 were found. The non-visceral tissues were also toxic after feeding with toxic algae, however, the toxicity was low and the profile was also similar to that of the toxic algae.

2.4.2 Shellfish containing PSP toxins

Although most filter-feeders are relatively insensitive to the STXs, there are differences among the various species of bivalves in the way they deal with and respond to the STXs. Mussels, for instance, appear in general to accumulate much higher levels of PSP toxins than oysters under similar circumstances. Subsequent laboratory feeding studies showed that mussels readily consumed concentrations of *Alexandrium* equal to or greater than those that caused oysters to cease pumping and close up. Electro-physiological investigations of isolated nerves from Atlantic coast bivalves demonstrated that those from oysters were sensitive to the toxins, while those from the mussels were relatively insensitive (Mons *et al.*, 1998).

The group of shellfish identified in cases of PSP consists mostly of bivalve molluscs. This group includes mussels, clams and, to a lesser extent, oysters, scallops and cockles in temperate zones. An extensive list of shellfish found to contain PSP toxins is given in Table 2.1.

In April 1991, the ormer *Haliotis (Eurotis) tuberculata* from the Galician coast of Spain was found to contain PSP toxins. In October 1993, the market for this mollusc was closed. Samples from December 1995 were contaminated with 252 ∂25 σg STX eq/100 g of meat by mouse bioassay analysis and 454 ∂86 σg STX eq (sum of STX and dcSTX converted to STX eq by conversion factors of 1.9 and 1.14, respectively)/100 g of meat by LC. No value below 140 σg STX eq/100 g of meat was detected by the mouse bioassay. The major component was dcSTX (83 to 100 percent) with STX in much smaller proportion. The epithelium carried 2.6 times more toxin than the muscle. Attempts at natural detoxification, keeping ormers under controlled laboratory conditions for three months, did not work. The elimination of epithelium and gut would result in around 75 percent less toxicity (Bravo *et al*., 1999).

Chlamys nobilis from the waters of the Hong Kong Special Administrative Region, China contained 320 σg STX eq/100 g soft tissue. Following the red tide from March to April 1998, high levels of PSP toxins were detected in *Perna viridis* from waters of Hong Kong Special Administrative Region, China (Zhou *et al*., 1999). In 5 percent of samples of shellfish caught along the Chinese coast from north to south, PSP toxins were found. Although the PSP toxin levels were low (only two samples exceeded the regulatory threshold limit), it indicated that PSP toxin producers existed in this area (Zhou *et al*., 1999).

Table 2.1 Shellfish found to contain PSP toxins

Type	Common name	Scientific name
Clams	purple clam	*Soletellina diphos (syn. Hiatula diphos)*
		Saxidomus giganteus
	Alaska butter clam	*Tapes (Amygdala) japonica*
	shortnecked clam	*Protothaca staminea*
	littleneck clam	*Siliqua patula*
	razor clam	*Mya arenaria*
	softshell clam	*Spisula solidai*
	thick through shell	*Spisula solidissima*
	surf clam	*Paphies subtriangulata* [#]
	(tuatua)	*Venerupis rhomboides*
	pullet carpet shell	*Ensis siliqua*
	pod razor-shell	*Donax trunculus*
	wedge-shell clam	*Scrobicularia plana*
	peppery furrow shell	*Chamalea striatula*
	striped venus clam	*Venerupis pullastra*
		(syn. Venerupis rhomboides)
		Amphichaena kindermani
		Arctica islandica [##]
		Mercenaria mercenaria [##]
		Mesodesma arctatum [##]
		Mytilus edulis
Mussels	blue mussel	*Mytilus californianus*
	California mussels	*Pinna bicolor* *
		*Mytilus chilensis***
		*Arctica islandica****
Oysters	ocean quahog	*Aulocomya ater* **
	cultured oyster	*Crassostrea gigas*
	common European oyster	*Ostrea edulis*
Cockles	common edible cockle	*Cerastoderma edule*
	Mediterranean cockle	*Acantocardia tuberculatum*
		Clinocardium nutalli
Gastropoda	ormer	*Haliotis tuberculata*
		Niotha clathrata
		Zeux scalaris
		Concholepas concholepas **
		*Argobuccinum ranelliformes***
Scallops	giant sea scallop	*Placopecten magallanicus*
	Japanese scallop	*Patinopecten yessoensis*
	bay scallop	*Argopecten irradians*
	bivalve warty Venus	*Venus verricosa*
		Callista chione
		Chlamys farreri *
		Pecten albicans *
	purple-hinged scallop	*Hinnites giganteus* ***
		Buccinum spp.[##]
		Colus spp.[##]
		Thais spp. [##]
		Homarus americanus [##]
Whelks		*Buccinum* spp.[##]
		Colus spp.[##]
		Thais spp. [##]
Lobsters		*Homarus americanus* [##]
	northern moonshell	*Lunatia heros* [##]

Source: Mons *et al*., 1998, except as indicated

* Takatani *et al*., 1997; ** Lagos, 1998; *** Shumway *et al*., 1988; [#] MacKenzie *et al*., 1996; [##] Todd (1997)

2.4.3 Other aquatic organisms containing PSP toxins

The grazing habits of two abundant copepod species from the Gulf of Maine, *Acartia tonsa* and *Eurytemora herdmani*, were compared using cultured isolates of *Alexandrium* spp., which differed in toxicity per cell and toxin composition and a non-toxic dinoflagellate *Lingulodinium polyedrum.* Toxicity of the dinoflagellates had no effect on the grazing efficiencies of the two copepod species. Neither species showed strong evidence of incapacitation or adverse effects from ingested toxins. *E. herdmani* accumulated higher levels of PSP toxins but also had fuller guts. The experiments with mixed dinoflagellates suggested that both copepod species have the ability to choose their prey items based on palatability and not on toxicity. Although the toxin retention efficiencies of copepods tested were low overall (5 percent), high levels of PSP toxins were accumulated in copepod grazers, supporting evidence that zooplankton may serve as PSP toxin vector to higher trophic levels (Teegarden and Cembella, 1996).

Three species of marine copepods (*Acartia tonsa, Centropagus hamatus, Eurytemora herdmani*), commonly co-occurring with toxic *Alexandrium* spp., appeared to be able to discriminate between toxic and non-toxic *Alexandrium* spp. cells by chemosensory means, suggesting that selective behaviour rather than physiological effects governs the grazing response of copepods. Feeding behaviour varied among copepod species, suggesting that grazing pressure on toxic *Alexandrium* spp. is not uniform throughout the zooplankton community (Teegarden, 1999).

In large volumes (20 litres), the adult pelagic harpacticoid copepod *Euterpina acutifrons,* incubated with a high toxic strain of *A minutum* (1 000 or 10 000 cells/ml) for up to five days, revealed only trace levels of PSP-toxins in the extracts analysed by LC. With both a low and a high toxic strain of *A. minutum* (1 000 and 10 000 cells/ml), 10 to 15 percent of copepods were inactive after one to two days. It is suggested that *E. acutifrons* may avoid feeding on the dinoflagellates after tasting a few cells (Bagøien *et al*., 1996).

Of the crabs involved in human PSP in Japan and Fiji, most are xanthid crabs *(Lophozozymus pictor),* though some other species are also involved (horseshoe crab and marine snail). These species share the common feature of living in coral reefs and feeding by surface grazing (Mons *et al*, 1998; Sato *et al*., 2000).

Out of 459 specimens of xanthid crabs collected in Taiwan Province of China during October 1992 and May 1996 and analysed for tetrodotoxin and PSP toxins, five specimens (*Zosimus aeneus, Lophozozymus pictor, Atregatopsis germaini, Atergatis floridus, Demania reynaudi*) were found to contain PSP toxins besides tetrodotoxin. The percentages of PSP toxins varied from 11 to 97 percent (the remaining 89 to 3 percent was tetrodotoxin). The toxin profile of the PSP toxins varied within the different species. The source of the PSP toxins was *A. minutum* (Hwang and Tsai, 1999).

Algal toxins can also cause mortalities in fish as they move through the marine food web. Some years ago, tons of herring died in the Bay of Fundy after consuming small planktonic snails that had been feeding on *Alexandrium.* From the human health point of view, it is fortunate that herring, cod, salmon and other commercial fish are sensitive to PSP toxins and, unlike shellfish, die before the toxins reach dangerous levels in their flesh. Some toxins, however, accumulate in the liver and other organs of the fish, and so animals such as other fish, marine mammals and birds that consume whole fish, including the viscera, are at risk. In 1987, 14 humpback whales died suddenly from exposure to a bloom of *A. tamarensis* in Cape Cod Bay (Massachusetts). Researchers later learned that the whales had eaten mackerel whose organs contained high concentrations of STX (Mons *et al*., 1998).

In May 1996, star fish *Asterias amurensis* collected in the estuary of the Nikoh River (Kure Bay, Hiroshima Prefecture, Japan) appeared to contain PSP toxins (in the mouse bioassay 8.0 MU/g for whole body and 28.7 MU/g for viscera). The PSP toxins were supposed to come via the food chain from toxic bivalves living in the same area. The starfish toxin comprised of highly toxic components. GNTX1, GNTX2, GNTX3, GNTX4, dc-GNTX3 and dc-STX were the major components and accounted for approximately 77 mole%, along with N-sulfocarbamoyl derivatives C1-C4. GNTX1 was present in the largest amount (37.4 mole%) (Asakawa *et al*., 1997).

Atlantic mackerel (*Scomber scombrus*) are lethal vectors of PSP toxins to predators. Mackerel appeared to retain PSP toxins (STX [96 percent], GNTX2 and GNTX3 [4 percent]) year-round. The toxin content of the liver (determined by LC) increased significantly with fish age, suggesting that mackerel progressively accumulate the toxins during their life. The toxin content of the liver also increased significantly during the summer feeding sojourn in the Gulf of St. Lawrence, Canada. Zooplankton was the likely source of the PSP toxins in mackerel. Mean toxin content was 17.4 nmol/liver and the mean toxicity was 112.4 σg STX eq/100 g liver wet weight (Castonguay *et al*., 1997).

Marine puffers (*Arothron mappa, A. manillensis, A. nigropunctatus, A. hispidus, A. stellatus, A. reticularis*) in waters of the Philippines contained considerable amounts of PSP toxins (major component STX) besides tetrodotoxin (TTX), another potent marine toxin present in finfish. The toxicity was detected in liver, intestine, muscle and skin (Sato *et al*., 2000). Freshwater puffers (*Tetraodon leiurus complex, Tetraodon suvatii*), collected from the northeastern province of Thailand appeared to contain PSP-toxins. Toxicity was highest in liver and varied with location and season of fish catch. Toxin profiles from eggs, liver, skin and muscle showed the presence of STX, neoSTX and dcSTX (Kungsuwan *et al*., 1997). Sato *et al*. (1997) identified STX in the freshwater puffer *Tetraodon fangi*, which caused food poisoning in Thailand. Tetrodotoxin was not detected in this species. Two species of freshwater puffers (*Tetraodon cutcutia, Chelonodon patoca*), collected from several locations in Bangladesh, showed lethality in the mouse bioassay (at 2.0 to 40.0 MU/g tissue as PSP). Toxicity of skin was generally higher than other tissues examined (muscle, liver, ovary). Analyses of *T. cutcutia* revealed the presence of STX, dcSTX, GNTX2 and GNTX3, dcGNTX2 and three unidentified components possibly related to PSP. No tetrodotoxin or related substances were detected (Zaman *et al*., 1997a).

2.5 Toxicity of PSP toxins

2.5.1 Mechanism of action

The pharmacological action of the PSP toxins strongly resembles that of TTX. Due to the almost identical action of STX and TTX, it was assumed that both molecules had the same interaction with the receptor. Much attention has been given to the elucidation of the mechanism via which the blockade of the voltage-gated sodium channel is achieved as STX and TTX are the only agents which block this channel in a selective manner and with high affinity.

The voltage-gated sodium channel is a protein of approximately 250 000 Da, which traverses the plasma membrane of many excitable cells and is characterized by uniform conduction, potential dependency and ion selectivity. Among these are all mammalian nerves, skeletal muscle fibres and most cardiac muscle fibres. Upon appropriate depolarization of the cell, a conformational change occurs in the sodium channel molecule such that an aqueous path opens to permit movement of Na^+ from the extra-cellular phase into the cell under the existing electrochemical driving forces. The inward sodium current is responsible for the rising phase of the action potential. Voltage-gated potassium channels are also present in the membrane, and when open,

they permit outward passage of intracellular K^+ and consequent repolarization. STX and several other PSP toxins block the voltage-gated sodium channel with great potency, thus slowing or abolishing the propagation of the action potential. However, they leave the potassium channel unaffected.

The 7,8,9-guanidine function has been identified as being involved in the channel blockade. The C12-OH (as hydrated ketone) is important, whereas the carbamoyl side chain contributes but is not vital to channel blockade. Several hydrogen bonds between the toxin molecule and the binding side add to the binding energy. There is a general agreement among the investigators on the kinetic aspects of the toxin binding. The averaged blocking time of the channel is not dependent on the toxin concentration, but on the dissociation velocity. The lifetime of the open channel, however, is reversibly correlated with the toxin concentration and depends on the association constant (Mons *et al*., 1998).

2.5.2 Pharmacokinetics

studies in laboratory animals

rats

A single intravenous (i.v.) dose of radiolabeled [^{3}H]-saxitoxinol (STXOL), an analogue of STX, given to male Wistar rats, was rapidly distributed to various tissues including the central nervous system (CNS). The rats had excreted 40 percent of the dose in urine within two hours and 80 percent after 48 hours. Half-lifetime ($t_{1/2}$) in plasma is 29.3 minutes. Radioactivity reached a maximum in most tissues, including brain, eight hours after dosing. In liver and gastro-intestinal tract (G.I.T.), radioactivity was low during early phase after dosing, and was highest 24 hours after dosing, suggesting an alternate route of elimination and excretion. STXOL was metabolized in various tissues. Ten minutes after dosing, 19 percent of activity in kidney extract was associated with unidentified metabolites, for lungs it was 28.5 percent and for heart it was 41.8 percent. After 48 hours, 75 percent of activity in these tissues was associated with unidentified metabolites. Minimum biotransformation was found in muscles (14.4 percent) 48 hours after dosing. In CNS 10 minutes after dosing, 31.8 percent of activity was associated with unidentified metabolites in brain and 37.4 percent in spinal cord. After 48 hours in the brain, 76 percent of activity was associated with unidentified metabolites. No STXOL metabolites were detected in urine (Naseem, 1996).

Rapid excretion in urine was observed in rats after i.v. administration of radioactively labelled STX at a sub-lethal dose (ca. 2 µg/kg). No radioactivity was detectable in faeces at any time. Four hours after injection, approximately 19 percent of the STX dose was excreted in urine. By 24 hours, approximately 58 percent of the administered dose was excreted. Average total urinary excretion of administered STX was approximately 68 percent for the full study period. No radioactivity was found in the faeces. The authors concluded that these results demonstrate that small quantities of non-metabolized STX can be detected in rat urine up to 144 hours after i.v. administration (Aune, 2001).

cats

Fourteen adult male cats (bw 2.5-5 kg) were anesthetized and permanently coupled to artificial ventilation. Then the cats received a single i.v. injection with 2.7 or 10 σg STX/kg bw. During four hours after injection, cardiovascular parameters such as blood pressure and electrocardiograms were recorded and urine and blood samples were collected. Then the cats were killed and STX levels in brain, liver, spleen, bile and medulla oblongata were measured. The low dose did not cause changes in hemodynamic parameters. However, the high dose drastically reduced blood pressure, caused myocardial failure and finally cardiac arrest. Administration of

dobutamine (2.5 σg/kg per minute) restored hemodynamics and allowed the cats to overcome the shock. STX was excreted only by urine; within four hours, 25 percent of the administered dose at 2.7 σg/kg and 10 percent of the administered dose at 10 σg/kg. Renal clearance at the high dose was 0.81 ml/min/kg and at the low dose 3.99 ml/min/kg. These data suggest that STX excretion mainly involves glomerular filtration. No PSP toxins other than STX were detected in urine, blood or tissues analysed, indicating that no biotransformation had occurred. STX was detected in intensely irrigated organs such as liver and spleen but also in the central nervous system (brain [1.81 ng/g of wet tissue at the high dose] and medulla oblongata [2.5 ng/g of wet tissue at the high dose]) showing that STX was capable of crossing the blood-brain barrier (Andrinolo *et al.*, 1999a).

observations in humans

Clinicians have observed that, if patients survive PSP for 24 hours either with or without mechanical ventilation, chances for a rapid and full recovery are excellent. Such observations suggest that toxin(s) responsible for PSP either undergo rapid excretion, metabolism or both. In spite of the fact that most PSP toxins are positively charged, they are readily absorbed through the gastrointestinal mucosa. Depending on the severity of poisoning, the symptoms vary somewhat. The determinants of the severity are the specific toxicity of the PSP toxin in the ingested food, the amount of food ingested, and the rate of elimination of the PSP toxin(s) from the body. If the amount of toxic food is high enough, the first symptoms occur within a few minutes (Mons *et al.*, 1998).

In patients from four outbreaks of PSP in Alaska during May and June 1994, PSP toxin levels of 2.8-47 nM and 65-372 nM in serum and urine, respectively, were detected at acute illness and after acute symptom resolution. Severe hypertension was observed in the patients although only nanomolar serum levels were detected. The PSP toxin profile differed between mussels and human biological specimens, suggesting human metabolism had occurred. Clearance of PSP toxins from serum was evident within 24 hours and urine was identified as a major route of excretion (Gessner *et al.*, 1997).

2.5.3 Toxicity to laboratory animals

acute toxicity

The toxicity of the PSP toxins is almost always expressed as STX or STX equivalents. The sulfocarbamoyl compounds are considerably less toxic than the other groups of PSP toxins. However, they might be converted to the more toxic carbamates under acidic conditions (Aune, 2001). The mouse is very sensitive to the PSP toxins when compared to species such as fish, amphibians, reptiles and animals of a low order. The LD_{50} values for the different routes of administration are shown in Table 2.2. The oral LD_{50} values for other species than the mouse are shown in Table 2.3.

Table 2.2 Acute toxicity of STX in mice (Mons et al., 1998)

Route	LD_{50} in σg/kg bw
oral	260-263
intravenous	2.4-3.4
intraperitoneal	9.0-11.6

Table 2.3 Oral LD50 values of STX in various species (Mons et al., 1998)

Oral route	LD_{50} in σg/kg bw
rat	192-212
monkey	277-800
cat	254-280
rabbit	181-200
dog	180-200
guinea pig	128-135
pigeon	91-100

Aside from mouse lethality bioassays which are used to determine the relative potency of all analogues compared to that of STX (see Table 2.4), the full biological actions have been studied for only 50 percent of the natural analogues. However, from those that have been studied, the cellular mechanism of action seems to be basically the same. The N-sulfocarbamoyl compounds are appreciably less toxic than their counterparts of the carbamoyl series but they are readily converted to the corresponding carbamoyl compounds under acidic conditions with increases in toxicity of up to 40-fold. Such conversion has some potential clinical and public health significance because weakly toxic shellfish containing N-sulfocarbamoyl toxins could cause disproportional severe poisoning once ingested. Experimentally, however, it has been found that the conversion occurs in artificial gastric juice of the mouse and rat at a pH of 1.1, but not in genuine gastric juice remaining at a buffered pH of 2.2 (Mons *et al*., 1998).

Table 2.4 Relative toxicity of PSP toxins in the mouse bioassay

Toxin	Relative toxicity
STX	1
neoSTX	0.5 - 1.1
$GNTX_{2/3}$[a]	0.39/1.09 - 0.48/0.76
$GNTX_{1/4}$[a]	0.8/0.33 – 0.9/0.9
dcSTX	0.43
dcneoSTX	0.43
B1	0.07 – 0.17
B2	0.07 – 0.09
C1 to C4	<0.01 – 0.14
dcGNTX1 to dcGNTX4	0.18 – 0.45

a = ζ/η epimeric mixture
Source: Usleber *et al*., 1997

type of toxic effects

In animal experiments effects of STX on the respiratory system, myocard, muscle and nervous tissue (both peripheral and central) have been studied (Mons *et al*., 1998).

effects on the respiratory system

If PSP intoxication occurs, the effects on the respiratory system are responsible for the fatal ending. The cause of death is asphyxiation due to progressive respiratory muscle paralysis. In animals (cat, rabbit) doses of 1-2 σg STX/kg bw administered intravenously caused a decreased respiratory activity reflected in both a decline in the amplitude and velocity. When the dose was raised to 4-5 σg STX/kg bw, a strong depression of the respiration occurred. By artificial respiration, death can be avoided. If the dose is not too excessive the respiration may return spontaneously. In animal experiments only peripheral paralysis has been noticed by direct effects on the muscle of the respiratory system. The respiratory centre of the nervous system is not inhibited, action potentials are sent off to the midriff and the middle rib muscles. Other investigators however suggest a central influence. The possibility of central effects on the respiratory neurones may therefore not be excluded. The occurrence of paresthesia and feeling of lightness are often connected with a central effect, however the peripheral effects on the nervous system may be the cause of these symptoms (Mons *et al*., 1998).

cardiovascular effects

In anesthetized animals doses above 1 σg STX/kg bw (i.v.) can already provoke hypotension (paralysis of muscles is already observed at lower dose levels). This cardiovascular effect is seldom observed in human cases of intoxications and is more likely the reflection of peripheral effects, although the central nervous system might be involved to a certain extent. About the peripheral action there are uncertainties. Apart from a direct effect on the muscle tissue the possibility of an axonal blockade of the sympathetic nervous system can not be excluded. Most investigators agree on the fact that there are no, or hardly any, direct cardial effects. As an exception a direct disturbance of the atrio-ventricular sinus conduction is mentioned (Mons *et al*., 1998).

neuromuscular effects

An intravenous dose of 1-2 σg STX causes a fast weakening of muscle contractions; both contractions by direct stimulation as contractions by indirect motoneuron stimulation are affected. The effects include all skeletal muscle tissues. This dose level induces also a decrease of the action potential-amplitude and a longer latency time in the peripheral nervous tissue. Both motor and sensory neurones are influenced but the sensory neurones are already inhibited at lower dose levels. By this influence on the sensory system, the numbness and the proprioceptive loss may be explained but not the paresthesia. No clarity has been achieved about the possible theory on the toxic mechanism and many scientific debates reflect this (Mons *et al*., 1998).

effects on the central nervous system

There are uncertainties about the existence of an effect of PSP toxins on the central nervous system. Most symptoms can be attributed to peripheral effects. However central effects can not be excluded. For example investigators reported the influence of STX on the hamstring reflex (Mons *et al*., 1998).

repeated administration

No data

mutagenicity

No data

reproduction/teratogenicity studies

No data

2.5.4 Toxicity to humans

acute toxicity

The level at which PSP intoxications occur in humans varies considerably. This variation is mainly due to individual difference in sensitivity and fluctuation in the method of determination. Oral intake causing mild symptoms varied from 144 to 1660 σg STX eq/person. Fatal intoxications were reported after a calculated consumption of 456-12400 σg STX eq/person. These values are only reconstructed from what remained of the toxic mussels and vary greatly. An oral consumption of 300 σg PSP toxin per person was in some cases reported as fatal, whereas others noted the absence of toxic symptoms after an oral dose of 320 ug toxin per person. In Alaska, PSP was fatal for one fisherman, while two others eventually recovered. The stomach contents of patients contained 370 ug PSP toxin (STX eq)/100 g (Mons *et al.*, 1998). Other sources report mild poisoning at doses of PSP toxins between 304 and 4128 µg/person, while severe poisonings are caused by doses between 576 and 8272 µg/person (Aune, 2001).

In 1987, an outbreak of PSP with 187 cases and 26 deaths was reported after consumption of a clam (*Amphichaena kindermani*) soup. The fatalities were the highest among young children (50 percent) compared with 7 percent in adults. Some of the children who died ingested an estimated dose of 140-160 MU/kg bw (Rodrigue *et al.*, 1990). Aune (2001) reported that the minimal lethal dose in this incident was estimated to be about 25 µg STX eq/kg bw for a child weighing 25 kg compared to 86-788 µg STX eq/kg bw in four adults who died.

The Australia New Zeleand Food Authority reported that 120 to 180 µg PSP toxins can produce moderate symptoms in humans, 400 to 1060 µg can cause death and 2 000 to 10 000 µg is more likely to constitute a fatal dose (ANZFA, 2001).

The mortality rate of PSP varies considerably. In recent outbreaks involving over 200 people in North America and Western Europe, no deaths occurred. However, in similar outbreaks in Southeast Asia and Latin America, death rates of 2 to 14 percent have been recorded. A large part of the difference is due to the fact that in the former cases intoxication often occurred in urban areas where victims already have access to hospital care, whereas in Southeast Asia and Latin America, intoxications often occurred in rural areas where such poisonings had never before been encountered by local people and health professionals (Mons *et al*., 1998).

toxic symptoms

Clinical symptoms of PSP in mild cases include a tingling sensation or numbness around lips, which appear mostly within 30 minutes. These effects are clearly due to local absorption of the PSP toxins through the buccal mucous membranes. These sensations then spread gradually to the face and neck. Prickly sensation in the fingertips and toes is frequent as are headaches, dizziness, nausea, vomiting and diarrhoea. Sometimes, temporary blindness is also observed. Most symptoms have a quick onset (hours) but may last for days, and are virtually invariant in all cases of paralytic shellfish poisoning. They precede distinct muscular weakness because sensory nerves, being thinner and having shorter internodes than motor nerves, are always affected first by any axonal blocking agents.

In moderately severe poisoning, paresthesia progresses to the arms and the legs, which also exhibit motor weakness. Giddiness and incoherent speech are apparent. Cerebellar manifestations such as ataxia, motor incoordination and dysmetria are frequent. Respiratory difficulties begin to appear as tightness around the throat. In severe poisoning, muscular paralysis spreads and becomes deeper. The pulse usually shows no alarming abnormality. Pronounced respiratory difficulty and death through respiratory paralysis may occur within 2 to 24 hours of ingestion (Mons *et al*., 1998).

Since STXs are charged, water-soluble molecules, it is probable that they do not penetrate the blood-brain barrier well and most of their effects are on peripheral nerves (Mons *et al*., 1998). In a study carried out in Alaska among patients suffering from PSP, hypertension also occurred corresponding with the ingested toxin dose (Gessner *et al*., 1997).

effects of alcohol consumption

The effect of alcohol consumption on PSP is still unclear. Some say that alcohol might be a protective factor against the adverse effects of PSP toxins but the mechanism through which alcohol might reduce the risk is unknown. Since the elimination of PSP toxins occurs at least in part through the urine, alcohol may influence illness by a diuretic effect. Alternatively, alcohol may cause hepatic enzyme induction. In a case-control study in Alaska, 47 outbreaks were studied for which the consumption histories of all persons were known. Alcohol consumption and eating cooked rather than raw shellfish were associated with a reduced risk of PSP. An association between illness and either the toxin level or dose ingested was not found (Mons *et al*., 1998).

treatment

The clinical management of poisoned victims is entirely supportive. If no vomiting had occurred spontaneously, induced emesis or gastric lavage should be used to remove sources of unabsorbed toxins. As the PSP toxins are strongly charged at the gastric pH, they would be effectively absorbed by activated charcoal. These steps are especially important in the management of child victims of poisoning, as the severity of the intoxication is directly dependent on the concentration of the toxins in the body. In the 1987 epidemic in Guatemala, the mortality rate in children up to six years of age was 50 percent while for adults it was 7 percent.

In moderately severe cases, maintenance of adequate ventilation is the primary concern. In uncomplicated PSP the airway is not obstructed by excessive excretion. As ventilatory failure is due to varying degrees of paralysis of the respiratory nerves and muscles, positive pressure assisted ventilation, when indicated, is desirable. Fluid therapy is essential to correct any possible acidosis. Additionally, it will facilitate the renal excretion of the toxins.

Time-honoured conservatively supportive management has proven effective. If the patient survives 18 hours, the prognosis is good, with complete and rapid recovery. Some say that even nine hours should be adequate for a physiological reduction of the toxins concentration to relatively harmless levels, except in those cases where the toxin concentration began at exceptionally high levels, or in victims with impaired renal function. Artificial ventilation and gastric lavage are still the only acceptable medical countermeasure against STX intoxication. However, in cases of severe intoxication, artificial ventilation may be inadequate (Mons *et al*., 1998).

Some studies in animals gave an indication that 4-aminopyridine may be useful as antidote for STX-intoxication. 4-Aminopyridine significantly reversed the respiratory rate, tidal volume and blood pressure to normal values in anesthesized STX-intoxicated rats. Furthermore, 4-aminopyridine, not only prolonged the survival time but also decreased the mortality of mice (71 to 43 percent) at a normally lethal dose (30 σg/kg i.p.) of STX (Chen *et al*., 1996). In guinea pigs, 4-aminopyridine was able to reverse the extent of cardiorespiratory infirmity and other sublethal effects of STX. At the point where cardiorespiratory performance was most seriously compromised (30 minutes after intramuscular STX injection) 4-aminopyridine was injected. Within minutes the STX induced diaphragmatic blockade, bradypnoea, bradycardia and depressed cortical activity were all restored to a level either comparable to, or surpassing, that of control. At the dose-level used to restore ventilatory function and cardiovascular performance, 4-aminopyridine produced no sign of seizures and convulsions. Although less secondary effects such as cortical excitant/arousal effect and transient periods of skeletal muscle fasciculation were

seen, these events were of minor concern in view of the remarkable therapeutic effects (Chang *et al*., 1997b).

STX-induced lethality in guinea pigs can be reversed by 4-aminopyridine when it is administered at the time of respiratory arrest. 4-Aminopyridine showed to facilitate recovery and reduce the amount of time guinea pigs are dependent upon artificial ventilation (Benton *et al*., 1998).

2.5.5 Toxicity to aquatic organisms

The acute and chronic effects of *A. tamarensis* on the shrimp *Neomysis awatchensis* were studied. Mortality rate in the presence of 9 000 cells/litre was 55 percent for *N. awatschensis*. The 96 hour LC_{50} for *N. awatschensis* was 7 000 cells/litre. In the presence of a cell-free filtrate 25 percent of *N. awatschensis* died after 96 hours. In a 62-day experiment, 37 percent of *N. awatschensis* died at a concentration of 900 cells/ml and the number of juveniles was 27 (only 16.4 percent of the number of juveniles in the control group). The first date of reproduction was prolonged for three days compared with controls. At 900 cells/ml length and weight of parent shrimps were 95.6 and 81.9 percent, respectively of control values. These last differences were not significant (Zhijun *et al*., 2001).

The hatching rate of fertilized eggs of the scallop *Chlamys farreri* was only 30, and 5 percent of controls at exposure for 36 hours to *A. tamarensis* cells or cellular fragments at concentrations of 100 and 500 cells/ml respectively. Exposure of the eggs to STX or cell contents (supernatant or re-suspended algal cells) did not elicit this inhibitory response. The alga also affected larvae at early D-shape stage of scallop. Survival rates began to decrease significantly at exposure for six days to concentrations ∅3000 cells/ml; no larvae could survive after 14-day exposure to 10 000 cells/ml. This study indicated that developmental stages before blastula were the most sensitive periods to *A. tamarensis* toxins. And the alga at the early exponential stage had the strongest effect on egg hatching comparing with other growth phases (Yan *et al*., 2001).

Most of the bivalve shellfish filter-feeders are relatively insensitive to the PSP toxins because many of them have nerves and muscles operated mainly by voltage-gated calcium channels and STX and other PSP toxins block only the voltage-gated sodium channel with great potency (high affinity). This enables them to continue feeding and thereafter they become highly toxic. The high tolerance of for example blue mussels to STXs and their continued feeding on toxic algae can result in exceeding the 80 σg STX level in less than one hour (Mons *et al*., 1998).

There are even some species of bivalves known to avoid toxic dinoflagellates. One species of particular interest is the northern quahog or hard clam, *Mercenaria mercenaria.* In a laboratory study in the presence of *A. tamarensis* the quahog first retracts its siphons and then completely isolates itself from the external environment by means of shell valve closure. The animals did not re-open their valves until the addition of clean seawater (Mons *et al*., 1998).

Blooms of *Alexandrium* can cause fish kills. For an adult herring 10-20 σg STX eq is a lethal dose (Teegarden and Cembella, 1996). When milkfish (*Chanos chanos*) fingerlings were exposed (without any aeration) to toxic *A. minutum* (1.4×10^4 cells/ml ~ 3.0×10^4 cells/ml) or toxic algal extracts (5.13×10^3 cells/ml ~ 2.05×10^4 cells/ml) for one day, noticeable edema, hyperplasia and necrosis of secondary lamellae in the gill were observed by light microscopical examination. Similar symptoms were also seen in fish treated with pure STX (6.5×10^{-2} σg/ml). At the same treatment, fingerlings showed a higher O_2 consumption rate and a higher demand of critical O_2 pressure. Treatment of milkfish fingerlings with non-toxic *A. minutum* cells or the algal cell extract did not cause any damage to the gills, nor a rise in O_2 consumption rate or critical O_2

demand. However, fingerlings died from suffocation at a cell concentration of 2.1 x 10^4 cells/ml of nontoxic *A. minutum* without aeration support in 24 hours (Chou and Chen, 2001b).

In a study on the sublethal effects of *Alexandrium*, as might occur during pre- and post-bloom conditions, copepods grazing on *Alexandrium*, were fed to larval mummichogs (*Fundulus heteroclitus*). The exposed larvae consistently showed reduced swimming performance and prey capture. In some trials exposed copepods were captured more easily than unexposed ones. The results suggest that *Alexandrium* impacts both fish and their prey, therefore facilitating transfer of the toxin through the food web (Samson *et al*., 1999).

A variety of zooplankton responses towards toxic flagellates have been reported, ranging from avoidance to ingestion of the algae or even active selection. Bagøien *et al*. (1996) showed that all developmental stages of the zooplankton species *Euterpina acutifrons* were inactivated by *A. minutum*, but the effect was faster and more intense on the nauplii. However, nauplii and copepods not moving within a given time are often defined as inactive, but in many cases they are not dead. Still, inactivation of adults also reached high proportions (80 percent was inactivated) towards the end of a five-day large volume experiment. Many of the copepods were then clearly dead but a considerable proportion of adult females remained active and were able to produce viable eggs. Only traces of PSP toxins were detected in adult copepods after five days of exposure to *A. minutum* suggesting that *E. acutifrons* avoids feeding on the dinoflagellates after tasting a few cells. Extrapolating these results to natural conditions suggests that even if the toxic effects are not acute under moderate to dense bloom conditions, a significant proportion of zooplankton population can be killed, assuming that the zooplankton do not avoid the affected area.

Dutz (1998) studied the effects of the PSP toxins-producing dinoflagellate *A. lusitanicum* on the reproductive success of the common calonoid copepod *Acartia clausi*. *Acartia clausi* did not show mortality when fed on *A. lusitanicum* and were also able to produce eggs. However, the egg production was reduced. It is suggested that the ingested toxins probably interfere with digestive processes or cause enhanced energy expenditure due to detoxification. Consequently less energy is available probably resulting in reduced fecundity. In Egyptian coastal waters, mortality of wild fish and of fish kept in aquariums with filtered seawater has been associated with dense blooms of *A. minutum* (Bagøien *et al*., 1996).

2.5.6 Toxicity to water fowl

An acute poisoning incident in captive herring gull chicks (*Larus argentatus*) fed a batch of store-bought scallops was described. The chicks developed a characteristic acute syndrome that had not until then been reported in birds and the cause of which remained to be identified. The authors suggested that it was a variant of PSP insofar as it was paralytic and caused by shellfish. However, analyses were negative for STX, brevetoxin and domoic acid (Gochfeld and Burger, 1998).

2.6 Prevention of PSP intoxication

2.6.1 Depuration

Various attempts have been made at detoxifying shellfish contaminated with paralytic shellfish poisons in an effort to reduce the duration of off market times. The most obvious method is to transfer shellfish to waters free of toxic organisms and allow them to self-depurate. However, transferring large quantities of shellfish is very labour intensive and costly. The rates of detoxification vary considerably between species and some species remain toxic for extended periods of time, for instance up to several months for *Crassostrea, Plactopecten, Spisula* and

others. The time required for elimination in mussel tissue also varies considerably. If the contaminating dinoflagellates have disappeared from the surrounding water there is a gradual decline in the amount of toxin. The toxicity of the mussel *Mytilus edulis* can decrease by 50 percent in 12 days in dinoflagellate free, salt water with a temperature of 15 to 20° C. The elimination time of PSP toxins from the clam *Saxidomus giganteus* however is much longer. It takes a year or longer after the exposure to the toxic dinoflagellates to lose the toxins (Mons *et al*., 1998). The rate of loss varies with season, and low water temperatures apparently retard toxin loss. However, the degree to which temperature affects the uptake and release of toxins is not clearly understood. Further, the rate of detoxification is highly dependent on the site of toxin storage within the animal (toxins in the gastrointestinal tract are eliminated much more readily than toxins bound in tissues) and on initial or peak level of toxicity. Mussels are known to accumulate PSP toxins faster than most other species of shellfish and also eliminate the poisons quickly. While oysters do not accumulate the toxic species as readily as mussels, they take considerably more time to detoxify (Mons *et al*., 1998*).*

Detoxification of PSP-toxins using temperature or salinity stress has been tried with marginal success. Instantaneous electrical shock treatments were found to accelerate toxin excretion in scallops. Reduced pH has been tried as a means of detoxifying butter clams but with no success. Chlorination has been used in France; however, this process alters the flavour of the shellfish and thus decreases marketability. However, ozonized seawater can be of value in detoxification of shellfish recently contaminated by the vegetative cell phase of toxic dinoflagellates. In a study during a red tide outbreak, it was shown that ozone treatment of the seawater prevents shellfish from accumulating PSP toxins. This activation could be achieved in a marketable species such as *Mya* within an economically feasible time frame. On the contrary, ozone is useless in detoxifying cysts or in bivalves that have ingested cysts or have the toxins bound in their tissue over long periods of time. Further, detoxification of algal toxins from shellfish, especially paralytic shellfish poisons, over long periods of time is not economically feasible. Ozone is not recommended as a practical or safe means of eliminating algal toxins from shellfish (Mons *et al*., 1998).

Cooking has also been proposed as a possible means of detoxifying shellfish contaminated with paralytic shellfish poisons. However, while cooking may reduce levels of toxins it does not eliminate the danger of intoxication. If the initial level of toxicity is low, cooking may effectively reduce toxicity to safe levels. Pan frying seems to be more effective than other methods of cooking. When clams or mussels are steamed or boiled, toxins lost from the tissues are contained in the cooking liquid rendering the fluids extremely toxic (Mons *et al*., 1998). Boiling of oysters only, for the usual home cooking times (98 ∀C for 10 minutes), reduced their toxicity by 68 to 81 percent. However, boiling by itself is not sufficient to detoxify extremely high toxic shellfish (Jeong *et al*., 1999).

Beringuer *et al*. (1993) studied the effects of operations carried out during the industrial canning process on the contamination of *Acanthocardia tuberculatum* (Mediterranean cockle) by PSP toxins. The observed effects of boiling and sterilizing averaged over 70.6 to 77.9 percent and 81.8 to 90.9 percent reduction of toxicity, respectively. Takata *et al*. (1994) investigated the reduction in toxicity of PSP-infected oysters (*Crassostrea gigas*) by heat treatment. The methods of heat treatment were boiling (at 98 °C for 5 to 60 minutes) and retorting (at 120 °C for 5 to 60 minutes). Boiling at 98 °C resulted in 53 to 88.3 percent detoxification, retorting at 120 °C in 57.4 to 100 percent detoxification. Boiling and retorting for 60 minutes resulted in more detoxification than boiling and retorting for five minutes.

Less detoxification after boiling and autoclaving of PSP-infected oysters was observed. Oysters having 17.4 or 29.8 MU PSP toxins were boiled, canned and autoclaved. The toxicity was reduced by about 20 percent after boiling and by less then 10 percent after autoclaving. The effectiveness

of canning as a means of reducing PSP-toxicity levels below quarantine levels is dependent upon the initial levels of toxicity and should be approached with great caution (Mons *et al*., 1998).

After both the boiled and the smoked canning process of oysters (*Crassostrea gigas*) contaminated with 185 to 778 σg eq STX/100 g, Jeong *et al.* (1999) measured a reduction to <80 σg eq STX/100 g. Mole % of toxin components in the shucked oysters was in the order of 25.1% GNTX1, 19.2 mole % GNTX3, 17.2 mole % of GNTX4 and 14.6 mole % of GNTX2. Trace amounts of C1, C2, STX and neoSTX were present. In case of specific toxicity, the major toxins were GNTX1-4. The sum of GNTX1, 2, 3 and 4 was >80 percent of total toxicity. STX and dc-STX were more thermostable than any other toxin component.

The standard canning process (steaming, cooking and sterilization) of pickled mussels and mussels in brine (*Mytilus galloprovincialis*) resulted in a 50 percent reduction of PSP toxicity in mussel meat. The decrease was not dependent on the toxin levels in the raw mussel meat. Total toxicity reduction did not fully correspond to toxin destruction, which was due to the loss of PSP toxins to cooking water and packing media of the canned product. The detoxification percentages can be affected by changes in the toxin profile due to toxin conversion (Vieites *et al*., 1999).

Indrasena and Gill (1999) studied the effects of a wide range of pH values (3-7) on the kinetics of thermal destruction for individual PSP toxins in scallop digestive glands. Most of the individual toxins degraded more rapidly when heated at higher temperatures and pH levels for longer times. PSP toxicity decreased rapidly at 130 ∀C at pH 6-7.

In a later study of Indrasena and Gill (2000a), mixtures of purified and partially purified PSP toxins including C1/2 and B1 toxins, GNTX1-4, neoSTX and STX were heated (90-130 ∀C) for different times (10 to 120 minutes) at different pHs (3-7) and analysed by LC. C toxins declined rapidly at low pH, and GNTX1/4 toxins decreased at high temperatures and high pH. GNTX 2/3 increased initially at low pH and then declined with subsequent heating, whereas STX increased consistently at pH 3 to 4. The integrated total toxicity declined at pH 6 to 7. The efficacy of thermal destruction was highly dependent on pH, with rapid thermal destruction of carbamate toxins at higher pH. Heating at low pH resulted in conversion of least toxic compounds to highly toxic compounds.

Variations in C-toxins (C1-2), GNTX1-4, STX and neoSTX in scallop digestive glands and a mixture of purified PSP toxins were studied during storage at –35, 5 and 25 ∀C at different pH levels by Indrasena and Gill (2000b). All toxins were stable at low pH (3-4) and –35 ∀C. C toxins were the most sensitive followed by GNTX1/4 for changes at all pH levels and at 5 and 25 ∀C. STX followed by neoSTX were the most stable at –35 and 5 ∀C, especially at pH 3-4.

With the exception of the methods reported by Berenguer *et al.* (1993) and Takata *et al.* (1994) there are hardly useful methods for effectively reducing PSP toxins in contaminated shellfish. Most of the methods tested have been unsafe, too slow or economically unfeasible, or have yielded products unacceptable in appearance and taste. Given the apparent global increase in harmful algal blooms and the continually growing interest in culture of bivalve molluscs, further efforts are needed to develop effective means of detoxifying shellfish contaminated with PSP toxins. Failing the development of any such methods, increased efforts will need to be devoted to monitoring shellfish for the presence of PSP toxins.

Takatani *et al.* (2003) suggested that microwave heating after pretreatment with salt and alkaline solution might be available for decomposition of PSP toxins in the oyster *Crassostrea gigas*.

Data on toxicity measured under different conditions for the adductor muscle of highly PSP-infested scallop *Patinopecten yessoensis*, were reviewed. In the adductor muscle, separated from live or fresh scallop, not any toxicity was observed even though the whole scallop contained levels as high as 2 900 MU/g. On the other hand, the adductor muscle separated from the frozen whole body, showed very small toxicity whose score depended on the different procedures, high especially in slow thawing over many hours. It can be concluded that the adductor muscle of the scallop *Patinopecten yessoensis* is safe for consumption only when it is prepared from live or fresh scallop with careful removal of toxic viscera, roe and the other organs (Murakami and Noguchi, 2003).

Within the EC Research Programme "Quality of Life and Management of Living Resources", research is ongoing on an accelerated detoxification system for live marine shellfish, contaminated by PSP toxins. The objective of this project is to determine the effects of micro-algae diet in order to accelerate the detoxification of live shellfish, namely oysters and clams, in a system that overcomes the problems of PSP contamination. The properties of the process are to speed up the shellfish detoxification kinetics with appropriate diets using the effect of non-toxic cell density (Anonymous, 1999a).

2.6.2 Preventive measures

At present the economic feasibility of efficiently detoxifying shellfish on a large scale in artificial systems is not promising. In areas prone to regular outbreaks of toxic algal species, culturists and commercial fishermen alike still depend on monitoring systems to warn of toxic shellfish and plan their activities accordingly. Through combined efforts of an intensive monitoring programme and culture of 'rapid release' species (e.g. *Mytilus edulis),* species known to avoid toxic dinoflagellates (e.g. *Mercenaria,* most oysters) or scallops, economic loss can be kept to a minimum.

Preventive measurements include regular inspection of seawater bodies in which the shellfish are grown on the possible appearance of toxic dinoflagellates especially in the season that blooms may occur. In addition, the presence of cysts of the dinoflagellates should be explored and the shellfish itself should also be inspected routinely. Therefore, the development of a rapid and reliable method for the detection of *Alexandrium* species before bloom formation is important (Mons *et al*., 1998). However, morphological identification continues to be controversial (Sako, 1999). Some of the morphological features may change with varying environmental conditions or growth stage. This necessitates the application of biological, biochemical, immunological and molecular biological techniques. Monoclonal antibody analyses and lectin-binding assays have also been applied. However, these analyses are based on phenotypic characters which may be affected by environmental factors (Adachi *et al.*, 1996). In order to resolve the phenotypic problems, the definition of genetic markers may be useful for classifying the *Alexandrium* species responsible for the production of PSP toxins. In a number of studies the ribosomal DNA (rDNA) sequences and internal transcribed spacers (ITS) (using DNA probes and whole-cell hybridization) were analysed (Adachi *et al.*, 1996; Sako, 1999).

Haley *et al*. (1999) reported a stream-lined method for a labour- and resource-intensive protocol for the isolation of *A. tamarensis* ribosomal DNA (rDNA). This method facilitated the detection of 10^{-4} ng/σl of *A. tamarensis* DNA. The kit enabled *A. tamarensis* to be isolated from the water sources with little signal degradation. This is a valuable technique for the rapid detection of *A. tamarensis*, even before cell numbers are large enough for morphological identification.

Guzmán *et al*. (2001) reported that prevention could be strengthened not only through a PSP monitoring programme but also through a strategy of specific training to specific target groups

and an appropriate information dissemination system. A strategy applied in southern Chile since 1997 included:

- training workshops oriented to diverse groups;
- design and dissemination of key information about harmful algal blooms and its effects, oriented to the general community; and
- specific activities with primary and high school teachers and students.

In case of contaminated shellfish, measurements have to be taken to prevent consumption and cases of human PSP intoxications should be reported to the health authorities as soon as possible. More information on regulations and monitoring will be presented in Chapter 2.8.

2.7 Cases and outbreaks of PSP

2.7.1 Introduction

The increased global distribution of paralytic shellfish poisoning is reported in Hallegraeff (1993). Until 1970, PSP-producing dinoflagellates blooms of *A. tamarensis* and *A. catenella* were only known from temperate waters of Europe, North America and Japan. By 1990, PSP was well documented from throughout the Southern Hemisphere in South Africa, Australia, India, Thailand, Brunei Darussalam, Sabah (Malaysia), the Philippines and Papua New Guinea. Of course, the increasing interest in the utilization of coastal waters for aquaculture is leading to an increased awareness of toxic algal species, but whether this is the only explanation remains uncertain.

2.7.2 Europe

The presence of PSP toxins in the coastal waters of ICES countries in Europe during the years from 1991 to 2000 is illustrated in Figure 2.2.

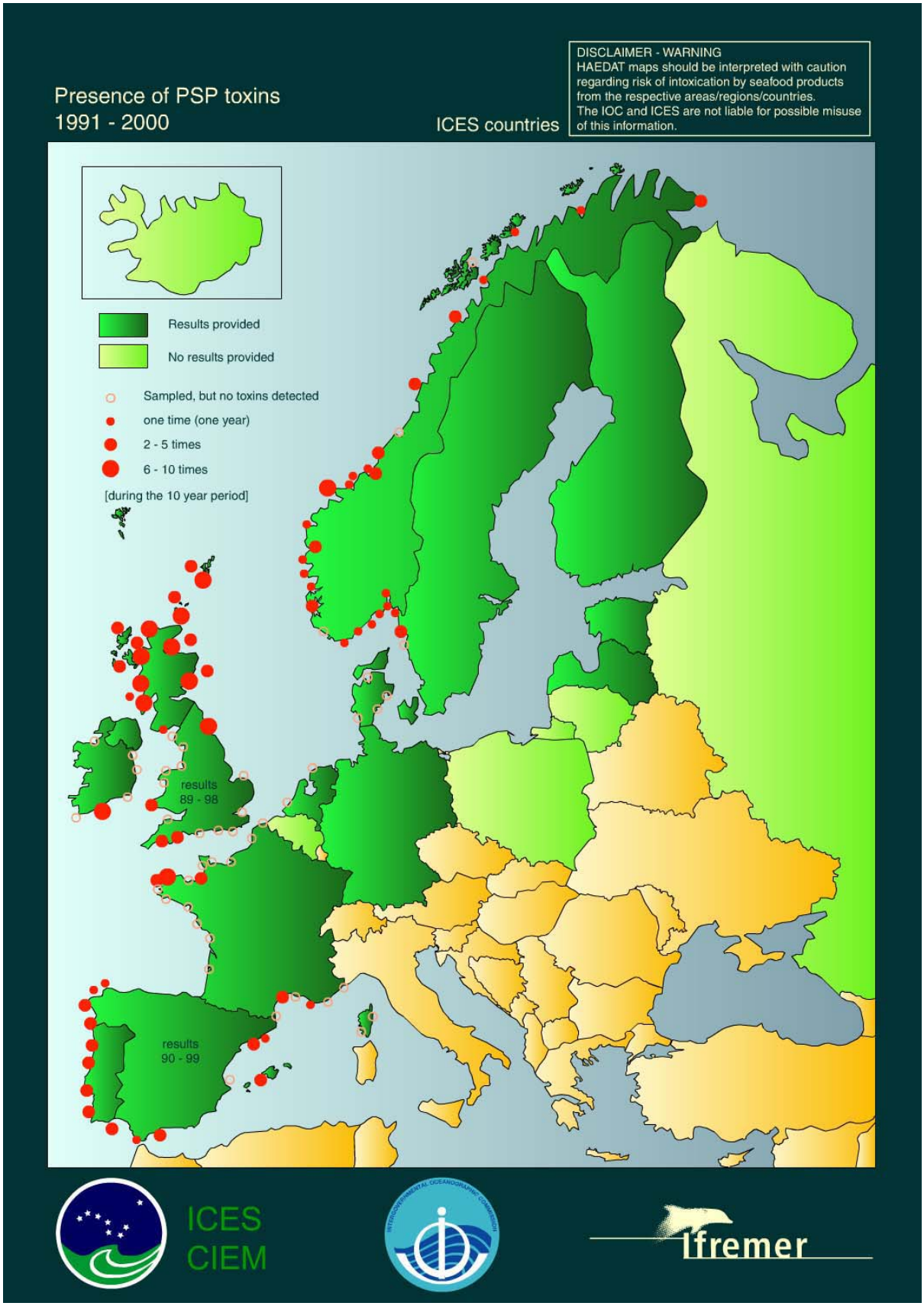
Denmark

At the east coast of Jutland, PSP was found in mussels in 1987 and in 1990 (and possibly also in 1988). The causing algae were *A. tamarensis* and *A. ostenfeldii* (CRL, 1995). A PSP episode took place in 1996 and a PSP-like episode followed in 1997. However, in the last case, the presence of PSP toxins was not confirmed (EU-NRL, 1998).

France

Most of the toxic episodes caused by marine biotoxins were related to diarrhoeic phycotoxins; but some minor toxic episodes were related to paralytic phycotoxins. These episodes occurred only at the northern Brittany coast and were always caused by *A. minutum.* Maximal toxicity in mussels and oysters was 400 ug STX eq/100 g meat. In late 1992, PSP toxins were found in mussels from the Atlantic coast without any occurrence of toxin producing algae in the water (CRL, 1995). At the end of 1998, *A. tamarensis* was detected at concentrations of up to 350 000 cells/litre and some production areas for clams, oysters and mussels were closed for two months (EU-NRL, 2000). In 2000, two areas in Brittany were closed due to the presence of PSP toxins (EU-NRL, 2001).

Figure 2.2 Occurrence of PSP toxins in coastal waters of European ICES countries from 1991 to 2000



Source: http://www.ifremer.fr/envlit/documentation/dossiers/ciem/aindex.htm

Germany

Since the beginning of the twentieth century, no poisonings related to PSP have been reported after the consumption of mussels collected in German waters. Since 1972 (PSP cases in Germany following consumption of contaminated Spanish mussels), mussels are regularly monitored for PSP toxins. Causative organisms like A. *tamarensis, A. minutum* and *A. ostenfeldii* occurred but only in limited numbers. In 1987, three cases of PSP occurred in Lower Saxonia which could, again, be attributed to the consumption of canned Spanish mussels (CRL, 1995). Since March 1992, viable cysts of a species very similar or identical to *G. catenatum* have been found in German coastal waters of the North and Baltic Seas. The toxicity of the strain has not (yet) been reported. In freshwater, PSP producing blue green algae have been isolated. Other species known to produce PSP toxins have not yet been reported in German waters (IOC, 1995).

Ireland

In Ireland, a PSP event was noted in Cork Harbour in July 1992 and persisted for one week only. The phytoplankton associated with this event was *Alexandrium tamarensis* (CRL, 1995). Following a bloom of *A. tamarensis* in Cork Harbour in June 1996 GNTX2 and GNTX3 were identified in wild mussels (*Mytilus edulis*) and cultivated oysters (*Crassostrea gigas*). Maximum recorded levels were 360 and 88 µg STX eq/100 g meat in *M. edulis* and *C. gigas*, respectively, determined by LC. The levels in *M. edulis* dropped by natural depuration, to 25 µg/100 g meat (by LC) within 12 days. The levels in *C. gigas* declined to 3 µg STX eq/100 g meat within 12 days in a depuration unit (Furey *et al*., 1998). In 1999 cysts of *A. tamarensis* were detected in surface sediments in Cork Harbour and viable cultures of *A. tamarensis* have been grown from these cysts (EU-NRL, 2000). During 2002, PSP toxins were detected above the regulatory limit in both mussels and oysters from Cork Harbour for a three-week period in July (EU-NRL, 2002).

Italy

Dinoflagellate red tides are a recurring phenomenon in the Adriatic Sea yet despite the presence of potentially toxic species such as *Alexandrium* spp., no cases of PSP in humans were reported until 1995. Along the northwest Adriatic coast of Italy, *A. minutum* was found at sampling stations in Emilia Romagna in 1994, 1995 and 1996 from April to July. In May 1994, some mussel samples showed PSP values greater than 80 µg STX eq./100 g meat but no public health problems were reported (Poletti *et al*., 1998). Some shellfish samples were subsequently also found to be contaminated by PSP along the Emilia Romagna coast as a consequence of the presence of *A. minutum* in seawater (CRL, 1995). PSP toxins at a level <40 µg STX eq./100 g of meat, associated with the presence of *A. minutum*, were detected in April and May 2001 in Friuli Venezia Giulia (EU-NRL, 2001). In late May and early June 2002, PSP toxins were detected in Sardinia (Golf of Olbia) due to the presence of *A. catenella* and *A. minutum*. In this case, STX levels were higher than 80 µg STX eq/100 g and a maximum of 2510 µg STX eq/100 g was reached (EU-NRL, 2002).

The Netherlands

PSP-producing algae have, until now, hardly been observed in the Netherlands. Although *Alexandrium* species were identified in the North Sea in 1989, shellfish containing PSP toxins were not found. In 1990, symptoms of paralysis were reported in some persons after consumption of shellfish from the Wadden Sea but it was subsequently determined that this was not related to PSP (Mons *et al*., 1998). Scallops with PSP toxins were once reported but this turned out to be a side-catch from the North Sea (CRL, 1995).

Norway

PSP contamination events in Norway are among the earliest recorded in Europe. PSP has been detected frequently in many localities and cases of human intoxication have been reported seven

times (1901, 1939, 1959, 1979, 1981, 1991 and 1992) with a total of 32 victims including two fatalities (Mons *et al.*, 1998). Results of a surveillance programme in 1994 showed different closures due to PSP. A similar pattern was found the following years (EU-NRL, 1998). In 1999, 11 percent of samples tested were positive for the presence of PSP toxins. Some cases of human illness, associated with mussels imported from Sweden, have been recorded (EU-NRL, 2000).

Portugal

From 1986 until 1990, there were occurrences of PSP outbreaks along the Portuguese coast north of Roca Cape. In 1991, the problem did not appear. In 1992, it appeared again but occurred also off the south coast of Lisbon and along the Algarve coast in concentrations of 100-500 σg STX eq/100 g of shellfish. In 1993, PSP was found almost all year round, covering the entire coast. Observed concentrations ranged from 113 σg STX eq/100 g of shellfish in December at the Northern coast up to 9145 σg STX eq/100 g at the Lisboan coast in September. In 1994, PSP outbreaks occurred off the south coast of Portugal and off the Algarve coast. The main causative organism was *Gymnodinium catenatum. Alexandrium lusitanicum* was also found to be responsible for PSP outbreaks at the Obidos Lagoon (IOC, 1995). During the last two weeks of October 1994, nine patients (six women and three men) were reported to suffer from PSP after consumption of molluscs (*Mytilus edulis*) from the west coast of Portugual (De Carvalho *et al*., 1998). In 1997, PSP was found in only one species, *Tellina crasa* (EU-NRL, 1998). *G. catenatum* at concentrations >3000 cells/litre were observed in the Algarve area and a precautionary closure was put in place (EU-NRL, 2000).

Spain

Mussel aquaculture is an important industry in the Galician Rias, located along the northwestern Atlantic coast of Spain. Since 1976, this region has been seriously affected by incidents of PSP. Spanish mussels contaminated with PSP have caused food poisoning in several other European countries as well as Spain. This occurred in 1976 when mussels exported by Spain caused paralytic shellfish poisoning in European countries including Germany, France, Switzerland and Italy. In total, 120 people in Western Europe were affected by PSP following consumption of these Spanish mussels (*Mytilus edulis*). However, there were no deaths (IPCS, 1984).

Another particularly bad episode occurred in 1993 when the toxic events lasted for an unusually long period. Many people became ill with unusual symptoms. The chemical analysis of the mussel samples taken revealed a complex toxin profile with both DSP and PSP toxins present. The observed PSP toxins were Bl and the decarbamoylated derivatives of STX, GNTX2 and GNTX3. Small amounts of STX and other (unidentified) PSP toxins were also observed. The contamination of the mussels was probably due to the dinoflagellate *A. catenatum* (Gago-Martinez *et al.*, 1996). Sporadic episodes of PSP lasting two to three weeks occurred in Galicia during 1995 to 1997 (EU-NRL, 1998). In 2000, there were some toxic events related to the presence of PSP toxins that led to the prohibition of harvesting of bivalves in some production areas in Galicia (EU-NRL, 2001).

Routine toxicity testing of bivalve warty Venus (*Venus verrucosa*) in January 1989 on the Mediterranean coast of southern Spain revealed rising levels of PSP toxins, probably related to the presence of high levels of naked dinoflagellate cells *(Gymnodinium catenatum*) in seawater samples. PSP toxins were detected in different mollusc species *(Venus verrucosa, Venerupis rhomboides, Callista chione, Acanthocardia tuberculatum)* from the affected areas in concentrations higher than 80 σg STX eq/100 g meat, which caused the collection and sale of molluscs to be banned by the regional health authorities. This incident affected the southern Mediterranean coast of Spain between Malaga and Algeciras, a span of approximately 150 km, probably reaching the coast of Morocco, which would explain the toxicity found in *A. tuberculatum* imported from Morocco in February 1989 (Mons *et al*., 1998*).*

During April and May 1995, the water in the harbour of Palma de Mallorca (Balearic Islands) appeared abnormally reddish brown as a result of the proliferation of the toxic dinoflagellate *Alexandrium minutum*, reaching concentrations up to $45x10^6$ cells/L. Mouse bioassays in natural populations of bivalve molluscs (*Mytilus galloprovincialis, Chamelea gallina* and *Lithopaga lithopaga*) produced positive results with a maximum of 1 170 STX eq/100 g tissue. The red tide recurred in the same area of the harbour in April 1996, although it lasted only ten days, and during three weeks in February 1997 (Forteza *et al*., 1998).

In October and November 1994, a PSP outbreak was observed on the Atlantic shores of Spain after a bloom of *G. catenatum* (Tahri Joutei, 1998). During 2002, there were short toxic events related to the presence of PSP toxins in Galicia (northwest Spain) due to the presence of *A. minutum* and A. *catenella.* In Andalusia, production areas, especially for *Acanthocardia tuberculata* and scallops, were closed due to the presence of *G. catenatum* (EU-NRL, 2002).

Sweden

Contamination with PSP in mussel farming in Sweden occurs at the end of spring and beginning of summer. PSP toxins have been detected in mollusc meat between 1985 and 1988, with the highest level of toxicity being reached in 1986 and 1987, i.e. 1 000 MU/100g meat. The species involved was *A. excavatum* (Mons *et al*., 1998). Samples were sent to Norway when performance of the bioassay was necessary, yielding high levels (approximately 300 µg STX eq/100 g) of PSP toxins in May 1997 (EU-NRL, 1998).

The United Kingdom of Great Britain and Northern Ireland

The first cases of PSP intoxication occurred in 1968 after consumption of local mussels (*Mytilus edulis*) containing 600-6 000 σg STX eq/kg from the northeastern coast of England; 78 people were admitted to hospital, however there were no deaths (IPCS, 1984). From then on, PSP has been detected very often with levels below 400 MU/100 g shellfish in 1972 and 1973 only. In 1977, toxicity reached 1 792 MU/100 g and from 1978 to 1981 PSP events were annual occurrences on the northeast coast spreading towards Scotland. However, during this entire period, the monitoring network prevented human intoxication. In 1990, the first cases of PSP were recorded along the west coast of Scotland with maximum toxin concentrations in the order of 16480 MU/100 g in mussels and scallops (Mons *et al.*, 1993).

In 2000/2001, low levels of PSP toxins were detected in some areas of England and Wales (EU-NRL, 2001). In Scotland, PSP toxins were detected from 2000 to 2001 in early May in mussels from aquaculture sites along the west coast, and in scallop gonad tissue from scallop fishing grounds in Orkney. Levels in scallop tissue rose to 158 µg STX eq/100 g by mid-May and restrictions on fishing activity were imposed. By the end of May, aquaculture sites in the Shetlands and scallop grounds on the east coast were also affected. Levels in mussels from the Shetlands reached 211 µg STX eq/100 g, in Orkney 2999 µg STX eq/100g, and on the west coast 220 µg STX eq/100 g. Restrictions on harvesting mussels from aquaculture sites were subsequently imposed. By mid-July, toxins were no longer detectable in mussels, although toxins in scallops and in scallop gonad tissue were still detectable in mid-October. In some areas, toxins in scallop tissues remained above 80 µg STX eq/100 g and fishing restrictions continued (EU-NRL, 2001).

In the period from 1 April 2002 to 31 March 2003, shellfish from 76 primary inshore production areas, 36 secondary areas and offshore fishing areas in Scotland were examined. A total of 5 409 mollusc samples were analysed. Out of all these samples, 1 690 were analysed for PSP. Twenty-one samples were positive for PSP (Anonymous, 2003c). In August 2001, the United Kingdom Food Standards Agency announced a ban on scallop fishing in the sea adjacent to Northern Ireland after the detection of PSP toxins in scallops (Anonymous, 2001a).

2.7.3 Africa

Morocco

In Atlantic Moroccan waters, *G. catenatum* has sometimes appeared in late summer and early autumn. The most important event was the bloom of October to November 1994, associated with a PSP outbreak that was also observed on the Spanish Atlantic shores and Portuguese waters in September and October 1994, respectively (Tahri Joutei, 1998). PSP toxin analysis in bivalve molluscs was carried out from 1994 to 1996. Along the Atlantic coast, PSP levels in *Mytilus galloprovincialis* in November 1994 showed a maximum of about 6 000 µg STX eq/100 g meat (Taleb *et al*., 1998).

Along the Mediterranean coast, *A. tuberculatum* was found to contain high levels of PSP toxins during all years in which there was monitoring (up to approximately 800 µg STX eq/100 g meat). However, other species, i.e. *Venus verrucosa*, *Callista chiona* and *Donax trunculus*, from the same site were only temporarily contaminated, generally from January to March in 1995, and PSP levels never exceeded 250 µg STX eq/100 g meat (Taleb *et al*., 1998).

South Africa

In 1969 and 1979, 6 and 17 cases of PSP, respectively, were reported after consumption of local black mussels (*Chloromytilus meridionalis*) containing up to 84 000 σg STX eq/kg. Two fatalities occurred (IPCS, 1984). Along the South African coast, *Gonyaulax catenella* is regularly present on the west coast and has caused several human deaths (Collins, 2001).

In March 1994 *A. tamarensis* was present in a bloom and tests revealed that mussels and oysters contained PSP toxins beyond the safe limit of toxicity (Anonymous, 2003a). Pitcher *et al*. (2001) reported the presence of PSP toxins in cultured abalone *Haliotis midae* on two West Coast farms in April 1999. Analysis of wild animals from the west coast also revealed the accumulation of PSP toxins in these gastropods. Toxin content (AOAC mouse bioassay) varied from non detectable to 1609 µg STX eq/100 g. The dinoflagellate *A. catenella* was the probable cause of the abalone toxicity.

Tunisia

More than 700 tonnes of cultured sea bass and sea bream and several species of wild fish were found dead in the lagoons of Burger and Gar el Melh. Mortality was probably caused by PSP toxins producing *Alexandrium minutum* and *A. tamarensis* and by a hemolytic substance producing *Girodinium aureolum* (Rhomdane *et al*., 1998).

2.7.4 North America

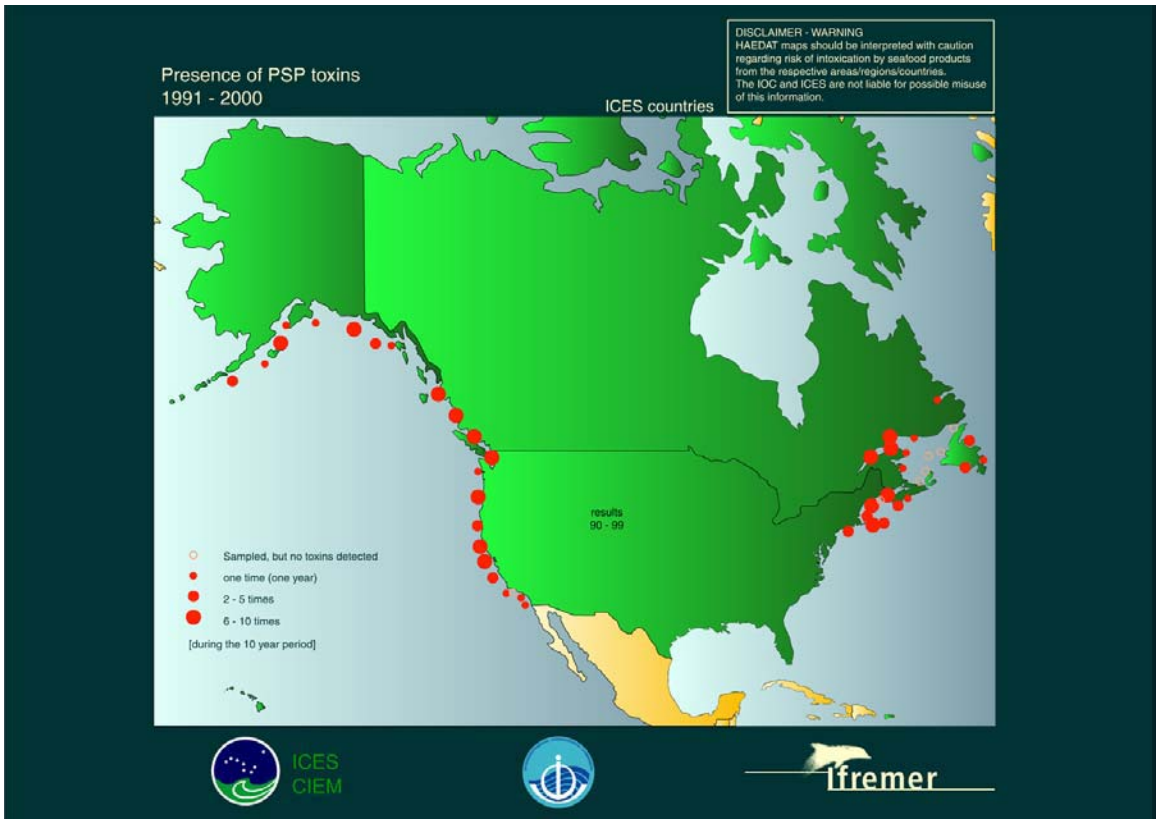
The presence of PSP toxins in coastal waters of ICES countries in North America from 1991 to 2000 is illustrated in Figure 2.3.

Canada

PSP has been documented in British Columbia since 1793 when four crewmen with Captain Vancouver's expedition became sick and one died shortly after eating shellfish from the coastal waters of British Columbia. PSP has been documented in the St. Lawrence Estuary (Quebec) and the Bay of Fundy (between New Brunswick and Nova Scotia) since the 1880s (Todd, 1997). In 1978 and 1982, two and five cases of PSP, respectively, were reported after eating local mussels or clams containing 430 000 and 21 000 σg STX eq/kg, respectively. One death occurred (IPCS, 1984). Overall, from 1880 to 1995, 106 PSP incidents involving 538 cases and 32 deaths have been documented. The largest concentration of illnesses occurred between 30 April and 18 August 1974 in the St. Lawrence Estuary demonstrating toxin levels up to 4 900 µg/100 g and involving

43 cases. The butter clam *Saxidomus giganteus* on the west coast can remain toxic throughout the year (Todd, 1997). In May 1999, two employees of a salmon farm in Herbert Inlet, British Columbia showed symptoms of PSP after eating wild scallops at the farm site (Anonymous, 1999b).

Figure 2.3 Occurrence of PSP toxins in coastal waters of North American ICES countries from 1991 to 2000



Source: http://www.ifremer.fr/envlit/documentation/dossiers/ciem/aindex.htm

The United States of America

East Coast

In the New England region, the first documented case of PSP was in the far eastern sections of Maine near the Canadian border in 1958. In 1972, a massive visible red tide of *A. tamarensis* stretched from southern Maine through New Hampshire and into Massachusetts, causing shellfish toxicity in southern areas for the first time. Thirty-three people were affected, however no one died. Low levels of toxicity were occasionally reported in southern Maine, west of Penobscot Bay prior to 1972, but present-day outbreaks in this area are far more numerous and involve much higher levels of toxicity. Virtually every year since the 1972 outbreak in Maine, New Hampshire and Massachusetts have experienced PSP outbreaks, a direct result of *Alexandrium* cysts being retained in southern waters once introduced there by massive blooms. In 1979, mussel beds were closed in Narragansett Bay, Rhode Island, when PSP was detected but no causative organism was ever identified. In subsequent years, PSP toxicity and *A. tamarensis* cells and cysts were documented in small embayments in Connecticut and Long Island. Cysts and motile cells of *A. tamarensis* have also been reported as far as south as New Jersey but no PSP toxicity has ever been detected that far south. Recent history suggests a gradual, southward dispersal of toxic *Alexandrium* species in the northeast of the United States over the last several decades. This is

certainly true with respect to the effects of the 1972 bloom, but some of the spreading can also be attributed to the detection of indigenous, low-toxicity populations in southern waters (Anderson, 1997). In 1980, 51 people were reported to be affected by PSP after consuming local mussels and oysters containing 3 000 to 40 000 σg STX eq/kg, however no one died (IPCS, 1984).

In June 1990, the Massachusetts Department of Public Health was notified that food-borne illnesses had occurred in six fishermen aboard a fishing boat in the Georges Bank area off the Nantucket coast. Onset of illness occurred after the men had eaten cooked blue mussels (*Mytilus edulis*) harvested in deep water about 115 miles from the island of Nantucket. Subsequently, 244 000 µg STX eq/g were detected in raw mussels and 4 280 µg STX eq/g in cooked mussels (Anonymous, 1991).

Connecticut, Long Island (New York) and New Jersey occasionally experience PSP toxins (or *Alexandrium*) at low levels but these areas seem to define the southern extreme of this organism's geographic distribution. The offshore waters of George's Bank experienced a serious PSP outbreak several years ago, leading to the extended closure of the surfclam fisheries and the demise of a fledgling roe-on scallop fishery (Boesch *et al*., 1997).

Since 1 January 2002, 10 cases of PSP were reported after eating pufferfish caught in waters near Titusville, Florida (Anonymous, 2002a).

West Coast

In Alaska, episodes of PSP have occurred for centuries – the first cases were reported in 1799. Between 1973 and 1994, 66 outbreaks of PSP occurred in Alaska and caused illnesses among 143 people. Eight persons had paralysis of a limb, eight required mechanical ventilation and two died. Most outbreaks occurred during late spring and summer (May to June), on Kodiak Island (the southern edge of the eastern half of the Aleutian Islands) and in southeastern Alaska (Gessner, 1996). During May and June 1994, five outbreaks of PSP involving 12 persons occurred, four who of whom required mechanical ventilation. One patient died. Most people had eaten mussels (*Mytilus edulis* or *Mytilus californianus*) (Gessner and Schloss, 1996; Gessner *et al*., 1997). In April 1995, August 1997 and December 1997, outbreaks of PSP were reported involving three, five and three patients respectively (Anonymous, 1995; 1997a; 1997b).

PSP has a long history in northwestern waters. Several PSP deaths occurred in 1942, which prompted the annual closure of the Strait of Juan de Fuca and the coast. By the 1970s, closures were occurring in the San Juans and Bellingham. In September 1978, heavy rains followed by warm "Indian Summer" weather produced a number of PSP illnesses from Sarragoto Passage to as far south as Vashon Island. Recurring outbreaks of *Alexandrium* occurred in the Puget Sound region of Washington in the late 1970s. In 1988, excessive PSP levels were detected in shellfish from Carr and Case Inlets in Puget Sound. (Boesch *et al.*, 1997 in Determan, 1998). In Puget Sound basin the moonsnails (*Polinices lewissi*), a predatory gastropod, collected from Agate Passage, contained an average of 145 µg STX eq/100 g viscera in September 1994. In October 1994, blue mussels (*M. edulis*) from Mystery Bay contained 652 µg STX eq/100 g whole body (Wekell *et al*., 1996). PSP is also a recurrent annual problem along the coast of Northern California and Oregon (Boesch *et al*., 1997).

2.7.5 Central and South America

Argentina

In the southern part of Argentina, PSP had been associated with blooms of *A. catenella* in the Argentine sector of the Beagle Channel and with blooms of *A. tamarensis* in the southern Atlantic coast to Uruguyan shores (Compagnon *et al*., 1998). The first recorded toxic bloom of *A. tamarensis* was at the Valdés Peninsula in 1980. Thereafter, a dramatic episode occurred in which two members of the crew of a ship died after eating contaminated mussels (Ferrari, 2001). Since that time the phenomenon has occurred periodically in the spring and summer seasons.

During an *A. tamarensis* bloom in 1993, only anchovy (*Engraulis anchoita*) obtained near Mar del Plata showed toxicity although the *A. tamarensis* was distributed over a broad area. PSP toxin levels in anchovy were 101 µg STX eq/100 g wet weight of viscera (by mouse bioassay), but the toxins did not accumulate to detectable levels in muscle tissue (Montoya *et al*., 1998).

During a bloom of *A. catenella* in the northeastern shore of Beagle Channel in the summer of 1991/1992, mussels (*Mitylus chilensis*) collected on 20 January 1993 contained 127 200 σg STX eq/100 g (Compagnon *et al*., 1998). In spring 1993, massive mortality of the mackerel *Scomber japonicus* in a restricted area of the Buenos Aires shelf known as Rincón, appeared to be caused by the incorporation of PSP toxins (Montoya *et al*., 1998). During November 1995 to May 1996, *A. tamarensis* cells and mussels (*Aulacomya ater*) obtained from eight sampling stations along the Valdés Peninsula contained up to a maximum of 490 fmol PSP toxins/cell and 631 σg STX eq/100 g respectively (Andrinolo *et al*., 1999b).

Brazil

Several outbreaks of *A. tamarensis* have been recorded in Brazilian waters. In addition, other PSP toxin producers were identified namely *A. catenella* and *G. catenatum*. Every year *A. catenella* blooms in the Tierra del Fuego fjords and shellfish toxicity has been registered since 1992. In 1998 *G. catenatum*, was observed along the coast of Santa Catarina State (Ferrari, 2001).

Chile

Reports of PSP due to mussel consumption in native people in the proximity of Ushuaia date from as early as 1886. *A. catenella* is mainly found in the three most southern regions of Chile. In two of these regions, 329 cases of PSP were reported from October 1972 through January 1997. Twenty six people died from PSP during these years. The bivalve species involved were *Aulacomya ater* and *Mytilus chilensis*. PSP toxin content varied from 1 555 to 96 000 MU/100 g (Lagos, 1998). Compagnon *et al.* (1998) reported a bloom of *A. catenella* in the early autumn of 1996 in the southern part of Chile resulting in PSP levels up to 113 259 σg STX eq/100 g of whole shellfish in the bivalve *A. ater* 25 days after the peak of the bloom (3.1×10^4 cells/L). High but diminishing levels persisted for six months after the peak of the bloom. High levels of PSP toxins were also detected in two carnivorous gastropod species *Concholepas concholepas* (9 164 and 737 σg STX eq/100 g in the digestive gland and foot muscle respectively) and *Argobuccinum ranelliformes* (14 057 and 31 σg STX eq/100 g in digestive gland and foot muscle respectively). The highest levels in carnivorous gastropods were reached five months after the bloom.

Up until 2001, PSP and DSP toxins have had a most severe public health and economic impact on Chile. As a consequence, all natural fish beds from 44 ∀SL southwards were closed and nationwide monitoring programmes were maintained (Suárez-Isla, 2001). In March 2002, one death and at least eight poisoning cases were reported after consumption of shellfish from the southern region of Chiloé. Catching and trading of shellfish around the whole island of Chiloé was prohibited and the prohibition included also the Ancud community, 100 km further north on Chloé (Carvajal, 2002).

Guatemala

In 1987, an outbreak of PSP involving 187 cases and 26 deaths was reported after consumption of clam soup. The fatalities were the highest among young children (50 percent) compared with seven percent in adults (Rodrigue *et al.*, 1990).

Mexico

In 1979, 20 cases of PSP were reported linked to consumption of local mussels and three people died (IPCS, 1984). A two-year study of the Tamiahua Lagoon from 1984 to 1986 revealed permanent blooms of *Pyrodinium bahamense* in estuaries, in areas with banks of *Crassostrea virginica*. These blooms were displaced hydrodynamically during the windy season from November to March. High densities of *P. bahamense* were then seen in mangrove areas with slow water currents. This phenomenon also occurred in other coastal lagoons from the Gulf of Mexico and the coastal lagoons of the Sian Ka'an Biosphere Reserve in the Mexican Sea (Gómez-Aguirre and Licea, 1998).

In November 1989, a coastal toxic event (from Chiapas to Oaxaca) was caused by *P. bahamanse* and contaminated oysters and mussels, which were implicated in 99 poisonings and three human deaths by ingestion (Orellana-Cepeda *et al.*, 1998). During May 1992, an extremely high PSP toxicity value of 23 000 MU/100 g was recorded in a single fanshell (*Pinna rugosa*) at Bahía Concepción (Peninsula of Baja California). No cases of human poisoning have been reported (Ochoa *et al.*, 1997).

During March 1993 and April 1994, densities of the dinoflagellate *A. catenella* reached 14 000 cells/litre in Bahía Concepción (Gulf of California). Following this, high PSP levels (up to 2 400 MU/100 g) were found in whole shellfish, mainly *Argopecten circularis*. The area was closed for shellfish harvesting. No human intoxications were reported (Lechuga-Devéze and Morquecho-Escamilla, 1998).

From 1995 to 1996, an outbreak of *P. bahamense* off Michoacán and Guerrero caused the death of six people and many illnesses. In the winter of 1995, high levels of PSP toxins were detected by mouse bioassay in oysters (*Ostrea iridiscens* 608-6 337 µg STX eq/100 g meat) and clams (*Donax* sp. 520 µg STX eq/100 g meat) in southwest Mexico. In November 1996, an exceptional bloom of *P. bahamense* occurred on a small area of the Pacific Coast of Mexico (Orellana-Cepeda *et al.*, 1998).

PSP caused 72 percent of the toxic events which occurred during the last decade of the twentieth century. Three of these PSP events represented 87 percent of the poisoning cases (460 individuals poisoned with 32 deaths, including the 1987 Guatemala incident). During the PSP events in Mexico, large numbers of marine animals such as fish and turtles were killed. At first, only STX produced by *G. catenatum* was reported as the responsible toxin. Since the 1987 event on the Guatemalan coast, *G. bahamense* dominated the toxic red tides on the Southeast Pacific coasts with dcSTX and STX as the principal components and low amounts of GNTXs. A different toxin profile was seen in Baja California Peninsula suggesting that *A. catenella* is the responsible species or the recently observed *A. tamiyavanichi* (Sierra-Beltrán *et al.*, 1998).

Trinidad

PSP was recorded for the first time in waters of Trinidad in 1994. Both the mouse bioassay and the receptor binding assay demonstrated the presence of PSP toxins in a meat extract of the mussel *Perna viridis*. The quantity of STX equivalents was 28 σg/100 g of meat, which is considerably lower than the 80 σg/100 g of meat considered to be unfit for human consumption. No human intoxications were reported (Ammons *et al.*, 2001)

Uruguay

Coinciding with PSP incidents in Brazil in 1980, 60 persons were affected with neurotoxic symptoms in Uruguay. Although PSP toxins reached high levels, the algal species was never identified. During the summer of 1991, PSP outbreaks were associated with *G. catenatum*. In coastal sediments high densities of this latter algal species were reported (Ferrari, 2001).

Venezuela

In 1979 and 1981, 171 and 9 people respectively were reported to be affected by PSP after consuming local mussels (*Perma perma*) containing 790 to 33 000 σg STX eq/kg. In total, 11 people died (IPCS, 1984).

2.7.6 Asia

China

The first published cases of PSP in China were in Zhejiang Province where 40 separate episodes resulted in 23 fatalities and 423 nonfatal intoxications between 1967 and 1979. The vector was the marine snail *Nassarius succinstus* (Zhou *et al*., 1999). In November 1986, PSP was reported in Dongshan (in the south of Fujian Province) with one fatality and 136 hospitalizations. The vector was the clam *Ruditapes phillipenensis* (Zhou *et al*., 1999). In February 1989, five persons showed PSP symptoms after eating the clam *Pinna pectinata*. In November 1989, four fishermen developed PSP symptoms after eating the snail *N. succinstus* (collected from Fuding in the north of Fujian Province) and one person died. The illnesses occurred in Huizhou but the shellfish probably originated from Daya Bay. In March 1991, there were two human fatalities and four cases of illness after eating the mussel *Perna viridis* collected from Daya Bay (Zhou *et al*., 1999).

A survey of the Guandong Province from 1990 to 1992 found PSP toxins in 33 edible marine organisms at levels up to 1 000 σg STX eq/100 g meat. In June 1994, five illnesses and one death were reported in Zhejiang Province following consumption of the snail *N. succinstus*.

Since shellfish monitoring is minimal along the Chinese coast, it seems likely that the above episodes underestimate the true extent of the PSP problem. From a few isolated algal bloom reports in the early 1970s, the problem has progressed to the stage where 40 to 50 red tides are now reported every year. Only a few are toxic but the trend is ominous and seems to be linked to increases in pollution in China's coastal waters during the same interval. The rapid expansion in shellfish aquaculture and the documented presence of PSP-causing algae in Chinese coastal waters suggest that shellfish toxicity will continue to be a major problem for many years (Anderson *et al*., 1996).

Sediment samples collected from 49 stations at 14 locations along the coasts of the South and East China Seas were checked for living dinoflagellate resting cysts. Cysts from *G. catenatum* were found in Dapeng Bay only but cysts from *A. tamarensis* were found in eight relatively contiguous locations from the mouth of the Pearl River through Guangdong and Fujian Provinces to Taizhou Bay in Zhejiang Province. The distribution is generally consistent with the region in which PSP events have been recorded (Qi *et al*., 1996). During 1996 and 1997, only four samples from three sampling sites, out of a total of 91 samples, contained PSP toxins. The highest level was 79 µg STX eq/100 g which was below the regulatory level of 80 µg/100 g (Zhou al., 1999).

Timor-Leste

One man died within several hours after ingestion of a specimen of the crab *Zosimus aeneus* in Timor-Leste. The total toxin concentration of the uneaten part of the crab was 163 µg STX eq/100 g tissue (GNTX2, GNTX3 and STX). The dose consumed by the victim was

calculated to be between 1 and 2 µg STX eq/kg bw. The victim's meal did not consist solely of the toxic crab and the possibility of other food items acting in a synergistic manner with the consumed PSP toxins cannot be ruled out (Llewellyn *et al*., 2002).

China, Hong Kong Special Administrative Region

There were three outbreaks of PSP in Hong Kong Special Administrative Region in 1992 but details on the number of patients affected and the clinical presentation are not available. It is likely that most outbreaks are not reported (Chan, 1995). In September 1996, a sample of *Chlamys nobilis* contained 320 σg STX eq/100 g soft tissue. Within 40 days after red tide events in March to April 1998, levels of PSP toxins up to 79 σg STX eq/100 g were found in *Perna viridis*, however, no cases of human poisoning were reported (Zhou *et al*., 1999).

India

In 1981, 98 cases of PSP were reported after consumption of local mussels. One fatality occurred (IPCS, 1984).

Japan

The first infestation of shellfish with PSP in Hiroshima Bay was reported in 1992 (Hamasaki *et al*., 1998). Industrially important bivalves such as scallop and oysters are frequently contaminated with PSP due to toxic dinoflagellates mainly such as *A. catenella*, *A. tamarense* and *G. catenatum* (Noguchi, 2003).

From July 1995 through October 1996, 6 out of 30 species of shellfish collected at Fukue Island, Nagasaki Prefecture were found to contain PSP toxins: *Pecten albicans* (scallop), *Chlamys farreri* (scallop), *Septifer virgatus* (mussel), *Pinna bicolor, Arca boucardi* and *Pseudochama retrove.* The two species of scallops (*P. albicans* and *C. farreri*) contained amounts above the regulatory limit of 400 MU/100 g. Only the digestive gland was toxic. The highest amount (13 380 MU/g) was found in *P. albicans*. In addition, *P. bicolor* contained an amount above the regulatory limit, namely 490 MU/100 g. The lowest values (below the regulatory limit) were found in *P. retrove* (320 MU/100 g), *A. boucardi* (230 MU/100 g) and *S. virgatus* (200 MU/100 g). Toxin profile in *P. albicans* featured the dominant presence of GNTXs in 1995. In 1996, however, low-toxicity components such as the C group were major, as was the case in the profile of *C. farreri*. The mossworm contained a low level of PSP toxins whose major components were dcGNTX2 and GNTX2, along with dcGNTX3 and GNTX3, differing clearly from the profile of its host of *C. farreri* (Takatani *et al*., 1997).

In March 1997, 20 people were poisoned after eating oysters collected at Fukue Island (Takatani *et al*., 1997). A bloom of *A. tamiyavanichii* occurred in Seto Inland Sea in 1999 and 2001 and attacked several oyster farms. On 28 November 2001, PSP toxins in cultured and wild oysters were 17.0 and 3.3 MU/g edible parts respectively (Nishio, 2003).

Malaysia

In 1977, 201 cases of PSP were recorded after eating local clams. Four fatalities occurred (IPCS, 1984). Until 1990, problems related to PSP were relatively simple being confined to the west coast of Sabah in Borneo. The toxin producer was the dinoflagellate *P. bahamense*. In early 1991, PSP occurred for the first time in Peninsula Malaysia when three people became ill after eating farmed mussels from Sebatu in the Straits of Malacca. *A. tamiyanavichi* was eventually confirmed as the toxin producer. In early 2000, PSP toxins and shellfish toxicity were reported for the first time from the east coast of Sabah, however the toxin producer has yet to be identified. In September 2001, six people became ill after eating clams harvested from a coastal lagoon on Kelatan on the east coast of peninsular Malaysia. One of the victims eventually died. High levels

of PSP toxins were detected in the clams by means of the mouse bioassay and receptor binding assay. A high density of *A. tropicale* and an unidentified *Alexandrium* species were found (Usup and Ahmad, 2001).

The Philippines

Marine puffers (*Arothron mappa, A. manillensis, A. nigropunctatus, A. hispidus, A. stellatus, A. reticularis*) collected in 1992 in the Philippine waters contained considerable amounts of PSP toxins (major component STX) besides TTX (Sato *et al*., 2000). In 1992, cultured mussels in the Philippines caused PSP in many people (Durborow, 1999). Between 1983 and 1998, 2 000 cases of PSP occurred with a mortality rate of 5.8 percent (Van Dolah *et al*., 2001).

Taiwan Province of China

In January 1986, two persons died and 30 showed signs of illness after consuming *Soletellina diphos*. In February 1991, eight cases of illness were registered after eating *Soletellina diphos* (Zhou *et al*., 1999). A survey of paralytic toxins in shellfish in the southern part of the island between August 1995 and March 1997 revealed both PSP and TTX in three types of shellfish namely *Niotha clathrata, Natica lineata and Natica vitellus* (Liao and Hwang, 2000). In Taiwan Province of China, PSP is distributed in purple clam, xanthid crabs and gastropods, and the toxic alga *A. minutum* appears from December to March (Hwang, 2003).

Thailand

In 1983, 62 cases of PSP were reported in Thailand after consumption of local mussels (*Mytilus* spp.) and one fatality occurred (IPCS, 1984). A freshwater puffer (*Tetraodon fangi*) poisoning case was reported in 1990. The puffer species appeared to contain STX. Tetrodotoxin was not detected (Sato *et al*., 1997).

2.7.7 Oceania

Australia

Monitoring from 1987 to 1997 in Port Philip Bay and Western Port Bay in Victoria occasionally resulted in very high levels of PSP toxins (maximum level 10 000 µg STX eq/100 g edible shellfish) (ANZFA, 2001).

New Zealand

An outbreak of shellfish poisoning occurred in New Zealand during January 1993. More than 180 cases were registered along with numerous instances of respiratory irritation from air-borne toxin in sea spray. Initially, the toxicity was characterized as NSP because PSP toxins were not very high in the most commercial shellfish. Subsequently, PSP toxins produced by *A minutum* were found to be responsible for at least part of the toxicity measured in shellfish (pipi, scallop, mussel and tuatua) (MacKenzie *et al*., 1996; Chang *et al*., 1997a).

Over the period January 1993 to July 1996, 0.5 percent of samples of shellfish taken around the coastline of New Zealand on a weekly basis exhibited PSP toxin levels above the regulatory limit during a total of 11 PSP events (maximum level 920 µg STX eq/100 g mussel). During the sampling period, there were no cases of human PSP poisoning (Sim and Wilson, 1997). *G. catenatum* caused extensive closures of North Island and South Island west coast shellfish areas in the summer of 2000 and 2001. The highest PSP level recorded in New Zealand has been 1 007 µg/100 g associated with *A. catenella* in the eastern Bay of Plenty in 1997 (Anonymous, 2003b).

Tasmania

Testing for PSP in mussels, oysters and scallops during September 1994 and July 1996 revealed maximum levels of 18 429, 699 and 83 µg STX eq/100 g edible shellfish, respectively, and average levels of 636, 123 and 60 µg STX eq/100 g edible shellfish, respectively (ANZFA, 2001).

2.8 Regulations and monitoring

2.8.1 Worldwide regulations

Several countries have already established or proposed regulations for PSP. Most regulations are set for PSP toxins as a group. In general, limits have often been set at 400 MU/100 g or 80 µg STX eq/100 g. Some countries indicate specific regulations for one of the PSP toxins, mostly STX. In most cases, existing regulations refer to shellfish. However, some countries mention molluscs generally or bivalves specifically as the types of products for which the maximum permissible levels of PSP toxins are set.

Many countries still use the standard mouse bioassay of the AOAC International as the method of analysis for official purposes. A directive came into force in the European Union in January 1993 stating that the total PSP content in molluscs has to be determined according to the "biological testing method in association, if necessary, with a chemical method for detection of STX. If the results are challenged, the reference method shall be the biological method".

Different concentration units are used to express the tolerance level: mouse units/g (MU/g) and σg/g (incidentally σg/ml). The latter unit seems to be less appropriate in the countries that use the mouse bioassay because they actually test for toxicity in the mouse. Expression of a tolerance level for PSP in µg/g would be valuable if the various PSP toxins exhibit the same toxicity, which is not the case. If the unit µg/g still would be preferred above MU/g, one might consider application of a toxic equivalence factor and expression of the concentration of the various PSP toxins (if these can be selectively measured) in concentration units of STX (Mons *et al*., 1998).

2.8.2 Europe

European Union

In EU Directive 91/492/EEC, a limit for PSP toxins in bivalve molluscs is laid down at 80 σg STX eq/100 g of meat. The official method of analysis is the (mouse) bioassay, if needed in association with a chemical detection method. If the results are challenged, the reference method is the bioassay (EC, 1991a).

Results of studies with *Acanthocardia tuberculatum* (Mediterranean cockle) led the European Commission to permit heating as a means to partially detoxify *A. tuberculatum*. It was allowed to harvest *Acanthocardia tuberculatum* when PSP levels in the edible parts exceed 80 µg STX eq/100 g tissue but are less than 300 µg STX eq/100 g. If analysis of the heated product has shown that STX levels are below the EU legal limit of 80 µg/100 g, this product may be marketed and sold for human consumption (EC, 1996). Most European countries also have monitoring programmes to check for PSP producing organisms.

2.8.3 Africa

Morocco

The limit for STX in molluscs is 80 µg STX eq/100 g. The method of analysis is the mouse bioassay (Fernández, 1998)

2.8.4 North America

Canada

Molluscs should contain less than 80 σg STX eq/100 g. The mouse bioassay is the required method of analysis. Products (soft shell clams and mussels) with levels between 80 and 160 σg STX eq/100 g may be canned (Shumway *et al*., 1995). Butter clams containing 300 to 500 µg STX eq/100g may be marketed after removing the entire siphon. Butter clams containing 80 to 300 µg STX eq/100 g may be marketed after removing the distal half of the siphon (Fernández, 1998).

In the western Bay of Fundy, eastern Canada, phytoplankton samples have been collected since 1988 at four stations. *Alexandrium* blooms occur annually from late May to August with the highest concentrations occurring from 9 to 21 July most years. Future plans include further refining and quality control, and exploring the temporal and spatial variability in the patterns more fully (Martin *et al*., 2001). A monitoring programme for a.o. *Alexandrium* spp. exists. Fishery product harvesting areas are closed when toxin levels in shellfish exceed tolerable limits (Hallegraeff *et al*., 1995).

The United States of America

The limit for PSP toxins in bivalves is set at 80 σg STX eq/100 g. The method of analysis is the mouse bioassay (Shumway *et al*., 1995). Shellfish with PSP levels over 80 µg STX eq/100 g tissue destined for canning or subjected to evisceration may be harvested (Fernández, 2000).

2.8.5 Central and South America

Argentina

The limit for STX in molluscs is 400 MU/ 100 g. The method of analysis is the mouse bioassay. Snails to be canned may contain up to 160 µg STX eq/100 g (Fernández, 1998). Argentina has a national monitoring programme for mussel toxicity in each coastal province involving regional laboratories and one fixed station in Mar del Plata (Ferrari, 2001).

Brazil

Brazil has implemented a pilot monitoring initiative for one year but does not have a national monitoring programme (Ferrari, 2001).

Chile

The limit for STX in molluscs is 80 µg STX eq/100 g. The method of analysis is the mouse bioassay (Fernández, 1998). Up until 2001, PSP and DSP toxins have had a severe public health and economic impact in Chile. Consequently, all natural fish beds from 44 ∀SL southwards were closed and nationwide monitoring programmes maintained (Suárez-Isla, 2001).

Guatemala

The limit for STX in molluscs is 80 µg STX eq/100 g. The method of analysis is the mouse bioassay (Fernández, 1998).

Mexico

In Mexico, a regulatory limit of 30 µg STX eq/100 g is valid. The method of analysis is the mouse bioassay (Aune, 2001).

Panama

The limit for PSP toxins in bivalves is 400 MU/100 g in Panama. The method of analysis is the mouse bioassay (Shumway *et al*., 1995).

Uruguay

The limit for STX in molluscs is 400 MU/100 g in Uruguay. The method of analysis is the mouse bioassay (Fernández, 1998). Uruguay has a national monitoring programme on mussel toxicity and toxic phytoplankton (Ferrari, 2001).

Venezuela

The limit for STX in molluscs is 80 µg STX eq/100 g. The method of analysis is the mouse bioassay (Fernández, 1998)

2.8.6 Asia

China, Hong Kong Special Administrative Region

The limit for PSP toxins in shellfish is 400 MU/100 g. The method of analysis is the mouse bioassay (Shumway *et al*., 1995).

Japan

The limit for PSP toxins in bivalves is 400 MU/100 g. The method of analysis is the mouse bioassay (Shumway *et al*., 1995).

Malaysia

Up until 1990, problems related to PSP incidents were relatively simple, being confined to the west coast of Sabah in Borneo. An effective shellfish toxicity monitoring programme was established by the Malaysian Department of Fisheries, which greatly reduced the occurrences of PSP despite recurring algal blooms. A PSP incident in 1991 on the Malaysian peninsula, linked to the consumption of mussels from Sebatu in the Straits of Malacca, prompted the government to establish an additional shellfish toxicity monitoring facility based at the Fisheries Research Institute in Penang. In September 2001, PSP cases following the consumption of clams from the east coast of the Peninsula Malaysia were reported. Logistical considerations warrant the establishment of a shellfish toxicity monitoring facility on the east coast of Peninsula Malaysia (Usup and Ahmad, 2001).

The Philippines

In the Philippines, a tolerance limit of 40 µg STX eq/100 g is valid (Aune, 2001).

Singapore

The limit for STX in bivalves is 80 σg/100 g. The method of analysis is the mouse bioassay (Shumway *et al*., 1995).

The Republic of Korea

The limit for gonyautoxins in bivalves is 400 MU/100 g. The method of analysis is the mouse bioassay and the LC method (Shumway *et al*., 1995).

2.8.7 Oceania

Australia

The limit in shellfish is 80 σg STX eq/100 g of shellfish meat. The method of analysis is the mouse bioassay (Shumway *et al*., 1995).

New Zealand

The New Zealand Biotoxin Monitoring Programme combines regular shellfish testing and phytoplankton monitoring. The regulatory limit in shellfish is 80 µg STX eq/100 g of shellfish meat (Sim and Wilson, 1997). Shellfish testing currently involves mouse bioassay screen testing with confirmatory testing (Busby and Seamer, 2001).

A new Biotoxin Monitoring Programme providing data that is highly accurate, in a shorter time and without the use of mouse bioassay's, is being developed. This new programme will implement test methods based on LC-MS providing chemical analytical data in place of bioassay screen test results. The development and implementation of new test methods are in discussion including funding, method validation, testing regulations, availability of analytical standards, comparison to existing tests, type of instrumentation and international cooperation (McNabb and Holland, 2001).

3. Diarrhoeic Shellfish Poisoning (DSP)

Diarrhoeic Shellfish Poisoning (DSP) in humans is caused by the ingestion of contaminated bivalves such as mussels, scallops, oysters or clams. The fat soluble DSP toxins accumulate in the fatty tissue of the bivalves. DSP symptoms are diarrhoea, nausea, vomiting and abdominal pain starting 30 minutes to a few hours after ingestion and complete recovery occurs within three days. DSP toxins can be divided into different groups depending on chemical structure. The first group, acidic toxins, includes okadaic acid (OA) and its derivatives named dynophysistoxins (DTXs). The second group, neutral toxins, consists of polyether-lactones of the pectenotoxin group (PTXs). The third group includes a sulphated polyether and its derivatives the yessotoxins (YTXs) (see Figures 3.1 and 3.2).

DSP toxins are produced usually by dinoflagellates that belong to the genera *Dinophysis spp*., however, the dinoflagellate genus *Prorocentrum* has also been found to be a producer of DSP toxins. DSP toxin production may vary considerably among dinoflagellate species and among regional and seasonal morphotypes in one species. The number of dinoflagellate cells per litre of water needed to contaminate shellfish is also variable. The most affected areas seem to be Europe and Japan. DSP incidences, or at least the presence of DSP toxins, appear to be increasing and DSP toxins producing algae and toxic bivalves are frequently reported from new areas.

3.1 Chemical structures and properties

The DSP toxins are all heat-stable polyether and lipophilic compounds isolated from various species of shellfish and dinoflagellates (Draisci *et al*., 1996a) (see Figure 3.1 and 3.2). Although diarrhoea is the most characteristic symptom of intoxication, several other effects may be of relevance and some of the toxins in the DSP complex (PTXs and YTXs) do not yield diarrhoea at all (Van Egmond *et al*., 1993). Re-evaluation of their toxicity will probably lead to these toxins being removed from their classification as DSP toxins (Quilliam, 1998a). The different chemical types of toxins associated with the DSP syndrome comprise:

a) The first group, acidic toxins, includes okadaic acid (OA) and its derivatives named dinophysistoxins (DTXs). OA and its derivatives (DTX1, DTX2 and DTX3) are lipophilic and accumulate in the fatty tissue of shellfish. These compounds are potent phosphatase inhibitors and this property is linked to inflammation of the intestinal tract and diarrhoea in humans (Van Apeldoorn *et al*., 1998; Hallegraeff *et al*., 1995). OA and DTX1 are also tumour promoters in animal test systems (Draisci *et al*., 1996a; Van Egmond *et al*., 1993). DTX1 was first detected in *Dinophysis fortii* in Japan; DTX2 was identified in shellfish in Ireland during a DSP episode (Van Egmond *et al*., 1993). DTX2 was isolated also from a marine phytoplankton biomass mainly consisting of *Dinophysis acuta* (James *et al*., 1999). A new isomer of DTX2, named DTX2B, was isolated and identified in Irish mussel extracts (James *et al*., 1997). DTX3 originally described a group of DSP toxin derivatives in which saturated or unsaturated fatty acyl groups are attached to the 7-OH group of DTX1. More recently it has been shown that any of the parent toxins, OA, DTX1 and DTX2, can be acylated with a range of saturated and unsaturated fatty acids from C_{14} to C_{18} (Hallegraeff *et al*., 1995; Wright, 1995). In a report of an EU meeting it was stated that chain length of the fatty acid can vary from C_{14} to C_{22} and that the number of unsaturation is varying from 0 to 6. The most predominantly fatty acid in DTX3 was palmitoyl acid (EU/SANCO, 2001). These acylated compounds also possess toxic activity. Since these compounds have only been detected in the digestive gland of contaminated shellfish, it has been suggested that they are probably metabolic products and not *de novo* products of toxin producing micro algae (Wright, 1995). Suzuki *et al*. (1999) demonstrated the transformation of DTX1 to 7-*O*-acyl-DTX1 (DTX3) in the scallop *Patinopecten yessoensis*.

The ester bond in the acylated compounds can be hydrolyzed by heating in 0.5 M NaOH/90 percent methanol solution at 75 ºC for 40 minutes. The ester bond in DTX3 was also easily hydrolyzed by lipase and cholesterol esterase (EU/SANCO, 2001).

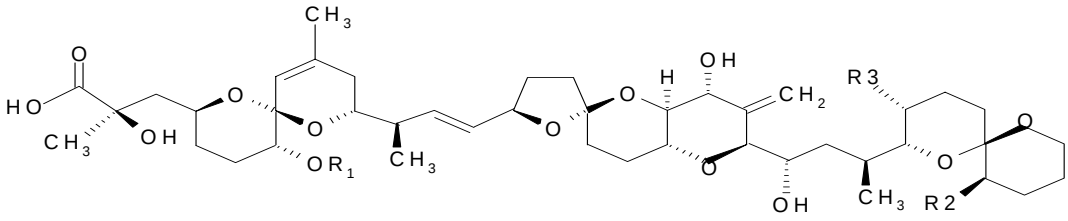
Two naturally occurring ester derivatives called diol esters were isolated from some *Prorocentrum* species. These diol esters did not inhibit phosphatase *in vitro*. However, it should be noted that these allylic diol esters may be somewhat labile and could be hydrolysed to yield the active parent DSP toxin (Hallegraeff *et al*., 1995). Draisci *et al* (1998) reported the detection of another OA isomer and called it DTX2C. The structure of DTX2C is not yet elucidated. The compound was isolated from *D. acuta* collected in Irish waters.

b) The second group, neutral toxins, consists of polyether-lactones of the pectenotoxin group (PTXs). Ten PTXs have been isolated until now and six out of these have been chemically identified; PTX1, -2, -3, -4, -6 and -7. Since PTX2 (PTX2,CH_3) is found in phytoplankton only (*D. fortii* in Japan and Europe) and never in shellfish, it is suggested that an oxidation occurs in the hepatopancreas of shellfish producing other PTXs (PTX1, CH_2OH; PTX3, CHO; PTX6, COOH) (Draisci *et al.*, 1996a; Yasumoto *et al*., 2001 and cited from Van Apeldoorn *et al*., 1998). Suzuki *et al*. (1998) demonstrated oxidation of PTX2 to PTX6 in scallops (*Patinopecten yessoensis*). Sasaki *et al*. (1998) identified PTX4 and PTX7 as spiroketal isomers of PTX1 and PTX6, namely *epi*-PTX1 and *epi*-PTX6, respectively. Two new artefacts, PTX8 and PTX9, were also isolated but their structures are not yet elucidated. Daiguji *et al*. (1998) isolated two new pectenotoxins from the greenshell mussel *Perna canalicus* from New Zealand and from *D. acuta* from Ireland and elucidated the structures as pectenotoxin-2-seco acid (PTX2SA) and 7-*epi*-pectenotoxin-2 seco acid (7-*epi*-PTX2SA), respectively. Suzuki *et al*. (2001) demonstrated that PTX2SA and 7-*epi*-PTX2SA (the most important PTX homologues in the New Zealand mussel *Perna canaliculus*) are converted from PTX2 by these mussels. This transformation is also expected to occur in the blue mussel *Mytilus galloprovincialis* as PTX2SA is also the predominant PTX homologue in this mussel species.

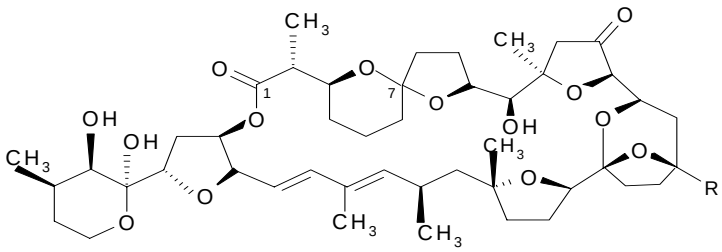
c) The third group includes a sulphated compound called yessotoxin (YTX), a brevetoxin-type polyether, and its derivative 45-hydroxyyessotoxin (45-OH-YTX) (Draisci *et al*., 1996a; Van Egmond *et al*., 1993). Yessotoxin was first isolated from the digestive organs from scallops (*Patinopecten yessoensis*) in Japan (Ciminiello *et al*., 1999) and is believed to be produced by microalgae. The yessotoxins do not cause diarrhoea. Yessotoxin attacks the cardiac muscle in mice after i.p. injection, while desulphated yessotoxin damages the liver (Van Egmond *et al*., 1993). In the digestive gland of Adriatic mussels (*Mytilus galloprovincialis*) besides yessotoxin, two new analogues of yessotoxin, homoyessotoxin and 45-hydroxyhomoyessotoxin were identified by Ciminiello *et al*. (1997; 1999). Tubaro *et al*. (1998) also detected homoyessotoxin in *M. galloprovincialis* from the Adriatic sea during a bloom of *Gonyaulax polyhedra* (=*Lingulodinium polyedrum*). Satake *et al*. (1997) and Satake *et al*. (1999) isolated YTX and 45,46,47-trinoryessotoxin from cultured cells of the marine dinoflagellate *Protoceratium reticulatum*. The production of yessotoxins by *P. reticulatum* differed from strain to strain. Ciminiello *et al.* (1998) detected again a new analogue of YTX, adriatoxin (ATX), in the digestive glands of DSP infested Adriatic mussels collected in 1997 along the Italian coast (Emilia Romagna). In addition, four further analogues of yessotoxin, carboxyyessotoxin (COOH group on C_{44} of YTX instead of double bond), carboxyhomoyessotoxin (COOH group on C_{44} of homoYTX instead of double bond) (Ciminiello *et al*., 2000a; 2000b), 42,43,44,45,46,47,55-heptanor-41-oxo YTX and 42,43,44,45,46,47,55-heptanor-41-oxohomo YTX (Ciminiello *et al*., 2001; 2002) in Adriatic mussels (*M. galloprovincialis*) were identified.

d) Unexplained human intoxication, with DSP symptoms, following the consumption of mussels from Killary, Ireland in 1995 was resolved by the isolation of a new toxin ($C_{47}H_{71}NO_{12}$), tentatively named Killary Toxin-3 or KT3 (Satake *et al*., 1998a). This toxin was later called azaspiracid (see Chapter 6).

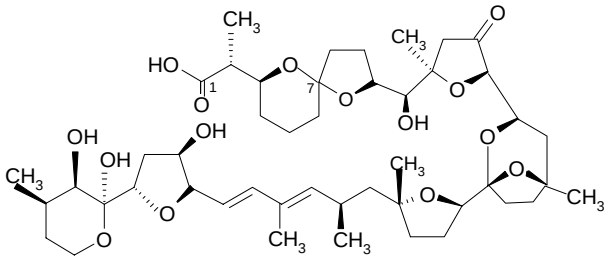
Figure 3.1 Chemical structures of okadaic acid, dinophysistoxins and pectenotoxins



	R1	R2	R3
okadaic acid (OA)	H	H	CH_3
dinophysistoxin-1 (DTX1)	H	CH_3	CH_3
dinophysistoxin-2 (DTX2)	H	CH_3	H
dinophysistoxin-3 (DTX3)	acyl	CH_3	CH_3



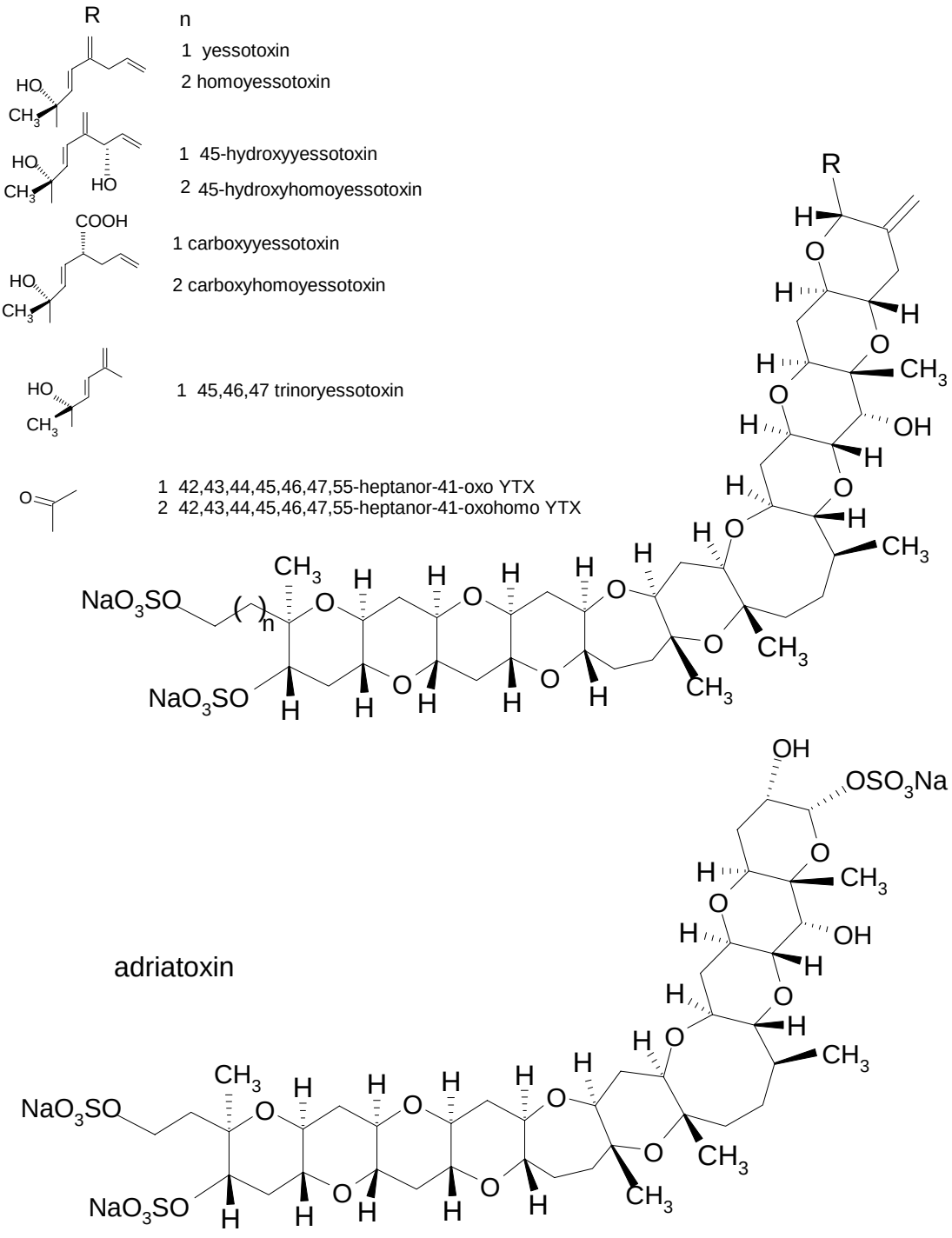
	R	C-7
pectenotoxin-1 (PTX1)	CH_2OH	*R*
pectenotoxin-2 (PTX2)	CH_3	*R*
pectenotoxin-3 (PTX3)	CHO	*R*
pectenotoxin-4 (PTX4)	CH_2OH	*S*
pectenotoxin-6 (PTX6)	COOH	*R*
pectenotoxin-7 (PTX7)	COOH	*S*



	C-7
pectenotoxin-2 seco acid (PTX2SA)	*R*
7-epi-PTX2SA	*S*

Source: Yasumoto *et al*., 2001

Figure 3.2 Chemical structures of yessotoxins and adriatoxin



Source: Ciminiello *et al*., 1998; 2002 and Yasumoto *et al*., 2001

3.2 Methods of analysis

3.2.1 Bioassays

in vivo assays

mouse bioassay

The most commonly used assay method is the mouse bioassay developed by the Japanese Ministry of Health and Welfare (Yasumoto *et al*., 1978; Japanese Ministry of Health and Welfare, 1981). Toxins are extracted from shellfish tissue using acetone and after evaporation the residue is dissolved in a small volume of 1% Tween 60. The extract is injected intraperitoneally into mice with a body weight of about 20 g and the survival is monitored from 24 to 48 hours. One mouse unit (MU) is defined as the minimum quantity of toxin needed to kill a mouse within 24 hours. The toxicity of the sample (MU/g whole tissue) is determined from the smallest dose at which two mice or more in a group of three die within 24 hours. In many countries, the regulatory level is set at 0.05 MU/g whole tissue. In this mouse assay, all DSP components are likely to be detected including those DSP toxins which do not cause diarrhoea (PTXs and YTXs) and have an unknown toxicity for humans. Other unknown toxin groups exhibiting ichthyotoxic and hemolytic properties may also cause mortality of mice in this bioassay.

Therefore major disadvantages of this assay are the lack of specificity (no differentiation between the various components of DSP toxins), subjectivity of death time of the animals, and the maintaining and killing of laboratory animals. In addition, this assay is time consuming and expensive, may give false positives because of interferences by other lipids (notably free fatty acids have shown to be very toxic to mice (Suzuki *et al*., 1996) and shows variable results between whole body and hepatopancreas extracts (Botana *et al*., 1996 and Van Egmond *et al*., 1993).

The problems observed with the original mouse bioassay of Yasumoto *et al*. (1978) have led to several modifications (Yasumoto *et al*., 1984a; Lee *et al*., 1987; Marcaillou-Le Baut *et al*., 1990). This, in turn, has led to a situation where different countries use different variants of the mouse assay, which calls for harmonization. In an attempt to standardize the methodology of the mouse bioassay, the EU has included directions on how to perform this assay, in its new directive on toxins of the DSP complex (EU, 2002a). The EU's Community Reference Laboratory on Marine Biotoxins has conducted an intercalibration exercise that showed reasonable agreement between EU National Reference Laboratories, when they applied the mouse bioassay on unknown shellfish extracts (CRL, 2001).

Fernández *et al*. (1996) warned that some bioassay procedures involve hexane washing steps to avoid false positive results when free fatty acids are present. The hexane washing step should be reconsidered taking into account the possible losses of low-polar DSP toxins, which may be solubilized in the hexane layer. This step must be avoided when analysing samples of unknown origin and with unknown DSP profiles.

suckling mouse assay

In this procedure, an extract of shellfish tissue is administered intragastrically to four to five day old mice. The degree of fluid accumulation in the gastrointestinal tract is determined after a four hour period by measuring the ratio of intestine mass to that of the remaining body. Ratio values above 0.8 to 0.9 indicate a positive reaction. The assay time is shorter than with the mouse bioassay but quantification of the results is much more difficult. Only diarrhoea causing substances (OA, DTXs) produce positive reactions. Detection limits for OA and DTX1 are 0.05 and 1 MU, respectively (Hallegraeff *et al*., 1995 and Van Egmond *et al*., 1993)

rat bioassay

This assay is based on diarrhoea induction in rats. The (starved) animals are fed with suspect shellfish tissue (mixed into the diet) and observed during 16 hours for signs of diarrhoea, consistency of the faeces and food refusal. The method is at best semi-quantitative and does not detect PTXs and YTXs (Hallegraeff *et al*., 1995 and Van Egmond *et al*., 1993). The test is still used routinely in the Netherlands and it is an officially allowed procedure in EU legislation.

Daphnia magna assay

An assay in *Daphnia magna* was developed and used to analyse OA in mussel extracts. This method was reported to be inexpensive and sensitive. The method can be used in replacement of the mouse bioassay for the screening of okadaic acid and some co-extracting toxins in mussels. The extraction method used allows okadaic acid and DTX1 to be determined. The *Daphnia* bioassay can measure OA levels 10 times below the threshold of the mouse bioassay method (Vernoux *et al*., 1994).

intestinal loop assays

Fluid accumulation in the intestine of intact rabbits and mice has been used to detect DSP. Suspensions of DSP toxins in 1% Tween 60 saline are injected into intestinal loops. A positive result is obtained when the ratio of the volume of accumulated fluid (ml) to the length of the loop (cm) was greater than 1.0 (Hungerford and Wekell, 1992)

The diarrhoeic activity of algal toxins in blue mussels is determined quantitatively in ligated intestinal loops of the rat by Edebo *et al*. (1988a). Hepatopancreas from toxic mussels is disintegrated by freeze-pressing, and the homogenized tissue suspended in an equal amount (w/v) of buffer or in the liquid recovered after steaming. Rapid fluid secretion is seen after injection of the suspension into ligated loops of rat small intestine; maximum is reached within two hours (about 300 mg of weight increase per cm of intestine). Within a range of 50-200 mg/cm dose-response relationship is close to linear. Average deviation from the mean is ∂ 9 mg/cm (SD= ∂ 4.9). Mussels yielding less than 100 mg/cm of weight increase per g hepatopancreas were allowed for human consumption, a quantity agreeing with the allowed level of okadaic acid. The minimum quantity of OA which produces significant secretion in the rat intestinal ligated loop test is approximately 0.5 σg.

in general

Although mammalian bioassays for DSP toxicity are applied worldwide, there are large differences in the performance of, for instance, the mouse bioassay (toxicity criterion: animal death; no consensus on appropriate observation time) among different countries, resulting in differences in specificity and detectability. A major problem is the fact that the mouse bioassay detects all DSP components and probably also other toxins. On the other hand, the rat bioassay detects only OA and DTXs because the criteria in this assay are soft stool, diarrhoea and feed refusal which effects are known to be caused by OA and DTXs only**.** In addition, there is an increasing pressure to replace mammalian bioassays, not only because they are considered less suitable for quantitative purposes, but also because of ethical reasons. In the EU, a recommendation with supportive and convincing documentation has recently been issued by representatives of government institutions in Germany, the United Kingdom and the Netherlands to the members of the Scientific Advisory Committee (ESAC) of the European Centre for the Validation of Alternative Methods (ECVAM) to stimulate the development of methodology that can replace the existing bioassays, not only for DSP, but also for PSP (Grune *et al*., 2003).

The European Commission has recognised the needs of the analytical community to develop methods alternative to animal testing. A relevant call for proposals in the Commission's Sixth

Framework Programme in Area 5: “Food Quality and Safety” appeared (EC, 2003) in which one of the objectives is to develop cost-effective tools for analysis and detection of hazards associated with seafood from coastal waters such as Diarrhoeic Shellfish Poisons, Yessotoxins, Pectenotoxins and Azaspiracid Shellfish Poisons. If granted, this will mean that progress can be expected in the coming years.

in vitro assays

cytotoxicity assays

An assay based on morphological changes in fresh rat hepatocytes when exposed to DSP toxins has been developed by Aune *et al*. (1991). Using this method, it is possible to differentiate between the diarrhoeic DSP toxins OA and DTX1, and the non diarrhoeic toxins PTX1 and YTX. OA and DTX1 induce irregular-shaped cells with surface blebs, PTX1 induces dose-dependent vacuolization and YTX does not cause changes in the shape of the cells but induces blebs on the surface. For OA and DTX1 the first signs appear at 0.5 σg/ml, for PTX at 5 σg/ml and for YTX at 10 σg/ml. This method is a valuable research tool in the separation between diarrhoeic and non-diarrhoeic DSP toxins. However, there are some disadvantages too as it is time consuming and confusing results may be obtained in the presence of mixtures of different algal toxins.

OA has high toxicity for KB cells (a human cell line derived from epidermoid carcinoma) apparent already after three hours of contact. Amzil *et al*. (1992) developed a method to determine the minimal active concentration (MAC) based on direct microscopic study of toxin-induced changes in cell morphology. A high correlation is found between the MAC of tested extracts and corresponding OA concentrations in mussel hepatopancreas as measured by LC (see Chapter 3.2.3).

Daiguji *et al*. (1998) reported that PTX2 showed cytotoxicity against KB cells at 0.05 µg/ml whereas pectenotoxin-2 seco acid and 7-*epi*-pectenotoxin-2 seco acid did not show cytotoxicity at a dose of 1.8 µg/ml. This implied that the cyclic structure of the PTXs is important to express cytotoxicity.

Tubaro *et al*. (1996b) developed a quantitative assay for OA using also KB cells. The method has shown itself to be effective in detecting OA in mussel samples at a detection limit of 50 ng/g digestive gland tissue in a 24 hours endpoint assay. The dose-dependent cytotoxicity assay is based upon the metabolic conversion of a tetrazolium dye (MTT) to yield a blue-coloured formazan product which can be read for absorbance with a microplate scanning spectrophotometer.

Pouchus *et al*. (1997) compared the activity of contaminated mussel extracts on KB cells by direct interpretation of morphological changes and by a colorimetric method estimating the number of viable cells after staining. The latter technique reveals interferences, not detected by the former, with mussel cytotoxins. The results show that the technique, based on determination of the minimal active concentration of DSP toxic extracts inducing morphological changes, is specific for OA and preferable to the determination of a 50 percent inhibition concentration (IC_{50}) by a cell culture method.

OA and related compounds in mussels possess a high toxicity to Buffalo green monkey (BGM) kidney cell cultures. A detection method for OA and related compounds based on the morphological changes in BGM cell cultures has been developed. A high correlation is found between the official mouse bioassay (Yasumoto’s bioassay, observation time five hours) and this cytotoxicity test conducted on naturally contaminated samples of *Mytilus galloprovincialis* (Croci *et al*., 1997, 2001).

Other cytotoxicity assays for DSP toxins make use of fibroblasts (Diogene *et al.*, 1995) as well as human cell lines (Oteri *et al.*, 1998; Fairy *et al.*, 2001; Flanagan *et al.*, 2001). Further endpoints used to assess the cytotoxicity of DSP toxins include neutral red uptake (Draisci *et al.*, 1998), vital staining (Flanagan *et al.*, 2001) and inhibition of cell aggregation and apoptosis (Fladmark *et al.*, 1998).

Cytotoxicity (hepatocytes, KB cells) assays seem to work well for OA and DTX1. However, their value in practice is to be awaited from ongoing inter-laboratory validation studies being carried out in the EU. Marcaillou-Le Baut *et al.* (1994) reported that results of the cytotoxicity assay with KB cells correlated well with results in the LC or the mouse test (by linear regression analysis).

3.2.2 Biochemical assays

immunoassays

There are several immunodiagnostic methods available for the detection of DSP toxins, configured as either RIA or ELISA tests, all of which incorporate antibodies prepared against a single diarrhoeic agent OA (Hallegraeff *et al.*, 1995). A radioimmunoassay (RIA) for OA has been developed by Levine *et al.* (1988) (Hallegraeff *et al.*, 1995). Antibodies to OA are prepared by immunizing rabbits with okadaic acid conjugated at the carboxy function to form an amide bond with an amino group of the immunogenic carrier, bovine albumin (using carbodiimide). Competitive binding of OA with ^{3}H-OA in the test system and measurement by scintillation counting allows detection of 0.2 pmoles of toxin (about 0.2 pg/ml). Structurally related marine toxins (a.o. maitotoxin, palytoxin and brevetoxin) do not inhibit binding of tritiated OA to the antibody.

Enzyme-linked immunosorbent assay (ELISA) test kits have been developed and are commercially available. The **DSP-Check®** ELISA test kit from UBE Industries, Tokyo, Japan has been used throughout the world for screening OA and DTX1 at a claimed detection limit of 20 ng/g. Reports about its performance in practice vary. Inconsistencies including false positive responses when applied to either phytoplankton or shellfish samples have been reported many times. However, in a comparative experiment with LC (method of Lee *et al.*, 1987), the **DSP-Check®** test kit was capable of detecting quantitatively DSP toxins in all tested contaminated samples containing only okadaic acid, provided that the parent toxins were within the range of detection and were not in the ester form (Vale and De M. Sampayo, 1999). The test was found to be more sensitive, specific and faster than LC. The monoclonal antibody in the **DSP-Check®** test kit cross-reacts with DTX1 at a level comparable to OA but PTXs and YTXs are not reactive (Hallegraeff *et al.*, 1995).

The **Rougier Bio-Tech®** ELISA test kit utilizes an anti-OA monoclonal antibody and an anti-idiotypic antibody which competes with OA for binding sites on the anti-OA antibody. The antibody in this test kit exhibits a much higher sensitivity (10-20 fold) for OA than either DTX1 or DTX2, and methyl-, diol- and alcohol derivatives of OA will also bind to the antibody, whereas DTX3 and brevetoxin-1 do not cross-react at all. This test kit has undergone extensive comparison with alternative analytical methods for DSP toxins such as HPLC and LC-MS and is found to be rather reliable for OA quantification in both mussel extracts and phytoplankton (Hallegraeff *et al.*, 1995)

Morton and Tindall (1996) compared the **DSP Check ®** test and the **Rougier Bio-Tech ®** test with LC (modification of method of Lee *et al.*, 1987) and found both ELISA kits to provide accurate estimations of okadaic acid in extracts which were free of methylokadaic acid. However, the **DSP Check ®** test underestimated quantities of total okadaic acid in extracts containing both analogues. Since outbreaks of DSP have been associated with okadaic acid, methyl okadaic acid

or a mixture of these and other related compounds, the ELISA kits may not accurately assess the total toxicity of shellfish samples.

The ELISA developed by **Biosense ®** for yessotoxin is new (see Direct cELISA YTX assay at www.biosense.com). In-house data show that this ELISA probably detects a multitude of yessotoxin analogues and an international inter-laboratory study to test its performance was planned for 2003 (Kleivdal, H. Personal information, 2002)

Garthwaite *et al.* (2001) developed an integrated ELISA screening system for ASP, NSP, PSP and DSP toxins (including yessotoxin). The system detects suspected shellfish samples. Thereafter the suspected samples have to be analysed by methods approved by international regulatory authorities. Alcohol extraction gave good recovery of all toxin groups.

Immuno technology has also been applied in the development of biosensors for DSP toxins. Botrè and Mazzei (2000) defined a biosensor as "a self-consistent bioanalytical device incorporating a biologically active material, either connected to, or integrated within, an appropriate physico-chemical transducer, for the purpose of detecting-reversibly and selectively-the concentration or activity of chemical species in any type of sample". Marquette *et al.* (1999) described a semi-automated membrane-based chemiluminescent immunosensor for okadaic acid in mussels. The sensor is integrated in a flow injection analysis system. Anti-OA monoclonal antibodies were labelled with horseradish peroxidase for their use in a competitive assay, in which the free antigen of the sample competes with OA, immobilized on commercially available polyethersulfone membranes. The authors investigated the operational stability of the sensor over 38 OA determination cycles and found a stable response for the first 34 measurements. In addition, the performance of five immunosensors (five different membranes) showed good repeatability for critically contaminated and blank mussel homogenates, with CVs of 12.6 and 7 percent respectively. It may be expected that the development and application of biosensors for the determination of toxins of the DSP complex will advance rapidly in the coming years.

Another antibody-based technique in DSP analysis is the application of immunoaffinity columns (IAC) to purify shellfish extracts prior to the determinative step in analysis procedures, usually LC. Puech *et al.* (1999) described the recent development and the characterization of IAC, which were elaborated using anti-okadaic acid monoclonal antibodies, for a specific retention of the OA group of toxins. The coupling yield and the stability of these columns were investigated as well as their capacity to remove interfering compounds. Cross-reactivity was observed between the antibodies and the DTX1 and the DTX2, allowing the detection of the different toxins in a single analysis. Different spiked or naturally-contaminated matrices (mussel digestive gland and algae) were tested, and recoveries varied from 55 to 95 percent according to the matrices. The IAC purification was then included as a step of a global IAC/LC/spectrofluorimetric detection method and the performance of the method was evaluated. Estimations of the linearity and the accuracy (percentages of the presumptive response for OA were in the range + 101 percent to 114 percent) were satisfactory in accordance with the method validation criteria. IACs have great potential as clean-up techniques in analytical methods, but their value in practice still has to be proven in inter-laboratory validation studies.

acid phosphatase assays

An assay for DSP based on acid phosphatase activity in the protozoan *Tetrahymena pyriformis* has been developed. Toxins are extracted from shellfish using acetone/ ether and cleaned up by silicic acid chromatography. *Tetrahymena* is cultured in the presence of the extract for 24 hours and the 50 percent acid phosphatase activity inhibitory concentration and the growth inhibitory concentration are determined and expressed as mouse unit equivalents (Van Egmond *et al*., 1993 and Hallegraeff *et al*., 1995)

The specific inhibition of protein phosphatase Type 1 (PP1) and Type 2A (PP2A) by certain DSP analogues (OA and DTX1) was used to develop a phosphatase radio assay using ^{32}P-phosphorylase. The assay is used directly on shellfish extracts and on fractions collected after HPLC separation of the toxins from digestive gland extracts. Although the original technique, which is coupled with toxin fractionation by LC is not widely used as regulatory tool, it has been used frequently in screening the phosphatase inhibition activity of putatively phycotoxic compounds and partially purified extracts of phytoplankton and shellfish. In its current format, this assay is based on the inhibition of PP1 by OA with a limit of detection as low as 10 fg OA/100 g tissue. A relatively rapid radioactive protein phosphatase (PP)-based assay has been developed and used by Honkanen *et al*. (1996a, b) to detect OA in oyster (*Crassostrea virginica*) extracts. In more than 320 assessments with spiked oyster samples, all samples containing ∅0.2 σg OA/g were positive. From the samples spiked with 0.1 σg OA/g, 16.7 percent were positive. Control samples and samples spiked with 0.02 σg OA/g were negative. A high correlation is seen between the results of this assay and LC.

Although the use of radiolabels in the PP assay leads to low limits of detection, colorimetric and fluorometric assays have been developed to allow a more widespread adoption of the PP assays (Quilliam, 1998a).

A colorimetric phosphatase-inhibition bioassay has been developed for the quantitative measurement of OA by Simon and Vernoux (1994). The assay uses an artificial substrate, *p*-nitrophenylphosphate, and a semi-purified protein phosphatase PP2A containing extract prepared from rabbit muscle. The lowest detectable concentration of OA is 4 ng/ml in aqueous solutions and 40 ng/ml (i.e. 100 ng of OA per g of mussel tissue) in crude methanol mussels extracts. The rapidity, accuracy, reproducibility (within the laboratory), specificity and simplicity of the procedure provide a simple way to assay OA in buffered or complex solutions.

Tubaro *et al*. (1996a) developed a colorimetric PP assay using *p*-nitrophenylphosphate and a commercially available PP2A preparation to assess the presence of OA in mussels. The assay, which is employed in the microplate format, is accurate and reproducible (within the laboratory). OA is detected in concentrations as low as 0.063 ng/ml in aqueous solutions and 2 ng/g in mussel digestive glands. Thirty naturally contaminated mussel samples were submitted to the PP2A inhibition assay as well as to an ELISA and a MTT cytotoxicity assay, with similar results. The assay is sensitive, rapid and does not require expensive equipment according to the authors.

Lower limits of detection are possible with fluorometric PP assays. Vieytes *et al*. (1997) developed a fluorescent enzyme inhibition assay for OA using 4-methylumbelliferyl phosphate and fluorescein diphosphate as substrates for enzyme PP2A. The detection limit of OA is 12.8 ng/g hepatopancreas in shellfish extracts. According to the authors this assay can also be used for very dilute samples, such as phytoplankton samples.

Fluorometric protein phosphatase inhibition assays have not only been shown to perform better than colorimetric assays, but also to agree well with the mouse bioassay and LC techniques (Quilliam, 1998a; Vieytes *et al.*, 1997; Mountfort *et al.*, 1999). However Mountfort *et al.* (2001) have modified the fluorometric assay to overcome the lack of sensitivity towards the ester derivatives of okadaic acid and analogues and to reduce significantly the incidence of false negatives observed previously. At the time of writing, a European collaborative study of the fluorometric protein phosphatase inhibition method was ongoing to establish the performance characteristics of this method for okadaic acid and DTX1. If the results are acceptable, the method will be standardized by CEN and it is likely that the method will subsequently be approved for regulatory purposes in the EU.

YTX inhibited the hydrolysis of *p*-nitrophenyl phosphate by PP2A. The IC_{50} was 0.36 mg/ml. The potency was lower than that of OA by four orders of magnitude. Hence, interference by YTX coexisting with OA in shellfish can be disregarded in the enzyme inhibition assay for OA or DTX1 (Ogino *et al*., 1997).

in general

Detection methods based on immunology (ELISA, RIA) are not yet fully developed and certainly not formally validated for all toxins involved. Nunez and Scoging (1997) reported that the ELISA assay detecting OA and/or DTX1 did not accurately detect low concentrations compared with the LC assay, the colorimetric phosphatase inhibition assay and the mouse bioassay. Gucci *et al*. (1994) did not find either a clear quantitative agreement between four different test methods for DSP (mouse bioassay, rat bioassay, ELISA test and LC method). Also Draisci *et al*. (1994) reported that the ELISA method did not give always quantitatively reliable results compared to the mouse bioassay and the LC method. Morton and Tindall (1996) compared the LC-fluorescence method with two commercially available ELISA test kits for the detection of OA and DTX1 in dinoflagellate cells (*Prorocentrum hoffmanium* and *P. lima*). Although false positive and false negative samples were not detected by the ELISA test kits, both test kits may underestimate total toxins present. Acid phosphatase inhibition assays also seem to work well for OA and DTX1. However, their value in practice will be ascertained by ongoing inter-laboratory validation studies being carried out in the EU.

3.2.3 Chemical assays

thin layer chromatography (TLC)

DSP toxins can be detected by thin layer chromatography (TLC). After a clean-up (silica gel column chromatography or gel permeation) of the extracts, fractions are applied directly to a silica gel plate and eluted with a toluene-acetone-methanol mixture. The acidic DSP toxins themselves appear as a weak UV-quenching spot at R_f 0.4. Both the diol esters and the free acid toxins give a characteristic pinkish-red stain after spraying with a solution of vanillin in concentrated sulphuric acid-ethanol and standing at room temperature for several minutes. The free acids produce a bright pinkish-red colour whereas the colour is duller with the diol esters. When clean material is applied to a TLC plate, 1 σg of the toxin could be detected; with cruder fractions, 2 to 3 σg is required before detection was possible (Hallegraeff *et al*., 1995). These rather high detection limits are a limiting factor for the use of TLC determining DSP toxins.

gas chromatography (GC)

Gas chromatography (GC) methods have been developed to detect and separate OA toxins. The toxins from diethyl ether extracts of dinoflagellate cultures are first isolated and purified using silicic acid, gel permeation chromatography and reversed-phase partition chromatography. GC analysis of trimethylsilyl derivatives of intact toxin and methyl esters is carried out with hydrogen flame ionization detection (Hungerford and Wekell, 1992). In practice this technique is rarely used.

liquid chromatography (LC)

The method described below is one of the most commonly used analytical techniques for determination of OA and DTX1. The original method (Lee *et al*., 1987) involves sequential extraction of shellfish tissue with methanol, ether and chloroform; derivatization with 9-anthryldiazomethane (ADAM); silica Sep-pak clean-up; determination by HPLC with fluorescence detection. The ADAM method is very sensitive for DSP toxins being able to detect 10 pg of the OA derivative injected on the column. The minimum detectable concentration in

shellfish tissue, however, is limited not by detector sensitivity but by chemical background, which can vary considerably between samples. The practical quantitation limit is about 100 ng/g tissue. If digestive glands only are used in the analysis this limit is equivalent to 10 to 20 ng/g for whole tissue of mussels.

Aase and Rogstad (1997) optimized the sample clean-up procedure for determination of OA and DTX1 with the ADAM derivatization method. The use of a solid-phase extraction silica column of 100 mg and washing solvents composed of dichloromethane instead of chloroform were proposed to minimize the effect of stabilizing alcohol.

The unstable nature of ADAM and its limited availability have led several researchers to look for alternative derivatization reagents including 1-pyrenyldiazomethane and 1-bromoacetyl-pyrene, N-(9-acridinyl)-bromoacetamide, 4-bromomethyl-7-methoxycoumarin, 2,3-(anthracenedicarboximido) ethyltrifluoro-methanesulphonate. The polyaromatic hydrocarbon reagents ADAM, 1-pyrenyldiazomethane (PDAM) and 1-bromoacetylpyrene (BAP) have proved to be the most successful as they are less prone to interferences from reagent and reaction artefact compounds (James *et al.*, 1997). The ADAM-LC method has been collaboratively studied in an inter-laboratory validation study conducted by the German Federal Laboratory for fish and fish products (GFL, 2001) for the determination of OA and DTX1 in mussel. This method is now in the stage of standardization by CEN and expected to become a European Standard in 2004. Whereas ADAM-LC seems to work reasonably well for OA and DTX1, this is not the case for the other toxins of the DSP complex.

DTX3 cannot be analysed directly by this method but must first be converted back to OA, DTX1 or DTX2 via alkaline hydrolysis. The diol esters of the DSP toxins as well as YTXs and some of the PTXs cannot be analysed by the ADAM-LC method (Hallegraeff *et al.*, 1995 and Van Egmond *et al.*, 1993).

For the determination of the yessotoxins and pectenotoxin-2, alternative LC procedures have been developed making use of a dienophile reagent DMEQ-TAD (4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalinyl)ethyl]-1,2,4-triazoline-3,5-dione) for fluorescent labelling (Yasumoto and Takizawa, 1997; Sasaki *et al.*, 1999). While the authors claim that these methods are superior to the mouse bioassay in rapidity, sensitivity and specificity, no inter-laboratory validations have yet been performed to establish their performance characteristics.

Fernández *et al.* (1996) warned that any procedure (LC-FD, LC-MS) used to characterize all the DSPs present in shellfish should take into account that the hexane layer, usually discarded, can be very rich in low-polar DSP toxins.

micellar electrokinetic chromatography (MEKC)

MEKC with UV detection was applied to the determination of non-derivatized DSP toxins. OA was detected in mussels spiked with 10 ng/g whole tissue, and the presence of OA and DTX2 was observed in the crude extract of the dinoflagellate *Prorocentrum lima* (Bouaïcha *et al.*, 1997a).

mass spectrometry

Hallegraeff *et al.* (1995) reported the analysis of diol esters of OA, DTX1 as well as DTX3 toxins. LC combined with electrospray ionization mass spectrometry (LC-ESI-MS) appears to be a sensitive and rapid method of analysis for DSP toxins. A detection limit can be achieved of 1 ng/g in whole edible shellfish tissue. Various analytical procedures continue to be developed for the determination of DSP toxins and recent reviews have described a comprehensive range of methods (Quilliam, 2001). DSP profiling of bivalves (scallops and mussels) with LC-MS has been

reported by Suzuki *et al*. (2000). They focussed on OA, DTX1 and PTX6. Negative ESI-mode was found to be much more efficient than positive ESI-mode.

Matrix effects in the DSP analysis with LC-ESI-MS have been tackled in different ways. Suzuki *et al*. (2000) successfully used an alumina B column for sample clean-up. Hummert *et al*. (2000) applied size exclusion chromatography (SEC) for the clean-up of raw extracts from algae and mussel tissue containing either microcystins or DSP toxins. Although it is likely that improvements were obtained, the article fails in demonstrating that matrix effects could be removed completely (recovery data are missing, spiking was not applied). Goto *et al*. (2001) paid more attention to the chemical properties of the different DSP compounds by applying different extraction solvents and solvent partitioning.

Ito and Tsukada (2001) conducted an explicit study on matrix effects. They demonstrated a better performance by applying the standard addition method to each separate sample, which however requires two LC-MS runs per analysis. An alternative method, where the response factor was based on one model sample, was less satisfactory. The study nicely demonstrated and emphasizes the matrix effect from shellfish extracts and demonstrates how that effect can be tackled for quantification purposes.

In the second half of 2002, an inter-laboratory study took place of a new LC-MS method for determination of ASP and DSP toxins in shellfish (Holland and McNabb, 2003). The eight participating laboratories generally obtained consistent sets of data for the broad group of analyte toxins down to low levels (< 5ng/ml, equivalent to 0.05 mg/kg). In general, sensitivity was adequate to achieve the LODs required. Most of the participating laboratories could detect the analyte toxins; greater differences were observed for quantitation of some toxins, especially when no analytical standards were present. The participants used different MS detection modes: some used single MS detection (SIM/SIR), others used tandem MS detection (MRM), and some used both. Although the use of MRM mode is attractive in order to enhance specificity, it requires additional care for quantitation. To sum up, the study was very stimulating and encouraging for those who are interested in using an alternative method for the mouse bioassays which are not supported by any statistical validation data, are well known to have a relatively high rate of false positives, have inadequate detection capability for some toxins and are ethically unacceptable for routine food monitoring. Additionally, method 40.105 (the method tested) can reliably detect ASP toxins and a range of other toxins and metabolites such as azaspiracids and pectenotoxin seco acids which may not respond in mouse assays (Holland and McNabb, 2003).

in general

Chemical methods (LC) are useful for identification and quantification of selected diarrhoeic toxins (usually OA or DTXs), and the first validated method for OA and DTX1 approaches the phase of standardization by CEN. For the other DSP toxins, some LC methods exist but have not yet been validated. The rapid developments in LC-MS methodology are promising; however, improvement and inter-laboratory studies will be necessary before these techniques can become generally accepted tools in regulatory analysis. A serious problem is that pure analytical standards and reference materials are hardly or not readily available, which hampers the further development and validation of analytical methodology for DSP toxins.

3.3 Source organism(s) and habitat

3.3.1 Source organism(s)

DSP toxins are produced by dinoflagellates that belong to the genera *Dinophysis spp*. and *Prorocentrum spp*. These algae, under favourable environmental conditions, may grow to large numbers and produce algal blooms.

The production of DSP toxins has been confirmed in seven *Dinophysis* species, *D. fortii* (in Japan), *D. acuminata* (in Europe), *D. acuta, D. norvegica* (in Scandinavia), *D. mitra, D. rotundata* and *D. tripos,* and in the benthic dinoflagellates *Prorocentrum lima, Prorocentrum concavum* (or *P. maculosum*) and *Prorocentrum redfieldi* (Viviani, 1992). Three other *Dinophysis* species, *D. caudata, D. hastata* and *D. sacculus,* are also suspected (Hallegraeff *et al*., 1995). Giacobbe *et al*. (2000) showed that *D. sacculus* contained OA and DTX1 concentrations of 110-400 and 8-65 fg/cell, respectively. Maximum DSP toxins (OA+DTX1 455 fg/cell) were found in early spring blooms. The authors suggested that the role of *D. sacculus* in harmful events in the Mediterranean area may be far from negligible despite their low toxicity.

Detection of DSP toxins in the heterotrophic dinoflagellates *Protoperidinium oceanicum* and *P. pellucidum* may reflect their feeding on *Dinophysis*. Toxin productivity varies considerably among species and among regional and seasonal morphotypes in one species. For example, *D. fortii* in northern Japan during March and June contains high concentrations of toxins and is associated with significant accumulation of toxins in shellfish. But the same species in southern Japan during May and July does show slight toxicity and shellfish is free from toxins (Hallegraeff *et al*., 1995).

Pan *et al*. (1999) reported the production of OA, OA diol ester, DTX1 and DTX4 by *Prorocentrum lima*. Caroppo *et al*. (1999) demonstrated the potential of the non-photosynthetic species *Phalacroma rotundatum* in the southern Adriatic Sea to produce OA, DTX1 and DTX2. The benthic dinoflagellate *Prorocentrum arenarium* isolated from the reef ecosystem of Europa Island (Mozambic Channel, France) (Ten Hage *et al*., 2000) and also *Prorocentrum belizeanum* from the Belizean coral reef ecosystem were found to produce OA (Morton *et al*., 1998). *Gonyaulax polyhedra* was implicated as responsible for YTX contamination in the Adriatic mussels by Tubaro *et al*. (1998). Satake *et al*. (1997) isolated YTX from cultured cells of the marine dinoflagellate *Protoceratium reticulatum*.

3.3.2 Predisposing conditions

The appearance of *Dinophysis*, even at low densities such as 200 cells per litre, can cause already a toxification of shellfish that is enough to affect humans (Botana *et al*., 1996). On the other hand, only blooms greater than 20 000 cells per litre were associated with DSP in the Dutch Wadden Sea. A study of *Dinophysis* in the Portuguese waters revealed that the time needed for shellfish to become toxic depends not only on the presence of toxic algae but also on the relative abundance of the non-toxic accompanying species (Aune and Yndestad, 1993). Not all DSP outbreaks are accompanied by macroscopic blooms of *Dinophysis spp*. or *Prorocentrum spp*. (Viviani, 1992). Toxicity of specific *Dinophysis* species varies spatially and temporally, and the number of cells per litre needed to contaminate shellfish is highly variable. Significant accumulation of cells in blue mussels (20 000 to 30 000 cells per digestive gland) resulting in a high toxicity in mussels in Norway, was seen at already 1 000 to 2 000 cells of *Dinophysis spp*. per litre of seawater (Aune and Yndestad, 1993).

In an isolated fjord in Sweden where levels of dissolved inorganic phosphate (DIP) and nitrogen (DIN) were relatively high, low OA levels in mussels (*M. edulis*) were detected. Deep water in this area was rich in dissolved silicate (DSi). Areas with low DIN/DSi and DIP/DSi ratios during the end of the summer coincided with low OA levels in mussels. High OA levels in mussels occurred in areas where DSi was almost totally depleted in July and remained low during the rest of the production season. Apparently the absence of silicate favours dinoflagellates including the DSP toxin producing *Dinophysis* spp. (Haamer, 1995).

In the north of the Gulf of California, Mexico, promoting factors for dinoflagellate dominance, such as disappearance of diatoms, low grazing pressure, probably nitrate-limited environment, a 20 to 23 ∀C temperature range and thermal stratification, were present in March 1993 and April to May 1994. During these periods maximal *D. caudata* densities of 75 to $90x10^3$ cells per litre were observed (Lechuga-Devéze and Morquecho-Escamilla, 1998).

P. lima is known from the benthos and plankton and is common in both warm and cool-temperate waters. Growth of cultures of *P. lima* (from Nova Scotia, Canada) was preceded by a prolonged lag phase. During the initial lag phase toxin levels per cell remained relatively high if nitrogen had been added to the medium. When cells began to grow total toxin level per cell generally decreased and remained between 5 and 10 pg. Cells of *P. lima* survived 0 ∀C for five weeks and recovered when brought to a higher temperature. During the cold period, some cell damage probably occurred with concomitant loss of toxins to the medium. Nitrogen concentration (NO_3^-) in the medium was used to limit growth or stress the cells physiologically. When growth was limited, increases in toxin associated with cells were recorded. Maximum accumulation of toxins in the cells occurred during the stationary phase. Ratios of OA/DTX1 were around five. Low OA/DTX1 ratios were associated with growing cells and higher ratios with cells in the stationary phase (McLachlan *et al*., 1994).

Pan *et al*. (1999) reported that DSP toxin synthesis by *P. lima* is restricted to the light period and is coupled to cell division cycle events. DTX4 synthesis is initiated in the G1 phase of the cell cycle and persists into S-phase ("morning" of the photoperiod), whereas OA and DTX1 production occur later during S and G2 phases ("afternoon"). No toxin production was measured during cytokinesis, which happened early in the dark.

3.3.3 Habitat

The DSP incidences, or at least the presence of DSP, appear to be increasing. This may be partly due to increasing knowledge about the disease and better surveillance programmes. However, it must be noted that toxin-producing algae and toxic molluscs are frequently reported from new areas (Aune and Yndestad, 1993). DSP was first documented in 1976 from Japan where it caused major problems for the scallop fishery. Between 1976 and 1982, some 1 300 DSP cases were reported in Japan, in 1981 more than 5 000 cases were reported in Spain, and in 1983 some 3 300 cases were reported in France. In 1984, DSP caused a shutdown of the mussel industry for almost a year in Sweden. The known global distribution of DSP includes Japan, Europe, Chile, Thailand, Canada (Nova Scotia) and possibly Tasmania (Australia) and New Zealand (Hallegraeff *et al*., 1995).

In Japan, *Dinophysis fortii* has been incriminated as the organism producing DSP toxins (Van Egmond *et al*., 1993 and Viviani, 1992). However, the OA-producing *Prorocentrum lima* occurred on the Sanriku coast of northern Japan. The dinoflagellate was distributed on the surface of the algae, *Sargassum confusum* and *Carpopeltis flabellata.* This *P. lima* strain grew well in T1 medium at 15 ∀C at which tropical strains do not grow, indicating that it is a local strain which adapted to cooler environments (Koike *et al*., 1998).

On European Atlantic coasts, other dinoflagellate species are also involved: *D. acuminata* and *D. acuta* in Spain , *D. acuminata, D. sacculus, P. lima* in France; *D. acuminata, P. redfieldii* and *P. micans* in the Netherlands (Van Egmond *et al*., 1993 and Viviani, 1992); *D. acuta, D. sacculus, D. acuminata, D. caudata* and *P. lima* in Portugal (Van Egmond *et al*., 1993), *D. acuta, D. acuminata, P. lima* and *P. concavum* in Ireland; *D. acuta, D. acuminata, D. norvegica, P. micans, P. minimum, P. lima* in Scandinavia; and *D. sacculus, D. acuminata, D. tripos, D. caudata* and *D. fortii* in the Adriatic sea (Van Apeldoorn *et al*., 1998; Ciminiello *et al*., 1997; Marasović *et al*., 1998; Giacobbe *et al*., 2000). Draisci *et al.* (1996a) reported the detection of PTX2 in *Dinophysis fortii* collected in the northern Adriatic Sea. This was the first report of such a toxin in Europe.

In the Gulf of Mexico, *D. caudata* was involved; in the Australian region *D. fortii, D. acuminata* and *P. lima* and at eastern Canada *D. norvegica* and *P. lima* (Van Apeldoorn *et al*., 1998). In Johor Strait, Singapore, *D. caudata* was the most frequent and abundant species from March 1997 to February 1998. Other dinoflagellates observed were *Prorocentrum micans* and *Protoperidinium* spp. (Holmes *et al*., 1999).

Phalacroma rotundatum which has the potential to produce toxins of the okadaic acid group, was observed in Japanese waters, in North West Spain (Ria Pontevedra) and along the southern Adriatic coast of Puglia (Italy) (Caroppo *et al*., 1999).

Along the Chinese coasts in the South and East China Sea, DTX1 and OA were detected in shellfish species implying that DSP toxins producers also exist in this area. Frequency and shellfish toxin levels in southern parts of the coast were greater than those in northern areas (Zhou *et al*., 1999).

D. acuminata and *Prorocentrum minimum* occurred in large numbers in the Peter the Great Bay (Sea of Japan, the Russian Federation) during summer 1995 and 1996 (Orlova *et al*., 1998).

The benthic dinoflagellate *Prorocentrum arenarium* isolated from the reef ecosystem of Europa Island (Mozambic Channel, France) (Ten Hage *et al*., 2000) and also *Prorocentrum belizeanum* from the Belizean coral reef ecosystem (USA) were found to produce OA (Morton *et al*., 1998).

3.4 Occurrence and accumulation in seafood

3.4.1 Uptake and elimination of DSP toxins in aquatic organisms

Diarrhoeic shellfish toxins associated with *Dinophysis* spp. and *Prorocentrum* spp. are readily accumulated by shellfish. However, little is known of the retention time of these toxins (Hallegraeff *et al*., 1995). A few studies described DSP toxin kinetics in bivalves under either natural or controlled laboratory conditions. Dynamics of DSP toxins were examined in juvenile and adult bay scallops by feeding cells of the epibenthic dinoflagellate *Prorocentrum lima* to scallops in a controlled laboratory microcosm. Analysis of DSP toxins in dinoflagellate cells and scallops tissues was performed by means of liquid-chromatography combined with ion-spray mass spectrometry (LC-MS). Juvenile and adult clearance rates were not inhibited by exposure to *P. lima* cells and no scallop mortalities were seen. Scallops could exceed regulatory toxin limits of 0.2 σg DSP toxin/g wet weight in less than one hour of exposure to high *P. lima* cell densities. Toxin saturation levels (2 σg DSP toxin/g wet weight) were attained within two days, however toxin retention was very low (under 5 percent). Although most of the total toxin body burden was associated with visceral tissue, weight-specific toxin levels were also high in gonads of adult scallops. Rapid toxin loss from gonads within the first two days of depuration indicated that the toxin was derived primarily from a labile (unbound) component within the intestinal loop section through the gonads. Detoxification of visceral tissue, however, followed a biphasic pattern of

rapid toxin release within the first two days of depuration, followed by a more gradual toxin loss over a two week period, suggesting that faecal deposition may be an important mechanism for rapid release of unassimilated toxin and intact dinoflagellate cells (Bauder and Grant, 1996).

Sedmak and Fanuko (1991) also observed two phases of DSP toxin release during a decontamination phase of mussels. There is first a rapid decrease in toxin content followed by a slow decrease with the toxicity remaining above the quarantine level of 0.5 MU/g hepatopancreas. The patterns of contamination and decontamination are specific for shellfish species and do not seem to depend on the type of dinoflagellate toxin.

Toxic scallops (*Patinopecten yessoensis*) cultivated in tubs in which filtered and sterilized seawater was circulated, with or without supply of planktonic diatoms as feed, showed a gradual decrease of DSP during cultivation (microbial assay method). DSP decreased to 30 percent of initial value within two weeks when dense cultures of *Chaetoceros septentrionelle* were supplied as the feed. Relatively high toxicity scores of DSP were detected in excrement of cultivated scallops. When other diatoms such as *Skeletonema costatum*, *Asterionella japonica*, *Rhabdonema* spp. and *Thalassiosira* spp. were supplied as feed not only the toxicity but also the amounts of glycogen, free amino acids and free fatty acids decreased, causing a deterioration in quality (Van Apeldoorn *et al*., 1998).

During decontamination of mussels *(Mytilus galloprovincialis*) from Galicia in northwestern Spain for 70 days under different environmental conditions (salinity, temperature, fluorescence, light transmission), fluorescence and light transmission appeared to have the most prominent effect on depuration. In most cases, there was an inverse relation between depuration and body weight. It could not be clearly concluded whether the DSP depuration evolved following 1- or 2-compartment kinetics (Blanco *et al*., 1999).

In a study on the feeding behaviour of the mussel *Mytilus galloprovincialis* on a mussel farm in the Gulf of Trieste (Italy) during a DSP outbreak, the mussels seemed to feed selectively on dinoflagellates rather than diatoms. Further selection was observed among different dinoflagellate genera and a preference for the genus *Dinophysis* was particularly evident. The mussels seemed to open the thecae of *Dinophysis* cells and digest them more easily than other dinoflagellates (Sidari *et al.*, 1998).

3.4.2 Shellfish containing DSP toxins

In Japan, the shellfish causing DSP were found to be the mussels *Mytilus edulis* and *M. coruscum*, the scallops *Patinopecten yessoensis* and *Chlamys nipponesis akazara*, and the short-necked clams *Tapes japonica* and *Gomphina melaegis*. Along the European Atlantic coasts, particularly *M. edulis* but also *Ostrea* sp. were contaminated with DSP toxins (Viviani, 1992).

In Japan and the Atlantic coast of Spain and France, the infestation ranges from April to September and the highest toxicity of shellfish is observed from May to August, although it may vary locally. By contrast, in Scandinavia, in February oysters have caused DSP and in October mussels have caused DSP. Data from the first DSP episode in the Adriatic Sea in 1989 indicated that the infestation period in some coastal areas ranged from May to November (Viviani, 1992).

Comparative analysis in various shellfish from one area in Japan revealed that the highest toxicity was found in blue mussels (*Mytilus edulis*) with less toxicity in scallops and very little in oysters. Differences in toxicity were also noted between mussels cultivated at different depths with concentrations differing by factors of two to three (Viviani, 1992). The highest toxicity was obtained in mussels from the upper level (3-6 m), whereas toxicity was reduced to half that level

at 6-8 m and 8-12 m (Botana *et al*., 1996). OA levels of 0.63 and 4.2 σg/g hepatopancreas in adjacent mussels were reported within the same mussel growing site and levels of 0.63 and 10 σg OA/g hepatopancreas in mussels grown at different depths along the same rope (Van Apeldoorn *et al*., 1998).

Spanish mussels from Galician Rias contained OA as the major toxin besides less polar DSP toxins. The levels of less polar DSP toxins never exceeded the OA levels. Highest low-polar DSP levels corresponded to the highest OA levels. The authors hypothesized that the low polar DSP toxins found in the hexane layer which is usually discarded, belong to the acyl-derivatives group (Fernández *et al*., 1996).

Data from DSP episodes in the Adriatic Sea showed that not all species of bivalve molluscs absorbed and concentrated the enterotoxin in their tissues to the same extent, although these species were living in the same habitat infested by microalgae. In particular, *Mytilus galloprovincialis*, *Chamelea gallina*, *Tapes decussata* and *Venus verrucosa* were monitored for DSP toxins by means of the mouse bioassay and DSP was detected only in mussels, although they were drawn from the same habitat in the Adriatic Sea. This uneven distribution of DSP will have an impact on developments of sampling plans for shellfish, as part of monitoring schemes for control purposes (Viviani, 1992). In *M. galloprovincialis* from the northern Adriatic sea, OA and DTX1 as well as YTX were detected (Ciminiello *et al.*, 1997). Cooking did not alter the toxicity of the contaminated shellfish but intoxication could be avoided if the digestive glands were eliminated beforehand (Viviani, 1992).

OA homologues in the alga *D. fortii*, the scallops *Patinopecten yessoensis* and the mussel *Mytilus galloprovincialis*, collected at the same site in Mutso Bay, Japan, were determined by liquid chromatography-fluorescence detection. Prominent toxins in scallops and mussels were DTX3 and DTX1, respectively, whereas only DTX1 was detected in *D. fortii*. Toxin contents in mussels were significantly higher than those in scallops indicating that mussels have a higher potential to accumulate OA homologues than scallops (Suzuki and Mitsuya, 2001).

Persistent low levels of DSP toxins were found in green mussels (*Perna viridis*) from the Johor Strait, Singapore. Six isomers of OA and five of DTX1 were detected and generally the levels of the isomers were higher than that of OA and DTX1. The highest concentration found was 97 ng/g mussel digestive tissue (wet wt) of an isomer of DTX1 (DTX1a). The maximum level of OA was 24 ng/g. These values were below the threshold limit for consumption (Holmes *et al*., 1999).

DSP was also widely distributed in different shellfish species along the Chinese coast. Twenty six out of 89 samples contained DTX1 or OA but only six samples contained levels above the regulatory limit for human consumption (20 σg/100 g soft tissue). The highest level of 84 σg/100 g was found in *Perna viridis* from Shenzhen (Zhou *et al*., 1999).

3.4.3 Other aquatic organisms containing DSP toxins

DSP toxins accumulate in mussels by plankton filter-feeding. However plankton filter-feeding is largely a non-selective process which is also used by certain fish and may therefore lead to accumulation of DSP toxins in fish. Predators can also accumulate significant amounts of toxin in only one meal given that many bivalve molluscs concentrate toxins in the digestive gland. OA may appear in predatory fish as a consequence of their preying on mussels and fish containing OA.

Cod fish in cages fed toxic mussels showed the highest concentrations of OA particularly in the liver (0.7 σg/g). Lower concentrations were noted in muscle and gonads. Whereas the mussels

used for feeding showed the presence of higher concentrations of DTX1 than of OA, DTX1 was nearly absent from fish tissue. After giving non-toxic feed the OA levels disappeared in one to two months time, least rapid from testis. Analysis of wild fish (cod, sea-cat, shark and herring) caught in Scandinavian waters in January to February 1992, when OA and DTX1 content of mussels in the vicinity was low, showed no OA. No OA was found in refined cod-liver oil (Van Apeldoorn *et al*., 1998).

Traditionally only filter-feeding molluscs are included in monitoring programmes. Shumway (1995) stressed the importance of including also higher-order consumers (such as carnivorous gastropods and crustaceans) in routine monitoring programmes, especially in regions where non-traditional species are being harvested. There are currently no records of DSP toxins in gastropods or crustaceans, but this is, undoubtedly, only because no one has looked for them. Based on the data above it cannot be excluded that DSP toxins also accumulate in higher-order consumers.

3.5 Toxicity of DSP toxins

3.5.1 Mechanism of toxicity

The discovery that OA acid caused long-lasting contraction of smooth muscle from human arteries was the first clue to elucidation of the mechanism of action of DSP toxins. Since smooth muscle contraction is activated by a sub-unit of myosin, it was supposed that the effect of OA was due to inhibition of myosin light chain phosphatase. Thereafter, OA was shown to be a potent inhibitor of the serine/threonine phosphatases PP1 and PP2A; PP2A is about 200 times more strongly inhibited than PP1. Protein phosphatases are a critical group of enzymes closely linked with many crucial metabolic processes within a cell. Phosphorylation and dephosphorylation of proteins is one of the major regulatory processes in eukaryotic cells. Processes as diverse as metabolism, membrane transport and secretion, contractility, cell division and others are regulated by these versatile processes. It is indicated that phosphatases, which are sensitive to OA, like PP1 and PP2A, are involved in entry into mitosis. It is suggested that diarrhoea in humans is caused by hyperphosphorylation of proteins that control sodium secretion by intestinal cells or by increased phosphorylation of cytoskeletal or junctional moieties that regulate solute permeability, resulting in passive loss of fluids (Van Egmond *et al*., 1993; Hallegraeff *et al*., 1995). Extensive structure-activity studies measuring the inhibition of protein phosphatase activity indicated that a free carboxyl group in the DSP molecule is essential for activity, since methyl and diol esters did not show phosphatase inhibition. However, the amide and reduced carboxyl (okadaol) derivatives are about half as active as OA, as are the naturally occurring DTX3 compounds (Hallegraeff *et al*., 1995).

3.5.2 Pharmacokinetics

studies in laboratory animals

<u>studies in mice with okadaic acid (OA)</u>

Adult Swiss mice received a single oral dose by gavage of 50 or 90 σg [^{3}H]OA/kg bw dissolved in 0.2 ml sterile water and methanol (50:50 [v/v]). Urine and faeces were collected over a 24 hour period and thereafter the animals were killed. At 50 σg/kg bw no clinical signs of toxicity were seen, whereas at 90 σg/kg bw diarrhoea was observed from eight hours onwards. No mortality occurred. Radioactivity was determined in the brain, lung, spleen, heart, liver and gall bladder, kidney, stomach, intestine tissue, intestine content, skin, blood, muscle, urine and faeces, and OA was analysed with LC (fluorescence detection) after derivatization with 9-anthryldiazomethane (ADAM). Both methods gave similar results indicating that OA was not very much metabolised. OA was absorbed from gastrointestinal tract as it was found mostly in intestinal tissue and

contents (49.2 percent of the dose) and urine (11.6 percent) after 24 hours. The high concentrations in intestinal tissue and contents after 24 hours demonstrated slow elimination of OA. OA was found in all tissues. The total amount of OA in organs at 50 σg/kg bw was low compared to the amount excreted in urine and faeces (11.6 and 6.6 percent of the dose, respectively) and by far lower than the amount in intestinal tissue plus contents. As the dose increased from 50 to 90 µg/kg bw, concentrations of OA in intestinal contents and faeces increased proportionally. The increase of OA in intestinal tissue at the higher dose correlated well with the diarrhoea observed. The fact that OA was present in liver and bile and all organs including skin and also fluids and the fact that concentrations in intestinal content were approximately two to seven fold higher than in faeces after 24 hours, confirmed that enterohepatic circulation occurred (Matias and Creppy, 1996a). This study also demonstrated that in acute OA intoxication, the concentration in intestinal tissue reaches cytotoxic concentrations in accordance with the diarrhoea seen (Matias *et al.*, 1999a). In recent studies in mice using the anti-OA antibody, OA was detected in lung, liver, heart, kidney and small and large intestines just five minutes after oral administration. OA was detected in liver and blood vessels for two weeks after dosing and in the intestines for four weeks (EU/SANCO, 2001).

Male and female adult Swiss mice received a single intramuscular (i.m.) injection with 25 σg [^{3}H]OA/kg bw dissolved in 0.1 ml sterile water and methanol (50:50 [v/v]). OA was detected in bile and intestinal contents one hour after injection. Its elimination pattern showed biliary excretion and enterohepatic circulation. Administration of cholestyramine, which prevents enterohepatic circulation, changed the cyclic elimination profile of OA (Matias and Creppy, 1996a).

observations in humans

No data

3.5.3 Toxicity to laboratory animals

acute toxicity

<u>studies with mussel extracts</u>

Toxicity of DSP toxins is usually measured by means of i.p. injection of extracts from contaminated mussels in mice. Although this is a crude comparison, it forms the basis of the most widely used screening and quality control methods.

When DSP toxins are given by the oral route, the lethal dose is 16 times higher than the i.p. dose but the symptoms are the same (Yasumoto *et al*., 1978).

Three to five mice (4 to 5 days old) receiving once orally by gavage 0, 0.05, 0.1, 0.2, 0.4 or 0.8 MU DSP toxins as 0.1 ml of a crude extract from contaminated scallops containing a drop of 1 percent Evans Blue solution per ml animals were kept for four hours at 25 ºC and killed. The whole intestine was removed and fluid accumulation was determined as the ratio of intestinal weight to that of remaining body weight (FA ratio). FA ratios in control, 0.05, 0.1, 0.2, and 0.4 MU groups were 0.072, 0.073, 0.09, 0.108 and 0.112, respectively. At 0.8 MU mortality occurred. The diarrhoeagenicity (as FA ratio) of the components of the crude mixture (OA, DTX1, DTX3, PTX1) in the suckling mice was as follows: OA and DTX1 had the same potency; diarrhoea was seen at doses ∅0.1 MU, with DTX3 diarrhoea was seen at doses ∅0.05 MU and PTX1 did not show diarrhoeagenicity at the doses tested (0.025 to 0.4 MU) (Hamano *et al*., 1986).

oral studies in mice with OA

After oral administration of 75 µg OA/kg bw to adult mice, the weight of the small intestines increased slightly within one hour (by fluid accumulation) but that of the liver decreased slightly. The lowest observed adverse effect level (LOAEL) in mice by acute oral administration was deduced to be 75 µg/kg bw (EU/SANCO, 2001).

Within one hour after oral administration of OA to mice, severe mucosal injuries in the intestine were seen. The injuries could be divided into three consecutive stages (Matias *et al*., 1999a):

- extravasion of serum into the lamina propria of villi;
- degeneration of absorptive epithelium of iliac villi;
- desquamation of the degenerated epithelium from the lamina propria.

Rat small intestine was stated to be the most sensitive and reproducible organ for studies of the diarrhoeic effects of marine toxins. When OA was injected in ligated loops from the middle duodenum of male rats (200 g) the following changes were seen within 15 minutes. Enterocytes at the top of the villi became swollen and subsequently detached from the basal membrane. Globet cells were not affected at the doses applied (1-5 σg OA). After 60 to 90 minutes, most of the enterocytes of the villi were shed into the lumen and large parts of the flattened villi were covered by globet cells. The degree of the damage was dose-dependent: 3 σg OA affected only the top of the villi, while 5 σg led to collapse of the villous architecture. Intravenous injection induced similar but less extensive changes (Van Apeldoorn *et al*., 1998).

oral studies in mice with DTXs

At oral doses of 100, 200, 300 or 400 µg DTX1 to mice 1/5, 0/5, 2/4 and 3/4 animals died respectively (Ogino *et al*., 1997).

oral studies in mice with PTXs

When oral doses of 25, 100, 200, 300 or 400 µg PTX2 were given to mice 1/4, 0/4, 1/5, 2/5 and 1/4 animals died respectively. This study did not show a dose-response. The oral toxicity of PTX2 is comparable to its i.p. toxicity (i.p. lethal dose in mice of PTX2 is 260 µg/kg bw) (Ogino *et al*., 1997).

At oral doses of 1.0, 2.0 and 2.5 mg PTX2/kg bw to mice, diarrhoea was seen in 1/5, 2/5 and 2/5 animals respectively. At 0.25 mg/kg bw, no diarrhoea was observed but the small intestine was swollen and filled with fluid (EU/SANCO 2001). Oral doses of 0.25 to 2.0 mg PTX2/kg bw in mice caused histopathological changes in liver as well as stomach and whole intestines. The oral dose of 0.25 mg PTX2/kg bw in mice is considered to be a LOAEL (EU/SANCO, 2001).

oral studies in mice with YTXs

At a maximum oral dose of 1.0 mg yessotoxin (YTX)/kg bw no lethality in mice was observed by Ogino *et al*. (1997). At this dose-level the mice gained weight during three days observation time (Yasumoto and Satake, 1998). Aune *et al*. (2002) reported that oral doses up to and including 10.0 mg YTX/kg bw did not cause mortality of female mice (not fasted). Microscopy revealed only moderate changes in the heart (slight intercellular oedema) at 10 and 7.5 mg/kg bw. At 5, 2.5 and 1 mg/kg bw no changes in the heart were seen by light microscopy. Ultramicroscopy revealed swelling of heart muscle cells leading to separation of the organelles. Effects were more pronounced close to the capillaries. These effects were dose-dependent and were only very slight at 2.5 mg/kg bw, which was the lowest dose examined by ultramicroscopy.

When YTX was given orally by gavage to four-day old suckling ddY mice at dose levels of 0.1, 0.2 and 0.4 σg/mouse as a 1 percent suspension in Tween 60 solution, no intestinal fluid accumulation was seen after four hours, whereas this phenomenon was seen at all dose-levels of OA or DTX1 (Ogino *et al*., 1997).

intraperitoneal studies

Thirty minutes to several hours after i.p. injection of DSP toxins in mice, inactivation and general weakness were seen and at sufficiently high concentrations mice died between one and a half and 47 hours. Concerning the effects reported after oral administration, it is of interest to compare the intraperitoneal (i.p.) toxicity of the different toxins in the DSP complex (see Table 3.1).

Table 3.1 Acute toxicity (lethal dose) of DSP toxins after i.p. injection in mice

Toxin	**toxicity (σg/kg bw)**	**pathological effects**
Okadaic acid (OA)	200	diarrhoea
Dinophysistoxin-1 (DTX1)	160-200[##]	diarrhoea
Dinophysistoxin-3 (DTX3)	500	diarrhoea
Pectenotoxin-1 (PTX1)	250	hepatotoxic
Pectenotoxin-2 (PTX2)	230-260[##]	hepatotoxic[*]
Pectenotoxin-3 (PTX3)	350	hepatotoxic[*]
Pectenotoxin-4 (PTX4	770	hepatotoxic[*]
Pectenotoxin-6 (PTX6)	500	hepatotoxic[*]
Yessotoxin (YTX)	100[**]	cardiotoxic[#]
	100-214[***]	
	286 [***] (LD_{50})	
45-OH yessotoxin (OH-YTX)	100	hepatotoxic[@]

Source: Van Egmond *et al*., 1993 and Ritchie, 1993 (except as indicated)

* presumed from the toxicity of PTX1
** fasted suckling male mice; at 80 σg/kg bw 1/3 mice died, at 100 σg/kg bw all 3 mice died (Ogino *et al*., 1997)
*** fasted male mice (Aune *et al*., 2002)
[#] data indicate damage to the heart
[##] Ogino *et al*. (1997)
[@] data indicate damage to the liver

Mice receiving an i.p. injection with 160 σg DTX1/kg bw died within 24 hours while suffering from constant diarrhoea (Van Egmond *et al*., 1993).

After intraperitoneal injections of 50-500 σg DTX1/kg bw into suckling mice (7-10 g) duodenum and upper portion of small intestine became distended and contained mucoid, but not bloody, fluid. Villous and submucosal vessels were severely congested at the higher concentrations. No discernible changes in organs and tissues other than the intestines were seen. At ultrastructural level, three sequential stages of changes of intestinal villi were observed as was seen after oral administration (see preceding page: oral studies in mice with OA) (Van Apeldoorn *et al*., 1998).

Marked dilation or destruction of Golgi apparatus suggests that DTX1 may directly attack this organelle (Van Apeldoorn *et al*., 1998)

OA and DTX1 induce also liver damage in mice and rats after oral as well as i.p. administration. The liver changes were expressed as degeneration of endothelial lining cells at the sinusoid. In addition, dissociation of ribosomes from the rough endoplasmic reticulum and autophagic vacuoles were seen in hepatocytes in midzone of hepatic lobuli. Haemorrhage in subcapsular region of the liver was observed. Furthermore OA, DTX1 and DTX3 induced damage to the epithelium in the small intestine after both oral and i.p. dosing (Van Apeldoorn *et al*., 1998).

PTX1 did not cause pathological findings in small and large intestine in suckling mice after i.p. injection but marked congestion of the liver and finely granulated surfaces of the liver were seen. Thirty to sixty minutes after i.p. injection of 1 000 σg/kg bw multiple vacuoles appeared around the periportal region of the hepatic lobules. Similar features were seen in livers from mice two hours after i.p. treatment with 500 or 700 σg/kg bw. Electron microscopy confirmed these light microscopic observations: Several portions of the microvilli of the hepatocytes became flat and the plasma membrane was invaginated into the cytoplasm. Within 30 minutes, vacuoles had increased in size and most of the cellular organelles had become compressed. Within 24 hours, almost all hepatocytes contained numerous vacuoles and granules and had become necrotic. Mice given i.p. 150-200 σg/kg bw showed only slight hepatic injuries after one hour (Van Apeldoorn *et al*., 1998).

YTX kills suckling mice at an i.p. dose of 100 σg/kg bw. Even at the lethal dose no intestinal fluid accumulation was seen in the suckling mice. Five week old male mice (bw 23-25 g) showed after i.p. doses above 300 σg YTX/kg bw normal behaviour for the first hours, but then suddenly dyspnea occurred and the mice died. No discernible changes in liver, pancreas, lungs, adrenal glands, kidneys, spleen or thymus were seen. Mice given i.p. 500 σg YTX/kg bw showed severe cardiac damage. Endothelial lining cells of the capillaries in the left ventricle were swollen and degenerated. Mice treated orally with 500 σg YTX/kg bw did not show changes (Van Apeldoorn *et al*., 1998).

Aune *et al*. (2002) gave i.p. injections of 0.1-1.0 mg YTX/kg bw in 1% Tween 60 to groups of three female white mice. At 1.0 mg/kg bw all three mice died and at 0.75 mg/kg bw two out of three mice died. Light microscopy revealed effects in the myocardium (slight intercellular oedema) at 0.75 and 1.0 mg/kg bw. Ultramicroscopy showed at 1.0 mg/kg bw swelling of myocardial muscle cells, separation of organelles, most pronounced near capillaries (no other dose-levels were examined by ultramicroscopy). The lethal effects seen at 0.75 mg/kg bw and higher, indicated a lower i.p. acute toxicity than reported in Table 3.1 above. Some of the reasons might be that in this study non-fasted female mice were used, whereas in the other studies fasted male mice of a different strain were used.

Male mice given 300 σg desulphated YTX (chemically prepared)/kg bw survived 48 hours. Desulphated YTX caused only slight deposition of fat droplets in the heart muscle. On the other hand, effects in liver and pancreas were seen. Within 12 hours after an i.p. dose of 300 σg/kg bw livers were pale and swollen. Fine fat droplets were found in all hepatocytes in the lobuli. Almost all mitochondria were slightly swollen and showed reduced electron density. Pancreatic acinar cells also showed degeneration. Disarrangement of the configuration of the rough endoplasmic reticulum was prominent within six hours. Mice treated orally with 500 σg desulphated YTX/kg bw developed fatty degeneration of the liver (Van Apeldoorn *et al*., 1998).

repeated administration

No data

reproduction/teratogenicity studies

Studies in pregnant mice demonstrated the transplacental passage of [^{3}H]-OA by measuring the radio-labelled compound 24 hours after oral administration of 50 σg/kg bw (dissolved in sterile water and methanol 50:50) at day 11 of gestation. Foetal tissue contained more OA than maternal liver or kidney: 5.60 percent of the administered label compared to 1.90 and 2.55 percent respectively as measured by scintillation counting and LC with fluorescent detection after derivatization with ADAM (Matias and Creppy, 1996b).

mutagenic activity of okadaic acid

OA did not induce mutations in *Salmonella typhimurium* TA 98 or TA 100 in the absence as well as the presence of a metabolic activation system, but it was strongly mutagenic in Chinese hamster lung cells without metabolic activation (mutagenic activity was comparable to that of 2-amino-N^{6}-hydroxyadenine, one of the strongest known mutagens). Diphtheria toxin resistance (DT^{r}) was used as marker of mutagenesis. Results indicated that OA increased the number of DT^{r} cells by induction of a mutation from the DT^{r} phenotype, and not by selection of spontaneously induced DT^{r} cells. The authors suggested that induction of DT^{r} mutation is not due to OA-DNA adduct formation, but probably operates via modification of the phosphorylation state of proteins involved in DNA replication or repair (Aune and Yndestad, 1993).

Using the ^{32}P-postlabelling method, DNA adduct formation was seen in two cell lines (BHK21 C13 fibroblasts and HESV keratinocytes) after treatment with OA for 24 hours (doses 0.01-5 nM). Low doses did not show adduct formation. Intermediate doses have given the most important number of adducts and with higher doses, the number of adducts decreased dose-dependently. Nineteen adducts were observed with BHK21 C13 cells and 15 with HESV cells. Ten adducts were similar in the two strains, while nine were specific of BHK21 C13 cell line, and five of HESV one (Fessard *et al*., 1996).

tumour promoting activity of okadaic acid and dinophysistoxin-1

OA and DTX1 are tumour promoters in two-stage experiments on mouse skin. OA and DTX1 do not activate protein kinase C as do the phorbol esters but inhibit the activity of protein phosphatase 1 and 2A, resulting in rapid accumulation of phosphorylated proteins. The effects of OA on protein phosphorylation in cellular systems emphasize the strong tumour-suppressing effect which PP1 and PP2A must have in normal cells. OA and DTX1 distinguish themselves from phorbol ester promoters by the fact that they do not bind to the same receptors. OA and DTX1 bind to a particulate fraction of the mouse skin. The binding sites of OA are also present in stomach, small intestine and colon, as well as in other tissues (Fujiki *et al*., 1988). OA and DTX1 and also PTX2 induce ornithine decarboxylase (ODC) in mouse skin (Fujiki *et al*., 1989). Furthermore OA induced ODC in rat stomach and enhanced the development of neoplastic changes (adenomatous hyperplasia and adenocarcinomas) in the rat glandular stomach after initiation with N-methyl-N'-nitro-N-nitrosoguanidine (Suganuma *et al*., 1992).

OA has been shown to promote morphological transformation of carcinogen (3-methyl-cholanthrene)-initiated BALB/3T3 cells. It was demonstrated that OA induced morphological transformation of BALB/3T3 cells also in the absence of an initiator (Sheu *et al*., 1995).

Induction of DNA adducts by okadaic acid was shown in Baby Hamster Kidney (BHK) cells, Human (HESV) keratinocytes and human bronchial epithelial cells. Also the induction of DNA adducts in zebra fish embryos was demonstrated. It was noted that the DNA adduct formation

increased with the dose at lower and intermediate (non cytotoxic) concentrations whereas higher concentrations caused toxic stress (Huynh *et al.*, 1998)

immunotoxicity of okadaic acid

The effect of OA on peripheral blood monocytes of humans *in vitro* by means of effects on the interleukin-1 (IL-1) synthesis was studied. OA induced a marked depression of IL-1 production in the monocytes at concentrations of 0.1-1.0 σg/ml. 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 this effect of OA is unknown (Aune and Yndestad, 1993).

in vitro toxicity

OA, DTX1, PTX1 and YTX were studied for their possible toxicity towards fresh rat hepatocytes by means of light and electron microscopy (Van Apeldoorn, *et al.*, 1998). OA was the most toxic. At 1 σg/ml blebs on the cell surface were seen. At increasing concentration blebs increased in size and number. At high concentrations the cells lost their circular appearance and became irregular. DTX1 showed at 2.5 σg/ml effects similar to those of OA, although to a lower degree. PTX1 gave quite different results. Morphological changes manifested themselves as small grooves on the cell surface and vacuoles in the cytoplasm in a dose-dependent pattern, starting at 7.5 σg/ml. Electron microscopy revealed invagination of the cell membrane and development of vacuoles.YTX was far less toxic. Between 25 and 50 σg/ml very tiny blebs on the cell surface were observed without changing the general spheric appearance of the cells. None of the four purified DSP toxins studied caused enzyme (lactate dehydrogenase) leakage from the cells.

Protein and DNA synthesis in Vero cells (from monkey kidney) were both inhibited by OA in a concentration-dependent manner (IC_{50} 3.3 x 10^{-8} and 5.3 x 10^{-8} M, respectively). RNA synthesis was inhibited with an IC_{50} of 8.2 x 10^{-8} M. The time lag before DNA and RNA synthesis inhibition occurred was longer (eight hours) than the time lag before protein synthesis occurred (four hours) indicating that protein synthesis is probably the main target and the first of OA's cytotoxic effect (Matias *et al.*, 1996).

In a later study (Matias *et al.*, 1999b), the effect of OA on the production of oxygen reactive radicals as possible inducers of impairment of protein synthesis was studied in the presence and the absence of oxygen radical scavengers (SOD+catalase, vitamin E and/or vitamin C). Lipid peroxidation appeared to be a precocious marker of OA exposure. The radical scavengers (partially) prevented the lipid peroxidation, but the inhibition of protein synthesis induced by OA was not reduced to the same level. This indicates that a more specific mechanism might be responsible for inhibition of protein synthesis.

In the cell free rabbit reticulocyte lysate specific mRNA is translated into globin. This was used to ensure that protein synthesis is a direct target of okadaic acid. Indeed in this system protein synthesis was also inhibited by OA in a concentration-dependent manner (Matias *et al.*, 1996).

Matias and Creppy (1998) studied the effect of OA on the five nucleosides (deoxycytosine, 5-methyldeoxycytosine, desoxythymidine, deoxyguanidine and deoxyadenine) in the DNA of Vero cells. At 7.5 ng OA/ml no significant inhibition of DNA synthesis was seen but hypermethylation of DNA was induced. The level of 5-methyldeoxycytosine increased from 3.8 to 7.8 percent, indicating possible interference with DNA regulation, replication and expression. Higher levels of OA inhibited DNA synthesis but failed to increase the rate of DNA methylation. Since OA is involved in tumour production, the most threatening effects are those possibly connected with DNA modification and/or regulation of gene expression, such as the rate of methylation. In other

terms, the risks for humans and animals may be more related to repeated exposure to low OA concentrations in seafood that could assault the DNA several times within a life span.

Primary cultures of liver cells of 11 days old chick embryos were exposed to PTX1 and the effects were examined by fluorescence microscopy. PTX1 reduced the cell size. Microtubules were reduced in number and lost their radial arrangement. Stress fibres (actin filament bundles) disappeared and actin became accumulated at the cellular peripheries. At exposure to concentrations Ω0.5 σg/ml for less than four hours these effects were reversible within 24 hours (Zhou *et al*., 1994).

The effect of OA on cultured human intestinal epithelial T_{84} cell monolayers was studied by measuring electrophysiological parameters, lactate dehydrogenase release, and $^{22}Na^{+}$ and [^{3}H] mannitol flux rates. Protein phosphorylation studies were carried out to identify potentially involved proteins. OA did not directly stimulate Cl^{-} secretion but increased the paracellular permeability of intestinal epithelia. This alteration may contribute to the diarrhoea of DSP poisoning (Tripuraneni *et al*., 1997).

YTX appeared to have an effect on the cytosolic Ca^{2+} levels of freshly isolated human lymphocytes. YTX modulated intracellular Ca^{2+} of human lymphocytes by producing a slight non-capacitative calcium entry and inhibiting the Ca^{2+} entry produced by emptying of internal calcium stores. OA did not cause these effects. The authors suggested interaction of YTX with plasma membrane calcium channels (De la Rosa *et al*., 2001).

3.5.4 Toxicity to humans

Shellfish containing more than 2 σg OA/g hepatopancreas and/or more than 1.8 σg DTX1/g of hepatopancreas are considered unfit for human consumption (Hallegraeff, 1995). The predominant symptoms in humans include diarrhoea, nausea, vomiting and abdominal pain. The onset of symptoms, which are never lethal, ranged from 30 minutes to a few hours after ingestion of the toxic shellfish, with complete recovery within three days. The intensity of the symptoms in humans depends upon the amount of toxin ingested. Hospitalization is usually not needed. Among the DSP toxins, OA, DTX1 and DTX3 are the most important in causing diarrhoea in humans (Aune and Yndestad, 1993). DTX2 was reported to be the predominant diarrhoeic DSP toxin in Ireland during a prolonged DSP episode (Carmody *et al*., 1996). Epidemiological data from Japan (1976-1977) indicated that as little as 12 MU was enough to induce a mild form of poisoning in humans (EU/SANCO, 2001). MU was defined as the amount of toxin (later defined as DTX1 in the Japanese study) killing a mouse by i.p. injection within 24 hours and 12 MU corresponded to 43.2 µg, which can be considered as a LOAEL for DTX1 (EU/SANCO, 2001). However Yasumoto *et al.* (1985) reported that the minimum dose of DTX1 for the induction of toxic symptoms in human adults was 32 µg. Fernandez and Cembella (1995) reported that 1 MU corresponded to approximately 3.2 µg DTX1 and 4 µg OA which means that the minimum dose for toxic effects in humans is 38.4 and 48 µg for DTX1 and OA, respectively. The probable human health problems associated with tumour-promoting, mutagenic and immunosuppressive effects shown in animals and experimental systems by OA and DTX1 cannot yet be quantified.

Concerning two other chemical groups of the DSP complex, the PTXs and YTXs, the situation is unsatisfactory. PTXs have a low diarrhoeic potential and YTXs do not induce diarrhoea in rodents, but both groups of toxins are lethal to mice at i.p. injection and they exert toxicity to liver and heart, respectively, in rodents. It is unclear whether PTXs or YTXs pose a health threat to consumers of contaminated mussels (Aune and Yndestad, 1993). In a pipi DSP event (56 cases of hospitalisation) in New South Wales, Australia in December 1997 (ANZFA, 2001) PTX-2 seco acids may have contributed to the gastrointestinal symptoms, vomiting or diarrhoea in humans

(Quilliam *et al*., 2000 in Aune, 2001). Burgess and Shaw (2001) reported that the patients consumed approximately 500 g of pipis containing 300 µg PTX-2SA/kg (~150 µg PTX-2SA/person~2.5 µg/kg bw for a 60 kg weighing person).

During a DSP episode in Norway in 1984, a few people were hospitalized with symptoms of severe exhaustion and cramps, in addition to the usual DSP symptoms. After intravenous injection of an electrolyte mixture, the patients recovered within a few days (Aune and Yndestad, 1993). In a recent incident in Norway, about 70 people were served blue mussels during the opening ceremony of a new mussel farm. Among the guests, 54 percent were intoxicated with typical DSP symptoms. DSP toxin levels in the left-over mussels were found to be around 55 to 56 µg OA eq/100 g mussel meat (Aune, 2001).

3.5.5 Toxicity to aquatic organisms

OA inhibited the growth of a variety of non-DSP producing microalgae at micromolar concentrations. The effects of DTX1 on microalgal growth were found to be equivalent to those of OA, and the effects of a mixture of both toxins were simply additive. The growth of the DSP toxin producing dinoflagellate *P. lima* was not affected (Windust *et al*., 1996).

Concentrations of ∅3 σM of both OA-diol ester and OA inhibited almost completely the growth of the diatom *Thalassiosira weissflogii*. (EC_{50} 2.2 and 1.0 σM for OA-diol and OA, respectively). This result is in contradiction with the accepted idea that only the free acid toxins, such as OA and DTX1, are potent phosphatase inhibitors. Substantially higher concentrations of DTX4 were required to detect any effect on the growth. The OA-diol ester was shown to be partially hydrolysed to OA (7 percent hydrolysed, 20 percent unchanged OA-diol ester and 73 percent was unidentified). This phenomenon does suggest that cells exposed to inactive DSP toxin esters could metabolically activate them. In an additional experiment both DTX4 and OA-diol ester were hydrolysed (2.0 and 2.7 percent, respectively, within five days) to OA spontaneously and not by mediation of the presence of *T. weissflogii*.

The inactive DTX4 can apparently be hydrolysed through uncharged, lipophilic intermediates ultimately to yield the active, free acid toxin OA (Windust *et al*., 1997).

Ichthyotoxicity of yessotoxin (YTX) and bisdesulfated YTX (dsYTX) was studied using killifish (*Oryzias latipes*). YTX was diluted in 0.1 ml methanol and the solution was diluted with water to 50 ml to prepare a 1.0 or 0.5 mg/L test solution. A killifish was placed in a beaker with test solution and observed for 24 hours. The assay was run in triplicate. Similarly, dsYTX was tested at 0.5 mg/L only. None of the fish exposed to 1 or 0.5 mg/L YTX died within 24 hours. Three fish exposed to 0.5 mg/L dsYTX died after six hours (Ogino *et al.*, 1997).

3.6 Prevention of DSP intoxication

3.6.1 Depuration

The rate of DSP toxin loss varies with the season. Low water temperatures apparently retard toxin loss; however, the degree to which temperature affects the uptake and release of toxins is unknown. The rate of detoxification is highly dependent on the site of toxin storage – that is toxins in the gastrointestinal tract (e.g. *Mytilus*) are eliminated much more readily than toxins bound in tissues. Information concerning bivalve molluscs reared in aquaculture showed that retention time of the toxin in *Mytilus edulis* varied from one week to six months. Studies with mussels reared in an aquaculture pond and in the laboratory showed that a highly toxic (three MU) level of DSP toxins dropped to acceptable levels more quickly in the aquaculture pond than in the

laboratory. It was suggested that the quality of food available to the mussels during detoxification may affect the rate at which toxins are eliminated (Hallegraeff *et al*., 1995).

The rate of removal of DSP toxin from shellfish (depuration rate) most likely depends upon the species and may be affected by such interrelated factors as feeding or pumping of the shellfish, temperature, salinity and the level of non-toxic algae and particulates. In Japan, DSP toxins decreased from 4.4 to 2.5 MU/g (by mouse bioassay) in one week and then to 0.5 MU/g by the next week. In the Netherlands, toxicity in mussels was no longer detectable by rat bioassay after four weeks at water temperatures of 14 to 15 ∀C (Hungerford and Wekell, 1992). At the coast of Sweden (water temperatures 1.4 to 3 ∀C) after the bloom had subsided, OA levels in mussels decreased in one week from 7.2 to 1.8 σg/g hepatopancreas as measured by LC with fluorescence detection (Edebo *et al*., 1988b). Except for a method to reduce PSP levels in Mediterranean cockles, there are currently no useful methods available for effectively reducing phycotoxins in contaminated shellfish. All methods tested until now (generally tested for reducing PSP toxins such as transfer of shellfish to waters free of toxic organisms for self-depuration, vertical displacement of mussels in the water column as a means of minimizing toxin accumulation, ozone treatment of the water, temperature or salinity stress, electric shock treatments, reduced pH or chlorination, cooking) appeared to be unsafe, too slow, economically unfeasible or yielded products unacceptable in appearance and taste (Hallegraeff *et al*., 1995). Only after very rigorous boiling (163 minutes at 100 ∀C) toxin denaturation occurs (Scoging, 1991).

Mussels *(M. galloprovincialis*) from Galicia in northwestern Spain contaminated with DSP toxins were transplanted to several uncontaminated sites having different environmental conditions (salinity, temperature, fluorescence, light transmission). The depuration kinetics of OA in each batch was monitored during a 70 day period. Fluorescence and light transmission appeared to have the most prominent effect on depuration. In most cases, there was an inverse relation between depuration and body weight. It could not be clearly concluded whether the DSP depuration evolves following 1- or 2-compartment kinetics (Blanco *et al*., 1999).

González *et al*. (2002) reported preliminary results about the instability of free okadaic acid in a supercritical atmosphere of carbon dioxide with acetic acid. Most of the toxin (up to 90 percent) was eliminated and the biological activity against phosphatase was also severely affected (up to 70 percent reduction). Detoxification of contaminated shellfish required a partial dehydration and the detoxification yield was lower than that obtained with the free toxin. Toxin content of partially freeze-dried mussel hepatopancreas containing 1 µg of OA/g was reduced to 51 to 57 percent after 190 minutes of exposure to the supercritical mixture.

3.6.2 Preventive measures

The prevention of shellfish-borne diseases requires monitoring of the marine environment and shellfish flesh. Frequent inspection of seawater around aquaculture facilities or shellfish farms for the presence of toxin producing strains of phytoplankton is an approach that is gaining support in several countries, and has received considerable impetus following the discovery that toxin-producing algae have been transferred in the ballast water of ships to completely new marine locations around the globe (Wright, 1995).

Data on the occurrence, type and concentrations of toxic algal species may indicate which toxins may be expected during periods of algal blooms and which seafood products should be considered for analytical monitoring. One problem is that certain algal species, which have never occurred in a certain area, may suddenly appear and then rapidly cause problems. Nevertheless, several countries have monitoring programmes to check for the occurrence of (toxic) phytoplankton species in areas where shellfish are grown. Some countries monitor the presence of only one or

two algal species, while others check for a long list of species. In some countries, the shellfish areas are closed when the number of cells of certain algal species exceeds certain concentrations according to the type of species. Other countries close their harvest areas only when the toxins have been detected in the shellfish. Closure of harvesting areas in Italy occurs when the presence of toxic algae in water and toxins in mussels are observed simultaneously (Hallegraeff *et al*., 1995).

The principal strategy to prevent DSP intoxication is effective monitoring of mussels with respect to DSP toxins so that contaminated products do not reach the market. However, the presentation above shows that weekly sampling may be insufficient for maximum protection of human health in endemic areas. A reliable sample plan is required in addition to efficient means of detection. However, several factors complicate efficient monitoring (Aune and Yndestad, 1993) including:

- cell numbers of toxin producing algae needed to produce toxic mussels vary considerably;
- time period of toxicity varies with region and episode;
- implicated toxins and algae may be different in different regions;
- simultaneous presence of DSP and PSP toxins complicates monitoring;
- toxicity of contaminated mussels can vary several fold with different depths even at the same sampling location;
- other seafood, like oysters, might also be contaminated, although at a lower level.

3.7 Cases and outbreaks of DSP

3.7.1 Europe

Occurrence of DSP toxins from 1991 to 2000 in coastal waters of European countries that are members of the ICES is illustrated in Figure 3.3.

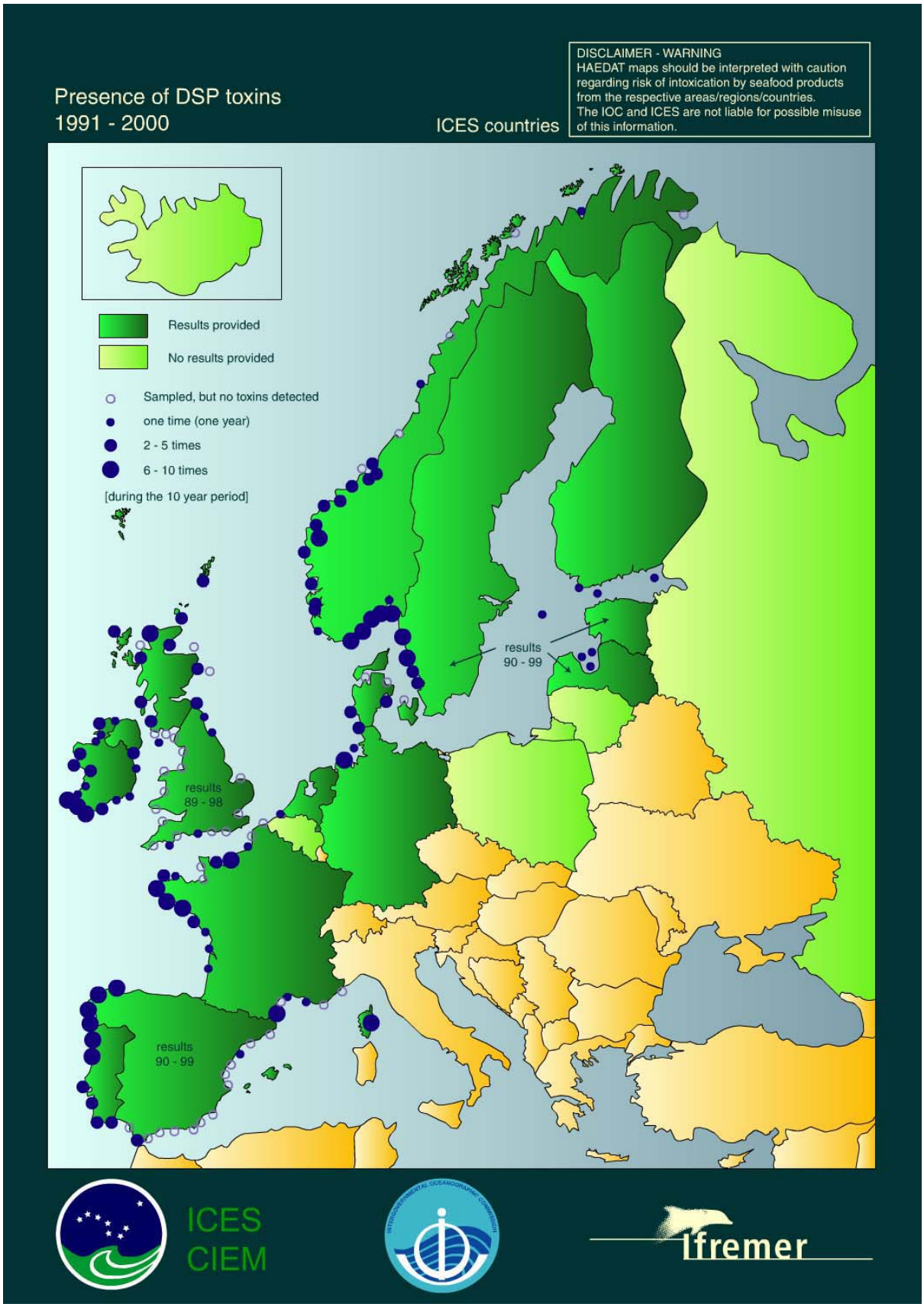
Belgium

During 1999, one out of 350 samples gave a positive result for DSP in the mouse bioassay (EU-NRL, 2000). In Antwerp in February 2002, 403 cases of DSP were reported after consumption of blue mussels imported from Denmark. The mouse bioassay for the presence of okadaic acid, dinophysis toxins, yessotoxin, pectenotoxins and azaspiracid showed a positive result. LC-MS techniques confirmed this result. In the mussels, 5.9 µg AZA/kg of meat was found (below regulatory limit), 229 µg free OA/kg of meat and 300 µg OA eq (conjugated OA or diol ester)/kg of meat. In spectrometry, a significant peak corresponding to pectenotoxin-2-seco-acid (PTX2-SA) was observed, but this toxin could not be quantified. The remainder of the imported mussels was withdrawn from sale (De Schrijver *et al*., 2002).

Croatia

Toxin analysis (mouse bioassay and LC) of *Mytilus galloprovincialis* from the Central Adriatic Sea (Kastela Bay) in the summer of 1994 led to identification of OA and DTX1. No health problems due to consumption of intoxicated seafood were registered (Orhanovic *et al*. 1996). During an intensive bloom in the summer of 1995 *M. galloprovincialis* were harvested from Kastela Bay. Mouse bioassay displayed a positive result for DSP toxins. LC analysis showed the presence of OA, the absence of DTX1 and DTX2, and suggested the presence of an unknown derivatized compound at high concentration. The origin of the mussel toxicity was traced to *D. sacculus*. (Marasović *et al*., 1998).

Figure 3.3 Occurrence of DSP toxins in coastal waters of European ICES countries from 1991 to 2000



Source: http://www.ifremer.fr/envlit/documentation/dossiers/ciem/aindex.htm

Denmark

In 1990, 170 σg OA/100 g of meat was detected in mussels on the north Danish coasts. Mussels from this area were imported by France, poisoning 415 persons (Van Egmond *et al*., 1993). Three toxic events took place during 1999. In the first two cases, domestic production areas were closed for some weeks because of the presence of DSP toxins in blue mussels. The third case was due to mussels caught in the North Sea. Two persons from the staff of a production company became ill because they ate the mussels before the mouse bioassay was carried out (EU-NRL, 2000). In 2000, OA was detected below the limit of 160 µg/kg whole mussel in samples from the East Coast of Jutland. This observation took place during a period where the fishery was restricted and/or closed due to high concentrations of the species *Dinophysis acuminata* (EU-NRL, 2001). During 2001, DSP toxins were registered in commercially fished blue mussels in concentrations exceeding the regulatory limits in three production areas. In 2002, much more DSP toxins were present than normal. Several production areas were closed for several weeks or months. DSP levels above the regulatory limit were detected in the Limfjord, on the east coast of Jutland, the Roskilde Fjord/Isefjorden and in the Wadden Sea, North Sea. In November and December 2002, people in Germany became ill due to the consumption of mussels containing OA from the production area Isefjorden. In Belgium (see above), people also became ill due to the consumption of Danish mussels (EU-NRL, 2002).

France

In several areas of France (Normandy, Loire-Atlantique, South Brittany, West Brittany, Mediterranean coasts), cases of DSP poisoning of shellfish consumers have been reported from 1978 onwards. In 1984 and 1985, mussels raised in France caused DSP symptoms in 10 000 and 2 000 people respectively (Durborow, 1999). Maximum algal densities are several thousand cells/litre on the Atlantic and Mediterranean coasts, whereas in the eastern part of the English Channel (north of the Seine estuary) densities can reach more than 100 000 cells/litre. The toxin produced is essentially OA (Van Egmond *et al*., 1993). Dinophysis species have been found in both Mediterranean and Atlantic coasts (EU-NRL, 1998); twenty seven production areas on the Atlantic and two production areas on the Mediterranean coast were closed due to the presence of DSP (EU-NRL, 2000). At the end of 1998 *A. tamarensis* was detected at concentrations of up to 350 000 cells/litre and some production areas for clams, oysters and mussels were closed for two months. Positive DSP results were obtained in shellfish from Ireland and Tunisia in 1999 (EU-NRL, 2000). In 2000, several production areas on the Atlantic coast and one area on the Mediterranean coast were closed due to DSP toxins (EU-NRL, 2001). Several DSP toxic episodes were observed in 2002. Along the southern Brittany coast and the coast near the Loire River, DSP toxins were recorded in shellfish very late in autumn 2002 and a few areas remained closed in December. This was the first time that so many DSP events were observed along the French Atlantic coasts (EU-NRL, 2002).

Germany

OA was detected in mussels from the Wadden Sea in 1987. *Dinophysis acuminata* was found regularly along the coasts of German Bight. Mussels causing DSP generally came from North and East Frisia between 1986 and 1989. In 1990, more than 1 000 *Dinophysis* cells/litre (max 25 000 cells/litre) were detected in the areas as mentioned above. The mussel beds were closed and no cases of human DSP poisoning occurred (Van Egmond *et al*., 1993). In 1998, OA was detected in September (EU-NRL, 1998). During 1999, two samples contained DSP toxins but below the regulatory limit (EU-NRL, 2000). There was one case of DSP intoxication in 2000 involving two elderly women (EU-NRL, 2001). During October 2001, increased cell density of *D. acuminata* (5.9×10^3 cells per litre) was observed at the East Frisian coast waters. DSP toxins in mussels increased and a ban was placed on mussel harvesting in the contaminated area (Anonymous, 2001b).

Greece

A total of 10 positive DSP samples were recorded during 1999. *D. acuminata* was detected at a concentration of 1 500 cells/litre (EU-NRL, 2000). During April to June 2000, one production area was closed due to the presence of DSP toxins (EU-NRL, 2001).

Ireland

The first events of DSP poisoning occurred in the 1980s. Mussels showed variable toxicity levels in 1984, and from 1987 to 1991. In 1988, *D. acuminata* (1500 cells/l) and *D. acuta* (240 cells/l) were detected on the southwest coast in Roaring Water, Dunmanus, Bantry, Kenmare and Dingle Bays and up to 200 σg OA/100 g and 25 σg DTX1/100 g of mussel meat was found. In Glengariff in 1990, an isomer of OA contributed to the residual toxicity observed in mussels after the total disappearance of OA, while toxicity during the winter was detected for the first time that year (Van Egmond *et al*., 1993). Both OA and DTX2were present in mussels in a DSP episode in 1991. Examination of similar mussel cultivation locations in 1994 showed that DTX2 was even more predominant (OA levels were less than 0.7 σg/g and max DTX2 levels 6.3 σg/g hepatopancreas) The toxicity in shellfish was seen soon after high cell counts of *Dinophysis acuta* (Carmody *et al*., 1996). In addition, a new isomer of DTX2, named DTX2B, was isolated and identified in Irish mussel extracts (James *et al*., 1997). In shellfish from Irish waters acyl-derivatives of OA and DTX2 were also detected (EU-NRL, 1996). Unexplained human intoxication with DSP symptoms following the consumption of mussels from Killary, Ireland, was resolved by the isolation of a new toxin ($C_{47}H_{71}NO_{12}$), tentatively named KT3, which represents a new class of polyether shellfish toxin later called azaspiracids (Satake *et al*., 1997) (see also Chapter 6).

During 1999, five percent of 1 800 samples tested for DSP/AZP were positive in the mouse bioassay (EU-NRL, 2000). In August 2000, 30 areas were closed for the harvesting of bivalve shellfish. People developed symptoms of DSP (Anonymous, 2000a). In 2001, 17 percent of samples were positive in the mouse bioassay compared to 3.4 percent in 2002. During 2002, the highest level of DSP toxins (OA, DTX2) found in oysters was 30 µg/kg. Seven percent of sampled mussels contained OA or DTX2 above the regulatory limit (EU-NRL, 2002).

Italy

On the Northern and Central Adriatic coast, *Dinophysis* spp. were present from 1989 onwards and DSP cases have been reported. A monitoring programme over the Italian shellfish banks was started in 1989. Species implicated were *D. sacculus, D. fortii* and *Dinophysis* spp., with maximum concentrations of 4 000 cells/l (Tubaro *et al*., 1992). Samples of toxic mussels and of algae of *Dinophysis* genus, both collected in occasion of algal blooms from the coastal area of Cesenato, have been analysed by ionspray LC-MS (LC-ISP-MS) for DSP toxins. OA was present in all mussel samples and its concentration (0.178-0.286 σg per g of edible tissue) exceeded the regulatory limit (0.16 σg per g of edible tissue). DTX1 was also found in some samples and its concentration (Ω0.076 σg per g of edible tissue) was lower than the amount which was thought to cause toxic effects in mice (0.13 σg per g of edible tissue). However, this toxin was never detected in toxic phytoplankton. LC-ISP-MS analysis of algal cells has for the first time unambiguously shown that *Dinophysis fortii* produced or transmitted OA to shellfish (Draisci *et al*., 1996b). As *Dinophysis* spp., particularly *D. sacculus*, are common species along the Italian coast, the presence in 1988 of large summer blooms (40 000 cells/l) in the briny lagoons of northeastern Sicily can be considered as a potential restriction to the expansion of aquaculture in these areas (Van Egmond *et al*., 1993). Salati and Meloni (1994) mentioned that *Dinophysis* spp. and *Prorocentrum* spp. are in fact common in Italian seas and that cases of DSP in Italy occurred in 1989, 1990 and 1991. The presence of PTXs has been recorded in the Adriatic Sea (EU-NRL, 1996). The occurrence of different DSP producing species such as *Dinophysis* spp.,

Lingoludinium polyedra and *Protoceratum reticulatum* in Italian waters was stated. A mixture of OA, low levels of DTX1, YTX and PTX has been found in phytoplankton and shellfish (EU-NRL, 1998).

In the digestive gland of mussels from the Adriatic Sea, besides YTX, 2 new analogues of YTX, homoyessotoxin and 45-hydroxyhomoyessotoxin were identified (Ciminiello *et al*., 1997). *Gonyaulax polyhedra* was implicated as responsible for the YTX contamination in these mussels (Tubaro *et al*., 1998).

During 1999, DSP toxins were detected in 350/900 samples of *Mytilus galloprovincialis* from the Northern Adriatic. The main problem area was Emilia Romagna. YTX always dominated over OA (EU-NRL, 2000). During 2000, DSP toxins were detected in *M. galloprovincialis* with 13 percent of the samples giving positive mouse bioassays. In the Emilia Romagna region, closures were enforced from late August through to end December, in the Veneto region closures were enforced from late October through to end December, and in Fruili Venezia Giulia closures were enforced in late December. The closures were mainly due to YTXs. In 2001, DSP toxins were detected in *M. galloprovincialis* with 18 percent of samples giving positive bioassay results. Closures were enforced in the Emilia Romagna region in January, February and early March, which was a continuation of the 2 000 closures, and also later during the period mid-June to late October. In Veneto, closures were enforced during July while in Friuli Venezia Giulia closures were enforced from January to mid-February and again from start of July to early August (EU-NRL, 2001). During 2002, DSP was detected in the Northern Adriatic Sea (Friuli Venezia Giulia, Veneto and Emilia Romagna coast). Harvesting was forbidden. In July, *Pecten maximus* samples from Scotland appeared to be positive for DSP (EU-NRL, 2002).

The Netherlands

The first reported cases of DSP in the Netherlands were in the 1960s (Fleming, 2003). From 1961, DSP has occurred on the Wadden Sea coasts. Maximal concentration of *D. acuminata* was 10 000 cells per litre in 1981 and dropped to only 80 cells/ per litre in 1986 and 1987 but with the same toxic effects. It seems that water temperature and mussel toxicity are strongly correlated. At 10 ∀C only 30 cells per litre would be required to maintain high mussel toxicity. Maximum level of *D. acuminata* occurs every year in August and September on the Wadden Sea coasts, with salinities of 30 $^{o}/_{oo}$ and a certain correlation with wind velocity. Densities above 10 000 cells/litre occur only when the wind is equal to or less than two on the Beaufort scale (Van Egmond *et al*., 1993). In 1998, it was reported that DSP producing species were present in Dutch waters, but no DSP toxins were found in bivalves (EU-NRL, 1998). In the summer of 2001, blooms of *D. acuminata* occurred in the Dutch Wadden Sea and mussels were contaminated. The blooms were caused by salt stratification and warm weather (Peperzak *et al*., 2002). A toxic event occurred in Grevelingen in the spring of 2002. Rat bioassay pointed to DSP. In the autumn of 2002, DSP was found in mussels from the Wadden Sea area (OA levels of 160-320 µg/kg by rat assay) (EU-NRL, 2002).

Norway

D. acuminata is often seen on Norwegian coasts. During a DSP outbreak in the Oslo Fjord in 1979, 1 900 cells/l were counted concomitant with proliferation of *Prorocentrum minimum. P. lima* blooms also occur sometimes in the Oslo Fjord (Van Egmond *et al*., 1993). Since 1984, DSP has been detected annually in mussels from the southeast and parts of the western coast of Norway. DSP has not been detected in oysters. *Dinophysis* spp. are regularly found in rather high numbers for long periods of the year. During 1984/85, widespread intoxications due to DSP occurred (Underdal, 1989).

Three to four hundred cases of DSP were recorded in 1984 in southeast Norway during a contamination period lasting from October 1984 to April 1985 at toxicity levels of approximately 7 σg OA-equivalents/100 g of hepatopancreas and 30 000 cells/litre of *D. acuta* and *D. norvegica*. *D. acuminata* and *D. acuta* blooms were seen in Skagerrak in 1985 and 1986, and DSP levels in mussels exceeded toxic thresholds in 1989 and 1990. Mussels from Arendal and Sognefjord showed multiple toxin patterns in 1985 and 1986; OA at Arendal and DTX1 and YTX at Sognefjord (Van Egmond *et al*., 1993). YTXs have also been reported in shellfish from Norway (EU-NRL, 1996). In Sognefjord DTX1 is the major DSP toxin, while OA is the relevant toxin in the rest of the coast (EU-NRL, 1998). Different closures due to DSP occurred in 1994. The same pattern was seen the following years (EU-NRL, 1998).

Mussel samples from four different locations along the Norwegian coast were found to be highly toxic in the mouse bioassay with symptoms indicating the presence of non-diarrhoeic toxins (cramps). Chemical analysis showed that OA and DTX1 were each present at one location but only a minor part of total toxicity could be attributed to these toxins. OA and DTX1 were absent at the two other locations. Incubation of extracts of samples from the four locations with freshly prepared rat hepatocytes indicated the presence of unknown toxin(s). Intraperitoneal and oral administration of purified mussel samples to baby mice showed that oral toxicity was 25 to 50 times lower than i.p. toxicity. The preliminary results indicate a large margin of safety between the amount of mussels consumed by humans and the large amounts of mussel extract needed to yield toxic effects in the intestine and liver in mice after oral exposure (Aune *et al*., 1998). During 1999, 135/473 samples gave positive results for DSP. On many occasions, YTX was the dominant toxin and in approximately 33 percent of cases, closures of production areas were due to the detection of YTX (EU-NRL, 2000). In 2000, 45 percent of 414 samples gave positive results in the DSP mouse bioassay. In late July, closures of production areas due to DSP toxins occurred in the southern part of Norway and the locations stayed closed until Easter 2001. Until October 2001, 26 percent of 915 samples were positive for DSP in the mouse bioassay (EU-NRL, 2001). Another species that probably caused problems in southern Norway (Lysefjorden) in 2000 and 2001 was *Gonyaulax grindleyi*. An indicator was the high rate of yessotoxin in cultured mussels. However, until now it cannot be said for sure that it was due to *G. grindleyi* (Hufnagl, 2001).

Portugal

DSP toxins have been detected in Portugal since 1987 but no human poisoning has occurred. At levels of 200 cells/litre of *D. sacculus* and *D. acuta* shellfish are contaminated after a brief latency period. On the north coast of Portugal, molluscs were contaminated by DSP toxins in 1988. The species involved was *D. acuta*. In 1988, the occurrence of DSP toxins was also reported after a *Prorocentrum lima* bloom in the Ria Formosa Lagoon. In 1989, *D. acuminata*, *D. sacculus* and *D. caudata* (around 1 600 cells/litre) caused DSP contamination on the Algarve coast (Van Egmond *et al*., 1993). OA was the main toxin responsible for DSP cases in Portugal (Gago-Martinez *et al*., 1993) but DTX2 was also recorded in shellfish and phytoplankton, and acyl derivatives of OA and DTX2 in shellfish (EU-NRL, 1996).

DSP episodes in southern Portugal have increased in frequency. *D. acuta* has been related with the occurrence of DTX2. Preventive closures have been due to DSP (EU-NRL, 1998). In 2000, OA and DTX2 were detected at high concentrations in the Aveiro Lagoon where the green crab, a shellfish predator, also accumulated these toxins. Several people became ill (EU-NRL, 2001). In the summer of 2001, an outbreak of DSP was reported after eating razor clams (*Solen marginatus*) containing 50 µg OA eq/100 g, harvested at Aveiro lagoon. All shellfish species tested in this region (except oysters) contained levels of OA and its esters above the regulatory limit (57-170 µg/100 g). One patient may have developed DSP after eating a large number of green crabs (*Carcinus maenas*, a shellfish predator) containing at least 32 µg OA eq/100 g (Vale and De Sampayo, 2002). In 2002, blooming of *D. acuminata* led to prolonged closures of wild intertidal

mussel and bentonic bivalve harvesting areas along the entire northwest coast. Recreational harvest of rock mussels, as well as cockles, caused several events of human poisoning. A maximum level of 1 860 µg total OA/100 g whole flesh was registered in wild mussels in September at Povoa do Varzim, connected to a dozen cases of severe gastroenteritis. Eighteen percent of 738 samples were positive in the mouse bioassay and PTXs were detected for the first time. So far, PTX1 and PTX2 levels have not exceeded 160 µg/kg by LC-MS. Contamination is mostly due to PTX2-SA (EU-NRL, 2002).

Spain

D. acuminata has been the main problem in the Spanish "Rias", except for some years (1989 and 1990) when *D. acuta* was the causative species involved in DSP outbreaks. The first confirmed DSP event was in 1978. *Gymnodinium catenatum* and *Dinophysis acuta* have sometimes entered the estuaries together and caused mixed PSP/DSP contaminations. In 1981, *D. acuminata* and *D. acuta* were associated with DSP events causing 5 000 cases of gastroenteritis throughout Spain. Other cases of DSP contamination were observed from 1982 to 1984. This phenomenon subsequently spread from 1989 to 1990 when *D. acuta* rather than appearing in September and October, was present from July to December reaching maximums of 7 000 to 22 000 cells/litre. *Prorocentrum lima* might have been associated with DSP contamination of mussels cultivated on ropes. In addition to OA, this species produced DTX1 and other compounds such as palytoxin (Van Egmond *et al*., 1993).

In 1993, a particularly bad episode occurred in Galicia, which lasted for an unusually long period. Analyses of Galician mussel samples revealed a very complex toxin profile with both DSP and PSP toxins present. Two DSP toxins, OA and DTX2, were detected. (Gago-Martinez *et al*., 1996). Intense DSP episodes led to prolonged closures in Galicia from April to December 1995. In 1996, closures took place only in January as a continuation of the 1995 episodes. In 1997, closures due to DSP occurred in spring, summer and/or autumn depending on the locations (EU-NRL, 1998). Fernández *et al*. (1996) reported, besides OA and DTX2, the occurrence of two polar derivatives of OA and DTXs in Spanish shellfish or phytoplankton: 7-*O*-acylesters containing a fatty acyl group attached to the 7-OH group and diol esters in which the carboxylic group of the toxins has been esterified. Throughout 1999 and 2000, there were different toxic events related a.o. to the presence of DSP toxins that led to the prohibition of harvesting of bivalves in some production areas (EU-NRL, 2000; EU-NRL, 2001). During 2002, toxic events occurred in Galicia (*D. acuminata* and *D. acuta*) and Andalucia (*D. acuminata*) causing long closure periods, and in Cataluña causing short closure periods (EU-NRL, 2002).

Sweden

Experimental mussel farming started in Sweden in 1971. During the developmental period from 1971 to 1980, mussels were harvested throughout the year. In 1983, DSP was observed among people consuming mussels. Consequently, a surveillance system to detect DSP toxins in mussels was launched in 1986. DSP toxin found in Swedish mussels is OA produced by *Dinophysis* spp. DSP toxin concentrations found in mussels from the outer archipelago are higher than in mussels from more sheltered waters. During the winter of 1989 to 1990, the harvest of farmed mussels was stopped for a long period due to high OA concentrations. The appearance of OA in mussels was ascribed to the inflow of water from the open sea containing toxic plankton. Appreciable concentrations of toxic dinoflagellates in offshore waters have been demonstrated in Skagerrak. The few observations of increased OA-concentrations in mussels in the sheltered fjords north of Orust occurred in connection with greater inflows of offshore water (Haamer, 1995). In 1997, OA and DTX2 were found, but isolated to minor cases (EU-NRL, 1998). During 1999, there was a peak in DSP toxicity in June and July. Closures of production areas are common in Sweden during the period September to March (EU-NRL, 2000).

The United Kingdom of Great Britain and Northern Ireland

The first occurrence of DSP in the United Kingdom was in 1997 when 49 patients presented symptoms 30 minutes after consuming mussels in two London restaurants (Durborow, 1999). In 1999, DSP events seemed to have become more frequent and prolonged (EU-NRL, 2000). At the beginning of 2000, DSP toxins were still detectable in mussels from Cornwall. Later that year, toxins were found in cockles from the southeast of England and from south Wales. Harvesting restrictions were enforced (EU-NRL, 2001). From July 2001 up to August 2002, there was an on-and-off ban on the harvesting of cockles in south Wales because of the presence of DSP toxins (Anonymous, 2002d).

Bans were put on shellfish harvesting in several parts of England in March 2002 (Hatchett, 2002). In September 2002, a ban was put on the harvesting of queen scallops from an area off the West Coast of the Isle of Man because of the presence of DSP toxins (Anonymous, 2002c). In Scotland in 2000 and 2001, DSP toxins were first detected on the west coast and subsequently in mussels from Shetland in late March. The outbreak was short lived. The toxins re-appeared in late May, and were detected in mussels and scallop hepatopancreas in several areas on the west coast. By mid-June, DSP was found in mussels at numerous locations, and was still being found in mid-October. Restrictions on harvesting were enforced at all sites affected (EU-NRL, 2001).

In the period from 1 April 2002 to 31 March 2003, shellfish from 76 primary inshore production areas, and 36 secondary areas and offshore fishing areas in Scotland were examined. A total of 5 409 mollusc samples were analysed, out of which 931 were analysed for DSP. It emerged that 66 samples were positive for DSP (Anonymous, 2003c). In Northern Ireland in 2001, positive DSP results were obtained in 25 oyster samples, 10 mussel samples, 1 cockle sample and 23 scallop samples (EU-NRL, 2001). The United Kingdom Food Standards Agency announced a ban on scallop fishing in the sea adjacent to Northern Ireland following these findings (Anonymous, 2001a).

3.7.2 Africa

South Africa

DSP was identified on the west coast of South Africa during autumn 1991 and on both the west and the south coasts during the autumn of 1992. The causative organism was *Dinophysis acuminata* (Pitcher *et al*., 1993).

3.7.3 North America

The presence of DSP toxins in North America during the years from 1991 to 2000 is illustrated in Figure 3.4.

Canada

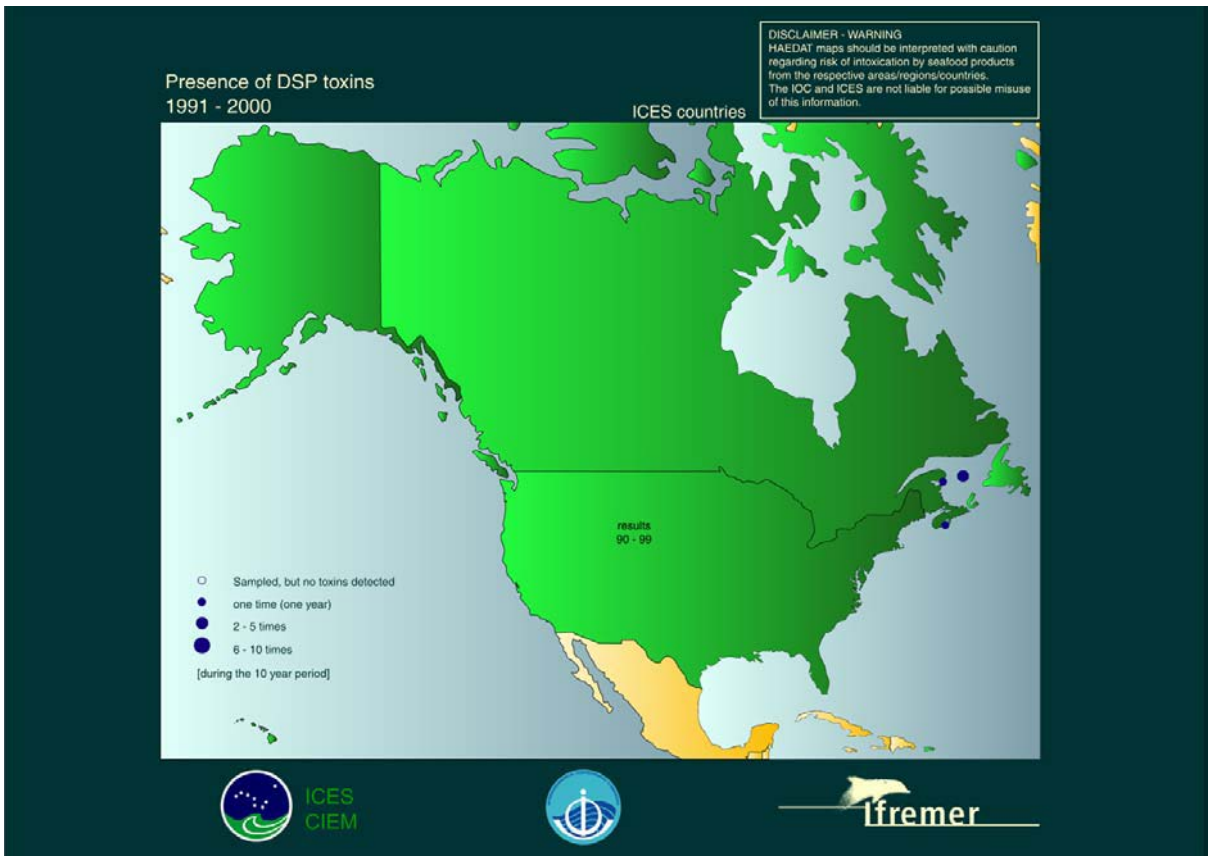
In 1989, DTX1 was isolated from Prince Edward Island mussels at a level of 0.15 µg/100 g digestive gland (Todd, 1997). In August 1990, 13 out of 17 persons in eastern Nova Scotia (Canada) developed gastroenteritis between one and eight hours after consuming boiled or steamed locally cultured mussels. *Dinophysis norvegica* was found in the digestive gland of some mussel samples and in low numbers in the water column at the harvest site. DTX1 appeared to be the toxin involved (Todd *et al.*, 1993). However, another dinoflagellate, *Prorocentrum lima*, which was found to be a producer of DSP toxins in unialgal culture, was isolated also from the toxic area (Marr *et al.*, 1992).

Based on the assumption that 100 µg DTX1/100 g of mussel soft tissue were present, the victims ingested between 1.4 and 6.0 µg DTX1/kg bw (Todd, 1997).

In Bonavista Bay, Newfoundland in October 1993, several persons developed diarrhoeic shellfish poisoning following consumption of mussels containing DTX1. Water samples contained *Dinophysis norvegica* up to 2000 cells/litre. Digestive tissue of the contaminated mussels revealed up to 40 000 cells of *D. norvegica* per mussel (McKenzie *et al*., 1994). The mussels contained up to 4 µg DTX1/100 g digestive gland (Todd, 1997).

Lawrence *et al*. (1998) studied microalgal populations at a mussel farm near Indian Point, Nova Scotia to establish the local source of DSP toxins accumulated in shellfish. In *Dinophysis*-rich samples, no DSP toxins were found by LC-MS nor by DSP-toxin antibody probing. However, cells of toxin producing *Prorocentrum lima* were found as epiphytes upon *Pilayella littoralis*, a macroalga which commonly fouls aquaculture lines in the region.

Figure 3.4 Occurrence of DSP toxins in coastal waters of North American ICES countries from 1991 to 2000



Source: http://www.ifremer/envlit/documentation/dossiers/ciem/aindex.htm

The United States of America

In the New York and New Jersey region, only sporadic cases of DSP were reported prior to 1980. The incidence increased to 31 cases during 1980, 210 cases in 1981, 1 332 cases during 1982 and 1 951 cases in 1983 (Stamman *et al*., 1987). Four episodes of DSP-like illnesses occurred between 1983 and 1985 in Philadelphia and Long Island, New York after consumption of clams and mussels. *Dinophysis* spp. or *Prorocentrum* spp. were involved although no chemical analysis was performed (Todd *et al*., 1993).

In 1989, high numbers of *D. acuta* were observed in discoloured water at Long Island. Analysis for OA revealed that mussels from two stations contained over 0.5 MU per 100 g. No cases of human intoxication were reported (Aune and Yndestad, 1993).

Two species of *Dinophysis* present in Maine coastal waters, *D. acuminata* and *D. norvegica*, are frequently found in high numbers from June to September. *Prorocentrum lima* was found only in the Frenchman Bay-Eastern Bay region. OA-like activity found in mussels was not at levels that present a human health issue (Van Dolah *et al*., undated). In 1998, the presence of *P. lima* along the coasts of Maine was reported (Stancioff, 2000).

Analyses of extracts from shellfish and phytoplankton from the Gulf of Mexico demonstrated the presence of OA (0.162 µg/g shellfish) and domoic acid (2.1 pg/cell phytoplankton). Domoic acid is the causative agent of amnesic shellfish poisoning (ASP). No cases of human poisoning have been reported from this area (Dickey *et al*., 1992a).

3.7.4 Central and South America

Argentina

D. acuminata and *D. fortii* were present but no toxicity was detected until 1999 when 40 persons were intoxicated in Patagonia. *P. lima* was confirmed in the plankton and DSP toxins in mussels (Ferrari, 2001).

Brazil

In 1990, several persons showed gastrointestinal distress and diarrhoea after eating mussels in Florianapolis. Plankton analysis and mouse bioassay supported the evidence of DSP and *D. acuminata*. (Ferrari, 2001).

Chile

Cases of gastrointestinal disorders were observed in Chile in 1970 and 1971, apparently associated with blooms of *Dinophysis* spp. (IPCS, 1984). A substantial DSP intoxication was reported in January 1991. Approximately 120 people became ill after ingestion of fresh mussels. *D. acuminata* was identified in the contents of fresh bivalves and in canned mussels. Toxic samples contained both OA and DTX1 (Aune and Yndestad, 1993). Zhao *et al*. (1993) detected DTX1 as the major toxin and OA as the minor toxin in mussels from Chile. Recently the presence of YTXs and related compounds in shellfish and phytoplankton in Chile was reported (Quilliam, 1998a).

Lagos (1998) also reported that DSP is present in Chile and well documented. Until 2001, PSP and DSP toxins have had severe public health and economic impacts in Chile. As a consequence, all natural fish beds from 44 ∀SL southwards were closed and nationwide monitoring programmes were maintained (Suárez-Isla, 2001). Uribe *et al*. (2001) reported the presence of DSP toxins in the Magellanic fjords (53∀19'S, 72∀30'W) in southern Chile in March 1998. DTX1 was found in *Mytilus chilensis* at a level of 6.5-58 µg/100 g of digestive gland. No OA was detected. *D. acuminata* was shown to be the causative algal species.

Mexico

Mouse bioassays with shellfish extracts were shown to be positive for DSP toxins in samples from Bahía Conceptión in the Gulf of California during the spring of 1992, 1993 and 1994. Samples from April 1994 showed the presence (by LC) of OA as well as DTX1. No human intoxications were reported. Dinoflagellate species known as DSP producers are often found in water samples from this area (Sierra-Beltrán *et al*., 1998).

During March 1993 and April 1994, densities of the dinoflagellate *D. caudata* reached a maximum of 74 to 90.10^3 cells/L in the Gulf of California (Punta Arena, Playa Escondia, Amolares and San Ignacio). However, no contamination of shellfish or human intoxications was reported (Lechuga-Devéze and Morquecho-Escamilla, 1998).

Uruguay

In 1990, several persons showed gastrointestinal distress and diarrhoea after eating mussels. Plankton analysis and mouse bioassay supported the evidence of DSP and *Dinophysis acuminata* (Ferrari, 2001). In January 1992, DSP was detected in shellfish harvested along the coast of Uruguay. At the same time, *D. acuminata* at concentrations up to 6 000 cells per litre occurred at La Paloma leading to a partial ban on shellfish harvesting (Aune and Yndestad, 1993).

3.7.5 Asia

China

DSP is widely distributed in different shellfish species along the Chinese coast. In 1996 and 1997, 26 out of 89 samples contained DTX1 or OA, but only six samples contained levels above the regulatory limit for human consumption (20 σg/100 g soft tissue). The highest level of 84 σg/100 g was found in *Perna viridis* from Shenzhen. No DSP poisoning of humans was reported in Shenzhen at that time (Zhou *et al*., 1999).

Japan

DSP was first documented in Japan in late June 1976 and 1977. A total of 164 persons were documented to have suffered severe vomiting and diarrhoea. Epidemiological data indicated that as little as 12 mouse units (MU) was sufficient to induce a mild form of poisoning in humans (Yasumoto *et al*., 1978). MU was defined as the amount of toxin (later defined as DTX1) killing a mouse by i.p. injection within 24 hours (EU/SANCO, 2001). The first dinoflagellate to be implicated was *D. fortii*. Between 1976 and 1982, some 1 300 DSP cases were reported in Japan (Hallegraeff, 1993).

India

A two year study (1984-1986) in India showed that diarrhoeic shellfish toxins were present in several shellfish examined. The levels ranged from 0.37 to 1.5 MU/g hepatopancreas. However, no reports of DSP episodes in the general population are known (Aune and Yndestad, 1993).

The Philippines

Five species of *Dinophysis* have been recently detected in the Philippines but no cases of human poisoning have been reported (Corrales and Maclean, 1995).

The Russian Federation

D. acuminata, *D. acuta*, *D. fortii* and *D. norvegica* have been identified in the far-eastern coastal waters of the Russian Federation. However, no cases of human poisoning have been reported (Aune and Yndestad, 1993).

3.7.6 Oceania

Australia and New Zealand

The dinoflagellate *P. lima* producing OA and methyl-OA was isolated at three locations on Heron Island, Australia. Cases of DSP were not reported (Morton and Tindall, 1995). In New Zealand waters (Northland and Marlborough Sounds), *P. lima* was also observed and appeared to produce

OA (Rhodes and Syhre, 1995). The presence of YTXs and related compounds in shellfish and phytoplankton in New Zealand was reported recently (Quilliam, 1998a).

A pipi (*Donax delatoides*) shellfish poisoning event (56 cases of hospitalization) in New South Wales, Australia occurred in December 1997 (ANZFA, 2001). According to Quilliam *et al*. (2000), PTX-2 seco acids may have contributed to the gastrointestinal symptoms, vomiting or diarrhoea in humans (Aune, 2001). Burgess and Shaw (2001) reported that the patients consumed approximately 500 g of pipis containing 300 µg PTX-2SA/kg (~150 µg PTX-2SA/person~2.5 µg/kg bw for a person weighing 60 kg).

Another poisoning incident with pipis occurred on North Stradbroke Island (Queensland) in March 2000 where an elderly woman became seriously ill after eating pipis from one of the local beaches. High levels of PTX-2SA were found in the pipis (Burgess and Shaw, 2001).

DSP toxins from the dinoflagellates *Dinophysis fortii* and *D. acuminata* have been detected in wild Tasmanian mussels (both OA and DTX1). However commercial Tasmanian shellfish have thus far proved negative for DSP toxins and no incidents of human poisoning are known (Hallegraeff, 1992).

During the period from September 1994 to July 1996, 0.7 percent of samples of shellfish collected around the coastline of New Zealand on a weekly basis, showed a DSP toxin level above the regulatory limit during a total of nine DSP events (maximum level 96 µg/100 g mussels). During the sampling period, there were three outbreaks of human DSP poisoning involving 13 cases (Sim and Wilson, 1997).

3.8 Regulations and monitoring

3.8.1 Europe

In 1996, the EU-NRL group agreed during the first Meeting of the EU National Reference Laboratories on Marine Biotoxins and Analytical Methods and Toxicity Criteria, that the mouse bioassay with the technique established by Yasumoto *et al*. (1978), with an observation time of 24 hours is currently the preferred method for the detection of the acute toxicity of acetone soluble DSP toxins. Based on acute toxic effects, a tolerable level of DSP toxins, including non-diarrhoeic acetone soluble toxins, of 80-160 σg of OA eq/kg of whole shellfish meat or 20-40 MU/kg of whole shellfish meat was agreed for EU member countries (EU-NRL, 1996).

In March 2002, the European Commission laid down the following rules (EC, 2002a):

- Maximum level of OA, DTXs and PTXs together, in edible tissues (whole body or any part edible separately) of molluscs, echinoderms, tunicates and marine gastropods shall be 160 σg OA equivalents/kg.
- Maximum levels of YTXs in edible tissues (whole body or any part edible separately) of molluscs, echinoderms, tunicates and marine gastropods shall be 1 mg YTX equivalents/kg.
- The mouse or the rat (not for yessotoxin) bioassay are the preferred methods of analysis for the toxins mentioned above. A series of analytical methods such as LC with fluorometric detection, LC-MS, immunoassays and functional assays such as the phosphatase inhibition assay can be used as alternative or complementary method to the biological assays, provided that either alone or combined they can detect at least the following analogues, that they are not less effective than the biological methods and that their implementation provides an equivalent level of public health protection;

OA and DTXs: an hydrolysis step may be required in order to detect the presence of DTX3
PTXs: PTX1 and PTX2
YTXs: YTX, 45 OH YTX, homo YTX, and 45 OH homo YTX.

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

Ireland

The Biotoxin Monitoring Programme in Ireland began in 1984 and was initially based on the screening of samples for the presence of DSP toxins by bioassays. In recent years, the detection of additional toxins, including DA and in particular the azaspiracids, has led to an increase in monitoring efforts and the programme now includes weekly shellfish testing using DSP mouse bioassay, LC-MS (okadaic acid, DTX2, azaspiracids) and LC (DA) as well as phytoplankton analysis. Regular reports of the results of sample analysis are sent to the regulatory authorities, health officials as well as the shellfish producers and processors. A Web-based information system is being developed to increase access to the information (McMahon *et al*., 2001)

Turkey

Regulation is based on mouse bioassay. No further information (Fernández, 2000).

3.8.2 North America

Canada

Hallegraeff *et al*. (1995) reported that in Canada monitoring for *Dinophysis* spp. and *Prorocentrum* spp. is carried out, and that closure of fishery product harvesting areas takes place when DSP toxin levels in shellfish exceed tolerable levels (i.e. >0.2 σg/g meat = 5 MU/100 g meat) using the mouse bioassay; not official).

The United States of America

No DSP has yet been confirmed so there is no DSP monitoring. The primary agency responsible for seafood safety and marine biotoxins is the Food and Drug Administration (FDA). The National Marine Fisheries Service (NMFS) of the National Oceanic and Atmospheric Administration (NOAA) has several marine biotoxin programmes, primarily focused on fish and wildlife. In the area of domestic food safety, cooperative programmes between the FDA and individual states exist. The National Shellfish Sanitation Programme provides guidelines for these cooperative agreements. Internationally, the FDA sets up memoranda of understanding with various countries to regulate imported seafood products programmes (APEC, 1997).

3.8.3 Central and South America

Argentina

Argentina has a national monitoring programme for mussel toxicity in each coastal province using regional laboratories and one fixed station in Mar del Plata (Ferrari, 2001).

Brazil

Brazil had a pilot monitoring initiative during one year but does not have a national monitoring programme (Ferrari, 2001).

Chile

Two types of monitoring are conducted in Chile. The National Health Service is responsible for detecting toxicity using a bioassay at 40 stations using monthly samples. In addition, the Fisheries Research Institute monitors toxicity in conjunction with universities. These are programmes that include measures of phytoplankton to understand more than just toxicity. However, there are problems with the methods since at times both PSP and DSP occur. The Ministry of Health, through the Regional Health Service, is responsible for the closure of contaminated harvesting areas. When DSP bioassay is positive, shellfish are quarantined. The National Fish Service (NSF) is responsible for seafood for export. At present, NSF has a memorandum of understanding with the USA and the EU to permit shellfish to be exported. No regulations exist for imported shellfish, since that is presently not a big market (APEC, 1997). Up until 2001, PSP and DSP toxins have had the most severe public health and economic impact in Chile. Consequently, all natural fish beds from 44 ∀SL southwards were closed and nationwide monitoring programmes maintained (Suárez-Isla, 2001). Regulation is based on mouse bioassay (no further information) (Fernández, 2000).

Uruguay

Uruguay has a national monitoring programme on mussel toxicity and toxic phytoplankton (Ferrari, 2001). Regulation is based on mouse bioassay (no further information) (Fernández, 2000).

Venezuela

Regulation is based on mouse bioassay (no further information) (Fernández, 2000).

3.8.4 Asia

China

No regulatory monitoring programme for toxins in shellfish and no regulations for algal biotoxins in seafood products exist in China. A major project on red tides has been funded that will include regular monitoring in two areas, one in the north and one in the south of the country. This will include bi-weekly monitoring of both plankton and shellfish (APEC, 1997).

Japan

Monitoring involving both plankton and shellfish is carried out. Researchers from Prefectural Fisheries Experimental Stations in major shellfish areas periodically collect plankton samples and carry out cell counts of key *Dinophysis* species. Shellfish are collected and assayed at least monthly during key seasons. When low levels of toxin are detected, monitoring frequency is increased and more stations are sampled. Tolerance level for DSP toxins in bivalves is set at 5 MU/100 g whole meat detected by the mouse bioassay (~0.2 µg/g). Information on shellfish toxicity is distributed through a well-defined network connecting governmental agencies, fisheries co-operatives, fishermen, mass media and the general public. Three weeks of toxicity levels below quarantine limits result in a lift of the shellfish harvesting ban (APEC, 1997 and Hallegraeff *et al*., 1995).

The Republic of Korea

The National Fisheries Research and Development Institute (NFRDI) collects and examines plankton samples in key areas on a bi-weekly basis from February to October. Over two hundred stations are sampled. Tests are run for ASP, but also for PSP and DSP. However PSP and DSP are not serious problems. DSP toxins were determined by means of LC (APEC, 1997). Monitoring for *Prorocentrum spp*. is carried out and fishery product harvesting areas are closed at concentrations

greater than 10^5 cells/litre. Furthermore, a tolerance limit for DSP toxins in shellfish of 5 MU per 100 g detected by the mouse bioassay is applied (Hallegraeff *et al*., 1995).

Thailand

Regulation is based on the mouse bioassay (no further information) (Fernández, 2000).

3.8.5 Oceania

Australia

Regulations for DSP recommend 16 to 20 µg OA eq/100 g shellfish meat. However, for 1995, it is stated that the maximum permitted levels for DSP were 20 to 60 µg OA/100 g shellfish meat or 2 µg OA/g hepatopancreas. It was not clear whether these figures were enforced values or recommended guidelines, and from where these figures were derived (Burgess and Shaw, 2001).

New Zealand

The New Zealand Biotoxin Monitoring Programme combines regular shellfish testing and phytoplankton monitoring. The regulatory level in shellfish is 20 µg OA eq/100 g of shellfish meat (Sim and Wilson, 1997). Currently shellfish testing involves mouse bioassay screen testing with confirmatory testing approved for OA and DTX1 (DSP ELISA Check Kit, PP2A, LC-MS), PTXs and YTXs (LC-MS) (Busby and Seamer, 2001).

A new Biotoxin Monitoring Programme providing data that is highly accurate, in a shorter time and without the use of mouse bioassays is being developed. This new programme will implement test methods based on LC-MS providing chemical analytical data in place of bioassay screen test results. The development and implementation of new test methods are in discussion including funding, method validation, testing regulations, availability of analytical standards, comparison to existing tests, type of instrumentation and international cooperation (McNabb and Holland, 2001).

4. Amnesic Shellfish Poisoning (ASP)

Amnesic shellfish poisoning (ASP), also known as domoic acid poisoning (DAP) because amnesia is not always present, was first recognised in 1987 in Prince Edward Island, Canada. At this time, ASP caused three deaths and 105 cases of acute human poisoning following the consumption of blue mussels. The symptoms included abdominal cramps, vomiting, disorientation and memory loss (amnesia). The causative toxin (the excitatory amino acid domoic acid or DA) was produced by the diatom species *Pseudo-nitzschia pungens* f. *multiseries* (=*Nitzschia pungens* f. *multiseries*) (Hallegraeff, 1995).

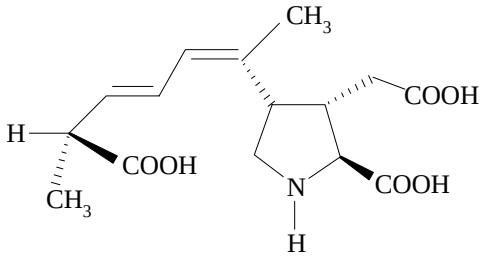
In September 1991, the unexplained deaths of pelicans and cormorants in Monterey Bay, California were attributed to an outbreak of DA poisoning produced by a related diatom *Pseudo-nitzschia australis*. This diatom was consumed by anchovies that in turn were eaten by the birds. In October 1991, extracts of razor clams from the coast of Oregon were found to induce DA acid-like symptoms in mice. These incidents prompted the regulatory authorities in the United States to conduct a massive survey of many marine species for the presence of DA. The toxin was found widely from California to Washington, and was also found unexpectedly in crabs, the first time this toxin was demonstrated in a crustacean. Since these incidents, global awareness of DA and its producing sources has been raised (Wright and Quilliam, 1995).

4.1 Chemical structures and properties

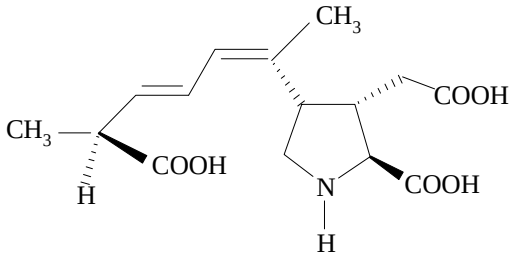
Amnesic shellfish poisoning (ASP) is caused by DA (see Figure 4.1), a naturally occurring compound belonging to the kainoid class of compounds that have been isolated from a variety of marine sources including macro- and microalgae (Wright and Quilliam, 1995). DA is a crystalline water-soluble acidic amino acid. It can be purified by a variety of chromatographic methods and contains a strong chromophore that facilitates detection by UV spectroscopy. DA was originally discovered as a product of a red macroalga *Chondria armata* and was later isolated from several other red macroalgae. However, these seaweeds were not the source of DA in the first reported ASP incident on Prince Edward Island in Canada in 1987. The source of DA in this outbreak of ASP was found to be the diatom *Pseudo-nitzschia* (formerly *Nitzschia*) *pungens* forma *multiseries*. DA is a potent neurotoxin and the kainoid class of compounds to which DA belongs, is a class of excitatory neurotransmitters that bind to specific receptor proteins in neuronal cells causing continual depolarization of the neuronal cell until cell rupture occurs (Wright, 1995).

Investigation of the kainoids present in *Chondria armata* resulted in the discovery, in minor amounts, of the geometrical isomers isodomoic acid A, B and C (see Figure 4.1) as well as domoilactones. None of these isomers, found in seaweed, were detected in extracts of plankton or shellfish tissue. However, three other geometrical isomers (isodomoic acids D, E and F) and the C5' diastereomer (see Figure 4.1) were isolated from both plankton cells and shellfish tissue (Wright and Quilliam, 1995; Ravn, 1995). The geometrical isomers can be prepared in the laboratory by brief exposure of dilute solutions of DA to UV light, and are therefore not considered to be *de novo* products of the plankton. Pharmacological studies indicate that these photoisomers bind less strongly to the kainate receptor proteins than DA itself suggesting that they are not as toxic as the parent amino acid. Formation of the C5' diastereomer is accelerated with warming. This C5' diastereomer shows almost the same binding efficacy to the kainate receptor as DA itself (Wright and Quilliam, 1995). Zaman *et al.* (1997b) reported the isolation of two new isomers of DA from the red alga *Chondria armata*, i.e. isodomoic acid G and H, (see Figure 4.1).

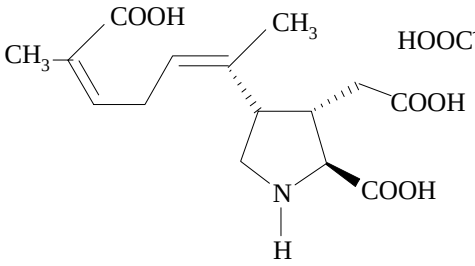
Figure 4.1 Chemical structures of domoic acid and its isomers



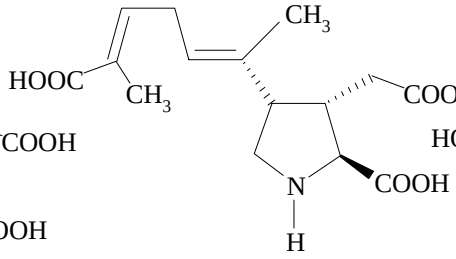
Domoic acid



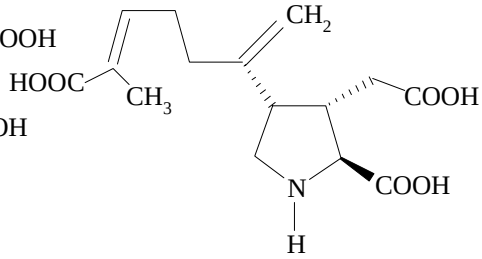
C5'-Diastereomer



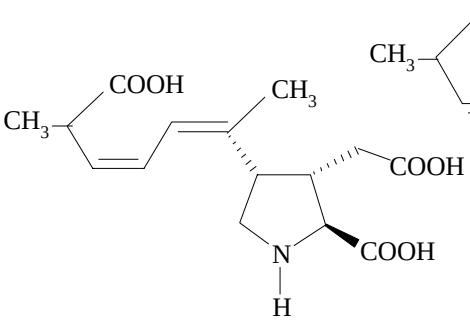
Isodomoic acid A



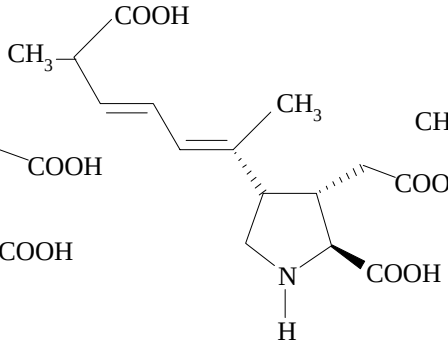
Isodomoic acid B



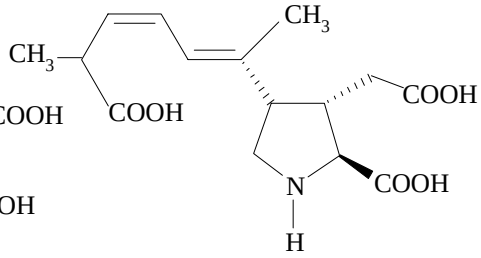
Isodomoic acid C



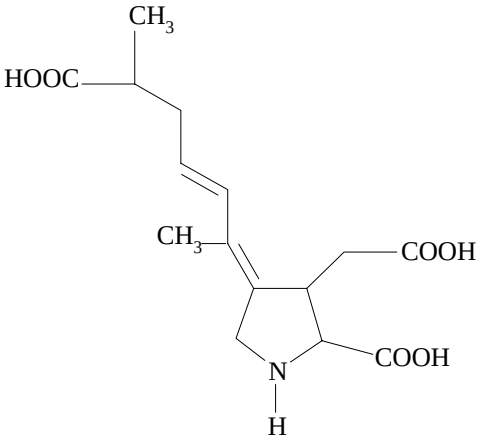
Isodomoic acid D



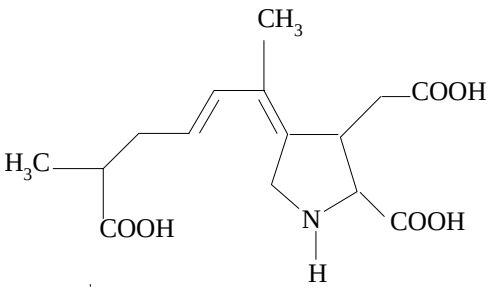
Isodomoic acid E



Isodomoic acid F



Isodomoic acid G



Isodomoic acid H

4.2 Methods of analysis

4.2.1 Bioassays

in vivo assays

mouse bioassay

The AOAC mouse bioassay for PSP toxins (AOAC, 1990) can also detect DA at concentrations of approximately 40 σg/g tissue. The PSP mouse bioassay involves acidic aqueous extraction of the tissue (whole animal or selected organs) followed by intraperitoneal injection of 1 ml of the extract into mice. The time from initial injection to mouse death (usually under 15 minutes) is recorded. This procedure was used when ASP toxicity was first identified in Canada in shellfish extracts. The first indications of toxicity associated with the ASP syndrome were revealed in the course of routine AOAC mouse bioassays for PSP toxicity. Skilled bioassay technicians noted that the aberrant symptoms of ASP were distinguishable from the classic symptoms of PSP intoxication and consequent death. The success of the approach during the first ASP incident was due in part to the high levels of toxin present in contaminated shellfish from the eastern Prince Edward Island. The typical sign of the presence of DA is a unique scratching syndrome of the shoulders by the hind leg, followed by convulsions. The time of observation must be extended from 15 minutes to four hours. Although the AOAC extraction procedure can yield substantial recovery of DA, the limit of detection of the AOAC bioassay procedure is not low enough to be used with confidence for regulatory purposes to quantify this toxin. The guideline value in mussels established in Canada, and subsequently adopted by most other countries that have set limits for ASP, is 20 σg DA/g of mussel tissue. For the routine detection of ASP toxins, the AOAC mouse bioassay has been superseded by LC methods using diode-array/UV or fluorometric detection which have been proven to be more sensitive and reliable tools (Fernandez and Cembella, 1995).

Tasker *et al*. (1991) pretended to have developed a behavioural rating scale from zero (normal) to seven (death), which these authors claimed to be consistently reproducible in mice injected intraperitoneally. These authors further claimed that the rating scale could be used to reliably quantize DA concentrations as low as 20 σg/mouse (~0.8 mg/kg bw).

in vitro assays

receptor binding assays

A competitive microplate receptor binding assay for DA using frog (*Rana pipiens*) brain synaptosomes was developed. The analysis of DA was based upon binding competition with radiolabelled-[^{3}H]-kainic acid for the kainate/quisqualate glutamate receptor. The method appeared to be sensitive (IC_{50} 0.89 nM ~ 0.3 σg) and showed high promises as a rapid automated assay for DA in contaminated seafood and toxic phytoplankton samples. Preliminary results with extracts of *Pseudo-nitzschia pungens* f. *multiseries* indicated good qualitative correlation with the fluorenylmethoxycarbonyl-HPLC method (Van Dolah *et al*., 1994). In 1995, the method was reported to be still in the latter stages of pharmacological trials (Wright and Quilliam, 1995). In 1997, Van Dolah *et al*. reported the further development of the receptor assay by replacement of frog brain by a cloned rat GLUR6 glutamate receptor to eliminate animals from the testing procedure. The limit of detection and selectivity of the assay were optimized through inclusion of the glutamate decarboxylase pretreatment step to eliminate potential interference due to high concentrations of endogenous glutamate in shellfish. The receptor binding assay of Van Dolah *et al*. (1997) is suitable for analysis of DA in seawater extracts from algae and for analysis of DA in shellfish.

hippocampal slice preparations

The effectiveness of *in vitro* hippocampal slice preparations was examined as a means of rapidly and specifically detecting DA. Extracellular neuronal responses were recorded from region CA1 of fully submerged, perfused adult Sprague-Dawley rat hippocampal slices, using reference standards in 50 to 1000 nM range. DA produced a rapid and reversible increase in amplitude of the orthodromic population spike and a decrease in field EPSP (extra-cellular potential from *stratum radiatum*). The results of this experiment indicated that the hippocampal slice preparation is a viable tool for detecting DA (Kerr *et al*., 1999; Saba *et al*., 1997).

4.2.2 Biochemical assays

immunoassays

An ELISA for the presence of DA in mammalian serum and urine was developed using polyclonal antibodies produced in rabbits. The method was effective in determining DA concentrations in rat urine, with a reported lower quantification limit of 40 ng/ml. DA levels in rat and monkey plasma could not be determined accurately using this antiserum in ELISA. This detection system had not been subjected to extensive collaborative testing for use as a routine technique (Cembella *et al*., 1995 and Smith and Kitts, 1994). The method above involved several steps and lacked the desired limit of detection. In addition, the method depended on the physical immobilization of DA on the microplate via a carrier protein. At present, CovaLink NH® microplates have been developed in which a projecting secondary amino group has been applied in ELISAs for coupling peptides, steroids, oligonucleotides, and DNA to the microplate well surface, directly and chemically. The use of these CovaLink microplates for simplification and improvement of the previous ELISA method, has resulted in two different versions of the ELISA, which are based on a physical and a chemical immobilization, respectively, of DA (Osada *et al*., 1995).

ELISAs for DA determination in human body fluids and in mussel extracts were developed by Smith and Kitts (1994) and Smith and Kitts (1995), respectively. The assays employed a polyclonal antiserum raised in mice against an ovalbumin-DA conjugate. The assay was used to quantify DA concentrations in human body fluids spiked with pure domoate. The lower quantitation limits were 0.2 σg/ml in urine, 0.25 σg/ml in plasma and 10 σg/ml in milk. The relative high quantitation limit in milk was probably due to the high fat content of the milk. The authors suggested that human milk samples may require extraction prior to analysis (Smith and Kitts, 1994). Recovery experiments in both aqueous and acid extracts of mussel tissue demonstrated that the DA concentration could be accurately measured to within 8 percent of the actual value. The limit of detection was 0.25 σg/ml of extract. This value represents 0.5 σg DA/g of mussel tissue when acid (AOAC) extracts are analysed (Smith and Kitts, 1995). Direct comparison of DA determinations with LC and ELISA analyses correlated well (r = 0.96), although the ELISA method resulted in higher values in most samples. It was suggested that this was partially attributed to a loss of DA in the solid phase extraction prior to HPLC or to the possible presence of DA isomers. DA isomers that do not co-elute with DA, are not determined in routine LC analyses. However, the ELISA method measures total DA content including a diastereoisomer and at least two *cis-trans* isomers (Smith and Kitts, 1995)

Garthwaite *et al.* (1998) used ovine antibodies raised against conjugates, linked through the secondary amino group of DA, together with activated-ester-derived conjugates of DA as the plate coater, to develop an indirect competitive ELISA for shellfish and seawater. The ELISA has a detection limit below 0.1 ng/ml, a limit of quantitation (LOQ) of 0.15 ng/ml and a working range of 0.15-15 ng DA/ml. The LOQ is equivalent to 38 µg DA/kg shellfish flesh, 500 times less than the regulatory limit of 20 mg/kg flesh. This ELISA was also shown to be appropriate for analysis

of DA in algal cultures and seawater collected from the field in New Zealand, and thus has the potential to provide early warning of developing algal blooms. Analogous compounds in seawater, such as kanaic acid, do not interfere with this assay.

The ELISA as developed by Garthwaite *et al.* (1998) has been commercialised by Biosense®, into a kit format, intended to be used in routine monitoring of DA levels in cultured bivalve molluscs to check compliance with the regulatory limits. According to the producer it is also applicable for quantification of DA in other matrices (see www.biosense.com "Direct cELISA ASP assay", 2003). Compared to the original procedure of 1998, the LOQ of the kit has been reduced to 10 σg/kg shellfish. Method validation of the Biosense® ELISA was ongoing at the time of writing this review, but a preliminary validation between reference laboratories in Scotland, Chile and New Zealand revealed excellent performance (Kleivdal, 2002)

Whereas the antibodies developed by Garthwaite were of polyclonal nature, Kawatsu *et al.* (1999) produced monoclonal antibodies against DA and applied them in an indirect competitive immunoassay. The range for quantitative determination of domoic acid and the LOQ in shellfish were estimated to be 0.15-10 ng DA/ml and < 40 ng DA/g respectively, so quite comparable with the performance characteristics of the ELISA of Garthwaite *et al.* (1998). The authors reported a recovery of DA at 103 percent (C.V. 4.5 percent) for DA added to extracts of shellfish at toxin levels of 0.02–0.2 ng/ml. The same research group also used the monoclonals as ligands in immunoaffinity chromatography which, in combination with LC, was successfully used to confirm the presence of DA in samples of commercial blue mussels (*Mytilus edulis*) (Kawatsu *et al*., 2000).

Garthwaite *et al.* (2001) developed a group ELISA for ASP, NSP, PSP and DSP toxins including yessotoxin as a screening system for contaminated shellfish samples. The system detected suspected shellfish samples. Thereafter the suspected samples have to be analysed by methods approved by international regulatory authorities. Alcohol extraction gave good recovery of all toxin groups.

A newer antibody-based approach is the use of biosensors. Traynor *et al.* (2002) have described the detection of residues of DA in bivalve molluscs with an immunobiosensor. In this application DA is bound to the sensor surface and use is made of polyclonal antibody raised to DA HAS conjugate. The authors reported that the assay was found suitable for rapid analysis of cockles, mussels, oysters and scallops. A limit of detection was found at 0.8 σg/g, and an intra assay C.V. of 8 percent was found at a level of 20 σg/g, the current legal limit for whole body. At the time of writing a large scale comparison with LC was underway. It may be expected that biosensor technology will become more widely applied in ASP determination in the near future.

4.2.3 Chemical assays

thin layer chromatography

DA can be determined by thin layer chromatography as a weak UV-quenching spot that stains yellow after spraying with a 1 percent solution of ninhydrin (Quilliam *et al*., 1998). Normal amino acids that are present in crude extracts will interfere and must be separated from DA. This can be accomplished for plankton samples by two dimensional TLC. Crude extracts of shellfish tissues cannot be analysed directly, as they are too complex. A clean-up procedure (strong anion change solid phase extraction [SAX-SPE] with minor modification) yields fractions that can be used directly or concentrated in vacuum before applying to a silica gel plate. Only one-dimensional TLC is required when this clean-up is used as almost all interfering amino acids are removed. The detection limit for DA is about 0.5 σg by this method, which permits detection in shellfish tissues

at about 10 σg/g. It is also possible to detect DA on the TLC plate using some other spray reagents. For example, after spraying a TLC plate with vanillin, a yellow colour with domoic (or kainic) acid forms first and changes to pink on standing (Wright and Quilliam, 1995).

Quilliam *et al* (1998) further studied TLC as a separation technique to detect DA, after extraction with aqueous methanol followed by SAX-SPE clean-up. He successfully applied the method to scallop, razor clam and anchovy samples contaminated with DA, and concluded the method should prove successful for the routine screening of shellfish tissues in those laboratories not equipped with an LC system. It should also be useful as a chemical confirmation method for DA in samples tested positive by assay methods such as immunoassay.

amino acid analysis

Crude aqueous extracts of plankton can be analysed directly by an amino acid analysing system. Using the buffer solutions and ion-exchange column normally used for the analysis of protein hydrolysates, DA elutes close to methionine. Absorbance measurement at 440 nm provides detection of amino acids with primary amine groups, while absorbance at 570 nm selectively detects imino acids such as proline and DA. The detection limit of this method for DA is about 1 σg/ml, with about 50 σl of extract injected on-column. Although the limit of detection of the amino acid analysis method is close to that of LC-UV methods, it is not as effective for samples containing a high concentration of free amino acids and the analysis time is much longer. Shellfish extracts can be analysed with this approach after the necessary clean-up and concentration of material (Wright and Quilliam, 1995).

liquid chromatography (LC)

DA can be analysed, as well as preparatively isolated, by either LC or ion exchange chromatography using UV absorbance detection. Reversed-phase LC-UV gives the fastest and most efficient separations. Use of an acidic mobile phase to suppress ionization of the carboxyl functions is recommended, and selective separation of DA and its isomers is best achieved with "polymeric-like" octadecylsilica phases. LC-UV is currently the preferred analytical technique for the determination of DA in shellfish and a method is available, formally validated for mussels in an AOAC collaborative study (Lawrence *et al*., 1991b). The detection of DA is facilitated by its strong absorbance at 242 nm. The LC-UV detection limit for DA is about 10-80 ng/ml, depending on the sensitivity of the UV detector that is used. The detection limit in tissue is dependent upon the method of extraction and clean-up. In the method of Lawrence *et al*. (1989a) DA is extracted from homogenized mussel tissue by boiling five minutes with 0.1 M HCl, similar as in the AOAC's PSP mouse assay extraction procedure. The mixture is cooled and centrifuged, and an aliquot of supernate is diluted, filtered and analysed by isocratic LC with UV detection at 242 nm.

If crude extracts (either acidic or aqueous methanol) are analysed without clean-up, the practical limit for quantitation is about 1 σg/g (Lawrence *et al.*, 1989a). This is suitable for most regulatory laboratories concerned with detecting contamination levels greater than 20 σg/g. However, interferences are commonly encountered that can give false positives with crude extracts. For example, it has been shown that tryptophan and some of its derivatives are often present in substantial concentrations in shellfish and finfish tissues and that these compounds elute close to DA. A photo-diode array detector can be used to examine UV spectra in order to confirm DA. An alternative approach is to prepare a chemical derivative and to analyse the sample with comparison to a known standard carried through the same procedure. Derivatization techniques involving reaction with phenyl- or butyl-isothiocyanate or esterification with isopropanol have been developed for this purpose (Lawrence *et al*., 1989b; Lawrence and Ménard, 1991). A more sophisticated technique of confirmation is to replace UV detection by electrospray mass spectrometry with selected ion monitoring (Lawrence *et al*., 1994). Application of this technique

offers the possibility of confirming the presence of DA down to levels of 0.1 σg/g without additional sample treatment.

An improved LC-UV analysis procedure was developed by Quilliam *et al.* (1995). In this procedure, an aqueous methanol extraction is applied in combination with SAX (strong anion exchange)-SPE (solid phase extraction) clean-up, leading to chromatograms free from interferences. Other advantages of the method of Quilliam *et al.* (1995) over the method of Lawrence *et al*. (1989a) seem more stable extracts, higher recoveries and a lower limit of detection (20-30 ng/g). A slight modification to the SPE (solid phase extraction) clean-up step in the method of Quilliam *et al*. (1995) was given by Hatfield *et al*. (1994). The standard 10 percent acetonitrile wash and 0.5 M ammonium citrate buffer in 10 percent acetonitrile eluting solution have been replaced with a 0.1 M sodium chloride in 10 percent acetonitrile wash and a 0.5 M sodium chloride in 10 percent acetonitrile eluting solution. This modified method permits the analyses of samples with complex matrices, such as crab viscera. Additionally DA appeared more stable in the elutes from the SAX SPE cartridges, permitting storage of the samples if analyses cannot be made immediately.

Thus far, LC methods of analysis for ASP, validated in formal collaborative studies, are scarce. The method of Lawrence *et al*. (1989a) is the only method in that respect by the AOAC.

The method of Quilliam *et al*. (1995) was planned to be studied in an AOAC collaborative study, but time constraints have prevented this collaborative exercise thus far. Instead, a European two-phase inter-laboratory validation study of the Quilliam method was conducted in 2002/2003 by the EU Community Reference Laboratory for Marine Biotoxins for a variety of shellfish and fish samples. The first phase (familiarization part) of this study was successfully completed. The second part was to be rounded off in 2003. The EU National Reference Laboratories for Marine Biotoxins participates in this validation study. If the study yields acceptable results, the method will be standardized by the European Committee for Standardization (CEN).

The method of Lawrence *et al*. (1989a) has been standardized by the CEN working group on biotoxins and is in the process of approval as provisional European Norm prEN 14176 (CEN, 2002c).

A rapid and sensitive automatic method for the determination of DA using LC with a column-switching system and UV-detection was reported. Interfering peaks resulting from matrix protein components are excluded by use of an especially designed reversed-phase LC column for pre-separation. The method is suitable for extracts from both mussels and algae. Sample material is extracted with pure water and the crude extract is injected directly. Application of a column-switching system eliminated the need for any further sample clean-up after extraction (Hummert *et al*., 1997).

A very sensitive procedure, based on reaction with 9-fluorenylmethylchloroformate to form the fluorenylmethoxycarbonyl (FMOC) derivative and LC analysis with fluorescence detection, has been developed for monitoring of DA in marine matrices such as seawater and phytoplankton. The detection limit is as low as 15 pg/ml for DA in seawater (Pocklington *et al*., 1990). This procedure has also been adapted to shellfish extracts (Wright and Quilliam, 1995).

Other derivatization techniques yielding fluorescent derivatives that are determined following LC separation, include the method of Sun and Wong (1999), where 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate is used, and the method of James *et al*. (2000a), where DA is derivatized with 4-fluoro-7-nitro-2,1,3,-benzoxadiazole (NBD-F). These various methods based on LC-fluorescence detection have not yet led to broad application.

capillary electrophoresis (CE)

This relatively simple method allows rapid, high resolution separations of complex polar compounds. A narrow bore fused silica capillary tube filled with buffer is connected between two liquid reservoirs. After a small volume of sample extract (typically 1-10 nL) is injected into the capillary, a differential voltage of 20-30 kV is applied at the ends of the capillary. Ionic substances migrate as narrow bands down the capillary, eventually passing by a detector (UV absorbance, fluorescence) (Wright and Quilliam, 1995). Nguyen *et al*. (1990) reported a detection limit of 2 σg/g (signal-to-noise ratio 5:1) of a treated extract of mussel tissue. During the extraction and extract treatment procedure, if care was taken to limit the amount of liquid introduced, the treated extract was five fold less concentrated than the tissues. The detection limit in wet tissues therefore was 10 σg/g. DA was readily separated from components of the mussel sample matrix in 10 minutes. With excellent mass detection limits, the CE method requires only 3 to 15 nl samples and will find applications where sample size is severely limited. Zhao *et al*. (1997) studied methods based upon capillary electrophoresis combined with UV absorbance detection. DA could be analysed using bare fused-silica capillaries in either the cationic or anionic mode with acidic or basic buffer systems, respectively. Highest performance, in terms of both separation efficiency and analysis time, was achieved with phosphate or borate buffers at a pH of approximately nine. The addition of η-cyclodextrin to the borate buffer permitted a separation of DA and several of its isomers (isodomoic acids) that was superior to that achieved with liquid chromatography. In addition, an extraction and clean-up procedure was developed and tested with mussels, clams and anchovies. A mass detection limit of 3 pg of DA injected and a method detection limit of 150 ng/g in tissues could be achieved. Comparison with LC showed that comparable precision and accuracy could be attained by the two techniques. The CE conditions developed by Zhao *et al.* (1997) were applied, with some slight modifications, by Piñeiro *et al*. (1999). In their study to optimise CE in combination with UV/diode array detection, the authors could clearly identify the presence of DA in some contaminated samples of razor clams and also in mussel tissue reference material (NRC CRM-ASP-MUS-b) (see Chapter 4.2.4.).

mass spectrometry

Electrospray has become the dominant technique for interfacing LC with MS, leaving behind the former mentioned methods such as (continuous flow) Fast Atom Bombardment (FAB) and Thermospray. Removal of the lipid content of the homogenates is probably the major point for solving the signal suppression so often experienced in shellfish extract analysis using LC-ESI-MS (ESI= Electrospray Ionisation).

Clean-up and LC-MS-determination experiments on domoic acid (DA), as well as confirmation experiments on many real-world samples were reported by Hess *et al*. (2001). They concluded that clean-up of mussel and scallop tissues with a SAX cartridge resulted in valid approaches for routine monitoring of DA in shellfish both for LC-UV and for LC-MS, preventing false positives. And they state "..it appears prudent to use SAX clean-up to avoid false negatives." (SAX = Strong Anion Exchange). On the other hand, Powel *et al*. (2002) concluded that an additional step with a SAX SPE cartridge did not significantly improve the recovery of domoic acid from sand crab samples, and Furey *et al*. (2001) even used a minimal sample clean-up not mentioning a matrix effect at all.

An example of extended use of LC-multiple MS was reported by Furey *et al*. (2001) in applying an Ion Trap mass spectrometer for qualitative and quantitative analysis of domoic acid. A handsome figure shows the fragmentation pathway for domoic acid. Good linearity was demonstrated for LC-MS $^{1-3}$ for calibration plots over 2-3 decades using extracts from spiked scallop tissue while it was found that MS^4 and MS^5 resulted in poor linearity.

In the second half of 2002, an inter-laboratory study has taken place of a new LC-MS method for determination of ASP and DSP toxins in shellfish (Holland and McNabb, 2003). The eight participating laboratories generally obtained consistent sets of data for the broad group of analyte toxins down to low levels (< 5ng/ml, equivalent to 0.05 mg/kg). In general, sensitivity is adequate to achieve the LODs required. Most of the participating laboratories could detect the analyte toxins; greater differences were observed for quantitation of some toxins, especially when no analytical standards were present. The participants have used different MS detection modes: some used single MS detection (SIM/SIR), others used tandem MS detection (MRM), and some used both. Although the use of MRM mode is attractive in order to enhance specificity, it requires additional care for quantitation. To summarize, the study was very stimulating and encouraging for those who are interested in using an alternative method for the mouse bioassays which are not supported by any statistical validation data, are well known to have a relatively high rate of false positives, have inadequate detection capability for some toxins and are ethically unacceptable for routine food monitoring. Additionally, method 40.105 (the method tested) can reliably detect ASP toxins and a range of other toxins and metabolites e.g. azaspiracids and pectenotoxin seco acids which may not respond in mouse assays (Holland and McNabb, 2003).

4.2.4 Reference materials

Helpful tools for analytical quality assurance are the certified materials that have been developed for ASP and that have been made available through the Certified Reference Materials Programme of the National Research Council, Canada (NRC, 2003). NRC provides DA certified calibration solution (NRC CRM-DA-d) and mussel tissue reference material for DA (NRC CRM-ASP-MUS-b). NRC CRM-DA-d is a certified instrument calibration solution prepared to aid the analyst in the determination of DA. It is available as a set of four ampoules, with each ampoule containing 0.5 mL of a solution of DA dissolved in acetonitrile/water (1:9 v/v) at a concentration of 100 σg/mL. This concentration is suitable for calibration of liquid chromatography experiments and for spiking shellfish control samples for recovery experiments. NRC CRM-ASP-MUS-b is an homogenized slurry of mussel tissue (*Mytilus edulis*) containing 36 ∂ 1 σg/g DA, as well as several related compounds. NRC CRM-ASP-MUS-b is also distributed in ampoules, each containing 8.1 ∂ 0.1 g of mussel homogenate.

4.3 Source organism(s) and habitat

4.3.1 Source organism(s)

ASP was reported for the first time in 1987 at Prince Edward Island in Canada and the toxin responsible for this syndrome has been identified as DA (Bates *et al.*, 1989). DA was originally isolated in the 1950s from the red macroalga *Chondria armata* (Ravn, 1995). Two decades later, DA was detected in the Mediterranean red macroalga *Alsidium corallinum* (Todd, 1993). Another diatom, *Amphora coffaeformis*, produces also DA (Lundholm *et al.*, 1994).

The origin of DA in the first ASP incident in 1987 was postulated to be the red alga *Chondria baileyana*, a species found in Prince Edward Island waters. However the diatom *Pseudo-nitzschia pungens* f. *multiseries* pointed out as the producer of DA. The outbreak of food poisoning in humans was traced to cultured blue mussels (*Mytilus edulis*) contaminated with identifiable fragments of *Pseudo-nitzschia pungens* f. *multiseries* (Bates *et al*, 1989).

DA is produced by *Pseudo-nitzschia pungens* f. *multiseries* in culture as well as under field conditions (Hasle and Fryxell, 1995). In the southwestern Bay of Fundy in Canada, *Pseudo-nitzschia pseudodelicatissima* appeared to be the organism producing DA which led to inacceptable levels of DA in shellfish destined for human consumption (Martin *et al.*, 1993).

Peculiarly, isolates of *P. pseudodelicatissima* from Galveston Bay (Texas), Massachusetts Bay (Massachusetts), Monterey Bay (California) and also from Denmark and Australia failed to produce DA (Bates, 2000).

ASP also occurred on the West Coast of the United States causing the death of pelicans and cormorants after eating anchovies contaminated with DA. In that region, the pennate diatom *Pseudo-nitzschia australis* appeared to be the source organism (Wright and Quilliam, 1995). *Pseudo-nitzschia australis* produced DA in culture as well as under field conditions (Hasle and Fryxell, 1995).

Both *Pseudo-nitzschia delicatissima* (syn. *Nitzschia actydrophila*) and *Pseudo-nitzschia seriata* were found to produce DA under culture conditions (Hasle and Fryxell, 1995). *Pseudo-nitzschia pungens* and *Pseudo-nitzschia multiseries* (syn. *P. pungens* f. *multiseries*) are now considered separate species because of morphological and genetic differences (Wright and Quilliam, 1995).

Rhodes *et al*. (1998) reported the detection of DA in cultures of *Pseudo-nitzschia fraudulenta* and *Pseudo-nitzschia turgidula* originating from New Zealand. *P. multistriata* was originally found in Japanese waters (Bates, 2000), but isolates from the Gulf of Naples were recently shown to produce DA (Sarno and Dahlmann, 2000). Kotaki *et al*. (2000) reported the isolation of a DA producing diatom from a shrimp-culture pond in Viet Nam, which was later identified as *Nitzschia navis-varingica* (Lundholm and Moestrup, 2000). Kotaki *et al*. (2000) compared cellular DA levels of *Nitzschia navis-varingica* with other DA producing *Pseudo-nitzschia* species. Cellular DA levels of *Nitzschia navis-varingica* were comparable to those in *P. multiseries* and *P. seriata*, less than in *P. australis*, but more than in *P. turgidula* and *P. pungens*.

4.3.2 Predisposing conditions for growth

Toxic *Pseudo-nitzschia* blooms may become a recurring phenomenon, and it is important to determine if there is any seasonal or spatial predictability. Since the first ASP incident in Canada in 1987, the autoecology of *P. pungens* f. *multiseries* has been intensively studied. Although it has been reported to dominate at colder temperatures, it is able to survive up to 30 ∀C (Villac *et al.*, 1993a). Optimal growth and photosynthesis of *P. pungens* f. *multiseries* were concluded to occur in the temperature range of 15-20 ∀C. However, monospecific blooms of *P. pungens* f. *multiseries* have been seen in late autumn or winter when the prevailing water temperature was low (-1 to 3 ∀C). Since the temperature for optimal growth is much higher than this, it is evident that factors other than temperature must have initiated the development of blooms (Van Apeldoorn *et al.*, 1999).

In studies of Lewis *et al.* (1993) the highest growth rates for *P. pungens* f. *multiseries* were observed at 20∀and 25∀C. The highest stationary phase cell concentrations occurred at 5∀to 15∀C and decreased at 20∀to 25∀C.

Upwelling of cold water with high nitrogen concentrations (such as found in Monterey Bay, California) might have stimulated the increase of *P. pungens* f. *multiseries* populations (Villac *et al.*, 1993b). A direct evidence of increased cell numbers with even small amounts of upwelling was reported. From 1991 to 1994 on the west coast of the United States, *P. australis* blooms were most common and persisted longer during late summer and autumn when hydrographic conditions were associated with the end of the upwelling season and were usually characterized by higher sea-surface temperatures, thermal stratification, and lower concentrations of nutrients. Nutrients might be even more important to phytoplankton growth than direct effects of seasonal temperature changes. *P. seriata*, another DA producing *Pseudo-nitzschia* species, was found from the Barents Sea near Spitsbergen in the north to Germany in the south (Kiel Bay in Baltic) and in the western

Atlantic from the west and east coasts of Greenland to 45∀N in Canada. *P. seriata* was apparently restricted to cold water and reached low latitudes only in winter. It is known in the Pacific from Alaska to British Columbia/Washington and it may be expected to occur also in northern Japan.

Within the genus *Pseudo-nitzschia*, production of DA varies greatly from one species to another, and it is vital to be able to distinguish species. The relationship between DA production by *Pseudo-nitzschia* spp. and environmental conditions is not yet clear.

Conditions in shallow regions with restricted circulation may provide the condition of stress with an excess of inorganic nitrogen needed to initiate DA production (Van Apeldoorn *et al*., 1999). Even toxin production of the same species can vary with the area. For example, *P. pseudodelicatissima* from the Bay of Fundy, New Brunswick, Canada, caused high levels of DA in molluscan shellfish, whereas isolates of this species from Galveston Bay (Texas), Massachusetts Bay (Massachusetts), Monterey Bay (California), Denmark and Australia failed to produce DA (Bates, 2000).

The peak of the *P. pungens* f. *multiseries* bloom in Canada in 1987 took place after an unusually dry spell in late summer, followed by severe rainstorm in early September. A relationship between pulses of nitrate availability and *P. pungens* f. *multiseries* peaks was found which was attributed mostly to freshwater runoff following the rains (Villac *et al.*, 1993a; Villac *et al.*, 1993b). Studies of population densities in Cardigan River Estuary (Prince Edward Island, Canada) indicated cell densities of approximately 3×10^5 per litre might be needed before shellfish exceed the 20 mg/kg DA tolerance level for human consumption (Dickey *et al.*, 1992a). The DA incident in the autumn of 1991 at the waters of Washington State on the west coast of the United States also occurred after a record hot, dry period lasting 45 days, followed by rain in mid-October (Horner and Postel, 1993).

In batch culture studies DA production by *P. multiseries* occurred only in the stationary phase and was not evident during exponential growth (Bates *et al.*, 1991). However in later studies (Bates, 2000), DA production by *P. multiseries* and *P. seriata* in culture studies were found to start during late exponential phase and continued more rapidly into stationary phase. In contrast, *P. australis* and *P. pseudodelicatissima* showed DA production during most of the exponential phase and not during stationary phase. The information for *P. pseudodelicatissima* is not entirely consistent because an isolate from Washington waters produced DA during late exponential as well as stationary phase (Bates, 2000). Kotaki *et al.* (2000) studied the dynamics of DA production by *Nitzschia navis-varingica* and found the same toxin dynamics as for *P. multiseries* and *P. seriata*, namely DA production beginning during late exponential phase and continuing more rapidly into stationary phase.

DA production by *P. seriata* also appeared to be temperature-dependent, with higher amounts produced at 4∀C than at 15∀C (Van Apeldoorn *et al.*, 1999).

Lewis *et al*. (1993) demonstrated that the rate of DA production by *P. pungens* f. *multiseries* in the stationary growth phase could be greatly reduced by a small decrease in temperature. However, even though the concentrations and rates of DA production were low at low temperatures, high cell yields could allow sufficient DA production to toxify molluscan shellfish. The studies of Lewis *et al.* (1993) further demonstrated that experimental photon flux densities had no apparent effect on the initial rate of DA production or on growth rate of *P. pungens* f. *multiseries*.

Bates *et al*. (1991) stated that the availability of extra-cellular nitrate or other nitrogen source and the presence of light were required in order to produce DA during the stationary phase. Nutrient

stress has also been implicated as a causative factor in eliciting DA production in *Pseudo-nitzschia pungens* f. *multiseries*.

The production of DA was greatly enhanced when a severe stress was applied to the algal population after a period of active growth. The production was accelerated by a factor of about three during the transition period from steady state to batch culture when growth was slowed and uptake of silicate or phosphate was diminished (Van Apeldoorn *et al*., 1999).

The *Pseudo-nitzschia* species *P. australis*, identified in the 1991 DA incident in Monterey Bay (California) on the west coast of the United States, *P. australis*, appeared to be a common inhabitant of these waters. At the time of the 1991 *P. australis* bloom, Monterey Bay area was completing its annual dry season; the waters were moderately stratified, the surface temperature was 13-14 ∀C, salinity was 30-33 and nutrients were relatively depleted. Nutrient depletion was most common in late summer and autumn following the period of seasonal upwelling (Villac *et al.*, 1993a). The relatively confined area of Monterey Bay has been characterized as a persistent "upwelling shadow" zone; a region in which water is trapped by a front along the coast restricting its offshore flow. Cellular levels of DA in *P. australis* were suggested to be correlated with silicate concentrations (Bates *et al.*, 1991). The effects of silicate and phosphate limitation on the production of DA by *P. multiseries* were examined in batch culture studies and continuous culture studies. Higher DA production was demonstrated under lower supplies of silicate even at the same growth rate. It is very likely that the natural blooms of *P. multiseries* producing high amounts of DA were severely silicate stressed. A preceding bloom of another diatom or a prolonged bloom of *P. multiseries* can deplete the silicate in the seawater. When the bloom of the toxin producing *P. multiseries* was at his height in Cardigan Bay, Canada in December 1987, silicate concentration was as low as 0.62 σM. DA peaked 10 days later. It was demonstrated that also phosphate limitation in the culture medium enhanced DA production. The concurrence of high rates of DA production at steady state with low rates of nutrient uptake and with high levels of adenosine triphosphate (ATP) further suggests that synthesis of DA required a substantial amount of ATP as a source of biogenic energy (Van Apeldoorn *et al*., 1999).

In a study of Pan *et al*. (1998), DA production by *P. multiseries* appeared to be enhanced when primary metabolism is stressed by limitation of Si, P and perhaps other essential nutrients such as vitamins and trace metals. These essential nutrients, when limiting, may:

- ∉# decrease primary metabolism, thereby making available necessary precursor(s), high energy compounds and cofactors, and
- ∉# favour the expression of genes involved in the biosynthesis of DA. In the case of Si and P limitation, DNA synthesis and the progression through the cell division cycle are slowed, perhaps prolonging or arresting the cells in the stage of the division cycle which is the most conductive to DA production.

However, N-limitation results in an insufficient pool of cellular free N, which restricts synthesis of this nitrogenous toxin. A continuous supply of photophosphorylated high-energy intermediates (e.g. ATP and NADPH) is needed for DA synthesis.

Subba Rao *et al.* (1998) reported that cultures of *P. multiseries* produced substantially higher levels of DA (230 fg/cell) upon enrichment with lithium (385.6 σM) than control cultures (135 fg/cell). Nitrogen, phosphate or silicate were not limiting in the cultures.

It was speculated that DA events might be limited to seasons when stratification and nutrient depletion occurred or limited to near shore regions where developing blooms depleted the dissolved nutrients. However field observations in Monterey Bay showed that DA was produced

by the large-celled *P. australis* at low cell densities and at moderate nutrient concentrations (Van Apeldoorn *et al*., 1999).

Axenic cultures of *Pseudo-nitzschia multiseries* were reported to produce less DA than the original bacteria-containing cultures. Bacterial strains isolated from two non-axenic *P. multiseries* clones were reintroduced individually into cultures of three axenic *P. multiseries* strains. The bacteria did not substantially affect division rates or cell yields. However, they caused a 2 to 95 fold enhancement of DA production (per cell basis) relative to the axenic culture, depending on the *P. multiseries* and bacterial strain used (Van Apeldoorn *et al*., 1999).

For a better understanding of the mechanism(s) of DA production by *P. multiseries*, more studies are needed to elucidate:

- the details of the biosynthetic pathway
- the regulation of enzymes involved in the pathway
- the relation between DA synthesis and the cell division cycle
- the cellular compartimentalization of DA biosynthesis
- other environmental factors that may trigger DA production

Furthermore these studies should be extended to other DA producing *Pseudo-nitzschia* species than *P. multiseries*, to confirm the commonality of these mechanisms (Pan *et al*., 1998).

4 3.3 Habitat

Pseudo-nitzschia species are widely distributed diatoms and according to Hasle and Fryxell (1995; unless given otherwise) the different species mentioned in Chapter 4.3.1 are found in the following waters:

P. multiseries:

Atlantic: waters of North America, Europe, and South America.
Pacific: waters of North America and Northeast Asia, Japan (Kotaki *et al*., 1999).

P. pseudodelicatissima:

Atlantic: waters of Europe and Africa (Denmark Strait to Northwest Africa, including Norwegian and Danish coastal waters, Skagerrak, Kiel Bay), Canada and USA (Arctic to Gulf of Mexico) and South America (Argentina) (Ferrario *et al*., 1999).
Pacific: California and British Columbia waters, coastal waters of Penn Cove, Washington (Trainer *et al*., 1998a).

P. australis

Atlantic: coastal waters of Spain, Portugal, Southwest Africa and Argentina.
Pacific: coastal waters of Peru, Chile, New Zealand, Mexico (Gulf of California) (Hernández-Becerril, 1998), west coast of North America from San Diego (California) to Puget Sound, Washington, British Columbia.

P. delicatissima

Atlantic: Norwegian coastal waters, Danish waters, Skagerrak, Northwest Africa, Rhode Island and Argentina (Negri and Inza, 1998) and coastal waters of the United States (Louisiana) (Parsons *et al*., 1998).
Pacific: California.

P. pungens

Atlantic: coastal waters of Argentina (Ferrario *et al*., 1999) and coastal waters of USA (Louisiana) (Parsons *et al*., 1998).
Pacific: coastal waters of the United States (Penn Cove, Washington) (Trainer *et al*., 1998a) and Mexico (Gulf of California) (Hernández-Becerril, 1998)
New Zealand coastal waters (Rhodes *et al*., 1998)
Sea of Japan, Peter the Great Bay, the Russian Federation (Orlova *et al*., 1998).

P. seriata

Barents Sea (ca. 80∀N), Norwegian Sea, North Sea, Norwegian coastal waters, Skagerrak, Kiel Bay, English Channel, Greenland to New Foundland (45∀N), Alaska, British Columbia.
Coastal waters of Argentina (Negri and Inza, 1998).

P. multistriata

Japanese coastal waters and in the Gulf of Napels, Italy (Bates, 2000).

P. turgidula

New Zealand coastal waters (Rhodes *et al*., 1998).

P. fraudulenta

New Zealand coastal waters (Rhodes *et al*., 1998).
Coastal waters of Argentina (Negri and Inza, 1998)

Nitzschia navis-varingica

Isolated from a shrimp-culture pond in Viet Nam (Kotaki *et al*., 2000; Lundholm and Moestrup, 2000).

4.4 Occurrence and accumulation in seafood

4.4.1 Uptake and elimination of ASP toxins in aquatic organisms

Shellfish (bivalve molluscs, gastropods, crabs, lobsters and others) accumulate phycotoxins either by direct filtration of the plankton cells or by feeding directly on contaminated organisms (e.g. carnivores and scavengers). Generalization regarding the uptake and retention of phycotoxins by shellfish should be avoided. Rate of accumulation of toxic algae (or toxin) by filter-feeding shellfish is species-specific and are, in most cases, directly related to the number of cells available to the animals. The rate of accumulation of toxic algae in individual shellfish in any given area is highly variable. The rate of elimination of the toxin varies with season and low water temperatures retard toxin loss; however, the degree to which temperature affects the uptake and release of toxins is not clearly understood. Furthermore, the rate of elimination is highly dependent on the site of toxin storage within the animal i.e. toxins in the gastrointestinal tract are eliminated much more readily than toxins bound in tissues. In the blue mussel (*Mytilus edulis*) and the oyster (*Crassostrea virginica*) the bulk of DA is resided in the gut.

The majority of information available concerns bivalve molluscs. DA was shown to depurate from mussels fairly rapidly (Villac *et al*., 1993a).

The level of DA in mussels in the absence of *Pseudo-nitzschia* is <1 σg/g. A minimum concentration of 2-4 x 10^5 *Pseudo-nitzschia* cells per L over a period of at least three to four weeks was needed to produce 20 σg DA per g mussels in eastern Prince Edward Island, Canada (Todd, 1993). When blue mussels (*Mytilus edulis*) were presented DA for 24 hours in dissolved form (125 nM; at 5∀C; salinity 28) or as food encapsulated in liposomes, <1% of dissolved DA

and up to 6 percent of food-borne DA was incorporated in mussel tissues. DA absorbed from solution was most concentrated in gills and kidneys, whereas DA ingested as food was most concentrated in digestive gland and kidneys. Gonad, muscle, foot and connective tissues retained the lowest concentrations of toxin. DA levels in mussel tissues did not decrease consistently over a depuration period of 48 hours, nor did DA appear to be transferred to any tissue for storage. Small amounts of DA were eliminated in faeces and larger amounts in dissolved form (Van Apeldoorn *et al.*, 1999).

Preliminary feeding studies with the New Zealand greenshell mussel (*Perna canaliculus*) fed *P. pungens* f. *multiseries* showed that the mussels were contaminated with DA but that the toxin was rapidly eliminated after feeding ceased. Under some conditions the rate of excretion was equivalent to the rate of ingestion and accumulation in tissues did not take place (Van Apeldoorn *et al.*, 1999).

When Pacific oysters (*Crassostrea gigas*) and Californian mussels (*Mytilus californianus*) were exposed continuously to the DA producing diatom, *P. pungens* f. *multiseries*, for 48 hours followed by a 120 hour clearance period, body burden was the greatest in the Pacific oyster after four hours of exposure (36.3 σg/g; highest level in soft tissue 32.9 σg/g). However, after four hours of exposure the Pacific oyster showed closure of their shell and body burden of DA decreased. At 120 hours of clearance, the gill, muscle and soft tissue still revealed detectable levels of DA. In the Californian mussel body burden of DA reached a maximum also after four hours of exposure (3.6 σg/g; highest level in the gill tissue 2.5 σg/g). No detectable levels in the mantle, gill or soft tissue were observed after 24 hours of clearance. In muscle tissue only trace levels were found after 24 hours of depuration (Van Apeldoorn *et al.*, 1999).

Depuration from razor clams is not very rapid. In razor clams, higher DA levels were concentrated in the edible muscular tissues and lower levels in the non-edible tissue parts. When depuration rates are low, low values of DA can be intoxicating, as is the case for razor clams. Therefore, the constant presence of DA producing diatoms at low densities might result in long-term high concentrations in the clam.

In anchovies, DA was found not only in the viscera but also in the fish muscle (Villac *et al.*, 1993a). The bay scallop (*Argopecten iradians*) was reported to take up DA up to levels of 60 σg/g in the digestive gland after approximately 84 hours of exposure to toxic *P. multiseries.* DA levels decreased to 5 σg/g after 48 hours of depuration (Douglas *et al*., 1997).

When sea scallops (*Placopecten magellanicus*) were fed *P. multiseries* cells with a high DA content (4-6.6 pg/cell) for 22 days, followed by 14 days of feeding with nontoxic microalgae, DA was incorporated within 24 hours with increased uptake after six days. DA was concentrated in tissues in the following order: digestive gland >> remaining soft tissue >> adductor muscle. A maximum of 3108 σg/g was recorded in the digestive gland; however, only trace amounts (0.7-1.5 σg/g) were found in the adductor muscle. At the end of the exposure period, 50.9 percent of the supplied DA had been incorporated into the tissues. DA level in the digestive gland 14 days after termination of the toxic diet, remained high, 752 σg/g. Throughout the experiment, there were no signs of illness or mortality of the sea scallops attributable to high DA loading. However, the destructive sampling of the scallops did not allow assessment of long-term effects (Douglas *et al*., 1997). Also the red mussel (*Modiolus modiolus*) retained DA for lengthy periods (Stewart *et al*., 1998).

Anatomical DA distribution was studied in scallops (*Pecten maximus*). In one study, only hepatopancreas, muscle and gonads were analysed. In a second study, hepatopancreas, muscle and gonads combined and the remaining soft tissues were analysed. In the first study 98.8 percent of

total DA content (in hepatopancreas, gonads and muscle) was localized in the hepatopancreas, and in the second study (when total tissue is included) 79.3 percent of total DA content was localized in hepatopancreas; negligible amounts were found in the gonads and muscle and about 14.5 percent in the remaining soft tissues (Arévalo *et al*., 1998).

Stewart *et al*. (1998) suggested the strong possibility that autochthonous bacteria might be a significant factor in the elimination of DA from molluscan species that eliminate DA readily. This was demonstrated in blue mussels *Mytilus edulis* and soft shell clams *Mya arenaria*. Stewart *et al.* (1998) suggested different mechanisms used by different shellfish in dealing with DA, i.e. freely available in blue mussels and soft shell clams but likely sequestered in the digestive glands of sea scallops and red mussels and, thus, largely unavailable for bacterial utilization.

Few data are available for retention times of toxins in crabs and carnivorous gastropods; the general trend in these organisms appears to be towards long-term retention. A retention time longer than two years was reported for *Siliqua patula* with a not defined *Pseudo-nitzschia* species as toxin source (Shumway *et al*., 1995).

A decrease in DA content from 50 σg/g to 5 σg/g within 72 hours was observed in blue mussels derived from the 1987 Canada incident (toxin source *P. pungens* f. *multiseries*), whereas in razor clams derived from the Monterey Bay (California) 1991 incident (toxin source *P. australis*) a decrease from 47.9 σg/g to 44.3 σg/g lasted over three months (Villac *et al*., 1993a). Dungeness crabs (*Cancer magister*) accumulated the toxin mostly in the viscera, although it can enter meat during cooking if the crabs were not eviscerated previously (Villac *et al*., 1993a). When Dungeness crabs were fed DA via contaminated razor clam meats, for six or nine days, analyses of the raw crabs indicated that DA was rapidly accumulated and was confined to the viscera, principally in hepatopancreas (22 σg/g). No DA was detected in either body or leg meats of the raw crab (Hatfield *et al*., 1995). Also studies of Lund *et al*. (1997) showed that Dungeness crabs absorbed DA (via contaminated clam meat) rapidly and accumulated DA only in the hepatopancreas. DA was effectively depurated from the hepatopancreas (via faeces) over a three-week period once the toxic feeding ceased. Depuration proceeded at a faster rate when crabs were fed toxin-free feed than when they were starved.

Arévalo *et al*. (1998) reported that the mean decrease in toxicity in standard total tissue scallop (*Pecten maximus*) samples (homogenized from 100 g of total tissue) not including the hepatopancreas, was 94 percent (ranging from 82.3 to 100 percent).

4.4.2 Shellfish containing ASP toxins

Cultured mussels (*Mytilus edulis*) sampled during the first outbreak of ASP poisoning in Canada (eastern Prince Edward Island) during autumn 1987 contained up to 790 σg DA/g wet tissue (whole mussel) (up to 1 280 and 1 500 σg/g in soft tissue and digestive gland, respectively) (Bates *et al*., 1989; Todd, 1997). During August-October 1988, DA was detected also in blue mussels and furthermore in soft-shell clams (*Mya arenaria*) from the southwest Bay of Fundy, Canada (Martin *et al*., 1993).

In October 1991, DA was detected in razor clams (*Siliqua patula*) from Oregon and Washington States in the United States. Levels peaked in the first week of December 1991 (maximum level in edible portion was 147 σg/g, average level was 106 σg/g for all Washington state beaches). The DA levels in the clams remained above the regulatory closure level of 20 σg/g for at least six months. DA levels declined to <10 σg/g by late spring of 1992. From the spring of 1992 until the spring of 1993, levels of DA were < 5 σg/g for most of the coastal sampling areas. DA appeared

to distribute itself throughout the various body parts of the razor clam. The highest level was found in the foot or "digger", followed by the body, viscera and siphon (or neck). The DA level in the razor clam foot reached 230 σg/g (Van Apeldoorn *et al*., 1999).

The bay scallops (*Argopecten iradians*) were reported to take up DA up to levels of 60 σg/g in the digestive gland after approximately 84 hours of exposure to toxic *P. multiseries*. DA levels decreased to 5 σg/g after 48 hours of depuration (Douglas *et al*., 1997). In autumn 1993, an unexplained mortality among sea scallops (*Placopecten magellanicus*) occurred in the Bay of Fundy, Canada. The digestive gland of the scallops appeared to contain 93.4 σg DA/g. Although some bivalve molluscs have been reported to contain high levels of DA without showing any symptoms, the spiny scallop (*Chlamys hastata*) died rapidly (within 12 hours) after exposure to cultures of toxic *P. multiseries* (Douglas *et al*., 1997). Substantial amounts of DA are found routinely in the digestive glands but not in the adductor muscles of offshore sea scallops in Canada at Georges Bank, Browns Bank and Bay of Fundy (Stewart *et al*., 1998).

4.4.3 Other marine organisms containing ASP toxins

In Dungeness crabs (*Cancer magister*) from Washington and Oregon States in the United States, DA was detected, but only in the viscera. DA levels in raw viscera of individual crabs from Washington State in December 1991 averaged 13 σg/g and ranged from 0.8 to 90 σg/g. The highest average levels of DA in Washington State crabs were found in Grays Harbor and Willapa Bay samples, 32 and 31 σg/g, respectively. By 1992, DA level averages were <5 σg/g in preseason samples of Dungeness crabs taken along the Oregon and Washington coasts, ranging from 0 to 71 σg/g. The highest levels of DA in 1992 (36-71 σg/g) were recorded in samples taken in January through April (Wekell *et al*., 1994a, b). The immediate source of DA for Dungeness crabs was unclear. These crabs were considered opportunistic predator-scavengers in the marine benthos. It is possible that Dungeness crabs prey on toxic subtidal razor clams, although some razor clams live in the “surf” zone and others persist in the subtidal regions. On the other hand, high DA levels were also observed in crabs taken from areas where few, if any, razor clams were found. Therefore other benthic sources of DA must also be considered (Wekell *et al*., 1994a).

DA was also found in benthic crustaceans, but the sources and pathways transferring DA to the benthic community have not been established and no studies were performed to determine how accumulated toxin might affect secondary consumers (Horner *et al.*, 1997).

In September 1991, water fowl (brown pelicans (*Pelecanus occidentalis*) and cormorants (*Phalacrocorax penicillatus*)) died in Monterey Bay, California, after eating anchovies (*Engraulis mordax*) contaminated with DA. Up to 485 σg/g DA was detected in the viscera of the anchovies. Frozen samples of anchovies harvested in April 1991, appeared to contain 270 σg/g DA in their viscera. By May, DA levels in frozen anchovy samples from the same area were less than 1 σg/g. Anchovies are primarily carnivorous but they will consume phytoplankton if other food sources are not available (Wekell *et al*., 1994a). McGinness *et al*. (1995) showed that the stomach of northern anchovies (*Engraulis mordax*) from Monterey Bay, Calfornia, (August 1992) contained nine different *Pseudo-nitzschia* species including four that have produced DA either under natural or under laboratory conditions. The study demonstrated that northern anchovies are able to filter pennate diatoms from the near-surface seawater.

In January 1996, the death of brown pelicans (*Pelecanus occidentalis*) at Cabo San Lucas on the tip of the Baja California Peninsula (Mexico) was ascribed to the feeding of mackerel (*Scomber japonicus*) contaminated by DA-producing *Pseudo-nitzschia* spp. (Sierra-Beltrán *et al*., 1997).

Over 400 Californian sea lions (*Zalophus californianus*) died along the central California coast during May and June 1998. DA produced by *P. australis* and transmitted to the sea lions via planktivorous northern anchovies (*Engraulis mordax*) was identified as the probable causative agent. Highest DA concentrations were found in the viscera (223 µg/g), exceeding values in body tissues by a factor 7. DA levels in sea lion faeces ranged from 136.5 to 152 µg/g by LC (requires correction from interference from an unidentified compound; receptor binding assay corresponded with LC values within a factor of two) (Lefebvre *et al*., 1999).

4.5 Toxicity of ASP toxins

4.5.1 Mechanism of action

The mechanism of action of DA is known on excitatory amino acid receptors and on synaptic transmission. Excitatory amino acids, most notably L-glutamate and L-aspartate, have long been considered to be the most likely neurotransmitters. These amino acids are known to act on several receptor types, the best characterized of which are named after the selective exogenous excitants N-methyl-D-aspartate (NMDA), kainate and quisqualate.

DA is a glutamate analogue and binds with high affinity to glutamate receptors of the quisqualate type. Glutamate and also NMDA subclass act to open membrane channels permeable to Na^+, leading to Na^+ influx and membrane depolarization. Only the channel opened by NMDA receptor accessible to kainate, quisqualate and to DA, is, in addition, highly permeable to Ca^{2+} and induced lethal cellular Ca^{2+} entry. Actions at NMDA receptors can be selectively antagonized by micromolecular concentrations of magnesium ions, organic antagonists such as D-2-amino-5-phosphonovalerate (APV) and dissociative anesthetics, such as phencyclidine (Viviani, 1992). In mice, kynurenic acid, administered intraperitoneally, protected powerfully and significantly against mussel-extract induced neurotoxicity. (Pinsky *et al*., 1989). Since drugs blocking the domoate-sensitive receptor are known, their use as antidotes for domoate poisoning has been considered (Laverty, 1993). DA is a two to three times more potent neuroexcitator than the structurally related kainic acid, and is up to 100 times more potent than glutamic acid. A synergistic effect between DA and other neurotoxic amino acids normally present in mussels is possible (Ravn, 1995). DA has two primary targets in the central nervous system; the hippocampal formation and its associated regions which are involved in processing memory, and the brain stem region of the area postrema and nucleus of the solitary tract associated with visceral function (Peng *et al*., 1994).

4.5.2 Pharmacokinetics

studies in laboratory animals

The absorption of DA after oral administration to rats is poor as was demonstrated by almost complete recovery in the faeces, suggesting that absorption of DA may be reduced in rodents compared to humans. After intravenous dosing to rats, thereby removing the impact of absorption, all DA was recovered in urine within 160 minutes and excretion was not affected by co-administration of probenecid. The results of this study indicated that DA was cleared from plasma by the kidneys and, more specifically, by the process of glomerular filtration. The plasma half-life in rats was 21.6 minutes compared to 114.5 minutes in monkeys. Clearly, the six-fold more rapidly elimination of DA in rats is at least partly responsible for the lack of sensitivity of the rat compared to the monkey (see also Chapter 4.5.3) (Iverson and Truelove, 1994). There was no evidence of any biotransformation of DA by the rodent or primate as it has always been recovered unchanged (Todd, 1993).

In female ICR mice, the clearance of DA from serum after single i.p. injections ranging from 0.25 to 4.0 mg/kg bw was more than 95 percent within two hours and complete clearance was seen after four hours (Peng and Ramsdell, 1996).

In adult rats, mice, monkeys and humans, DA poorly penetrates the blood-brain barrier. However DA has been shown to be very toxic not only to newborn but also to foetal mice *in utero* where DA clearly induced hippocampal excitotoxicity. Recently, DA was reported to be extremely neurotoxic to neonate rats at exposure levels 40 times lower by body weight than those reported for adult animals. Since the blood-brain barrier is incomplete during neurodevelopment, this fact may explain why neonates show a high sensitivity to DA (Mayer, 2000).

4.5.3 Toxicity to laboratory animals

acute toxicity

The toxic effects of DA have been studied using mice, rats and cynomolgus monkeys. The toxin induces very characteristic symptoms in mice and rats following intraperitoneal injection. The most characteristic symptoms include a unique scratching of the shoulders by the hind leg, followed by convulsions and often death. More subtle effects include hypoactivity, sedation-akinesia, rigidity, sterotypy, loss of posteral control and tremors (Wright and Quilliam, 1995).

A series of publications on the neurotoxic effects caused by DA, showed that the agonist produced a loss of neurons in the CA1, CA3 and CA4 area of the hippocampus, oedema and neuronal degeneration in the arcuate nucleus and vacuolated and pyknoted cells in the inner layer of the retina. The hippocampal lesions were identical to those found in human ASP victims (Iverson and Truelove, 1994).

<u>mice</u>

An intraperitoneal LD_{50} value in the mouse of 3.6 mg DA (via mussel extracts)/kg bw was reported. (Todd, 1993). The earliest neuroexcitatory effect on behaviour in female ICR mice was seen at a single i.p. dose of 0.5 mg/kg bw, in the form of hyperactivity. Stereotypic scratching behaviour was seen at doses ∅1.0 mg/kg bw. At 2.0 mg/kg bw and higher, convulsions and seizures occurred. At 4.0 mg/kg bw prolonged convulsions and seizures were observed and 4/9 mice died within 24 hours. The induction of c-*fos* mRNA in brain was detected at 1.0 mg/kg bw, whereas increased Fos immunostaining was localized in the dentate granule cells and the pyrimidal cells of the hippocampal formation at 0.5 mg/kg bw. These results indicated that Fos expression in the hippocampus was a sensitive biomarker for the neuroexcitatory effects of DA, being induced at doses lower than those eliciting stereotypic scratching behaviour (Peng and Ramsdell, 1996).

DA toxicity at different pHs in female CD mice after i.p. injection was measured as the onset times for scratching behaviour, seizure activity and death. At pH 3.7 the onset times were 12, 40 and 55 minutes, respectively. DA toxicity was lowest at pH 3.7 and highest at pH 7.4. Onset time for scratching behaviour was not affected by pH at three different doses (8.5, 11.5 and 14.5 mg/kg bw) whereas the onset times for seizure activity and death were significantly affected. The pH effect diminished as the dose of DA increased (at 14.5 mg/kg bw toxicity was the same at pH 3.7 and 7.4) (Nijjar and Madhyastha, 1997).

Studies in mice showed that toxic actions of DA are generally insensitive to NMDA (*N*-methyl-d-aspartate) receptor antagonists, although the onset of some responses could be delayed by CPP (3-(2-carboxypiperazine-4-yl) propyl-1-phosphonic acid). Domoate and kainate toxicity were reduced by drugs (6,7–dinitroquinoxaline-2,3-dione=DNQX and 2,3-dihydroxy-6-nitro-7-

sulfamoyl-benzo[*F*]quinoxaline=NBQX) that interact preferentially with non-NMDA receptors but do not distinguish between different kainate binding sites. The newly developed isatinoxime NS-102 selectively antagonized both the behavioural and pathological toxicity of DA relative to kanaic acid (Tasker *et al.*, 1996).

rats

Oral doses required more than 10 times as much toxin to achieve the same effects as i.p. doses. Mice and rats tolerated, orally, 30-50 mg DA/kg bw without observable effects (Todd, 1993).

Rats given DA as toxic mussel extracts orally, developed mastication and seizures when DA equivalent was ∅70 mg /kg bw and mortality occurred at doses ∅80 mg/kg bw. At 60 mg DA equivalents/kg bw the rats showed some clinical signs (flaccidity, head on floor and inactivity) but no excitation. Histopathological lesions of the central nervous system were seen only at 80 mg/kg bw (Tryphonas *et al.*, 1990a). The inability of the rat to vomit precluded what appeared to be a very sensitive clinical sign of DA intoxication in humans.

Rats showed scratching, crab-like walking, "praying", loss of balance and seizures after single i.p. doses of 2.0-7.5 mg/kg bw of crude DA (purity 85 percent) (one to four animals per group). At 1 mg/kg bw the animals were unaffected. In the rats given ∅2 mg/kg bw histopathological lesions were apparent in the amygdala, cortex, hippocampus, hypothalamus, olfactory system and retina (Tryphonas *et al.*, 1990b).

Sobotka *et al.* (1996) reported that i.p. doses less than 1 mg DA/kg bw produced already measurable behavioural effects in adult rats without apparent signs of neurological dysfunction or neuropathology. Slightly higher doses than 1 mg/kg bw, 1.32 mg/kg bw and 2.25 mg/kg bw, produced not only behavioural effects, clinical signs of neurotoxicity and occasional morbidity, but also hippocampal damage. DA induced convulsions at 2.25 mg/kg bw and caused effects on brain function that are still reflected six to eight days later in altered fatty acid metabolism and gliosis (Appel *et al.*, 1997).

Adult Sprague-Dawley rats given intraperitoneally 1 mg DA/kg bw in saline showed significantly increased serum T3 and T4 levels (determined by enzyme immunoassay) at 30, 45 and 60 minutes (end of the study) after injection. The serum T3 and T4 levels did not decrease during the 60 minutes after injection. After five minutes the animals showed significantly increased serum TSH levels (determined by radioimmunoassay) which remained elevated during 60 minutes after injection (Alfonso *et al.*, 2000).

Xi *et al.* (1997) reported that, after intraperitoneal injection, neonatal rats were approximately 80-fold more sensitive to DA induced scratching and approximately 40-fold more sensitive to DA induced seizures and lethal effects than adult rats. The i.p. LD_{50} for postnatal day two and day ten rats was 0.25 and 0.7 mg/kg bw, respectively. Mayer (2000) stated that during neurodevelopment in neonates the blood-brain barrier is incomplete and this fact might explain the higher sensitivity to DA.

Also Doucette *et al.* (2000) reported that DA was a very potent neurotoxin in newborn rats and the potency decreased progressively with increasing age (interpolated ED_{50} = 0.12, 0.15, 0.30 and 1.06 mg/kg bw at post-natal day 0, 5, 14 and 22, respectively).

In studies in rodents, the effects induced by extracts of contaminated mussels were compared with the effects induced by pure DA and the effects induced by extracts of non-contaminated mussels. The extract of contaminated mussels appeared to be the most potent formulation. This was

ascribed to potentiation of the excitatory effect of DA by glutamate and aspartate; both are excitatory amino acids found in mussel extracts (Mariën, 1996).

monkeys

Cynomolgus monkeys (*Macaca fascicularis*) received single oral doses of 0.5 to 10.0 mg DA/kg bw as mussel extracts or as crude or purified DA isolated from mussels. All animals receiving mussel extracts (~5.89-6.62 mg DA/kg bw) developed anorexia, salivation, retching, vomiting, diarrhoea and prostration as early as two hours after dosing for as long as 70 hours. All monkeys recovered. With the exception of diarrhoea and prostration, monkeys given crude or purified DA (5 to 10 mg/kg bw) developed similar clinical signs. In addition these animals showed licking and smacking of lips and empty mastication. One monkey given 10 mg/kg bw purified DA vomited one hour after dosing. At 0.5 mg/kg bw purified DA no clinical signs were seen. Mild to moderate histopathological central nervous system lesions consistent with neuroexcitation were seen at doses of 5 to 10 mg/kg bw (Tryphonas *et al*., 1990a).

In addition, Iverson and Truelove (1994) reported that oral doses of 0.5 mg DA/kg bw elicited no effect in cynomolgus monkeys but that 5.0 mg/kg bw caused vomiting, mastication and yawning. A single oral dose of 1.0 mg DA acid/kg bw to cynomolgus monkeys caused vomiting, gagging and yawning, but single doses of 0.75 or 0.5 mg/kg bw did not result in overt effects. Evidence of neurotoxicity, at the light microscopic level, was absent at 5.0 mg/kg bw orally, and present at 10 mg/kg bw orally (Todd, 1993).

When cynomolgus monkeys (one animal per dose) were given intravenously (i.v.) 0.025, 0.05, 0.2 or 0.5 mg/kg bw or intraperitoneally (i.p.) 4 mg/kg bw DA obtained from cultured mussels, clinical signs of neurotoxicity were seen preceded by a short pre-symptomatic period (two to three minites) and an even shorter prodromal period (0.5 to one minute). The symptomatic period was characterized by persistent chewing with frothing, varying degrees of gagging, and vomiting. At the higher doses also abnormal head and body positions, rigidity of movements, loss of balance and tremors were observed. Duration of the symptoms was dose-dependent. Excitotoxic lesions consisting of vacuolation of the neuropil, astrocytic swelling and neuronal shrinkage and hyperchromasia were detected in the area postrema, the hypothalamus, and the inner layers of the retina at an i.v. dose of 0.5 mg/kg bw and an i.p. dose of 4 mg/kg bw. It could be concluded that DA is neuroexcitotoxic and strongly emetic at single i.v. doses of 0.025-0.2 mg/kg bw. At higher doses (0.5 mg/kg bw i.v. and 4 mg/kg bw i.p.) DA is strongly excitotoxic (Tryphonas *et al*., 1990c).

DA given i.v. at doses as low as 12.5 σg/kg bw induced readily observable clinical signs (gag response) in monkeys. On a mg/kg bw basis, this level is 1 000 fold lower than that observed in the mouse bioassay using the clinical response as endpoint (Iverson and Truelove, 1994).

Schmued *et al*. (1995) applied a degeneration-specific histochemical technique (de Olmos' cupric silver method) to reveal degeneration within the brains of DA-dosed (i.v. 0-4 mg/kg bw) cynomolgus monkeys. This method revealed degenerating neuronal cell bodies and terminals not only within the hippocampus, but also within a number of other 'limbic' structures including the entorhinal cortex, the subiculum, the piriform cortex, the lateral septum and the dorsal lateral nucleus of the thalamus. The pattern of degeneration generally correlated with those regions containing high densities of kainate receptors. Slikker *et al*. (1998) quantified the abundance of the silver grains yielding continuous dose-response data. The authors applied this quantitative histochemical approach besides the currently used safety factor (SF) approach (in this case using SF 300) and the benchmark approach, for estimating acceptable doses of DA. Assuming 5 percent oral absorption of DA and a human body weight of 70 kg, the acceptable dose would be achieved if subjects ate 200 g of seafood containing 10, 12 and 6 mg DA/kg of seafood respectively

repeated dose toxicity

mice

Mice from two strains (outbred and seizure-sensitive inbred strain, respectively) received single or four intraperitoneal injections (every other day for seven days) with either sub-symptomatic (0.5 mg/kg bw) or symptomatic sub-lethal (2.0 mg/kg bw) doses of DA in order to investigate the possibility of enhanced toxicity (observable behavioural responses) after repeated exposure. The serum DA levels did not differ following single or repeated exposures. Both strains showed comparable concentration dependent toxic responses. The study did not provide evidence that short-term repeated exposures altered DA clearance from serum or led to a more sensitive or greater neurotoxic response than single exposure (Peng *et al*., 1997)

Single intraperitoneal injections of DA into mice have been shown to impair learning function on the place version of the Morris water maze task and the eight arm radial maze task. In the present study mice were examined for their spatial working memory on a delayed matching-to-sample task after single as well as repeated i.p. exposure. Groups of nine to ten male DBA mice received single or four intraperitoneal injections (with 48 hours interval times) of 0, 1.0 or 2.0 mg DA/kg bw in sterile phosphate buffered saline as vehicle. The animals in the single dosed groups received 3 additional injections with the vehicle with 48 hour interval times. During one hour after the last injection mice were evaluated for symptomatic toxicity (hypoactivity, sedation, hyperactivity, scratching, loss of balance control, tremors-convulsions, death). Toxicity scores showed a dose-dependency, but did not differ after single and repeated exposure. Impaired spatial working memory on the delayed matching-to-sample task was seen in the single dosed groups; greatest impairment at a single dose of 2.0 mg/kg bw. This means that the animals were unable to form a memory that persisted for 24 hours and hence were incapable to utilize the prior day's experience. The repeated exposure groups did not perform as poorly as the single dosed groups. This indicates that multiple pathways are utilized for the working memory tasks and that the animals appear to be able to accommodate by unknown processes following repeated DA exposures (Clayton *et al*., 1999).

rats

Three groups of 10 male and 10 female rats received daily, orally by gavage, 0, 0.1 or 5.0 mg DA/kg bw dissolved in water for 64 days. No clinical abnormalities were observed. Haematology and clinical chemistry did not show abnormalities. Organ weights did not reveal abnormalities. Histopathology of several tissues (including eyes and brain) and immuno-histochemistry of selected sections of hippocampus and retina were unremarkable. DA determinations in urine and faeces revealed that absorption was approximately 1.8 percent of the administered dose (Truelove *et al*., 1996).

monkeys

Three cynomolgus monkeys received daily, orally by gavage, 0.5 mg DA/kg bw dissolved in water, for 15 days and then for another 15 days 0.75 mg/kg bw. After the 30-day treatment period the monkeys were killed. Body weight, food and water consumption were recorded, clinical observations were made, haematology and serum chemistry were performed, histopathology of all major organs (including brain and retina) and glial fibrillary acid protein immunohistochemistry were carried out. All examined parameters remained unremarkable. DA concentrations in urine and serum were measured at several time points. Absorption in the monkeys appeared to be 4 to 7 percent (compared to 1.8 percent in rats) and the plasma half-life was 114.5 minutes (compared to 21.6 minutes in rats) (Truelove *et al*., 1997).

reproduction/teratogenicity

Groups of five pregnant CD-1 mice received by intravenous injection 0 or 0.6 mg DA/kg bw (25 percent of the convulsive dose) on day 13 of gestation and were allowed to deliver spontaneously. EEG was monitored in developing progeny during postnatal days 10, 20 and 30 for residual effects of intrauterine DA exposure. Hippocampal excitotoxicity appeared to be induced as a consequence of increasing neuronal calcium influx through kainate receptor activation. Histological changes suggested progressive hippocampal damage, but without overt clinical seizures. The progeny showed significantly reduced seizure thresholds to an additional dose of DA, given post-natally (Dakshinamurti *et al*., 1993).

Nine to 15 pregnant rats per group received intraperitoneally during days 7 to 16 of gestation 0, 0.25, 0.5, 1.0, 1.25, 1.75 or 2.0 mg DA/kg bw, respectively. On day 22 of gestation, the dams were killed and foetuses were examined for developmental changes and for visceral anomalies. No signs of maternal toxicity were observed up to doses of 1.25 mg/kg bw. At the dose of 2 mg/kg bw, six out of nine dams died after two doses. The remaining three dams on this dose-level aborted after three doses. At 1.75 mg/kg bw, six out of twelve dams aborted prior to Caesarean section. At 1.0 and 0.5 mg/kg bw a reduction in live foetuses/litter were seen. However, this effect was neither dose-related, nor associated with an increased incidence of resorptions plus dead foetuses. A statistically significant increased incidence of retarded ossification of the sternebrae was observed at 1.25 mg/kg bw, but this effect was not seen at any other dose level. At the lowest dose level of 0.25 mg/kg bw no maternal or foetal toxicity was seen. No teratogenic effects were observed in this study (Khera *et al*., 1994).

mutagenicity

Rogers and Boyes (1989) investigated the mutagenicity of DA in a hepatocyte mediated assay with V79 Chinese hamster lung fibroblasts. The genetic endpoints measured were: mutation to 6-thioguanine resistance at the HPRT locus; mutation to ouabain resistance at the Na^+, K^+-ATPase locus; sister chromatid exchanges and micronuclei frequency. No significant cytotoxicity was seen. None of the genetic endpoints was significantly affected by exposure to DA at dose levels of 27.2 and 54.4 σg/ml with or without metabolic activation by freshly isolated rat hepatocytes.

in vitro studies

An *in vitro* study with isolated rat cardiomyocytes showed that DA inhibited the action of extra-cellular ATP, a putative neurotransmitter that elevates intracellular Ca^{2+} in the cardiomyocytes and is considered to regulate the heart function (Nijjar *et al*., 1999)

DA induced the death of cultured neurons of chick embryonic retina and also inositol triphosphate (ip_3) accumulation 4 to 7x above basal levels, both in a concentration and Ca^{2+}-dependent manner (Duran *et al*., 1995).

4.5.4 Toxicity to humans

Anecdotal evidence has indicated that Japanese islanders once prized seaweed extracts containing DA as a very useful tonic. The red alga *Chondria armata* containing DA has been used for treatment of roundworm disease for centuries and as insecticide (Higa and Kuniyoshi, 2000). Trials were apparently undertaken to test the anthelmintic properties of DA and single 20 mg doses of unknown purity were administered to adults and children without harmful effect (Iverson and Truelove, 1994).

However, DA is toxic to both the central and peripheral nervous systems of humans. DA is an emetic causing gagging and vomiting, likely through its effect on the vomit centre in the area

postrema of the brain. It produces a syndrome of axonal sensorimotor neuropathy, amnesia, seizures, coma and death. Because of its impact on memory, among other ill effects, DA intoxication was named amnesic shellfish poisoning (ASP) (Todd, 1993 and Watters, 1995).

In the first ASP outbreak in 1987 at Prince Edward Island in Canada, 107 cases were reported. The first symptoms were experienced 15 minutes to 38 hours (median 5.5 hours) after mussel consumption. The most common symptoms were nausea (77 percent), vomiting (76 percent), abdominal cramps (51 percent), headache (43 percent), diarrhoea (42 percent) and memory loss (25 percent). There was a close correlation between memory loss and age; those under 40 were more likely to have diarrhoea and those over 50 to have memory loss. Memory loss was predominantly short-term. The most severely ill were hospitalized, of which 12 were treated in intensive care units (ICU). Eight of these were ∅65 years old and the other four had pre-existing illnesses (diabetes, chronic renal feature or hypertension). The ICU patients demonstrated confusion, coma, mutism, seizures, chewing motions, grimacing, hiccups, lack of response to painful stimuli, uncontrolled crying or aggressiveness, profuse respiratory secretion, and unstable blood pressure or cardiac arrhythmias. Fourteen patients showed persistent neurological defects. Eye problems were noted in several of these, including disconjugate gaze, diplopia and ophthalmoplegea, but these resolved within 10 days (Todd, 1993). In addition they manifested seizures, myoclonus, anterograde memory deficits, decreased glucose metabolism in the medial temporal lobes on positrone-emission tomography (PET) scanning, and EMG changes of pure-motor or sensori-motor axonopathy. Four out of the 14 patients remained in coma and died. Post-mortem examination revealed necrosis and neuronal loss predominantly in the hippocampus and amygdala (Teitelbaum *et al*., 1990). Amounts of DA consumed, ranged from 15 to 20 mg/person for an unaffected person to 295 mg/person for a case with severe neurological symptoms. Assuming that average body weight is 50 to 70 kg the unaffected male person ingested 0.2-0.3 mg DA/kg bw. Some persons showed mild symptoms (mainly gastrointestinal) after consuming 60 to 110 mg DA, equivalent to 0.9 to 2.0 mg/kg bw. The most serious cases (severe neurological deficits) consumed 135 to 295 mg, equivalent to 1.9 to 4.2 mg DA/kg bw (Todd, 1993).

Effects due to long-term exposure of humans to low concentrations of DA in mussels or fish are not known (Van Apeldoorn *et al*., 1999). One 84-year old man showed status epilepticus after acute DA intoxication. After a "silent" year, he developed temporal lobe epilepsy. Three and a half year after the acute intoxication the patient died due to pneumonia. Post-mortem examination revealed severe bilateral hippocampal sclerosis. This indicated that the human hippocampus is vulnerable to kainate-receptor excitotoxicity (Cendes *et al*., 1995).

4.5.5 Toxicity to aquatic organisms

In a laboratory study, DA appeared to be toxic to the marine copepod (*Tigriopus californicus*) at low concentrations. LC_{50} (24 hours) was found to be 8.62 σM (Van Apeldoorn *et al*., 1999). In autumn 1993, an unexplained mortality among sea scallops (*Placopecten magellanicus*) occurred in the Bay of Fundy, Canada. The digestive gland of the scallops appeared to contain 93.4 σg DA/g. Although some bivalve molluscs have been reported to contain high levels of DA without showing any symptoms, the spiny scallop (*Chlamys hastata*) died rapidly (within 12 hours) after exposure to cultures of toxic *P. multiseries* (Douglas *et al*., 1997).

Chlamys hastata might be the source of crab toxicity. The swimming scallops *Chlamys hastata*, when exposed to DA by feeding on *Pseudo-nitzschia multiseries*, lost motor or escape responses and would fall easy to prey bottom dwelling scavengers such as crabs (Whyte *et al*., 1997).

In a laboratory study, sea scallops (*Placopecten magellanicus*) were fed *P. multiseries* with a high content of DA (4-6.6 pg/cell) for 22 days, followed by 14 days of feeding with nontoxic

microalgae. No signs of illness or mortality were observed during this study despite the high DA loading. However, the destructive sampling of the scallops did not allow assessment of long-term effects (Douglas *et al*., 1997).

The physiological effects of DA on the marine invertebrates Pacific oyster (*Crassostrea gigas*) and California mussel (*Mytilus californianus*) which were known to accumulate this neurotoxin, were investigated. The oysters and the mussels were exposed continuously to the DA producing diatom *P. pungens* f. *multiseries*, for 48 hours followed by a 120 hour clearance period. The Pacific oyster rapidly accumulated significant soft tissue burdens of DA resulting in a generalized stress response characterized by shell closure four hours after introduction of the algae, hemolymph acidosis and an acute transient hypoxia. The Californian mussel appeared to increase its ventilatory flow resulting in a mild non-compensated respiratory alkalosis (Van Apeldoorn *et al*., 1999).

DA in *Pseudo-nitzschia multiseries* caused feeding inhibition of the rotifer, *Brachionus plicatilis*, with subsequent reduced nutritional condition and loss of fecundity (Whyte *et al*., 1997).

Over 400 Californian sea lions (*Zalophus californianus*) died along the central California coast during May and June 1998. DA produced by *P. australis* and transmitted to the sea lions via planktivorous northern anchovies (*Engraulis mordax*) was identified as the probable causative agent (Levebvre *et al*., 1999).

4.5.6 Toxicity to water fowl

In September 1991, water fowl (brown pelicans (*Pelecanus occidentalis*) and cormorants (*Phalacrocorax penicillatus*)) died in Monterey Bay, California, after eating anchovies (*Engraulis mordax*) contaminated with DA (Horner *et al*., 1997). In January 1996 brown pelicans (*Pelecanus occidentalis*) died at Cabo San Lucas on the tip of the Baja California Peninsula, Mexico. The death of these birds was the result of feeding on mackerel (*Scomber japonicus*) contaminated by DA-producing *Pseudo-nitzschia* spp. (Sierra-Beltrán *et al*., 1997).

4.6 Prevention of ASP intoxication

4.6.1 Depuration

To date there have been no useful methods devised for effectively reducing phycotoxins in contaminated shellfish. All methods tested have been unsafe, too slow or economically unfeasible, or have yielded products unacceptable in appearance and taste (Shumway *et al*., 1995). Mussels were reported to take up DA rapidly but also depurated rapidly, while other bivalves retained DA for longer periods. Depuration of DA by razor clams is a long-term process (Horner *et al*., 1997). Depuration of DA from starved mussels and clams was relatively rapid (43 to 15 σg/g at 13 ∀C in 24 hours with traces remaining for up to six days in Passamaquaddy Bay, Canada, and 130 to 20 σg/g at 15 ∀C in four to six days in the Cardigan River, Canada). Complete depuration, however, in the natural habitat may take longer. DA concentrations in Cardigan Bay area, eastern Prince Edward Island, Canada declined to negligible levels in 40 to 50 days (Todd, 1993).

Whole scallops (*Pecten maximus*) flesh contaminated with DA, showed a 43 percent decrease (mostly in hepatopancreas) in DA content after 180 days of frozen storage (-20 ∀C). During frozen storage, there was a transfer of DA from the hepatopancreas to the rest of the body, with a net average decrease in the whole product. Subsequently pickling of the scallops flesh or packing with brine and canning after frozen storage did not cause a further decrease of the DA content. During canning there was a notable transfer of toxin from the scallops to brine or the pickling

medium (more than 30 percent of total DA content in canned product) (Leira *et al*., 1998). Sea scallops (*Placopecten magellanicus*) and red mussels (*Modiolus modiolus*) were reported to retain DA for lengthy periods (Stewart *et al*., 1998). The DA level in the digestive gland of sea scallops was found to be only slightly lower at the end of a 19-month depuration study than it was at the beginning (Stewart *et al*., 1998). Dungeness crabs (*Cancer magister*) accumulated the toxin mostly in the viscera, although it can enter meat during cooking if the crabs were not eviscerated previously (Villac *et al*., 1993a). When contaminated whole crabs (22 σg DA/g confined to hepatopancreas) were cooked in fresh or salted water, visceral DA was reduced by 67 to 71 percent. After cooking, DA was detected not only in hepatopancreas (6.4 σg/g) but also in the body (1.9 σg/g) and leg (1.1 σg/g) meats. However, the majority of the DA was extracted out and diluted into the cook water. When cooked crabs were held for one or six days at 1 ∀C, DA was detected in hepatopancreas (6.1 to 8.2 σg/g) and body meats, but not in leg meats. Body meats proximal to the viscera contained higher DA levels (1.5 to 2.1 σg/g) than those distal (0.57 to 0.92). When cooked crabs were held for 90 days at -23 ∀C, DA was detected in the viscera (7.6 σg/g), body (0.67 to 0.79 σg/g) and leg (0.38 σg/g) meats. The storage conditions of cooked crabs had some effect on DA distribution, but no effect on the total DA content in each crab (Hatfield *et al*., 1995).

In laboratory studies, Lund *et al*. (1997) showed that DA was effectively depurated from the hepatopancreas of Dungeness crabs over a three-week period once the toxic feeding of DA via contaminated clam meat ceased. Depuration proceeded at a faster rate when crabs were fed toxin-free clam meat than when they were starved.

Depuration studies on the west coast of Canada with Dungeness crabs indicated that, if the crabs were placed in filtered seawater, DA levels dropped rapidly within a few weeks, but in harbour water in cages (without access to contaminated shellfish), DA levels fluctuated but did not go down (Todd, 1993).

Arévalo *et al*. (1998) reported that the mean decrease in toxicity in standard total tissue scallop (*Pecten maximus*) samples (homogenised from 100 g of total tissue) after removing the hepatopancreas, was 94 percent (ranging from 82.3 to 100 percent).

4.6.2 Preventive measures

Commercial harvest areas and aquaculture facilities are adversely and often unpredictably affected by toxic blooms. One problem is that certain algal species, which have never occurred in a certain area, may suddenly appear and then rapidly cause problems. Therefore preventive measures can hardly be taken. Extensive monitoring of the marine environment and the possibly contaminated fishery products together with regulations (see Chapter 4.8) will be required to prevent (shell)fish poisoning incidents. Data on the occurrence of toxic algal species may indicate which toxins may be expected during periods of algal blooms and which fishery products should be considered for analytical monitoring. In the case of domestic produce, several countries stopped the harvest of fishery products if levels of the toxin exceeded the limits and a waiting period was established until the concentrations of the toxin are below the acceptable limit (Shumway *et al*., 1995). Within the genus *Pseudo-nitzschia,* DA production can vary greatly with the species and it is vital to be able to distinguish species (Van Apeldoorn *et al*., 1999). Toxin concentrations in the fishery products can also vary with the species of fishery product involved and with the area of harvest. Harvested fishery products containing too much toxin were usually destroyed. Toxic doses are often estimated from left-over toxic seafood but these may not be always representative of the ingested food (Shumway *et al*., 1995).

Direct analysis of phytoplankton samples for DA will probably remain the fastest and most reliable method to confirm the presence of DA (Van Apeldoorn *et al*., 1999). Although the need for routine phytoplankton monitoring often has been stressed, phytoplankton monitoring has been implemented only in a few areas (Horner *et al.*, 1997).

It is relatively easy to recognize *Pseudo-nitzschia* in mixed plankton using light microscopy. However, identification and enumeration between different *Pseudo-nitzschia* species in natural populations is often difficult and time consuming because of the need for detailed morphological observations often requiring scanning or transmission electron microscopy. Therefore large subunit ribosomal RNA (LSU rRNA)-targeted fluorescent DNA probes for discrimination among a variety of *Pseudo-nitzschia* species, a.o. collections from Monterey Bay, California, were developed. Probes were applied using both whole cell and sandwich hybridization techniques (Miller and Scholin, 1998, 2000; Parsons *et al*., 1999; Scholin *et al*., 1997, 1999).

In New Zealand, identification of toxic *Pseudo-nitzschia* species was performed using FITC-conjugated lectins. The binding of FITC-conjugated lectins to specific sugars has been shown to be a suitable assay for differentiating between some micro-algal species. The differential binding of the various FITC-conjugated lectins allowed the discrimination of the New Zealand species isolates from *P. multiseries, P. pungens* and *P. australis* using epifluorescence microscopy, but not the discrimination of *P. delicatissima* or *P. pseudodelicatissima*. The results differed from those reported for *Pseudo-nitzschia* species from Galician (Spanish) waters suggesting variability in the production of surface sugars by these diatoms depending on geographical origin and/or environmental conditions. Therefore, ribosomal RNA-targeted oligonucleotide probes are still the preferred method of *Pseudo-nitzschia* species identification (Rhodes, 1998).

Monitoring of intertidal shellfish may be not a particularly sensitive method for detecting of low but significant levels of DA in plankton, missing all but the most extreme events (Van Apeldoorn *et al*., 1999). This is caused by the differences in uptake and depuration in the shellfish species. Mussels, for instance, were reported to take up DA rapidly but also depurated rapidly, while other bivalves retained DA for longer periods. Depuration of DA by razor clams is a long-term process (Horner *et al*., 1997). However, Whyte *et al*. (1997) claimed that studies of the uptake and depuration of DA by the mussel *Mytilus californianus* had shown that this species was adequate as a sentinel organism in a weekly testing programme, despite the relatively rapid elimination of DA by this species.

4.7 Cases and outbreaks of ASP

4.7.1 Europe

The presence of ASP toxins in European coastal waters is illustrated in Figure 4.2.

Belgium

During 2000 and 2001, 151 and 154 samples respectively were tested for ASP. No toxic episodes were detected in shellfish produced in Belgium (EU-NRL, 2001).

Denmark

Investigations in Danish waters have shown that *P. seriata*, a widely distributed species in colder areas of the Northern hemisphere, produced DA in concentrations similar to those found in *Pseudo-nitzschia pungens* f. *multiseries* in Canada (1-20 pg/cell). During the survey five species and one subspecies of *Pseudo-nitzschia* were found in Danish waters: *P delicatissima, P. fraudulenta, P. pseudodelicatissima, P. pungens, P. pungens* f. *multiseries* and *P. seriata*. Isolates of *P. seriata* appeared to contain DA. In isolates of three other *Pseudo-nitzschia* species detected

during this survey, *P. pseudodelicatissima, P. delicatissima* and *P. pungens*, no DA was present. *P. seriata* is one of the most common species of *Pseudo-nitzschia* in the North Atlantic. These findings support the idea that toxic and non-toxic strains occur within the same species of the diatom (Van Apeldoorn *et al*., 1999).

In 1992, a large bloom was detected due to *P. pseudodelicatissima* (around 16 x 10^6 cells/litre). No DA was detected. In 1993, another bloom was detected with a lower density (about 5 x 10^5 cells/litre) and small concentrations of DA were detected (EU-NRL, 1996).

France

In May 2000, DA levels in *Donax trunculus* above the regulatory limit were detected (27 to 53 µg/g tissue) on the French coast (Western Brittany). The causative algal species were *P. pseudodelicatissima* and *P. multiseries* (Amzil *et al*., 2001). In 2002, one episode was observed on a part of the Mediterranean coast in late April and early May, but it did not last a very long time. The toxin levels were below 50 µg DA/g of flesh (EU-NRL, 2002).

Ireland

Very high concentrations of DA, up to 3000 µg/g in scallop hepatopancreas, were detected in Ireland in December 1999. DA was detected in scallops from production areas on all Irish coasts. In other species no DA was detected (EU-NRL, 2000).

During 2000 and 2001, 738 and 500 samples respectively were analysed for ASP toxins. ASP toxins above the regulatory limit were detected in 83 percent of the scallops in the hepatopancreas but only in 8 percent and 1 percent in the gonad and adductor muscle, respectively (EU-NRL, 2001). During 2002, 620 ASP analyses were carried out. For the first time ASP toxin levels above the regulatory limit were detected in mussels from a production area in the northwestern Ireland. In scallop samples, ASP toxins were detected at a number of locations. Maximum DA level in adductor muscle was 33.8 µg/g and 2 percent of adductor muscle samples showed a DA level above the regulatory limit. Maximum DA level in the gonad was 79.9 µg/g and 10 percent of gonad samples had a DA level above the regulatory limit. Maximum DA level in total tissue was 574 µg/g and 31.7 percent of total tissue samples had a DA level above the regulatory limit (EU-NRL, 2002).

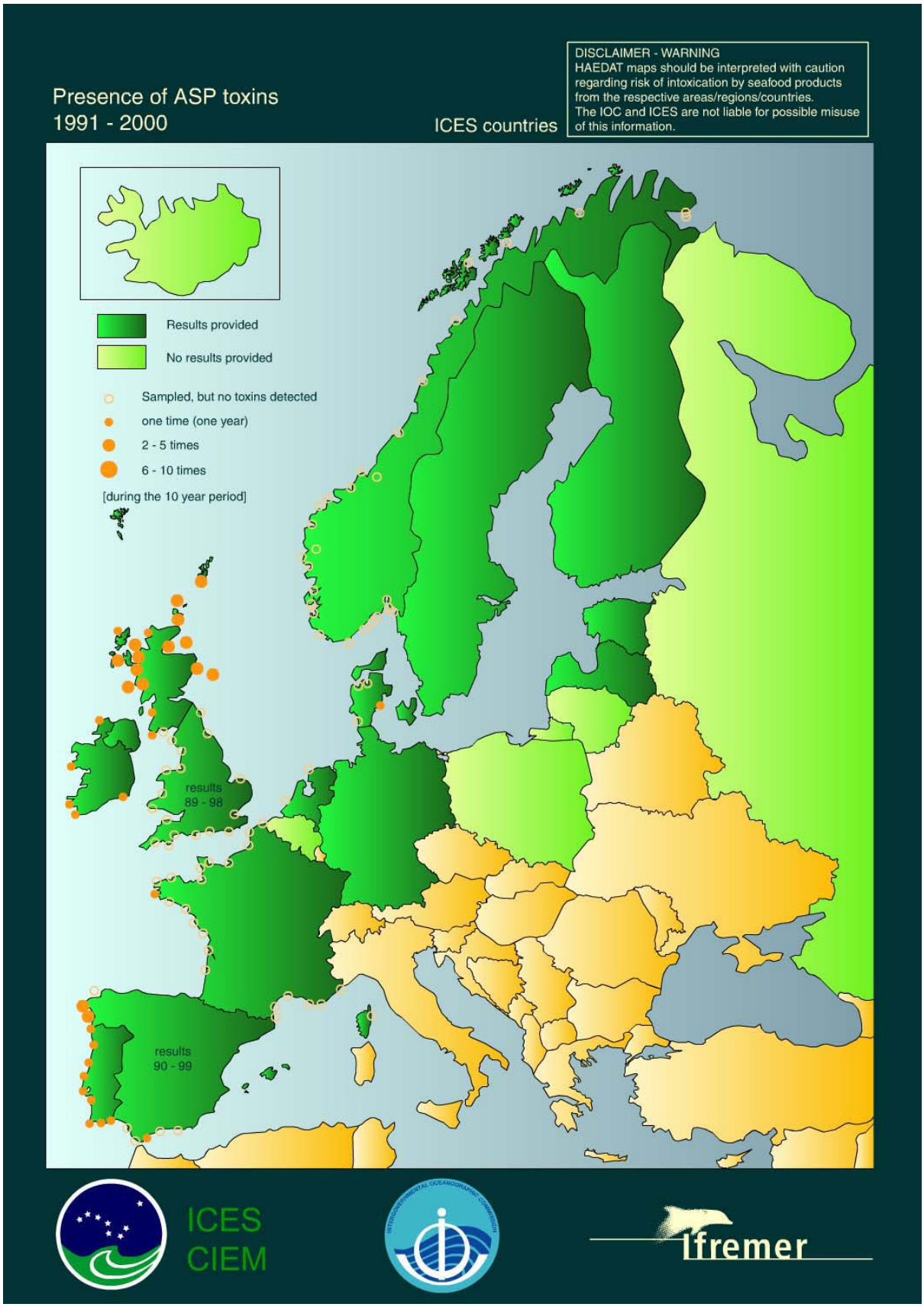
Italy

In the Gulf of Naples, high concentrations (up to 4x10^6 cells/litre) of *Pseudo-nitzschia* species (*delicatissima* and *pseudodelicatissima*) occurred sometimes (Montresor *et al*., 2000). In July 2002, DA above the regulatory limit was detected in *Pecten maximus* from Scotland (EU-NRL, 2002).

The Netherlands

In the Dutch Wadden Sea, *Pseudo-nitzschia*-like pennate chain-forming diatom species with cell numbers ranging from 10^2 to 10^5 per litre were detected between November 1993 and July 1994. Elecron microscopy of cultured isolates and field samples revealed the majority to be *Pseudo-nitzschia pungens*. At the beginning of June 1994, *P. fraudulenta* was also present and occasionally *P. delicatissima* was detected. One isolate showed the characteristic morphology of *P. multiseries*. The isolate of *P. multiseries* produced DA; after 55 days of growth about 19 pg/cell was measured. The *Pseudo-nitzschia* species found in Dutch coastal waters have not yet been implicated in shellfish poisoning (Van Apeldoorn *et al.*, 1999). In 2001, DA producing algae were detected but ASP toxins were not detected in shellfish (EU-NRL, 2001). In spring 2002, a single sample of *Spisula* contained levels up to 5 mg DA/kg (LC-UV method). Resampling three days later resulted in the absence of DA (EU-NRL, 2002).

Figure 4.2 Occurrence of ASP toxins in coastal waters of European ICES countries from 1991 to 2000



Source: http://www.ifremer.fr/envlit/documentation/dossiers/ciem/aindex.htm

Norway

Despite high abundances of *Pseudo-nitzschia* species no outbreaks of ASP have been reported (EU-NRL, 1998). In 2000 and 2001, DA was detected in mussels and scallops, however never above the regulatory limit even during a bloom of *Pseudo-nitzschia* (>4 000 000 cells/litre) (EU-NRL, 2001).

Portugal

In 1996, DA was detected in very small amounts (<20 σg/g) in almost every bivalve species all around the Portuguese coast for short periods scattered in time and coincident with the occurrence of *Pseudo-nitzschia* spp., mainly *P. australis* in concentrations below 10^5 cells/litre. The first detected occurrence of DA in bivalves over 20 σg/g was in smooth callista (*Callista chione*) in 1995 (EU-NRL, 1998). In 1999, a total of 960 samples were tested for the presence of ASP toxins. DA was detected in 102 samples but the regulatory limit was exceeded only in two samples (EU-NRL, 2000). In 2000 and 2001, DA was detected in scallops from a wild fishery (EU-NRL, 2001). DA in Portuguese shellfish is a recurrent event that affects several shellfish resources several times a year, mainly in spring and autumn. Levels as high as twice the regulatory limit are not unusual. When several shellfish species are exploited in the same restricted area, common cockle (*Cerastoderma edule*) and carpet shell (*Venerupis pullastra*) are usually among the most toxic, followed by pepper furrow shell, clam, mussel, oyster and razor clam. In whole sardines, DA was detected in levels exceeding sometimes the regulatory limit. Fortunately, toxicity is restricted to the gut content, and does not accumulate in muscle tissue (Vale and De Sampayo, 2001)

Spain

In October 1994, DA was detected for the first time in cultured mussels (Ω20µg/g) from Galicia in northwest Spain, coincident with a bloom of *P. australis*. Four other *Pseudo-nitzschia* spp. found in Galician waters (*P. fraudulenta, P. cuspidata, P. pungens, P. delicatissima*) were cultured and found not to produce DA. Other species identified in these waters, but not yet cultured were *P. multiseries, P. subpacifica* and *P. pseudodelicatissima* (Fraga e*t al*., 1998). In April 1995, DA was again detected in mussels (> 20 µg/g) (Galician Rias) coincident with the occurrence of *Pseudo-nitzschia australis*. In August and October 1995 (Galician Rias) and in September and November 1996 (Galician Rias), DA in mussels was detected coincident with the occurrence of *Pseudo-nitzschia* spp. Quick detoxification of the mussels occurred in 1996.

From September to December 1995 and during all of 1996, DA was detected in scallops (Galician Rias) coincident with the occurrence of *Pseudo-nitzschia* spp. Slow detoxification occurred.

It was reported that a few of the events were highly virulent, produced toxin levels in mussels and other shellfish near the guideline value, and usually occurred in restricted areas. Since 1995, collection of scallops is banned in most areas of Galicia because of the presence of ASP toxins (EU-NRL, 1998).

In 1999, 4 953 ASP analyses by LC were carried out. During 1999, different toxic events which were related to the presence of a.o. ASP toxins occurred leading to the prohibition of harvesting of bivalves in some production areas (EU-NRL, 2000). During 2000 and 2001, there were many closures because of the presence of DSP and ASP toxins (EU-NRL, 2001). During 2002, there were short toxic ASP events in Galicia and Andalucia (except for scallops). Production areas were closed due to the presence of *Pseudo-nitzschia* spp. (EU-NRL, 2002).

The United Kingdom of Great Britain and Northern Ireland

In 1997, traces of DA were detected for the first time in Scotland (Shetland). No details were available (EU-NRL, 1998). In July 1999, a scallop fishing area of 8 000 square miles on the west coast of Scotland was closed following the discovery of ASP toxins (Wyatt, 1999). *P. australis* was found to be the source of DA in king scallops (*Pecten maximus*) and queen scallops (*Chlamys opercularis*) in 1999 and 2000 (Bates, 2000). The main toxicity event during 1999 was the closure of 8 000 km^2 of scallop fishing grounds after the detection of DA. Low levels of DA were detected in mussels but concentrations of up to 250 µg/g were detected in scallop gonads and up to 500 µg/g in total tissue (EU-NRL, 2000). During 2000 and 2001, ASP toxins above the regulatory limit were found in whole scallops and in scallop gonad tissue from all major scallop fishing areas around Scotland. Restrictions were placed on fishing activities in all affected areas (EU-NRL, 2001).

In the period from 1 April 2002 to 31 March 2003, shellfish from 76 primary inshore production areas, 36 secondary areas and offshore fishing areas in Scotland were examined. A total of 5 409 mollusc samples were analysed. Out of this total, 2 788 samples were analysed for ASP toxins and 115 samples were determined positive (Anonymous, 2003c).

In Northern Ireland, ASP toxins above the regulatory limit were detected in scallops in 2001 (EU-NRL, 2001). The United Kingdom Food Standards Agency subsequently announced a ban on scallop fishing in the sea adjacent to Northern Ireland (Anonymous, 2001a).

4.7.2 North America

The presence of ASP toxins in North American coastal waters is illustrated in Figure 4.3 below.

Canada

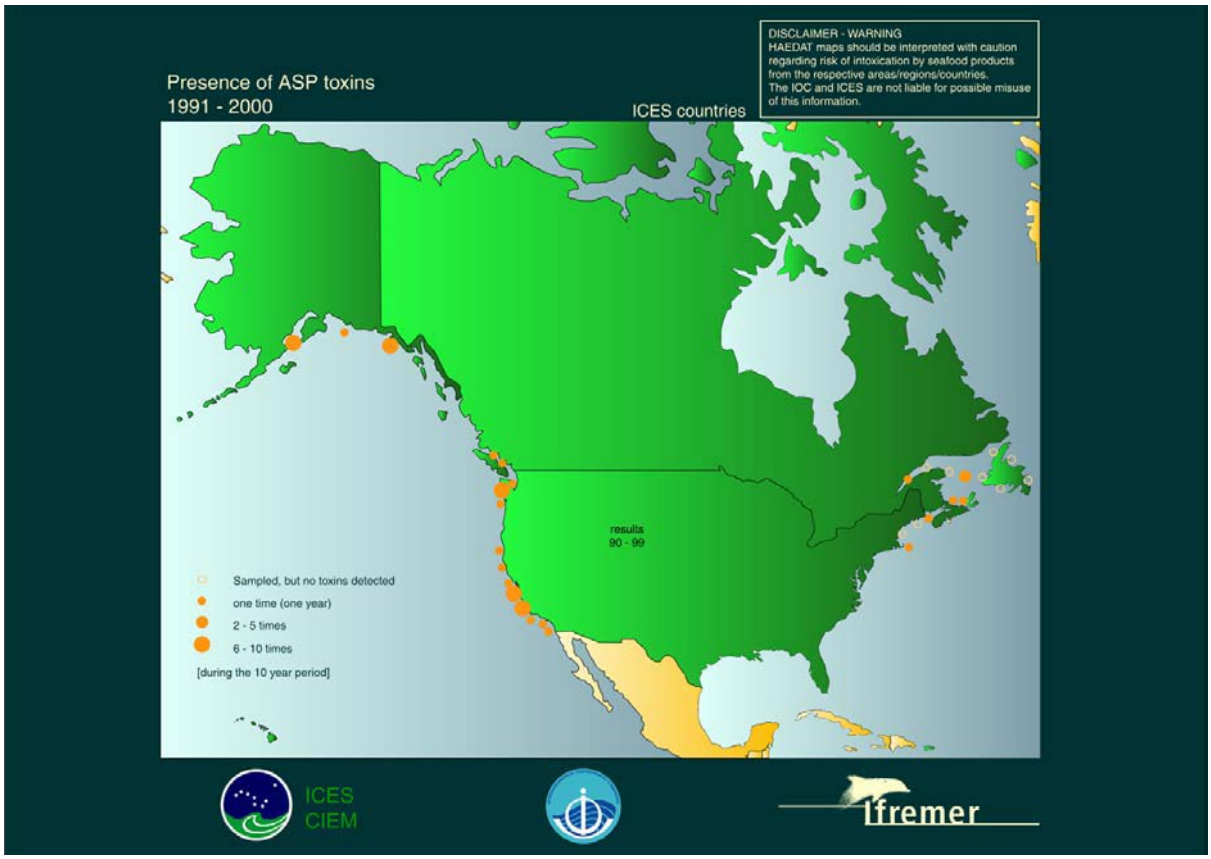
The first report of ASP in Canada dates from 1987. An outbreak of food poisoning during November and December 1987 was traced to cultured blue mussels (*Mytilus edulis*) from the Cardian Bay region of eastern Prince Edward Island. Three deaths and 105 confirmed cases of acute intoxication were recorded following consumption of mussels from this area. The toxin was identified as DA and the source organism appeared to be the pennate diatom *Pseudo-nitzschia pungens* f. *multiseries*. A plankton bloom at the time of the outbreak consisted almost entirely of this diatom and a positive correlation was found between the number of *P. pungens* f. *multiseries* cells and DA concentration in the plankton. This toxic shellfish incident was the first known outbreak of human intoxication due to ingestion of DA (Bates *et al.*, 1989). It is not known whether ASP occurred earlier than 1987 but prior to 1980 the mussel industry was in its infancy. However, there was one incident in 1984 in Calgary, Alberta, in which 12 persons consumed Prince Edward Island mussels and developed vomiting, diarrhoea and blurred vision 1.5 to three hours later; the symptoms lasted from one to seven days. No etiologic agent was identified (Todd, 1993). In the years after the 1987 incident, blooms have occurred but have been less extensive. Only in the autumn, DA levels in shellfish sometimes reached the guideline value at which harvesting areas had to be closed for a short period. In October 1991, a limited bloom was recorded from northern Prince Edward Island (Todd, 1997).

Since 1988, phytoplankton samples have been collected at four stations in the western Bay of Fundy. Blooms of *P. pseudodelicatissima* in the Bay of Fundy tend to occur during late summer with highest concentrations observed during 1988 and 1995 ($> 1.0x10^6$ cells/litre), during late August and early September, leading to closure of shellfish harvesting areas (Martin *et al.*, 2001).

During August to October 1988, DA levels greater than the acceptable levels for human consumption (20 σg/g) were detected in soft-shell clams (*Mya arenaria*) and blue mussels (*Mytilus edulis*) from the southwestern Bay of Fundy, New Brunswick, resulting in the closure of some shellfish harvesting areas. *P. pseudodelicatissima* was found to be the source of DA and was detected in all plankton tows (collected since 1987) where DA was found. *P. pseudodelicatissima* was detected throughout the year with higher concentrations in June/July followed by the highest concentrations in September when water temperatures were elevated. The highest concentration (1.2×10^6 cells/litre) of *P. pseudo-delicatissima* was measured during 1988 and persisted throughout the water column for a longer period than during 1987, 1989 and 1990. Analysis of nutrients (chlorophyll a, salinity, nitrate, phosphate, silicate at surface and 10 and 1 m above bottom; measured, however, during 1989 and 1990 and not in 1988) did not reveal an obvious correlation between *P. pseudodelicatissima* and nutrient concentrations (Martin *et al.*, 1993).

In adductor muscles of offshore sea scallops from Georges Bank, Browns Bank and Bay of Fundy no DA was found, but substantial amounts (10-200 σg/g) were routinely found in the digestive glands. Only the adductor muscles were available for sale because the digestive glands usually contain paralytic shellfish poisons (PSP). In April and May 1995, sea scallops on Georges Bank showed DA levels in their digestive glands in excess of 1 300 σg/g and up to 150 σg/g in the roe, while Brown Bank scallops had more than 2 500 σg/g in their digestive glands. The single highest individual value recorded for Brown Banks was 4 300 σg/g of scallop digestive gland in 1995. The source of DA in this 1995 episode was not discovered (Stewart *et al.*, 1998). In September 2000, DA-contaminated mussels were found on the east coast (Mos, 2001).

Figure 4.3 Occurrence of ASP toxins in coastal waters of North American ICES countries from 1991 to 2000



Source: http://www.ifremer.fr/envlit/documentation/dossiers/ciem/aindex.htm

The United States of America

Alaska

In Alaska, no severe problems with DA have existed although potentially toxic *Pseudo-nitzschia* spp. had been identified in Alaskan waters. Approximately 3 000 samples, primary commercially valuable shellfish and finfish, have been tested since 1992. The highest DA value was 11.1 σg/g (for a razor clam) with only 17 values above 2 σg/g (Horner *et al*., 1997)

West Coast

In late October and November 1991, razor clams (*Siliqua patula*) living in the surf zone on Pacific coast beaches in Washington and Oregon contained DA at levels in the edible parts (i.e. foot, siphon, and mantle) as high as 154 σg/g (wet weight). Therefore recreational and commercial harvest of the clams was forbidden (Van Apeldoorn *et al*., 1999). Twenty-four cases of illness of humans were reported in the State of Washington with mild gastrointestinal symptoms and one complaint of memory deficit but the occurrence of ASP was never confirmed. DA levels were still above the harvest closure measure of 20 σg/g at least until May 1992. Other molluscan shellfish, including oysters grown commercially in coastal embayments, and mussels, never became toxic (Horner *et al*., 1997).

Subsequently, DA was found in the viscera of Dungeness crabs (*Cancer magister*) in coastal waters of California, Oregon and Washington. As a consequence, this important commercial fishery was closed for several weeks until investigators determined that proper cleaning of the crabs before cooking kept DA out of the edible meat (Horner and Postel, 1993). The source of DA in razor clams and Dungeness crabs during these incidents was not determined (Horner *et al*., 1997).

Trainer *et al*. (1998b) suggested that *P. pungens* might be the species responsible, at least partially, for the accumulation of DA in razor clams at levels above 20 µg/g in coastal areas of Washington State in 1991. Since razor clams depurate DA slowly from their tissues, chronic exposure to low levels of DA may be sufficient to result in its accumulation.

In Hood Canal in western Washington in autumn 1994, a bloom of *P. pungens*, *P. multiseries* and *P. australis* persisted for more than six weeks. Mussels, the sentinel organism in this state to test for algal toxins, contained ~10 σg DA/g (wet weight) and the phytoplankton ~14 σg/g wet weight (Horner *et al*., 1997).

A bloom of *Pseudo-nitzschia* spp. was observed in Penn Cove, Washington in July and August 1997. Levels of DA in mussels up to 3 µg/g were measured. In seawater DA levels ranged from 0.1-0.8 µg/L. Four species of *Pseudo-nitzschia* were detected, namely *P. pungens, P. multiseries, P. australis* and *P. pseudodelicatissima*. Highest *Pseudo-nitzschia* concentration was 13 x 10^6 cells/litre on 28 July 1997 (Trainer *et al*., 1998a).

In the autumn of 1998, elevated DA levels prompted a coast-wide closure of the Washington razor clam fishery. In April 2001, a spike in marine toxin levels suspended razor clam digging in the Twin Harbours area but subsided enough for resumption of digging within a month. In October 2002, all three ocean beaches north of Grays Harbour, Washington, which were tentatively scheduled to open in the beginning of October, remained closed until further notice after the detection of DA in razor clams exceeding the Federal standard of 20 µg/g (Ayres, 2002). At the end of December 2002, toxin levels had declined but were still above 20 µg/g. This means that razor clams digging on Washington beaches was not allowed during the season that runs through the spring of 2003 (Ayres, 2003).

In early September 1991 (18-27 September 1991), more than 100 brown pelicans (*Pelecanus occidentalis*) and cormorants (*Phalacrocorax penicillatus*) in Monterey Bay, Central California died or suffered from unusual neurological symptoms which were attributed to the neurotoxin DA. The source was identified as a bloom of the pennate diatom *P. australis* (Horner *et al*., 1997).

At the peak of this incident, DA levels in coastal waters were 10 σg/l and abundances of *P. australis* exceeded 10^6/L. Approximately 100 σg DA/g (wet weight) was found in *P. australis*. Remnants of *P. australis* frustules and high levels of DA were found in the stomach contents (40-50 σg/g) of affected birds. DA was detected in viscera (up to 190 σg/g) and flesh (up to 40 σg/g) of local anchovies, a principal food source of seabirds. Some authors reported DA levels in viscera of anchovies up to even 485 σg/g (Van Apeldoorn *et al*., 1999).

During the autumn of 1991, besides *P. australis* at the Monterey Bay, California, other *Pseudo-nitzschia* spp. were also present at several sites on the USA west coast from Southern California to the mouth of the Columbia River (Newport, Coos bay, Ilwaco). In the autumn of 1992, in addition to *P.australis*, other potentially DA producing *Pseudo-nitzschia* spp. were present in Monterey Bay (*P. delicatissima, P. pungens* f. *multiseries* and *P. pseudodelicatissima*) but no report of a DA outbreak was reported.

There is a strong evidence from the literature that the *Pseudo-nitzschia* species found in 1991 and 1992, except *P. australis*, have been part of the diatom community of the west coast at least since the 1940s (Van Apeldoorn *et al*., 1999).

Over 400 Californian sea lions (*Zalophus californianus*) died along the central California coast during May and June 1998. DA produced by *P. australis* and transmitted to the sea lions via planktivorous northern anchovies (*Engraulis mordax*) was identified as the probable causative agent. In contrast to fish, the blue mussel (*Mytilus edulis*) collected during the mortality period of the sea lions contained no DA or only trace amounts (Lefebvre *et al*., 1999; Scholin *et al*., 2000).

In late August and early September 2000, a large bloom of *Pseudo-nitzschia* with very high ASP toxin levels occurred along the coast of California (Monterey Bay). During this bloom anchovies, sardines and krill accumulated enough DA to be harmful to animals consuming them (Anonymous, 2001c).

In May 2002, sardines, anchovies, crabs and shellfish along the Californian coast contained high levels of DA. Authorities advised against harvesting or eating them (Anonymous, 2002e). In April and May, a growing number of marine mammals and birds have been dying along the Californian coast. About 70 dolphins have washed up on state beaches, while more than 200 sea lions and 200 seabirds have become sick or died. Up to 380 µg/g DA was found in mussels from Santa Barbara waters. No human illnesses have been reported (Anonymous, 2002f).

East Coast

The DA-producing diatom *P. pungens* f. *multiseries* was isolated in Massachusetts Bay near Boston. It produced DA levels ranging from undetectable to 0.21 pg/cell. *P. pseudodelicatissima* was also isolated but did not produce detectable levels of DA. These findings provided at least one probable source for DA accumulation in mussels from Nantucket in January-February 1991. The fact is that the occurrence of DA in Nantucket shellfish at about half the regulatory limit was never traced to a causative organism (Villareal *et al*., 1994).

Pseudo-nitzschia species are often present in great abundance in Louisiana coastal waters, including areas where there are oyster beds. A multi-year study in the shelf and estuarine waters from Louisiana showed the presence of *Pseudonitzschia* spp. in 73 percent of the shelf samples

and in 20 percent of the estuarine samples. At least six *Pseudonitzschia* species were present of which *P. multiseries* had the greatest potential of causing an outbreak of DA poisoning (Parsons *et al*., 1998). There have been no known outbreaks of ASP in Louisiana, possibly because isolated cases have not been recognized, or oysters did not become toxic (Dortch, 2002).

Gulf of Mexico

Extracts from shellfish and phytoplankton from the Gulf of Mexico indicated the presence of DA in phytoplankton (2.1 pg/cell). The marine diatom *Pseudo-nitzschia pungens* f. *multiseries* was first observed as the dominant species in a scanning electron microscopy study of plankton from Offats Bayou, Galveston Bay, Texas on 25 February 1989. In the waters around Galveston Bay *P. pungens* f. *pungens* appears to be the most abundant during the warmer months, to be gradually replaced by *P. pungens* f. *multiseries* when autumn and winter storms occur. However, viable cultures of both forms have been established from water as warm as 29.5 ∀C (Dickey *et al*., 1992a).

Direct evidence for the accumulation of ASP toxins in Gulf shellfish has not been obtained. *Pseudo-nitzschia pungens* f. *multiseries* has been observed only in low densities in Galveston Bay. DA production from the Galveston Bay isolate (cell vs. whole culture) of *Pseudo-nitzschia pungens* f. *multiseries* is equivalent to that reported from Canadian isolates. All of the culture clones of this form isolated from Galveston Bay have produced DA in the stationary and senescent growth phases. The concentrations of ASP toxins in the Gulf of Mexico phytoplankton were not considered to be a public health hazard (Dickey *et al*., 1992a).

Pseudo-nitzschia spp. were extremely abundant (up to 10^8 cells/L; present in 67 percent of 2 159 samples) from 1990 to 1994 on the Louisiana and Texas continental shelves and moderately abundant (up to 10^5 cell/L; present in 18 percent of 192 samples) over oyster beds in Terrebonne Bay estuary in Louisiana in 1993 and 1994. On the shelf there was a strong seasonal cycle with maxima every spring for 5 years and sometimes in the autumn, which were probably related to river flow, water column stability and nutrient availability. In contrast, in the estuary no apparent seasonal cycle in abundance was observed. The *Pseudo-nitzschia* spp. was not routinely identified during this study. However, toxin producing *P. multiseries* has been identified previously from Galveston Bay, Texas (see paragraph above), and cells from a bloom on the shelf in June 1993 were identified by scanning electron microscopy as *P. pseudodelicatissima*, which is sometimes toxic. There have been no known outbreaks of ASP in this area (Van Apeldoorn *et al*., 1999).

4.7.3 Central and South America

Argentina

In the winter of the year 2000, ASP was detected in Mar del Plata. The dominant species was *P. australis* and the toxin was registered in mussel and in fish (*Engraulis anchoita*) and two massive mortality episodes of seabirds were reported (Ferrari, 2001).

Chile

ASP is possibly a threat to Chile, since the diatom *Nitzschia pseudoseriata* (*Pseudo-nitzschia australis*), one of the postulated causative organisms producing DA, has been described frequently in phytoplankton sampling in Chilean waters (Lagos, 1998).

The percentage of shellfish samples with DA levels exceeding the regulatory limit of 20 σg/g has increased steadily since 1997. Up until 2001, no cases of ASP intoxications in humans were recorded but the situation is a potential threat to public health (Suárez-Isla, 2001).

Mexico

In January 1996, 150 dead brown pelicans (*Pelecanus occidentalis*) were found within a period of five days at Cabo San Lucas on the tip of the Baja California Peninsula. The death of these birds was the result of feeding on mackerel (*Scomber japonicus*) contaminated by DA-producing *Pseudo-nitzschia* spp. (Sierra-Beltrán *et al*., 1997).

Ochoa *et al*. (1997) reported that the Baja California Peninsula has witnessed several toxic algal blooms from 1991 to 1996 among which *Pseudo-nitzschia* spp. Bahia Magdalena was considered as an ideal site for aquaculture exploitation and huge projects are underway. At Bahia Magdalena the presence of DA in shellfish was suggested during winter 1994 and 1995. The DA levels were well below the guideline value but continuous monitoring was recommended. In February 1996, a bloom of *Pseudo-nitzschia* spp. was also observed but no toxin was detected.

During January and February 1997, mass toxicity and mortality of marine organisms occurred in the Gulf of California, affecting 766 common loons (G*avia immer*) and 182 sea mammals belonging to four different species. In the stomach of common dolphins (*Delphinus capensis*), a remainder of *Pseudonitzschia* (frustules) and sardine (*Sardinops sagax*) was found. LC analysis of tissues showed the presence of DA and some of its isomers. DA and its isomers were also detected in diatom samples from the sardine stomach. *P. australis* was identified as the toxin producer (Sierra-Beltrán *et al*., 1998).

4.7.4 Asia

Japan

From 1991 onwards, ASP toxin screening of cultured bivalves and of diatoms has been carried out in Japan. DA has not been detected in industrially important shellfish from 1991 to 1994, or in diatoms except for a *Pseudo-nitzschia pungens* sample (0.01 pg of DA per cell) collected from a red tide which occurred in Hiroshima Bay in August 1994. On the other hand, large amounts of DA were detected in the red alga *Chondria armata* occurring in Kagoshima Prefecture, Southern Japan. In this area the xanthid crab *Atergatis floridus* contained 10 mg DA/kg. Since the crab feeds on seaweeds, it is suggested that the DA may have originated from the food web (Van Apeldoorn *et al*., 1999).

4.7.5 Oceania

Australia, Tasmania and New Zealand

An Australian-wide taxonomic survey for species of the potentially toxic diatom genus *Pseudo-nitzschia* was carried out. The dominant bloom-forming *Pseudo-nitzschia* species in Australian coastal waters were *P. fraudulenta* (New South Wales), *P. pungens* f. *pungens* and *P. pseudodelicatissima* (Tasmanian and Victorian waters). *P. pungens* f. *multiseries* was detected on only one occasion and only as a minor component (5 percent of total biomass) of a dense *P. pungens* f. *pungens* bloom in a New South Wales estuary. *P. australis* was never detected in Australian waters. Cultured diatom populations of *P. pseudodelicatissima* from Tasmanian and Victorian coastal waters were consistently non-toxic. Cultures of *P. pungens* f. *pungens* from Australia (Hallegraeff, 1994) and Tasmania (Hallegraeff, 1994) were also non-toxic. *P. fraudulenta* has proved also non-toxic (Hallegraeff, 1994). Traces of DA have been detected in some scallop viscera by both LC and mass spectrometry, but the concentrations in edible shellfish products were all well below 20 σg/g of shellfish meat (Hallegraeff, 1994). In New Zealand, DA was not identified in 150 greenshell mussel (*Perna canaliculus*) samples and in plankton samples taken during *Pseudo-nitzschia* bloom periods (Van Apeldoorn *et al*., 1999). During the summers of 1992 and 1993, DA was detected in the marine biotoxin programme of New Zealand at low

levels in phytoplankton samples from Otago to Northland. *P. pungens* has been found in low numbers (up to 3 000 cells per litre) at the Bay of Islands, the Hauraki Gulf and Bay of Plenty (Smith *et al*., 1993). Both *P. pungens* and *P. pseudoseriata* have been detected in New Zealand waters but ASP has never been clearly associated with shellfish from the Pacific Ocean. Chemical analysis of shellfish samples has identified low levels of DA. The highest level (16.5 σg/g) came from Manukau Harbour. Other detectable levels were well below 20 σg/g (Bates *et al*., 1993).

Over the period from January 1993 to July 1996, 0.3 percent of samples of shellfish taken around the coastline of New Zealand on a weekly basis showed an ASP toxin level above the regulatory limit during a total of eight ASP events (maximum level 600 µg/g scallop). During the sampling period there were no outbreaks of human poisoning cases (Sim and Wilson, 1997).

The highest levels of DA found in New Zealand until early 2003 were 187 µg/g in green mussels from Marborough Sounds (December 1994), 72.4 µg/g in scallops M&R from Tauranga Harbour (December 1994), 210 µg/g in whole scallops from Whangaroa Bay (November 1993) and 600 µg/g in scallop gut from Doubtless Bay (December 1994) (Anonymous, 2003b).

4.8 Regulations and monitoring

4.8.1 Europe

In Member States of the European Union, a guideline value of 20 mg/kg is valid for the total ASP toxin content in the edible parts of molluscs (the entire body or any part edible separately). The analytical method to be used involves LC. If a sample, as defined in an Annex, contains more than 20 mg DA/kg, the entire batch shall be destroyed (EC, 2002b).

For bivalve molluscs belonging to the species *Pecten maximus* and *Pecten jacobeus*, scientific studies have shown that with a DA level in the whole body between 20 and 250 mg/kg, under certain restrictive conditions, the DA level in the adductor muscle and/or gonads intended for human consumption is normally below the limit of 20 mg/kg. In the light of these recent studies, it is appropriate to lay down, only for the harvesting stage and only for the bivalve molluscs belonging to the species mentioned above, an ASP toxin level with respect to the whole body higher than the limit of 20 mg/kg. No harvesting of *Pecten maximus* and *Pecten jacobeus* must be allowed during the occurrence of an ASP active toxic episode in the waters of the production areas (EC, 2002b).

A restricted harvesting regime of molluscs with DA concentration in the whole body higher than 20 mg/kg can be initiated if two consecutive analyses of samples, taken between one and no more than seven days, show that DA concentration in whole mollusc is lower than 250 mg/kg and that the DA concentration in the parts intended for human consumption, which have to be analysed separately, is lower than 4.6 mg/kg. The analyses of the entire body will be performed on an homogenate of 10 molluscs. The analysis on the edible parts will be performed on an homogenate of 10 individual parts (EC, 2002b).

Denmark

Monitoring of shellfish by regulations takes place since 1993 (Ravn, 1995). Monitoring for *Pseudo-nitzschia pungens* takes place. At approximately 5 x 10^5 cells/litre fishery product harvesting areas are closed (Shumway *et al*., 1995).

Ireland

The Biotoxin Monitoring programme in Ireland began in 1984 and was initially based on the screening of samples for the presence of DSP toxins by bioassays. In recent years, the detection of

additional toxins including DA and particularly the azaspiracids has led to an increase in monitoring effort and the programme now includes weekly shellfish testing using DSP mouse bioassay, LC-MS (okadaic acid, DTX2, azaspiracids) and LC (DA) as well as phytoplankton analysis. Regular reports of the results of sample analysis are sent to regulatory authorities, health officials, and shellfish producers and processors via fax and mobile telephone messages. A Web-based information system is being developed to increase access to information (McMahon *et al*., 2001).

4.8.2 North America

Canada

In Canada, a regulation came into force in 1988 including a guideline value of 20 mg DA/kg of mussel. Fishery product harvesting areas are closed when toxin levels in shellfish exceed the guideline value. The analytical method to be used involves LC. Monitoring for *Pseudo-nitzschia pungens* takes place (Shumway *et al*., 1995). Since 1988, phytoplankton samples have been collected at four stations in the western Bay of Fundy. The dataset represents more than 70 000 records between 1988 and 2001. Future plans include further refining and quality control and exploring the temporal and spatial variability in the patterns more fully (Martin *et al*., 2001).

The United States of America

In the USA, a not-official guideline value of 20 mg DA/kg for bivalves exists. The analytical method to be used involves LC. For cooked crab (viscera and hepatopancreas) a guideline of 30 mg DA/kg is valid. The analytical method to be used involves LC (Shumway *et al*., 1995). The Department of Marine Resources conducted a limited sampling programme for DA. Information from adjacent Canada is available on an up-to-date basis. Closures will be made once DA levels reach 20 mg/kg. Shellfish exported to EU countries must be accompanied by a health certificate (Shumway *et al*., 1995).

4.8.3 Central and South America

Argentina

Argentina has a national monitoring programme of mussel toxicity in each coastal province involving regional laboratories and one fixed station in Mar del Plata (Ferrari, 2001).

Brazil

Brazil had a pilot monitoring initiative during one year but a national monitoring programme has not been established (Ferrari, 2001).

Chile

National monitoring programmes for shellfish and phytoplankton are maintained (Suárez-Isla, 2001).

Uruguay

Uruguay has a regular national monitoring programme on mussel toxicity and toxic phytoplankton (Ferrari, 2001).

4.8.4 Oceania

Australia

Monitoring based on regulations has taken place since 1993 for mussels and algae (Ravn, 1995).

New Zealand

Monitoring by regulations has taken place since 1993 for shellfish. The analytical method to be used involves LC (Ravn, 1995). The regulatory limit is 20 mg DA/kg of shellfish meat (Sim and Wilson, 1997).

The New Zealand Biotoxin Monitoring Programme combines regular shellfish testing and phytoplankton monitoring. Currently shellfish testing for ASP toxins involves mouse bioassay screen testing with confirmatory testing (LC and LC-MS) (Busby and Seamer, 2001).

A new biotoxin monitoring programme that will provide data that is highly accurate in a shorter time and without the use of mouse bioassays is being developed. This new programme will implement test methods based on LC-MS providing chemical analytical data in place of bioassay screen test results. The development and implementation of new test methods are in discussion including funding, method validation, testing regulations, availability of analytical standards, comparison to existing tests, type of instrumentation and international cooperation (McNabb and Holland, 2001).

5. Neurologic Shellfish Poisoning (NSP)

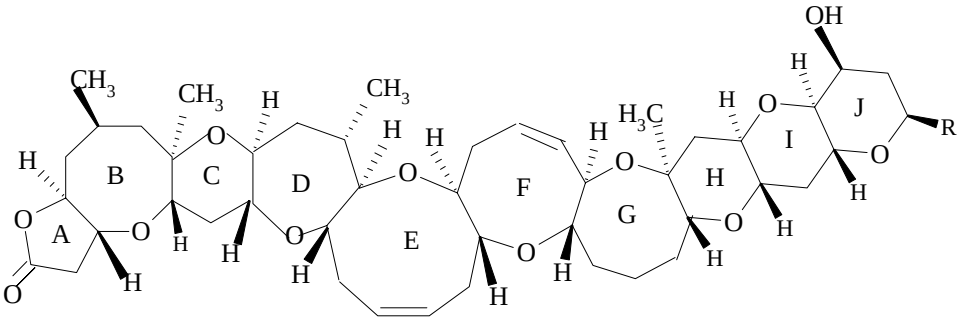
Neurologic or neurotoxic shellfish poisoning (NSP) is caused by polyether brevetoxins produced by the unarmoured dinoflagellate *Gymnodinium breve* (also called *Ptychodiscus breve,* since 2000 called *Karenia brevis*). The brevetoxins are toxic to fish, marine mammals, birds and humans, but not to shellfish. Until 1992/1993, neurologic shellfish poisoning was considered to be endemic to the Gulf of Mexico and the east coast of Florida, where "red tides" had been reported as early as 1844. An unusual feature of *Gymnodinium breve* is the formation by wave action of toxic aerosols which can lead to asthma-like symptoms in humans. In 1987, a major Florida bloom event was dispersed by the Gulf Stream northward into North Carolina waters where it has since continued to be present. In early 1993, more than 180 human shellfish poisonings were reported from New Zealand caused by an organism similar to *G .breve*. Most likely, this was a member of the hidden plankton flora (previously present in low concentrations), which developed into bloom proportions triggered by unusual climatic conditions (higher than usual rainfall, lower than usual temperature) coincident with an El Niño event (Hallegraeff, 1995).

5.1 Chemical structures and properties

The NSP toxins, called brevetoxins, are tasteless, odourless, heat and acid stabile, lipid-soluble, cyclic polyether neurotoxins produced by the marine dinoflagellate *G. breve* (or *P. brevis*). The molecular structure of the brevetoxins consists of 10 to 11 transfused rings; their molecular weights are around 900. Ten brevetoxins have been isolated and identified from field blooms and *G. breve* cultures (Benson *et al.,* 1999) (see Figure 5.1). These brevetoxins show specific binding to site-5 of voltage-sensitive Na^+ channels leading to channel activation at normal resting potential. This property of the brevetoxins causes the toxic effects (Cembella *et al.,* 1995). PbTx-2 is the major toxin isolated from *G. breve*.

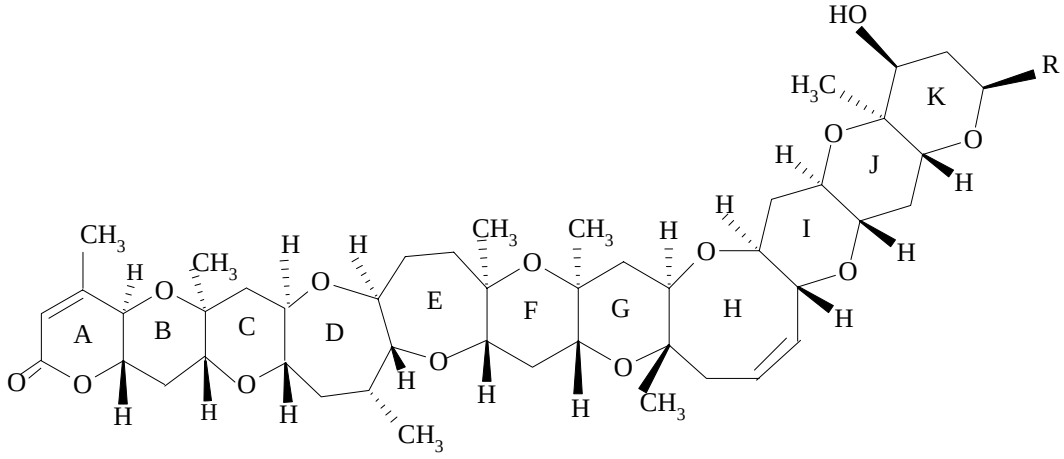
Four brevetoxin analogues (see Figures 5.2 and 5.3) were isolated from contaminated shellfish. The shellfish was derived from NSP incidents in New Zealand. The brevetoxin analogues were analysed in cockles (*Austrovenus stutchburyi*) (BTX-B1) (Ishida *et al.,* 1995) and greenshell mussels (*Perna canaliculus*) (BTX-B2, BTX-B3 and BTX-B4) (Morohashi *et al.,* 1995, 1999; Murata *et al.,* 1998) and differed from brevetoxins isolated from dinoflagellate cultures. Apparently BTX-B1, BTX-B2, BTX-B3 and BTX-B4 are metabolites formed by the shellfish itself as they were not found in field blooms or *G. breve* cultures. The presence of BTX-B2, BTX-B3 and BTX-B4 in *Perna canaliculus* does suggest that metabolic pathways in this species are more complicated than those in cockles (*Austrovenus stutchburyi*). However, the major toxins in shellfish were left unelucidated because of the extreme difficulty in isolation (Morohashi *et al.,* 1999).

Figure 5.1 Chemical structures of type A and B brevetoxins (Hua et al., 1996)



Type 1 (A) brevetoxins:

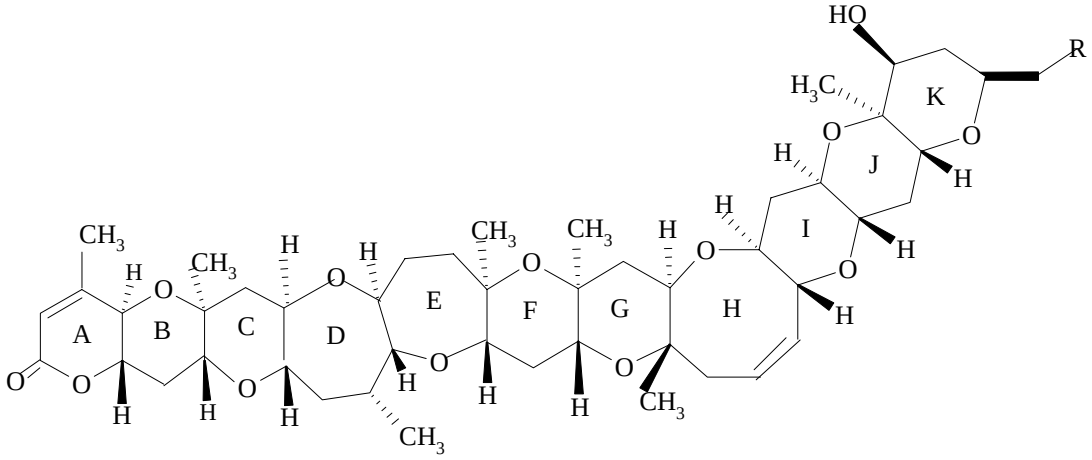
	PbTx-1,	R = $CH_2C(=CH_2)CHO$
	PbTx-7,	R = $CH_2C(=CH_2)CH_2OH$
	PbTx-10,	R = $CH_2CH(CH_3)CH_2OH$



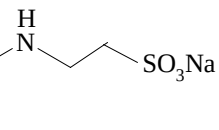
Type 2 (B) brevetoxins:

PbTx-2	R = $CH_2C(=CH_2)CHO$
oxidized PbTx-2	R = $CH_2C(=CH_2)COOH$
PbTx-3	R = $CH_2C(=CH_2)CH_2OH$
PbTx-8	R = CH_2COCH_2Cl
PbTx-9	R = $CH_2CH(CH_3)CH_2OH$
PbTx-5	the K-ring acetate of PbTx-2
PbTx-6	the H-ring epoxide of PbTx-2

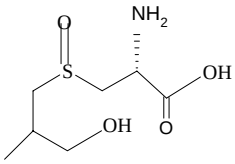
5.2 Chemical structures of brevetoxin analogues BTX-B1, -B2 and –B4 isolated from contaminated shellfish



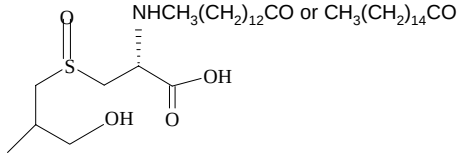
BTX-B1 R =



BTX-B2 R =

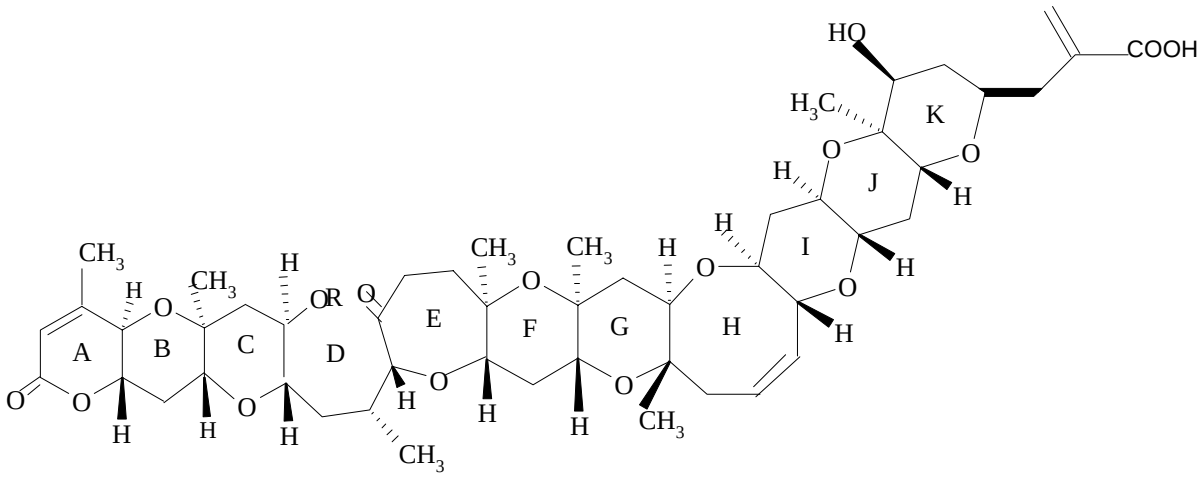


BTX-B4 R =



Source: Yasumoto *et al*., 2001

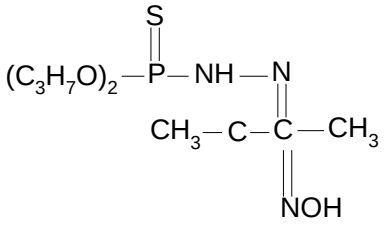
Figure 5.3 Chemical structure of brevetoxin analogue BTX-B3 isolated from contaminated shellfish



Source: Yasumoto *et al*., 2001 BTX-B3 R = $CH_3(CH_2)_{12}CO$ or $CH_3(CH_2)_{14}CO$

In addition to brevetoxins, some phosphorus containing ichthyotoxic compounds resembling anticholinesterases, have also been isolated from *G. breve*. One example is an acyclic phosphorus compound with an oximino group in addition to a thiophosphate moiety, namely *O*,*O*-dipropyl(*E*)-2-(1-methyl-2-oxopropylidene)phosphorohydrazidothioate-(*E*)oxime (Van Apeldoorn *et al*., 2001).

Figure 5.4 Phosphorus containing ichthyotoxic toxin isolated from G. breve.



5.2 Methods of analysis

5.2.1 Bioassays

in vivo assays

mouse bioassay

The mouse bioassay involves the evaluation of toxicity by intraperitoneal injection of the crude lipid extract of shellfish into mice. Results are expressed as mouse units (MU) (Hokama, 1993). One MU is defined as the amount of crude toxic residue that on average will kill 50 percent of the test animals (20 g mice) in 930 minutes (Dickey *et al.*, 1999). The currently accepted method is the American Public Health Association (APHA) procedure from 1985 based on diethylether extraction of shellfish tissue. After the detection of NSP in New Zealand in 1993, a management strategy to monitor NSP toxins was developed by the MAF Regulatory Authority. The sample preparation method used was based on acetone extraction of these lipophilic components followed by partitioning into dichloromethane. This procedure was very effective in extracting unknown lipid-soluble toxins from shellfish containing NSP toxins and presented certain advantages as compared with the APHA protocol (simpler and more suitable for rapid and quantitative separation of organic and aqueous phases of the extract and greater extraction efficiency). However, following the discovery of a novel bioactive compound (gymnodimine) produced by the dinoflagellate *Gymnodinium mikimotoi*, a common species in New Zealand waters during neurotoxic events, the authorities returned to the diethylether extraction procedure of the APHA. Gymnodimine is not extractable by diethylether but it causes very rapid mouse deaths when the dichloromethane procedure is used. Since gymnodimine is not considered to present a risk to human health, the monitoring programme now employs diethylether extraction as a means of discriminating gymnodimine activity from NSP toxicity (Fernandez and Cembella, 1995).

Basically, any detectable level of brevetoxins per 100 g shellfish tissue was considered potentially unsafe for human consumption. In practice, a residue toxicity ∅20 MU per 100 g shellfish tissue was adopted, and remains as the guidance level for prohibition shellfish harvesting (Dickey *et al.*, 1999).

The problems with the mouse assay are that it requires large numbers of animals, uses relatively large amounts of tissue extracts, the results are interpreted subjectively and it lacks specificity (Hokama, 1993).

fish bioassay

Mosquito fish (*Gambusia affinis*) bioassays are conducted in 20 ml seawater (3.5 percent salinity) using one fish per vessel with toxin added in 0.01 ml ethanol. Each LD_{50} was determined by preparing triplicate 2-fold serial dilutions of each toxin. Lethality was assessed after 60 minutes and median lethal dose was determined using the tables in Weil from 1952 (Baden *et al.*, 1988). The fish bioassay is generally used to determine the potency of either the contaminated seawater or crude and purified toxin extracts (Viviani, 1992).

in vitro assays

neuroblastoma cell assay

The toxins responsible for NSP exert their toxic effects by binding to a certain class of biological receptors namely to voltage-sensitive Na^+ channels. This highly specific interaction with naturally occurring receptors forms the basis of the neuroreceptor assay. Any modification to a toxin molecule, which interferes with its binding to the receptor and thus its detection in a receptor-based assay, would also compromise its ability to elicit a toxic response. Detection is therefore based on its functional activity rather than on recognition of a structural component, as is the case of an antibody-based assay. Moreover, the affinity of a toxin for its receptor is directly proportional to its toxic potency. Thus, for a mixture of congeners, a receptor-based assay will yield a response representative of the integrated potencies of those toxins present (Cembella *et al.*, 1995).

A tissue culture technique using an established mouse neuroblastoma cell line (Neuro-2a) has been developed for the assay of site-5 Na^+ channel activating toxins a.o. brevetoxins. This detection method is based on end-point determination of mitochondrial dehydrogenase. The detection limit for PbTx's is 0.25 ng/10 σl tissue extract. PbTx can be detected within four to six hours but the detection limit can be decreased with an incubation time of 22 hours. The method was further modified and simplified by incorporating a colorimetric procedure based upon the ability of metabolically active cells to reduce a tetrazolium compound namely MTT (=3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) to a blue-coloured formazan product (Manger *et al.*, 1993; 1995).

The most potent brevetoxin PbTx-1, could be detected in the MTT cell bioassay at levels substantially below the intraperitoneal LD_{50} in mice after four to six hours of exposure. For comparison the LD_{50} for PbTx-1 in mice is 0.01 mg/20 g animal, intraperitoneal injection, correlating with 0.1 mg/100 g tissue extract or equivalent to 1 ng/10 σl sample in neuroblastoma cells (Manger *et al.*, 1993).

Other methods have used XTT (a soluble formazan reagent) for colorimetric determination. (Yasumoto *et al.*, 1995). The neuroblastoma cell assay can be used for detection of brevetoxins in contaminated shellfish tissue but this assay cannot distinguish between individual brevetoxins (Hua *et al.*, 1995).

Fairey *et al.* (1997) reported a further modification of the receptor-binding assay in neuroblastoma cells of Manger *et al.* (1995), to a reporter gene assay that utilizes luciferase-catalyzed light generation as an endpoint and a microplate luminometer for quantification. A c-*fos*-luciferase reporter gene construct was stably expressed in the N2A clone of mouse neuroblastoma cells, the assay parameters were optimized and the sensitivity of this reporter gene assay to several algal toxins that activate or inhibit sodium channels was evaluated. PbTx-1 caused a concentration-dependent and saturable increase in luciferase activity. Although additional characterization of this assay is still required to evaluate performance with different fish and shellfish matrices, algal pigments and other classes of algal toxins, the assay as presented met or exceeded the sensitivity of existing bioassays for sodium channel active algal toxins.

Voltage-gated sodium channels are integral, neuronal membrane proteins. A purified membrane protein can be incorporated into a lipid bilayer by formation of a vesicle in its presence, and this process is termed "reconstitution". Once the appropriate phospholipids for functional reconstitution of sodium channels have been elucidated, the reconstituted channel can be used as a tool for the measurement of specific binding of algal toxins. Specific binding of PbTx-3 to purified rat brain sodium channels which were reconstituted into phospholipid vesicles, was demonstrated. This demonstration of specific binding of sodium channel toxins paves the way toward development of a highly specific functional assay for the presence of these toxins in biological tissue (Trainer *et al.*, 1995).

synaptosome binding assay

The synaptosome assay is a competitive binding assay in which radiolabeled NSP toxin and/or its derivatives compete with unlabeled NSP toxin for a given number of available receptor sites in a preparation of rat brain synaptosomes. The percent reduction in radiolabeled NSP binding is directly proportional to the amount of unlabelled toxin present in an unknown sample (Poli *et al.*, 1986). As is the case with the immunoassay (see Chapter 5.2.2), both PbTx-2 and PbTx-3 displaced ^{3}H-PbTx-3 in an equivalent manner. However, oxidized PbTx-2 did not replace ^{3}H-PbTx-3 as was the case in the immunoassay (Baden *et al.*, 1988).

Van Dolah *et al.* (1994) developed a high throughput synaptosome binding assay for brevetoxins using microplate scintillation technology. The microplate assay can be completed within three hours, has a detection limit of less than 1 ng and can analyze dozens of samples simultaneously. The assay has been demonstrated to be useful for assessing algal toxicity, for purification of brevetoxins and for the detection of brevetoxins in seafood.

An AOAC Peer-Verified Method trial on the microplate receptor assay of Van Dolah *et al.* (1994) for PbTx in oysters is in progress (Quilliam, 1999).

Whitney *et al.* (1997) reported the complex behaviour of marine animal tissue in the rat brain synaptosome assay. Extracts of manatee, turtle, fish and clam tissues appeared to contain components that interfere by co-operative, non-competitive inhibition of ^{3}H-PbTx-3 specific binding and increased non-specific binding to synaptosomes. Whitney *et al.* (1997) developed a correction method for these problems.

hippocampal slice assay

Kerr *et al.* (1999) investigated *in vitro* rat hippocampal slice preparations as a means of rapidly and specifically detecting the marine algal toxins STX, brevetoxin and DA in shellfish tissue or finfish and identified toxin-specific electrophysiological signatures for each. It was concluded that hippocampal slice preparations are useful in detection and analysis of marine biotoxins in contaminated shellfish tissue.

5.2.2 Biochemical assays

immunoassays

At a time when only the structures of PbTx-2 and PbTx-3 were known, a competitive radioimmunoassay (RIA) to detect PbTx-2 and PbTx-3 with a detectability of 2 nM was developed. Detectability has been improved later to approximately 1 nM (Trainer and Baden, 1991). Utilizing bovine serum albumine (=BSA)-linked PbTx-3 as complete antigen, an antiserum was produced in goats. The RIA technique for PbTx is based on the competitive displacement of ^{3}H-PbTx-3 from complexation with the antibody. Both PbTx-2 and PbTx-3 were detected in

approximately equivalent manners. However, oxidized PbTx-2, which was not toxic in either the fish or mouse bioassay, did also displace PbTx-3 in RIA, an indication that potency was not reflected in competitive displacement assays using this antibody (Trainer and Baden, 1991).

Work has also advanced in the preparation of a reliable monoclonal antibody enzyme-linked immunosorbent assay (ELISA). Trainer and Baden (1991) developed an ELISA method utilizing brevetoxin coupled to either horseradish peroxidase or to urease with a goat antibody to purified brevetoxin. A potential ELISA system for brevetoxin detection from extracts of dinoflagellates or fish has been established with a limit of detection of 0.04 pM. The toxin can be linearly quantified from 0.04 to at least 0.4 pM brevetoxin per well. In initial trials BSA-linked PbTx-3 was used as the antigen and an antiserum was produced in goats, which was found to bind competitively to PbTx-2 and PbTx-3 (Cembella *et al.*, 1995). Since the assay is structural rather than functional, the antibody also binds to non-toxic PbTx derivatives with similar binding activity. When keyhole limpet hemocyanin (KLH) was used instead of BSA, more efficient antibody production occurred (Baden *et al.*, 1988). Recent studies on epitopic recognition using naturally occurring and synthetic brevetoxin derivatives with two different anti-PbTx sera indicated that single antibody assays may not be adequate for detecting NSP toxin metabolites. Tests are being developed to utilize more than one antibody specifically for recognition of different regions of the polyether ladder (Baden *et al.*, 1988; Levine and Shimizu, 1992; Poli *et al.*, 1995; Trainer and Baden, 1991). In a later study (Baden *et al.*, 1995) further modifications of the ELISA method are reported which resulted in improved specificity and detectability. Brevetoxin in fish tissue could not be measured until 1995 by the ELISA because brevetoxin is covalently conjugated via well-known cytochrome P450-monooxygenase detoxification pathways, and glutathione-S-transferase activities are also induced. Normal tissue extraction will not release bound toxin in fish tissue. The ELISA was entirely satisfactory for detecting and quantifying brevetoxins in dinoflagellate cells, requiring as few as 10 to 50 cells. Shellfish tissue could be analysed with ELISA but at the expense of the detectability. The modifications and alternative techniques reported by Baden *et al.* (1995) made it possible to use the ELISA for brevetoxin detection in dinoflagellate cells, in shellfish and fish seafood samples, in seawater and culture media, and in human serum samples.

Naar *et al*. (1998) reported the improved development of antibody production to PbTx-2 type brevetoxins and developed a new radioimmunoassay. The detection limit for PbTx-3 was 0.33 picomoles with a detectability range between 0.01 and 1100 picomoles. In a later study, Naar *et al*. (2001) described the production and characterization of mice polyclonal and monoclonal antibodies (MAbs) specific for PbTx-2 type toxins using PbTx-3-carrier-conjugates prepared at the nanomolar level in a reversed micellar medium. The authors considered this first report on MAbs production to PbTxs most promising for the development of MAb-based assays to poorly available marine polyether-type potent neurotoxins. In 2002, Naar *et al*. reported the development of a competitive ELISA for the detection of brevetoxins in seawater, shellfish extract or homogenate, and mammalian body fluid (urine and serum without pretreatment, dilution or purification) using goat anti-brevetoxin antibodies obtained after immunization with keyhole limpet hemocyanin-brevetoxin conjugates, in combination with a three-step signal amplification process. The detection limit for brevetoxins in spiked oysters was 2.5 µg/100 g shellfish meat.

Garthwaite *et al.* (2001) developed a group ELISA for ASP, NSP, PSP and DSP toxins including yessotoxin as a screening system for contaminated shellfish samples. The system detects suspected shellfish samples. Thereafter the suspected samples have to be analysed by methods approved by international regulatory authorities. Alcohol extraction gave good recovery of all toxin groups.

5.2.3 Chemical assays

MEKC detection

Micellar electrokinetic capillary chromatography (MEKC) with laser-induced fluorescence (LIF) detection was used to measure four brevetoxins (PbTX-2, PbTx-3, PbTx-5, PbTx-9) at sub-attomole levels. Brevetoxins were isolated from cell cultures and fish tissue and the method detection limit in fish tissue was approximately 4 pg/g (Shea, 1997).

Electrospray LC/MS

Reversed-phase liquid chromatography-electrospray ionization mass spectrometry was successfully applied to separation and identification of brevetoxins associated with red tide algae. The detection limits for PbTx-9, PbTx-2 and PbTx-1 were 600 fmol, 1 pmol and 50 fmol, respectively. Furthermore a number of unknown compounds (totally six components were detected) among which possibly an isomer of PbTx-9, were detected. An advantage of this method is that co-eluting compounds can be much more readily noticed and possibly identified via mass spectral information (Hua *et al*., 1995).

In a follow-up study, the application of this reversed-phase liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) method was expanded for the first time to investigate the distribution of brevetoxin compounds in red tide blooms collected from Sarasota Bay, Florida. PbTx-2, PbTx-1 and PbTx-3 were detected at 60, 10 and 5.7 σg/L levels, respectively, in natural red tide bloom samples. This distribution differed quantitatively from that found in red tide culture extract samples. PbTx-9 was not detected either in red tide bloom extracts or in red tide culture extracts (Hua *et al*., 1996).

Ionspray LC/MS

An ion-spray LC-MS method was developed by Quilliam in 1996. Mass detection limits as low as 10 pg (10 femtomole) can be achieved using selected ion monitoring of the $(M+H)^+$ ions. All principal toxins as well as some new minor components in a crude extract of *G. breve* were detected with this method. Recently the method was extended to the more polar metabolites identified in New Zealand shellfish. Analyses by LC-MS can be very rapid (as low as two minutes in some cases) and can be totally automated (Quilliam, 1998b).

LC/MS/MS

A fish tissue procedure based on gradient reversed-phase LC/tandem mass spectrometry (LC/MS/MS) was used for the detection of PbTx-2 in fish tissue. The detection limit in fish flesh was at least 0.2 ng/g (Lewis *et al*., 1999).

5.2.4 In general

The brevetoxins are a multi-component family of toxins. In addition to 10 brevetoxins, four metabolites have been identified, occurring in cockles and greenshell mussels in the New Zealand case. Whereas these metabolites are not ichthyotoxic, they exhibit also a potency to activate Na channels. Depending on the type of analytical method employed, they therefore may have a significant effect on the results of analytical measurements and thus on the comparability of the various analytical methods. In whelks and clams from a contaminated area in Florida, USA, the presence of metabolites was also demonstrated (Poli *et al*., 2000). In this study indeed a different sensitivity to metabolites between the RIA assay and the receptor binding assay was seen. Dickey *et al*. (1999) reported that the neuroblastoma cytotoxicity assay appears to overestimate the composite toxicity due to increased sensitivity to brevetoxin metabolites as compared to the mouse bioassay. Furthermore, the extraction solvents used in the different assay methods could

have affected the test results probably due to a higher polarity of the brevetoxin metabolites than that of the parent toxin (Personal communication S. Hall). For example Dickey *et al*. (1999) showed that, in a cytotoxicity assay (in mouse neuroblastoma cells), a 2.5-fold and 4-fold greater PbTx-3 equivalent toxicity was yielded with methanol and acetone extracted samples, respectively, than with diethylether extracted samples. The discrepancy in estimates of PbTx-3 equivalent toxicity and the moderate correlation of different assays appear to result in part from: a) the presence and temporal distribution of metabolites in shellfish; b) the efficiencies of the different extraction solvents; and c) the different sensitivity of the assay systems to the brevetoxin metabolites. All in all, this may have important implications for seafood safety and regulation because the active metabolites are likely to be the true cause of NSP (Poli *et al*., 2000).

Each of the methods of analysis that are used to determine brevetoxins suffers from certain disadvantages (see also Hannah *et al*., 1998):

- The mouse bioassay, although still commonly used, is not specific and uses experimental animals.
- The ELISA uses antibodies raised against PbTx-2 only, is not able to assay fish tissue and has only a limited sensitivity to shellfish tissue.
- The neuroblastoma cell assay, although sensitive, suffers from interferences.
- The receptor binding assay is rapid, sensitive and specific, but radiolabeled compounds are needed.
- A sensitive and specific LC/MS method is available, detecting individual components, but the method requires very expensive equipment.

Furthermore reference materials (both calibrants and matrices) for the brevetoxins and their metabolites are chronically lacking.

5.3 Source organism(s) and habitat

5.3.1 Source organism(s)

The motile form of *G. breve* produces several neurotoxins, collectedly called brevetoxins (Viviani, 1992). Ten brevetoxins have been isolated and identified from field blooms and *G. breve* cultures (see Figure 5.1) (Benson *et al.,* 1999). Four brevetoxin analogues (see Figures 5.2 and 5.3) isolated from contaminated shellfish only, and not from field blooms or *G. breve* cultures, were considered to be metabolites formed from the brevetoxins within the shellfish (Ishida *et al.,* 1995; Morohashi *et al.*, 1995, 1999; Murata *et al.,* 1998). Besides the neurotoxic brevetoxins *G. breve* also produces, in a lesser amount than the brevetoxins, hemolytic toxins. Massive fish kills seen during Florida red tides are mainly due to exposure to neurotoxic brevetoxins with a possible contribution of the hemolytic fraction. The *G. breve* organism is relatively fragile and is readily broken down in wave action along beaches releasing the toxins. During an active in-shore red tide, the aerosol of contaminated salt spray will contain the toxins and organism fragments both in the droplets and attached to salt particles and can be carried in land depending on wind and other environmental conditions (Fleming and Baden, 1999).

Furthermore, brevetoxin-like toxins were produced by four algal species belonging to the class *Raphidophyceae* (raphidophytes). Three neurotoxic compounds were isolated from *Chattonella antiqua* cultures, namely CaTx-I, CaTx-II and CaTx-III, which appeared to correspond to brevetoxins PbTx-2, PbTx-3 and oxidized PbTx-2 (same Rf values at thin layer chromatography and same retention times in HPLC). The quantity of each toxin fluctuated according to the age and growth stage of the culture.

Five neurotoxic components were tentatively identified from cultures of the red-tide producing species *Fibrocapsa japonica*, namely FjTx-I, FjTx-II, FjTx-IIIa, FjTx-IIIb and FjTx-IV. These

neurotoxic components corresponded with PbTx-1, PbTx-2, PbTx-9, PbTx-3 and oxidized PbTx-2, respectively. The quantity of each component also fluctuated with the age and growth stage of the culture.

In 1995 an unusual large-scale red tide of *Heterosigma akashiwo* occurred in Kagoshima Bay, Japan causing massive fish kills. Four neurotoxic components, HaTx-I, HaTx-IIa, HaTx-IIb and HaTx-III corresponding to PbTx-2, PbTx-9, PbTx-3 and oxidized PbTx-2, respectively, were isolated. Four neurotoxic components were isolated from *Chattonella marina* and were identified to be PbTx-2, PbTx-3, PbTx-9 and oxidized PbTx-2 (Van Apeldoorn *et al*., 2001).

5.3.2 Predisposing conditions for growth

G. breve blooms on the west coast of Florida occur from summer to winter, and most frequently in the autumn. Changes in bloom occurrence correlate with wind and sea surface temperature. Blooms typically were initiated offshore in the summer when the winds are weakest. However, they appear and continue at the coast during the autumn, a period of strong easterly (offshore) winds (Stumpf *et al.*, 1998).

G. breve blooms may also be transported inshore by currents. *G. breve* blooms consume low levels of nutrients. In coastal bays, the blooms may last longer if provided with additional nutrients from human-made sources. It was once believed that *G. breve* stayed almost exclusively in the Gulf of Mexico from Yucatan to the Texas coast (sightings have also occurred in Alabama, Mississippi, and Louisiana waters). Researchers have delineated a gigantic "dead zone" of low-oxygen waters in the Gulf of Mexico at depths of 0.5 to 20 m. After the Great Mississippi Flood of 1993, which poured huge amounts of agricultural nutrients from Midwest farms into the Gulf, the size of the dead zone doubled from 3 500 square miles to 7 000 square miles. In recent years, transport of the dinoflagellate from the Gulf has documented. In 1987 and 1988, the Gulf Stream carried *G. breve* to the east coast of Florida and pushed it farther north to North Carolina. In January 1998, *G. breve* was again transported from the Gulf of Mexico to Palm Beach County on Florida's east coast (Tibbetts, 1998).

C. marina belonging to the raphidophytes and reported to produce brevetoxins also, occurred in brackish coastal areas rich in organic material (Hallegraeff and Hara, 1995). Optimal growth was seen at temperatures of 20 to 25 ∀C, salinities of 20-30 ‰, light intensities of 60 to 140 σE m^{-2} s^{-1} and at pH 7.5 to 8.5. Growth did not occur at temperatures below 15 ∀C or above 30 ∀C, and at salinities below 10 ‰ (Van Apeldoorn *et al*., 2001).

H. akashiwo, also belonging to the raphidophytes and producing brevetoxin-like toxins, was found in coastal and brackish water in the Pacific and Atlantic (Hallegraeff and Hara, 1995). *H. akashiwo* blooms require metals, such as iron and manganese, in addition to nitrogen, phosphorus and vitamin B_{12}. Runoff, formation of bottom water having low oxygen content, and wind-induced turbulence of bottom sediments supplied these nutritive substances.

H. akashiwo has a high growth potential (up to five divisions per day) causing the production of red tides in a short period. Raphidophytes occurred in Japanese coastal waters where about 16 ∀C is the minimum water temperature. In 1991, for the first time, raphidophytes, namely *C. antiqua*, *C. marina* and *F. japonica*, were also found in Dutch coastal waters, where, except in summer, the temperature is well below the temperature of Japanese coastal waters. During warm periods, the same conditions prevail in the Dutch Wadden Sea and the estuarine south of the river Rhine as in Japan. Species adapted to the cooler environment of the North Sea may be present. Optimal growth of raphidophytes occurred in Japan at salinities varying from 11 to 20 ‰ which is the same range as measured in the Dutch Wadden Sea and the estuary south of the river Rhine. The

small bloom of *Chattonella* in May 1993 in the south of the central North Sea at salinity 25 to 28‰ was therefore not expected. Even cysts of raphidophytes may be present in Dutch coastal waters. Investigations revealed that encystment took place frequently whenever environmental conditions are unfavourable for 'normal' growth. Encystment-stimulating factors such as nutrient depletion, the presence of solid surfaces for cyst adhesion and low light intensities occasionally occur in Dutch coastal waters (Van Apeldoorn *et al*., 2001).

C. marina and *C. antiqua* have a diplontic life cycle in which smaller pre-encystment cells were observed besides cysts. However, these cells and cysts are not known from Dutch coastal waters possibly for lack of an adequate sampling scheme. *C. antiqua* grew maximally at 25 ∀C, at salinities between 25 and 41‰ and under light intensities above 0.04 ly min^{-1} (1 ly=700W). At the pH range tested (7.6 to 8.3) no significant effects on growth of *C. antiqua* were seen and maximal growth was observed.

Temperature and salinity affected also the shape and motility of *C. antiqua* cells. Light intensity did not influence morphology at the range of intensities tested (20-180 σ$E/m^2/s$) whereas good motility was seen at 60-180 σ$E/m^2/s$. Growth of *C. antiqua* was supported by nitrate and ammonium, and by urea to a limited extent, but not by glycine, alanine and glutamate. Orthophosphate served as a good P source but not glycerophosphate. Fe^{3+} promoted growth as did vitamin B_{12}. Glucose, acetate and glycolate did not improve growth in the light nor in the dark (Van Apeldoorn *et al*., 2001).

5.3.3 Habitat

G. breve occurs regularly in the Gulf of Mexico but *G. breve* or *G. breve*-like species have also been reported from the West Atlantic, Spain, Portugal, Greece, Japan and New Zealand. It is uncertain whether the *G. breve*-like species occurring outside the Gulf of Mexico and the Western Atlantic region should be assigned to *G. breve* or if represent different, closely related species (Smith *et al*., 1993; Taylor, *et al*., 1995). An atoxic form of *G. breve* was found in Inland Sea, Japan (Viviani, 1992).

In New Zealand *G. breve* was identified in 1993 in waters off the North Auckland coast following the NSP incident at Orewa and in the Bay of Plenty. *G. breve* was also present in the Coromandel region (cell counts up to 70 000 per litre) and in Bream Bay (cell counts up to 100 000 per litre) in January 1993. Cell counts declined during February and March to less than 300 cells per litre in April in Coromandel (Smith *et al.*, 1993).

In the summer of 1995 to 1996, a severe aerosol toxin problem was reported in South Africa viz. in False Bay, which later spread to the coastal resort of Hermanus in Walker Bay. The aerosol toxin was linked to a bloom of a toxic dinoflagellate species *Gymnodinium*, first recorded in False Bay in 1988. Despite the species having bloomed on several occasions since then, the noxious effects in humans were never before as evident as in 1995-96. Faunal mortalities were however small, with the exception of larval mortalities experienced by several land-based abalone farmers in the Walker Bay area. Along the South African coast, the dinoflagellate *Gymnodinium nagakiense* is usually implicated in NSP. Most outbreaks have been reported from False Bay, where they are responsible for the olive-green discolouration of the seawater during autumn (Van der Vyver , 2000).

The presence of *Heterosigma akashiwo*, and *Fibrocapsa japonica* in coastal waters of Tampa and Florida Bays in Florida was demonstrated in 1986-87. In addition, *Chattonella* species (*subsalsa* and *marina*) were reported to be present in 1990. All these species are known to produce brevetoxin-like toxins. The presence of these species in Florida waters extended their distribution

to warm temperate regions at lower salinities (<32‰) and higher temperatures (>28 ∀C) than previously reported (Van Apeldoorn *et al*., 2001).

In the Peter the Great Bay (Sea of Japan, the Russian Federation) massive blooms of *Heterosigma akashiwo* and *Chattonella* sp. were recorded in May-September 1995-1996 (Orlova *et al*., 1998). In Japan, *Fibrocapsa japonica* formed heavy red-tides in the coastal areas of Ehime Prefecture in 1972 and this raphidophyte was later reported from Atsumi Bay, the Seto Inland Sea and Harima Nada. *F. japonica* has also been reported from the Dutch part of the North Sea and from New Zealand, at the east and west coasts of the North Island and east coast of the South Island (Van Apeldoorn *et al*., 2001). In addition, Hallegraeff and Hara (1995) reported that *F. japonica* occurred in coastal waters of Australia, California, North America and France.

Off the coast in the Hauraki Gulf in New Zealand *F. japonica* and the fish killing *Heterosigma akashiwo* appeared to dominate red blooms which were reported during October and November 1992. *F. japonica* persisted in low numbers in the Hauraki Gulf and Bay of Plenty through to mid January 1993 (Smith *et al.*, 1993).

Red tides of *H. akashiwo* occurred in temperate and subtropical embayments in Japan, the Republic of Korea, Singapore, Canada, New Zealand, England, eastern and western areas of North America and Bermuda (Van Apeldoorn *et al*., 2001). According to Hallegraeff and Hara (1995) *H. akashiwo* is a problem organism for finfish aquaculture in British Columbia, Chile, New Zealand and possibly Singapore.

Heavy red tides formed by *Chattonella antiqua* were reported from the coastal regions of Japan (Khan *et al*., 1996). Also in Southeast Asia, *C. antiqua* caused massive fish kills (Hallegraeff and Hara, 1995).

In Boston Bay, Southern Australia high levels of brevetoxins were found in the livers of farmed bluefin tuna fish (*Thunnus maccoyii*) sampled at different times, at a mortality episode. *Chattonella marina* was found in the water column (Munday and Hallegraeff, 1998). According to Hallegraeff and Hara (1995) *C. marina* occurred in brackish coastal areas from India, Australia and Japan, which were rich in organic material. Several extensive blooms caused by potentially toxic *Chattonella* spp. cells occurred from the German Bight to the almost north Skagen between late March and first half of May 1998, 2000 and 2001 (Douding and Göbel, 2001)

5.4 Occurrence and accumulation in seafood

5.4.1 Uptake and elimination of NSP toxins in aquatic organisms

There are little quantitative data on rates of accumulation and depuration of brevetoxins in bivalves. Oysters accumulate the toxins in less than four hours in the presence of 5 000 cells/ml and depurate (60 percent) the accumulated toxins in 36 hours. Potency of depuration is species-specific and highly variable, even under controlled laboratory conditions (Viviani, 1992).

Crassostrea virginica depurated brevetoxins two to eight weeks after a bloom. Biotransformation is species-specific and may lead to more potent derivatives. When Gulf toad fish (*Opsanus beta*) received orally ^{14}C- PbTx-3 in fishmeal slurry, 72 hours later the hepatobiliary system contained 40 percent of body burden confirming the key role of this system in detoxification and elimination of brevetoxin. Muscle tissue contained 27 percent of body burden, followed by gastrointestinal tract with 25 percent. When Gulf toad fish (*Opsanus beta*) received intravenously (via an implanted indwelling cannula in caudal vein) 0.5 σg ^{3}H-labelled PbTx-3/kg bw, radioactivity in blood declined rapidly with a $T_{1/2}$ of 29 minutes. Toxicokinetics were best described by a three

compartment open model with the central compartment representing blood. Distribution to tissues was rapid. One hour after dosing radioactivity was detected in all tissues examined with highest proportions in muscle, intestine and liver (40.2, 18.5 and 12.4 percent of body burden). Through 96 hours radioactivity in liver remained constant (7.8 percent), while levels in bile, kidney and skin increased (34.5, 13.8 and 6.7 percent, respectively) and levels in all other tissues decreased, particularly in muscle (15.9 percent). Approximately 24 percent of the administered radioactivity had been excreted into the gall bladder by 96 hours. Extraction of the bile revealed both aqueous-soluble and organic-soluble metabolites of PbTx-3 (>94 percent of radioactivity in bile). No metabolites have been identified (Van Apeldoorn *et al.*, 2001)

Immature red fish (*Scianops ocellatus*) receiving orally 1.5 or 2.5 σg PbTx-3/100 g bw in a fishmeal slurry by gavage showed significantly increased activity of the hepatic P450 enzyme ethoxyresorufin *O*-deethylase (EROD) at the high dose. The activities of the hepatic P450 enzyme pentoxyresorufin *O*-depentylase (PROD) and the cytosolic enzyme, glutathione *S*-transferase (GST) were not affected. Total cytochrome P450 was not higher in treated fish. (Van Apeldoorn *et al.*, 2001)

In the striped bass (*Morone saxatilis*) the effects of PbTx-2 on xenobiotic metabolizing enzymes and the possible identification of potential biomarkers of exposure were examined. Seven striped bass were exposed orally by gavage for four days to a 0.5 g/100 g body weight of a toxin laden slurry (~50 σg/100 g bw). A negative control group received control slurry and a positive control group received intraperitoneally η-naphthoflavone (5 mg/100 g bw). Hepatic microsomal and cytosolic fractions were assayed for EROD, UDP glucuronosyl transferase, microsomal epoxide hydrolase, and four isozymes of glutathione-S-transferase (GST). No significant effect on body weight was seen in PbTx-2 treated fish. In PbTx-2 treated fish a larger hepatosomatic index was seen and both microsomal and cytosolic proteins in the liver were significantly lower. PbTx-2 caused a three fold increase in EROD activity whereas η-naphthoflavone caused a 30-fold increase. PbTx-2 caused a 35 and 50 percent increase in the activity of two gluthathione S-transferase (GST) isozymes. These increases seen in GST isozymes make them potentially useful biomarkers. PbTx-3 induced cytochrome P-450IA, a key Phase I enzyme, and glutathione S-transferase, an important Phase II enzyme. Possible pathways of metabolism include epoxidation at the H-ring double bond, hydrolysis of the epoxide to form the hydrodiol, cleavage of the A-ring lactone, and formation of glutathione conjugates either at the alcohol functionality of PbTx-3 or at Phase I metabolites (Van Apeldoorn *et al.*, 2001).

5.4.2 Shellfish containing NSP toxins

Major seafood containing brevetoxins is shellfish (Viviani, 1992). Several species (such as oysters, clams and mussels) have been reported to accumulate brevetoxins. While fish, birds and mammals are all susceptible to brevetoxins, oysters, clams and mussels are not susceptible to these toxins and may appear perfectly healthy (Fleming and Baden, 1999).

PbTx-2 and PbTx-3 were detected in the oyster *Crassostrea gigas* in New Zealand (Ishida *et al.*, 1996). Four brevetoxin analogues were detected (see Figures 5.2a and 5.2b) viz. BTX-B1 in cockles (*Austrovenus stutchburyi*) (Ishida *et al.*, 1995) and BTX-B2, BTX-B3 and BTX-B4 in greenshell mussels (*Perna canaliculus*) (Morohashi *et al.*, 1995, 1999; Murata *et al.*, 1998). These analogues were found only in contaminated shellfish and not in *G. breve* field blooms or *G. breve* cultures and therefore were considered as brevetoxin metabolites formed by the shellfish itself (Morohashi *et al.*, 1999). This shellfish from New Zealand was derived from NSP incidents. BTX-B1, BTX-B2 and BTX-B4 did not show ichthyotoxicity but they retained their potency to activate Na channels (Ishida *et al.*, 1995; Murata *et al.*, 1998; Morohashi *et al.*, 1999). BTX-B3

did not kill mice at intraperitoneal injection of 300 σg/kg bw (Morohashi *et al.*, 1995). No data on the ichthyotoxicity of BTX-B3 are available.

Whelks (*Busycon contrarium*) and clams (*Chione cancellata* and *Mercenaria* spp.) collected from Sarasota Bay, Florida (an area in which NSP occurred in three people in 1996) were analysed for brevetoxins by a radioimmunoassay and a receptor binding assay. Activity consistent with brevetoxins was seen in the shellfish samples. HPLC analysis of the shellfish extracts demonstrated the presence of PbTx-2 and PbTx-3 as well as the presence of conjugated metabolites of PbTxs. The structure of these metabolites was not yet determined (Poli *et al.*, 2000).

5.4.3 Other aquatic organisms containing NSP toxins

Brevetoxins from *G. breve* were traced under laboratory conditions, through experimental food chains from the dinoflagellate, through copepod grazers, to juvenile fish. Three different combinations of copepods and species of juvenile fish were used:

a) the copepod *Temora turbinata* and the spotted majarra, *Euchinostomus argenteus*, and the striped killifish, *Fundulus majalis.*
b) the copepod *Labidocera aestiva* and the pinfish, *Lagodon rhomboides*
c) the copepod *Acartia tonsa* and the spot, *Leiostomus xanthurus*

None of the four fish species died after eating copepods fed on *G. breve*.

In the experiment under (a) brevetoxins (PbTx-2 and -3) in the fish were detected only when copepods were fed on cultures with $600x10^3$ *G. breve* cells/L. With cultures of $8x10^3$ and $20x10^3$ cells/L no toxin was found in the fish. Roughly a 10 percent transfer from copepods to fish (viscera) over a two hours digestion time was found. Also in experiments under (b) transfer of the brevetoxins from copepod to fish (viscera) was observed within two hours (after 40-50 minutes of feeding with copepods). Toxin level in viscera decreased up to eight hours; no toxin was detected in fish muscle tissue. Under (c) again toxin in the fish was detected. Highest toxin level in fish viscera was measured after two hours, while after two to six hours to 25 hours toxin transferred to fish muscle (Tester *et al.*, 2000).

Brevetoxins have been quantitatively detected in Muir birds from the coast of California, in some tuna samples from Australia and in menhaden and mullet from the coast of Florida (Bossart *et al.*, 1998; Quilliam, 1999).

5.5 Toxicity of NSP toxins

5.5.1 Mechanism of action

Brevetoxins are depolarizing substances that open voltage gated sodium (Na^+) ion channels in cell walls. This alters the membrane properties of excitable cell types in ways that enhance the inward flow of Na^+ ions into the cell; this current can be blocked by external application of tetrodotoxin (Fleming and Baden, 1999). The brevetoxins act on binding site 5 in a 1:1 stoichiometry (Rein *et al.*, 1994). The toxin appears to produce its sensory symptoms by transforming fast sodium channels into slower ones, resulting in persistent activation and repetitive firing (Watters, 1995).

Conformational analysis revealed that the unsaturated H-ring of brevetoxin B (see Figure 5.1) favours the boat-chair conformation as does the saturated G-ring of brevetoxin A (see Figure 5.1). Upon reduction, the H-ring of brevetoxin B shifts to a crown conformation. This subtle change in

conformational preference induces a significant change in the gross shape of the molecule, which is believed to be responsible for the loss of binding affinity and toxicity (Rein *et al.*, 1994).

Respiratory problems associated with the inhalation of aerosolized brevetoxins are believed to be due in part to opening of sodium channels. In sheep, bronchospasm could be blocked by atropine. In addition, there appears to be a role for mast cells; in sheep the bronchospasm could be effectively blocked by cromolyn and chlorpheniramine. It was reported that brevetoxin could combine with a separate site on the gates of the sodium channel, causing the release of neurotransmitters from autonomic nerve endings. In particular, this can release acetylcholine, leading to smooth tracheal contraction, as well as massive mast cell degranulation (Fleming and Baden, 1999).

Since brevetoxins are also enzymatic inhibitors of the lysosomal proteinases known as cathepsins found in phagocytic cells such as the macrophages and lymphocytes, it is also possible that acute and chronic immunologic effects (including the release of inflammatory mediators that culminate in fatal toxic shock) may be associated with exposure to aerosolized brevetoxins, especially with chronic exposure and/or susceptible populations (Bossart *et al.*, 1998) although Fleming and Baden (1999) doubt on the cathepsin mechanism.

5.5.2 Pharmacokinetics

studies in laboratory animals

oral administration

Male F344 rats received a single oral dose of ^{3}H-labeled PbTx-3 and were killed after six, 12, 24, 48, 96 or 192 hours. Tissues were collected and analysed for radioactivity. Another group of animals received a bolus dose of ^{3}H-PbTx-3 orally and urine and faeces were collected at 24 hour intervals for a period of seven days. PbTx-3 distributed widely to all organs and concentrations decreased gradually with time. Highest PbTx-3 level was found in the liver at all sampling times. Based also on the intravenous studies below, it can be concluded that the liver received PbTx-3 from the portal as well as the hepatic circulation and so continued to accumulate PbTx-3. Seven days after receipt of the oral bolus dose approximately 80 percent of the dose was excreted via urine and faeces, with equivalent amounts in each. However, during the first 48 hours, more PbTx-3 was cleared through the faeces, whereas afterwards, most toxin was cleared through urine (Cattet and Geraci, 1993).

intravenous administration

Intravenous studies in male Sprague-Dawley rats with ^{3}H-labeled PbTx-3 showed a rapid clearance of PbTx-3 from bloodstream (less than 10 percent remained after one minute) and distribution to the liver (18 percent of the dose after 30 minutes), skeletal muscle (70 percent of the dose after 30 minutes) and gastrointestinal tract (8 percent of the dose after 30 minutes) ($T_{½}$ distribution phase approx. 30 seconds). Heart, kidneys, testes, brain, lungs and spleen each contained less than 1.5 percent of the dose. By 24 hours radioactivity in skeletal muscle decreased to 20 percent of the dose while radioactivity in liver remained constant and radioactivity in stomach, intestines and faeces increased suggesting biliary excretion as an important route of elimination. By day six, 14.4 percent of radioactivity had been excreted in urine and 75.1 percent in faeces, with 9.0 percent remaining in carcass. Thin layer chromatography of urine and faeces indicated biotransformation to several more polar compounds.

Studies with isolated perfused livers and isolated hepatocytes confirmed the liver as site of metabolism and biliary excretion as an important route of toxin elimination. PbTx-3 was excreted into bile as parent toxin plus four more-polar metabolites, one of which appeared to be an epoxide

derivative. Whether this compound corresponds to PbTx-6, to the corresponding epoxide of PbTx-3 or to another structure is unknown (Van Apeldoorn *et al*., 2001).

dermal application

The *in vitro* percutaneous penetration of ^{3}H-labeled PbTx-3 in human and guinea pig skin was examined and the effects of three vehicles (water, methanol and dimethylsulfoxide=DMSO) were compared. Epidermal surfaces with PbTx-3 in water were occluded for the entire duration (48 hours) of the experiment in order to reduce evaporation. Epidermal surfaces with PbTx-3 in methanol or DMSO were exposed to ambient conditions (incubation of diffusion cells at 36 ∀C). Total penetration through the isolated human skin was 0.43, 0.14 and 1.53 percent of the dose with water, methanol and DMSO as vehicle, respectively. Total penetration through guinea pig skin was 1.5, 3.4 and 10.1 percent of the dose with water, methanol and DMSO as vehicle, respectively. Penetration through guinea pig skin was significantly faster than through human skin with methanol and DMSO as vehicles. Analysis of the receptor fluid indicated that more than 80 percent of radioactivity was associated with unchanged PbTx-3 (Kemppainen *et al.*, 1989).

Dermal penetration and distribution of ^{3}H-labeled PbTx-3 into pig skin (0.3-0.4 σg/cm^{2} of skin) was studied in *in vivo* and *in vitro* studies. DMSO was used as vehicle. In the *in vivo* studies the application site was covered with a non-occlusive protective patch. In the *in vitro* studies the epidermal surfaces were exposed to ambient air (22 ∀C). *In vivo* studies revealed a mean cutaneous absorption of 11.5 percent of the administered dose during 48 hours of topical application (calculated by dividing percentage of dose excreted following topical administration by percentage of dose excreted following subcutaneous administration and multiplying by 100). In *in vitro* studies mean cutaneous absorption during 48 hours after application was 1.6 percent (based on accumulation of radioactivity in receptor fluid) or 9.9 percent (based on receptor fluid and dermis). Penetration through the epidermis into the dermis was rapid; maximal dermal accumulation was seen at four hours (9.1 percent *in vivo* and 18 percent *in vitro*). At 24 hours the amount in the dermis decreased to 2.3 and 15 percent *in vivo* and *in vitro*, respectively. In the *in vitro* study, more than 95 percent of radioactivity in the receptor fluid was unchanged PbTx-3 (Kemppainen *et al.*, 1991).

intratracheal instillation

Because a major route of human exposure to brevetoxins is via the respiratory tract, an intratracheal study in rats with PbTx-3 was performed to study the toxicokinetics of this brevetoxin.

^{3}H-Labeled PbTx-3 was administered to male F344 rats by intratracheal instillation. The animals were killed at 0.5, 3, 6, 24, 48 or 96 hours after exposure and urine, faeces and tissues were collected. Over 80 percent of the dose was cleared rapidly (within 0.5 hour) from the lung and distributed throughout the body, chiefly to the carcass (skeletal muscle) (49 percent), intestines (32 percent) and liver (8 percent); only 6 percent was found in the lung after 0.5 hour. Blood, brain and fat contained the lowest levels. About 20 percent of the initial level in tissues was retained for seven days. The majority of PbTx-3 was excreted within 48 hours in faeces and urine with approximately twice as much in faeces (60 percent) as in urine (30 percent). The identity of metabolites has not been determined. The results of this study suggested that the potential health effects associated with inhaled brevetoxins might extend beyond the transient respiratory irritation seen in humans exposed to sea-spray during red tides (Benson *et al.*, 1999).

5.5.3 Toxicity to laboratory animals

acute toxicity

Table 5.1 Acute toxicity of brevetoxins in Swiss mice

brevetoxins	route	observation time (hours)	LD_{50} value σg/kg bw	vehicle	reference
PbTx-1	intraperitoneal	24	> 100	0.9% saline + 0.1% Tween 60	Dechraoui *et al.*, 1999
PbTx-3	oral (females)	24	520	0.9% saline	Baden and Mende, 1982
PbTx-3	intraperitoneal (females)	24	170	0.9% saline	Baden and Mende, 1982
PbTx-3	intravenous (females)	24	94	0.9% saline	Baden and Mende, 1982
PbTx-	oral (females)	24	6 600	0.9% saline	Baden and Mende, 1982
PbTx-2	intraperitoneal (females)	24	200	0.9% saline	Baden and Mende, 1982
PbTx-2	intravenous (females)	24	200	0.9% saline	Baden and Mende, 1982

Table 5.2 Acute intraperitoneal toxicity of brevetoxin analogues in mice

brevetoxin analogues	route	survival time	minimum lethal dose σg/kg bw	vehicle	reference
BTX-B1	intraperitoneal	< 2 hours	50	methanol	Ishida *et al.*, 1995; 1996
BTX-B2	intraperitoneal	< 1 hour	306	water	Morohashi *et al.*, 1999; Murata *et al.*, 1998
BTX-B3	intraperitoneal	no deaths within 24 hours	>300	unknown	Morohashi et al., 1995
BTX-B4	intraperitoneal	6-24 hours	100	1% Tween 60	Morohashi *et al.*, 1999

symptoms of poisoning

Brevetoxins produce a variety of centrally and peripherally mediated effects *in vivo*; these include a rapid reduction in respiratory rate, cardiac conduction disturbances, and a reduction in core and peripheral body temperatures (Van Apeldoorn *et al.*, 2001).

In orally dosed mice, PbTx-3 caused tremors, followed by marked muscular contractions or fasciculations, Straub tail phenomenon, a period of laboured breathing and death. Mice injected

with PbTx-3 exhibited the SLUD syndrome i.e. salivation, lacrimation, urination and defecation. Hypersalivation was the most pronounced symptom, while copious urination and defecation were also common. Compulsory chewing motions and rhinorrhea were occasionally present at higher dosages. Intravenous dosing to mice produced immediate effects whereas intraperitoneal and oral dosing caused latent (30 minutes and 5 hours, respectively) responses. The two-fold more potency of PbTx-3 after intravenous dosing compared to intraperitoneal dosing pointed to partial detoxification or excretion in the bile during the first passage to the liver (Baden and Mende, 1982). In rats, gasping-like respiratory movements, head-bobbing, depression, ataxia, and, in some animals, the development of a head tilt were observed (Van Apeldoorn *et al.*, 2001).

Brevetoxin analogues BTX-B2 and BTX-B4 caused paralysis of hind limbs, diarrhoea, dyspnea and convulsion after intraperitoneal injection in mice (Morohashi *et al.*, 1999) and BTX-B1 irritability, hind and/or hind-quarter paralysis, severe dyspnea and convulsions prior to death due to respiratory paralysis (Ishida *et al.*, 1995; 1996).

antidotes

In a prophylactic study conscious tethered (catheters in carotid artery and jugular vein) male rats were pre-treated with 1 ml of anti-brevetoxin IgG (PbAb) or control IgG by a 10 minutes intravenous infusion. Twenty minutes thereafter the rats were infused with PbTx-2 (25 σg/kg bw = sublethal dose) over one hour. Rats pre-treated with control IgG showed signs of brevetoxin toxicity. These signs were absent in rats pre-treated with PbAb. In a therapy study rats were infused over 1 hour with 100 σg/kg bw PbTx-2 (=LD_{95}) followed immediately by 2 ml of either PbAb or control IgG infusion over 30 minutes. During PbTx-2 infusion, both groups showed signs of brevetoxin intoxication. Rats treated with control IgG died within six hours. In rats treated with PbAb, respiratory rates began to return toward baseline almost immediately and fewer neurological signs were seen. After 24 hours, nearly all neurological signs had disappeared and both core and peripheral temperatures had returned to normal. All animals treated with PbAb survived at least eight days. There was a time differential between two groups of signs, suggesting high and low accessibility compartments for the antibody representing probably central and peripherous nervous system (Van Apeldoorn *et al.*, 2001).

intravenous dosing

The intravenous LD_{50} in mice of the hemagglutinative fraction separated from red tides of *Chattonella marina*, appeared to be 2-4 mg/kg bw. The mice showed respiratory paralysis (Van Apeldoorn *et al*., 2001).

Groups of four male rats received after surgical preparation and a 24 hour recovery, an intravenous infusion during one hour with vehicle only or with 12.5, 25, 50 or 100 σg PbTx-2/kg bw and were monitored for six hours or until death. All animals at the 100 σg/kg bw dose level died within two hours. One out of four animals at 50 σg/kg bw died during the six hours study; the remainder of the animals survived. Within 90 minutes the respiratory rates at 12.5 σg/kg bw fell to near 60 percent of baseline value and at 25, 50 and 100 σg/kg bw to 20 percent of baseline value. Recovery to normal respiratory rates occurred six hours after exposure except in the 50 σg/kg bw group which recovered to only 60 percent of baseline value. During the first two hours, dose-dependent decreases in core body temperature occurred in all treated groups and a significant decrease in peripheral body temperature was seen in all but the 12.5 σg/kg bw group. An average decrease in peripheral body temperature of 0.5 ∀C was seen in the 12.5 σg/kg bw group. Blood gas values remained normal, except terminally. Electrocardiography showed at doses ∅25 σg/kg bw heart block, premature ventricular contractions and idioventricular rhythms (Van Apeldoorn *et al.*, 2001).

Catheterized male Hartley guinea pigs received an intravenous infusion with PbTx-3 at a rate of 0.63 σg/kg/min until death of the animal. The mean time until respiratory failure was 25 minutes. The mean dose of PbTx-3 at that time was 15.8 σg/kg. PbTx-3 caused lactic acidosis of unknown etiology which began early in the infusion period and was compensated for by increased minute volume. Airways resistance was not increased, nor was dynamic compliance decreased during intoxication, suggesting that neither central airways (upper airways, trachea and second-third generation airways) nor peripheral airways responded significantly (Van Apeldoorn *et al*., 2001).

intraperitoneal dosing

Intraperitoneal injection of the hemagglutinative fraction separated from red tides of *Chattonella marina*, in mice at a dose of 2.5 mg did not cause any abnormal sign (Van Apeldoorn *et al*., 2001).

repeated dose toxicity

No data

teratogenicity/reproduction

No data

mutagenicity

No data

in vitro studies with brevetoxins

The effects of PbTx-3 on various parameters of hepatic metabolism were evaluated in mouse liver slices. PbTx-3 inhibited oxygen consumption and increased Na^+ content and presumably intracellular Na^+ concentration of liver slices. PbTx-3 also activated a pathway that mediated K^+ efflux. No effect of PbTx-3 on the Na^+-K^+ pump activity was observed. The effect of PbTx-3 on liver slices Na^+ content was abolished by the sodium channel blocker tetrodotoxin. Tetrodotoxin also antagonized the inhibition of oxygen consumption. The effect of PbTx-3 on K^+ movements was not affected by tetrodotoxin, suggesting that two distinct ion channels or pathways were activated by PbTx-3. The results of this study suggest that PbTx-3 can induce effects in the liver that appear to be similar to those observed in nerve and muscle membranes (Van Apeldoorn *et al*., 2001).

The effects of PbTx-3 on hepatic cell structure were studied also in mouse liver slices. Light microscopy revealed hypertrophy and increased vacuolation of hepatocytes, and an increase in basophilia in the perivenous area of the lobules. Ultra-structurally, the vacuolation was related to swelling of the rough endoplasmic reticulum with water and/or protein retention without accumulation of fat droplets. Accumulation of proteins and/or degranulated ribosomes accounts for the increased basophilic reaction of the cells, especially in the perivenous area, an area where lipids are normally processed. Swelling in smooth endoplasmic reticulum, degranulation of rough endoplasmic reticulum, the deformities and lytic cristae in the mitochondria, and the presence of active lysosomes are evidence of the effects of PbTx-3 upon liver cells (Van Apeldoorn *et al*., 2001).

Positive inotropic and arrhythmogenic effects on isolated rat and guinea pig cardiac preparations were seen at concentrations between 1.25×10^{-8} and 1.87×10^{-7} M PbTx-2. The studies suggested that PbTx-2 is a potent cardiotoxin and exerted its effects by increasing sarcolemnal sodium permeability, and by releasing catecholamines from sympathetic nerve endings (Van Apeldoorn *et al*., 2001).

Crude preparations of brevetoxin produce airway contraction; however, it was unknown if this mechanical response was coupled to changes in airway smooth muscle membrane potential, either to direct action on the airway smooth muscle cell membrane or indirectly via the release of endogenous acetylcholine at peripheral nerve terminals. Therefore membrane potentials and contractility of *in vitro* canine trachealis smooth muscle preparations were measured before and during exposure to either the crude toxin (0.01-1.2 σg/ml), or the purified fractions PbTx-2 and PbTx-3 (0.01-0.07 σg/ml). Membrane potentials in cultured airway smooth muscle cells were similarly studied. The crude fraction of brevetoxins produced concentration-dependent depolarizations in airway smooth muscle preparations *in vitro* as did the purified fractions PbTx-2 and PbTx-3 however with an approximately 10-fold higher potency than the crude brevetoxins. In all cases, depolarizations stabilised within four minutes. There was no significant difference in concentration-response relationship between PbTx-2 and PbTx-3. The effects of crude and purified toxins were fully reversed within 30 minutes of their washout from tissue bath. The results of this study suggested that brevetoxins did not produce direct depolarizing effects on airway smooth muscle cells, as brevetoxins were without any significant effect in *in vitro* preparations treated with tetrodotoxin, or in cultured cell preparations. Brevetoxin induced bronchoconstriction is probably due to the depolarizing effect of endogenous acetylcholine, which is released from peripheral nerve terminals, on the airway smooth muscle cell (Van Apeldoorn *et al*., 2001).

5.5.4 Toxicity to humans

oral exposure

When brevetoxins are accumulated in shellfish, consumption of the raw or cooked shellfish can cause NSP, a toxic syndrome somewhat similar to PSP and ciguatera intoxication but less severe. The symptoms of NSP occur within 30 minutes to three hours, last a few days and include nausea, vomiting, diarrhoea, chills, sweats, reversal of temperature, hypotension, arrhythmias, numbness, tingling, paresthesias of lips, face and extremities, cramps, bronchoconstriction, paralysis, seizures and coma. No mortality or chronic symptoms are reported (Cembella *et al.*, 1995; Fleming *et al.*, 1995; Tibbets, 1998). Treatment is primarily supportive (Fleming and Baden, 1999).

dermal exposure

Due to the relative fragility of the *G. breve* organism (*G. breve* is a "naked" organism having no outer shell of polysaccharide plates like other dinoflagellates) it is easily broken open in the rough surf releasing the toxins. During swimming direct contact with the toxic blooms may take place and eye and nasal membrane irritation can occur (Cembella *et al.*, 1995; Fleming and Baden, 1999; Tibbets, 1998).

inhalation exposure

Due to the relative fragility of the *G. breve* organism, inhalation exposure to brevetoxins may also occur causing respiratory distress, as well as eye and nasal membrane irritation. (Cembella *et al.*, 1995; Fleming and Baden, 1999; Tibbets, 1998).

G. *breve* toxins stimulate post-ganglionic cholinergic fibres which may result in respiratory irritation, conjunctival irritation, copious catarrhal exudates, rhinorrhea, non-productive cough, and bronchoconstriction when exposed to aerosolized surf or its red tides. Some people also report other symptoms such as dizziness, tunnel vision and skin rashes. In the normal population, the irritation and bronchoconstriction are rapidly reversible by leaving the beach area or entering an air conditioned area. However, asthmatics are apparently particularly susceptible. Furthermore, there are anecdotal reports of prolonged lung disease, especially in susceptible populations such as the elderly or those with chronic lung disease (Fleming and Baden, 1999; Watters, 1995).

Furthermore PbTx is supposed to cause chronic immunosuppression, possibly mediated by interaction with an additional pharmacological target, cysteine cathepsins, present in immune cells and involved in antigen presentation (Van Dolah *et al*., 2001).

PtBx-3 was indicated to be the primary toxin responsible for respiratory discomfort in humans (Benson *et al.*, 1999).

5.5.5 Toxicity to aquatic organisms

C. marina strongly inhibited the proliferation of marine bacteria, *Vibrio alginolyticus*, in a plankton/bacteria co-culture. The growth inhibition of bacteria caused by *C. marina* was related to the density and the metabolic potential of *C. marina*. Ruptured plankton showed no toxic effect on the bacteria. Furthermore, the toxic effect of *C. marina* on *V. alginolyticus* was completely suppressed by the addition of catalase and superoxide dismutase. In addition to these radical scavenging enzymes, a chemical scavenger, sodium benzoate, also had a protective effect. These results suggest that oxygen radicals are important in the toxic action of *C. marina* (Van Apeldoorn *et al*., 2001).

Incubation of the sea urchin (*Lytechinus variegatus*) and the sea trout (*Cynoscion nebulosis*) in the sea-surface microlayer collected off the Florida Keys, particularly when taken from slicked areas, affected early embryogenesis of both the invertebrate and the fish. Samples of underlying subsurface water elicited almost no adverse responses in cultured embryos. Results from a partial toxicity identification evaluation procedure indicated that an organic compound containing a non-polar functional group was the primary determinant of toxicity. While subsequent GC-MS failed to identify a specific compound, it did rule out common xenobiotics such as organochlorine pesticides, as potential toxicants. Preliminary tests indicated that two of the most toxic sea-surface microlayer samples contained a brevetoxin. However the identification of any toxic agent remains speculative without a complete toxicity identification evaluation (Van Apeldoorn *et al*., 2001).

According to Viviani (1992) fish usually start to die when *G. breve* counts reach the 250 000 cells/litre range. However, other authors report that fish kills will occur at counts of ∅100 000 cells/litre (Landsberg and Steidinger, 1998).

Ichthyotoxic symptoms included violent twisting and corkscrew swimming, pectoral and caudal fin analysis progressing to a loss of equilibrium, and subsequent respiratory paralysis and death. These symptoms are believed to begin with the binding of PbTx-3 to specific receptor sites in fish excitable tissues (Van Apeldoorn *et al*., 2001).

Toxicity tests with five to six month old juvenile red sea bream (*Pagrus major*) were performed in 1-l cultures of *Chattonella antiqua*, *Fibrocapsa japonica* and *Heterosigma akashiwo*. In the early growth phase *C. antiqua* was hardly toxic to the red sea bream until cell density reached approximately 1.95×10^3 cells/ml. In low density cultures (on the second day) fish did not die but showed abnormal movements for about 30 to 45 minutes, recovered gradually and swam normally within a few hours. Beyond that point the increase in toxicity appeared to be a function of cell density. The highest toxicity per cell was seen during early to mid-log-arrhithmic growth phase. In the late logarithmic growth phase, there was a gradual decrease in toxicity. In the early logarithmic phase CaTx-II (~PbTx-3) content was 14 times higher than the PbTx-3 content in the logarithmic growth phase of *C. marina* cultures whereas the CaTx-III (~oxidized PbTx-2) content was only two times higher than the oxidized-PbTx-2 content in *C. marina*. As PbTx-3 is 10 times more ichthyotoxic than oxidized PbTx-2 *C. antiqua* appears to be much more ichthyotoxic than *C. marina*. No toxicity of *F. japonica* cultures to the red sea bream was detected until cell density reached 4.1×10^3 cells/ml. Ichthyotoxicity also appeared to vary with the growth phases and

increased with age; it was highest on the eighth day. Thereafter toxicity began to decline to low levels as the cells entered the early stationary phase. In *H. akashiwo* cultures the red sea bream showed no abnormal behaviour at a cell density of 34 000 cells/ml but exhibited a violent paralysis, leading to death, when the cell density surpassed 120 000 cells/ml. When exposed to a red tide of *H. akashiwo* at a cell density of 30 000 cells/ml, the red sea bream showed a transient, but not fatal, paralysis. The red tide in Kagoshima Bay in Japan killed fish at a cell density >100 000 cells/ml (Van Apeldoorn *et al*., 2001).

Medaka fish (*Oryzias latipes*) eggs which were micro-injected (six to eight hours post-fertilization) with doses of 0.1-8.0 ng PbTx-1/egg, showed a dose-dependent inhibition of hatching (half maximal effect at about 3 ng/egg) and larval survival (half maximal effect at about 4.5 ng/egg). A dose-related increase in muscular activity (hyperkinesis) was seen after embryonic day four at doses from 0.1 to 0.9 ng/egg onwards. Upon hatching morphologic abnormalities were found at the following LOAELs: 1.0 to 3.0 ng/egg lateral curvature of spinal column, the severity of which was dose-related; 3.1 to 3.4 ng/egg herniation of brain and meninges through defects in the skull; and 3.4 to 4.0 ng/egg malpositioned eye and lack of a frontal skull. Hatching abnormalities (head-first instead of tail-first) were seen at doses ∅2.0 ng/egg and doses ∅4.1 ng/egg produced embryos which failed to hatch (Kimm-Brinson and Ramsdell, 2001).

H. akashiwo red tides caused damage to fish culture operations in Japan (yellow tail and red sea bream for the Seto Inland Sea), New Zealand, British Columbia and Chile (salmon) (Van Apeldoorn *et al*., 2001).

Several extensive blooms caused by potentially toxic *Chattonella* sp. cells occurred from the German Bight to the almost north Skagen between late March and first half of May 1998, 2000 and 2001 and caused fish killing (Douding and Göbel, 2001).

In April and May 1996, an estimated 1 700 tonnes of cultured bluefin tuna (*Thunnus maccoyi*) were killed in South Australia after a bloom of *Chattonella marina* (Van Apeldoorn *et al*., 2001).

Toxicity of PbTx-1, 2, 3, 6 and 9 for female mosquito fish (*Gambusia affinis*) was studied. The LC_{50} (24 hour) values were 2.57, 14.3, 15.8, 77.7 and 31.4 nM for PbTx-1, 2, 3, 6 and 9, respectively (Rein *et al.*, 1994).

A neurotoxic, a hemolytic and a hemagglutinative fraction were isolated from red tides of *Chattonella marina*. Juvenile red sea bream (*Pagrus major*) were exposed to the three fractions (0.02 percent) in beakers of seawater. The fish died within seven to nine minutes at exposure to the neurotoxic fraction showing conspicuous edema on their second lamellae. At exposure to the hemolytic and hemagglutinative fractions fish died within 20 to 50 minutes with a marked mucous release on their gill filaments (Van Apeldoorn *et al*., 2001).

Exposure of red sea bream (*Pagrus major*) to *C. marina* red tide water significantly decreased the heart rate, presumably resulting in anoxia from reduced blood circulation in the gill. Since atropine restored the depressed heart rate, the cardiac disorder seemed to occur neurogenously in association with the intrinsic cardiophysiology of the fish. The heart rate of fish is largely controlled by the vagal nerve. The vagal nerve has a parasympathetic character and depresses the heart rate under depolarization. It has been reported that the function of the vagal nerve is inhibited by atropine. Neurotoxin fractions of *C. marina* depolarised the vagal nerve of fish, and hence induced the reduction of the heart rate. Histological examination showed little branchial damage due to neurotoxin fractions (Van Apeldoorn *et al*., 2001).

During the autumn and winter from 1987 to 1988, the third year of an eight-year larval fish study, a bloom of *G. breve* occurred in the coastal waters of North Carolina. Densities of nine species of larval fishes (*Paralichthys albigutta, Citharichthus spilopterus, Micropogonias undulatus, Lagodon rhomboides, Brevoortia tyrannus, Paralichthus lethostigma, Leiostomus xanthurus, Mugil cephalus, Myrophis punctatus*) in the years 1987 and 1988 were compared to their densities in the two seasons prior to and five seasons after the bloom. No severe impact on the larval fish community as a whole was evident during the year of the bloom. However, there were species specific differences in response to the presence of *G. breve*. Two species (*Micropogonias undulatus* and *Lagodon rhomboides*) showed generally normal, or above normal densities, both during the bloom and for the remainder of the larval recruitment season. Two species (*Paralichthus albigutta* and *Citarichthus spilopterus*) had consistently low densities throughout their normal period of recruitment, suggesting that their estuarine recruitment may have been impacted by the effects of *G. breve* even after the bloom ended. The remaining five species (*Brevoortia tyrannus, Paralichthus lethostigma, Leiostomus xanthurus, Mugil cephalus* and *Myrophis punctatus*) had low densities during the bloom, but increased markedly later in the season (Warlen *et al*., 1998).

In 1996, at least 149 manatees (*Trichechus manatus latirostris*) died in an unprecedented epizootic along the southwest coast of Florida. At the same time a bloom of *G. breve* was present in the same area. Exposure of the manatees occurred via inhalation and oral ingestion (Bossart *et al.*, 1998). One of the likely vectors for the toxin is being filter-feeding sea squirts (Marsden, 1993). Grossly, severe nasopharyngeal, pulmonary, hepatic, renal, and cerebral congestion was present in all cases. Nasopharyngeal and pulmonary edema and haemorrhage were also seen. Consistent macroscopic lesions were catarrhal rhinitis, pulmonary haemorrhage and edema, multi-organ hemosiderosis, and non-suppurative leptomeningitis. Immunohistochemical staining using a polyclonal primary antibody to brevetoxin, showed intensive positive staining of lymphocytes and macrophages in the lung, liver and secondary lymphoid tissues. Additionally, lymphocytes and macrophages associated with the inflammatory lesions of the nasal mucosa and meninges were also positive for brevetoxin. These findings implicate brevetoxicosis as a component of and the likely primary etiology for the epizootic. The data suggested that mortality resulting from brevetoxicosis might not necessarily be acute but might occur after days or perhaps weeks after inhalation and/or ingestion of brevetoxins. Neurological signs including muscle fasciculations, incoordination, and inability to maintain a righting reflex were reported from four manatees rescued alive from the epizootic. Immunohistochemical staining with interleukin-1-η-converting enzyme showed positive staining with a cellular tropism similar to brevetoxin. This suggests that brevetoxicosis may initiate apoptosis and/or the release of inflammatory mediators that culminate in fatal toxic shock (Bossart *et al.*, 1998).

Brevetoxin (PbTx-3) was shown to be bound to isolated nerve preparations from manatee brain with similar affinity as that reported for a number of terrestrial animals. *In vitro* studies with ^{3}H-PbTx-3 showed binding to manatee brain synaptosomes with high affinity and specificity. The binding was saturable, there was competition of specific binding, and temperature dependence (decreased toxic-receptor affinity and lower measured percentages of specific binding as temperature increases from 0 to 37 ∀C) (Van Apeldoorn *et al*., 2001).

The brevetoxin analogues (or metabolites) found in New Zealand cockles (*Austrovenus stutchburyi*) (BTX-B1) and in New Zealand greenshell mussels (*Perna canaliculus*) (BTX-B2 and BTX-B4) did not show ichthyotoxicity against the fresh water fish *Tanichthys albonubes* at 0.1 mg/L unlike brevetoxins (Ishida *et al.*, 1995; Morohashi *et al.*, 1999; Murata *et al.*, 1998).

Mortality among the double-crested cormorant (*Phalacrocorax auritus*) caused by brevetoxins, was observed along the Florida gulf coast (Fleming and Baden, 1999). Brevetoxin was the cause of a summer mortality in common murres (*Uri aalge*) in California (Fleming and Baden, 1999).

5.5.6 Toxicity studies with a phosphorus containing G.breve component

Besides potent brevetoxins, some phosphorous containing toxic components have also been isolated from *G. breve*. One phosphorus containing (ichthyotoxic) component was isolated and subsequently its structure has been determined. The chemical name is *O,O*-dipropyl(*E*)-2-(1-methyl-2-oxopropylidene)-phosphorohydrazidothioate(*E*)oxime. This component has a chemical structure similar to an organothiophosphate (Van Apeldoorn *et al*., 2001).

mice

The acute intraperitoneal toxicity (i.p.) of the above mentioned oxime (synthetic) and some analogues was investigated in mice with special attention to acetylcholinesterase (AChE) inhibition (IC_{50}) in both cerebral and peripheral tissue. The oxime appeared to be a more potent inhibitor of AChE *in vivo* than the analogues, whereas higher toxicity is associated with some analogues suggesting involvement of other factors than AChE inhibition, affecting the toxicity. The mice exposed to the oxime and its analogues exhibited hyperactivity, tremors and convulsions which were not very severe. Generally these symptoms appear in animals exposed to AChE agents when more than 40 percent inhibition of brain AChE is observed. In this study, brain AChE activity was inhibited by 36.6 percent after intraperitoneal dosing of the oxime. Such inhibition might cause only mild symptoms (Van Apeldoorn *et al*., 2001).

rats

Anesthetized male Wistar rats received a single intravenous injection with 16, 24, 48 or 72 σg/kg bw of the oxime. A dose-dependent cardiovascular depressant activity was observed as demonstrated by a dose-dependent decrease in mean arterial blood pressure as well as in heart rate. A time related recovery was only seen at the two lowest doses (16, 24 σg/kg bw). At higher doses the toxin caused irreversible hypotension and bradycardia. The animals died of cardiac arrest immediately after intravenous administration of 72 σg/kg bw. The effects were not accompanied by constriction or spasm in tracheobronchial response. The hypotension and bradycardia occurred even in artificially ventilated rats. The cardiovascular effects were antagonized by tetraethylammonium while blockade of cholinergic and histaminergic receptors or inhibition of prostaglandin synthesis failed to modify these effects. These findings indicated that the cardiovascular effects are probably mediated through ζ-adrenergic and ganglionic blockade accompanied by modulation of K^+ channel activity (Van Apeldoorn *et al*., 2001).

cats

An intravenous study with the oxime in anesthetized cats was performed to study the effects on mean arterial blood pressure, ECG pattern, unit discharge of baroreceptors and respiratory activity. Intravenous doses of 0.25 to 1.5 mg/kg caused a dose-dependent fall in blood pressure which was associated with bradycardia. Initial respiratory apnoea followed by increased rate and depth of respiration (hyperapnoea) was seen. The hypotensive effect was accompanied by a decrease in aortic baroreceptor activity per heart beat recorded from the cervical aortic afferents. The ECG showed atrioventricular conduction block, arrhythmia and depression of S-T segment and T wave which indicated coronary insufficiency. The vasodepressor property of the toxin is presumably muscarinic in nature as atropine counteracted the vasodepression (Van Apeldoorn *et al*., 2001).

fish

The oxime, appeared to be very ichthyotoxic (0.9 mg/L against *Lebistes reticulatus*) (Van Apeldoorn *et al*., 2001).

5.6 Prevention of NSP intoxication

5.6.1 Depuration

detoxification of shellfish

The loss rate of toxins from bivalves depends upon the site of accumulation, which may differ between phycotoxins. Scallops are the most intensively studied species and a two-phase detoxification was suggested: an initial rapid loss similar to the accumulation rate followed by a slower phase. During this process, the toxin profile may change between tissues such as kidney and mantle, with toxic transfer between tissue compartments or organs before excretion or secretion into the environment. The most usual way of depurating bivalves is self-depuration, achieved by moving shellfish stock to clear water. Cooking and freezing is ineffective. One of the most promising treatments appeared to be ozone which has been shown to assist in the depuration of mussel tissue of NSP (Van Apeldoorn *et al*., 2001).

Oysters accumulate brevetoxins in less than four hours in the presence of 5 000 cells/ml, and depurate 60 percent of the accumulated toxin in 36 hours. Potency of depuration is species-specific and highly variable, even under controlled laboratory conditions. Commercial bivalves are generally safe to eat one to two months after the termination of any single bloom episode. Canning cannot be a way to decrease brevetoxin concentration in bivalves (Viviani, 1992).

In *Crassostrea virginica* depuration of brevetoxins occurs two to eight weeks after the bloom has dissipated. Using a half-factorial experimental design, *G. breve* cells were cultured and fed to Pacific oysters (*Crassostrea gigas*) at rates of between 10 and 25 million cells per oyster over 24 hour periods. Thereafter the oysters were detoxified in various laboratory tanks over five-day periods. Mouse bioassays showed initial levels between 25 and 100 mouse units (MU) per 100 g drained oyster meat with larger oysters accumulating more toxin than the smaller ones. Experimental factors were temperature (15 and 20 ∀C), salinity (24 and 33-34 ‰), filtration (5 µm) versus no filtration, and treatment with ozone (to a redox potential of 350 mV in the shellfish tanks) versus passive UV light sterilization. Two experiments compared oysters that had been fed *G. breve* over five days (5.0 or 3.5 million cells per oyster/day) with those fed for 24 hours (10 or 25 million cells per oyster). With the exception of one (four tanks), all treatment combinations resulted in an initial decline of the brevetoxin level reaching a minimum <20 MU per 100 g by day three regardless of the initial toxin level or whether the toxin had accumulated over one or five days. The three-day period of decline was followed by a period of minimal reductions. None of the experimental factors had any statistically significant effect on the final toxin levels suggesting that oysters will detoxify regardless of the conditions once they are placed in an environment free from toxic algae. Final brevetoxin levels just above 20 MU per 100 g (20.6 MU per 100 g) were observed in some samples of four treatments (Van Apeldoorn *et al*., 2001).

decrease of G.breve cells and reduction of toxins

Cell cultures of *G. breve* in artificial seawater were subjected to microwave irradiation at 2 450 MHz. Irradiation was for four 60 seconds intervals separated by five minutes intervals of cooling at 25 ∀C. A decrease in number of cells was seen. As a function of power (0-0.113 kJ/ml culture) the decrease in surviving cells was about 14 percent. A pronounced decrease or threshold effect was evident at energy levels above 0.08 kJ/ml of culture (Van Apeldoorn *et al*., 2001).

The effect of ozonated artificial seawater on *G. breve* cells and toxins was studied. When artificial seawater which was ozonated for 60 seconds, was added to *G. breve* cultures the number of surviving cells decreased approximately 80 percent ($t_{1/2}$=10 seconds). When cultures of *G. breve* in artificial seawater were directly ozonated for 60 seconds, non intact cells were found ($t_{1/2}$=2.5 seconds). Experiments carried out in artificial seawater demonstrated that extracted *G. breve* toxins (PbTx-1, -2, -3, -5, -7 and -9) reintroduced into artificial seawater as well as toxins in whole cell cultures of *G. breve* in artificial seawater at exposure to ozone for 0, 1, 5 or 10 minutes, displayed a marked reduction as ozone exposure increased. Total toxin concentration was reduced 99.9 percent after 10 minutes ozonisation as determined by HPLC analysis. Bioassays with the fish *Cyprinodon variegatus* confirmed the toxin reduction. In both experiments (with extracted brevetoxins and brevetoxins from whole cells) a slight increase of PbTx-7 was seen after one minute of ozone exposure probably caused by the reduction of PbTx-1, the aldehyde form of type A brevetoxins, to PbTx-7, the alcohol form of the same skeleton. As ozone exposure was increased to five minutes, total amounts of all toxins were reduced (Van Apeldoorn *et al*., 2001).

Doucette *et al.* (1999) studied the role of algicidal bacteria active against *G. breve*. Two bacterial strains isolated from the Gulf of Mexico appeared to be lethal to *G. breve*. The algicidal activity of one of these two strains was characterized. The strain was isolated from waters without *G. breve* cells, suggesting that such bacteria are part of the ambient microbial community and are not restricted to areas of high *G. breve* abundance. The bacterial strain examined, produced (a) dissolved algicidal compound(s) that was (were) released into the growth medium, and the algicide was effective against the four Gulf of Mexico *G. breve* isolates tested as well as to a closely related HAB species that also occurred in this region: *G. mikimotoi*.

Pérez *et al*. (2000) remarked the discovery of several algae that produce allelopathic chemicals, termed APONINs, which can affect adversely *G. breve*. A dynamic computer model, based on laboratory studies, was designed to evaluate the effectiveness of treating a *G. breve* bloom with the allelopathic alga, *Nannochloris oculata*, which produces the cytosolic substance APONIN-3, a substance that retards the growth rate of *G. breve*. However, certain boundary conditions and/or assumptions are involved. Under the conditions used, the model showed that *N. oculata* would eradicate a *G. breve* outbreak in a little less than two days. The model demonstrated that *N. oculata* is a potential management method for *G. breve* blooms, but further experiments need to be performed.

detoxification of brevetoxins

Brevetoxins containing an aldehyde functional group on the terminal "tail" side chain, are easily converted to dimethylacetal structures in acidic solutions, while acid reaction to form the methyl ester at the head-side lactone ring proceeds slowly. Reactivity of brevetoxins to acid attack shows the following order: PbTx-1>PbTx-2>PbTx-9. Under basic conditions, head-side lactone ring opening initiated by hydroxide ion attack proceeds to completion in 120 and 50 minutes for PbTx-2 and PbTx-9, respectively, while that for PbTx-1 did not reach completion after 120 minutes. Base hydrolysis proceeds faster than acid hydrolysis under comparable acidic or basic conditions. However, these acid and base hydrolyses can be reversible reactions and they may be not reliable for detoxification purposes. Brevetoxins are easily oxidized by potassium permanganate through double bond addition and then cleavage. Brevetoxin oxidation treatment is an irreversible process and proceeds relatively fast, so it can be a good means of brevetoxin detoxification (Hua and Cole, 1999).

5.6.2 Preventive measures

Toxic blooms of *G. breve* are generally detected by visual confirmation (water discolouration and fish kills), illness to shellfish consumers and/or human respiratory irritation with actual toxicity

verified through time-consuming chemical analyses for brevetoxins within shellfish samples and by mouse bioassays. The exact environmental conditions that lead to harmful algal blooms is poorly understood. As a consequence it is extremely difficult to predict the occurrence and magnitude of a bloom, thereby ensuring an 'after-the fact' management strategy dependent upon accurate water-quality evaluation. Monitoring programmes relying on microscopic identification and enumeration of harmful taxa in water samples generally suffice for preventing human intoxication. However microscopic based monitoring requires a high level of taxonomic skill, usually takes considerable time, and can be highly variable among personnel. Therefore, an alternative and/or complimentary evaluation system for predicting bloom occurrence and dynamics is highly desirable. Diagnostic pigment signatures and in vivo optical density spectra can effectively differentiate among most phylogenic groups of micro- and macroalgae, and sometimes, taxa with a variety of habitats. If such diagnostic pigments and/or spectra would allow for detecting the presence of harmful taxa prior to bloom status, a rapid, objective, and economical 'biomarker' protocol could be developed. The gyroxanthin-diester may be a diagnostic pigment for *G. breve* within Florida coastal waters. This pigment only has been reported from *Gyrodinium aureolum*, *Gymnodinium galatheanum* and *G. breve*. Of these taxa, only *G. breve* can be considered as a warm water taxon and would be expected to occur in Florida coastal waters. Additionally, gyroxanthin-diester was a minor, yet stable, component of the total carotenoids in *G. breve*, being consistently detectable and quantifiable in populations exposed to all irradiance treatments.

The utility of photopigments and absorption signatures to detect and enumerate *G. breve*, was evaluated in laboratory cultures and in natural assemblages. The presence of gyroxanthin-diester provided for delineation of *G. breve* from other taxa within phytoplankton assemblages in Florida. In addition, the high correlation of this carotenoid with *G. breve* cell abundance allowed tracking of bloom development and senescence. However, the gyroxanthin-diester provides only a minor contribution to the cellular absorption and has absorption maxima similar to those of other carotenoids and chlorophyll *c* and its presence does not dramatically alter the absorption spectrum of a mixed assemblage. The technological advances in computer-based instrumentation will stimulate the increased usage of bio-optical methodologies for potentially detecting and characterizing harmful plankton (Van Apeldoorn *et al*., 2001).

Kirkpatrick *et al*. (2000) collected pigment and spectral absorption data from natural blooms in the eastern Gulf of Mexico between August 1995 and August 1997. Quantifying gyroxanthin-diester and chlorophyll *a* allowed the estimation of the fraction of the biomass in mixed populations associated with *G. breve*. Subsequent regression of the *G. breve* similarity indexes to the *G. breve* biomass fractions yielded a significant linear correlation. The liquid waveguide capillary cell appeared to be a promising technology for automating this technique.

Microphotometric methods were compared with conventional spectrophotometric methods for the assessment of spectral absorption of monospecies cultures. The feasibility of using microphotometry as a means of characterizing spectral absorption coefficients of a.o. *G. breve* was demonstrated. Subsequently, an approach for the detection of *G. breve* in a mixed population on the basis of spectral absorption signatures was evaluated. The development of improved hyperspectral *in situ* or air-borne sensors may enhance the ability to monitor the presence and evolution of harmful algal blooms. The phases of *G. breve* blooms include: a) offshore initiation; b) transport to mid-shelf; and c) growth. Several aspects of the biology and ecology of *G. breve* make it a likely bloom species to be detected and tracked via remote sensing. While a cell count of 5 000 cells/litre is sufficient to require closure of shellfish beds to harvesting, generally, visual detection of *G. breve* blooms by eye can be made only when cell concentrations approach 10^6 cells/litre, by which time respiratory irritation, shellfish contamination and fish kills already are manifested. While biomass concentration is patchy, chlorophyll *a* values from >1 to 100 mg/m^3

make the resultant discoloured surface water detectable by colour sensors onboard satellites (Van Apeldoorn *et al*., 2001).

A minimum detection level of approximately 100 000 cells/litre was reported by remote sensing; 10 times more sensitive than visual detection. In this case, there could be a minimum of three to six days between bloom biomass detection and population growth to levels known to cause massive fish kills. In the mean time the presence of *G.breve* can be verified (Van Apeldoorn *et al*., 2001).

5.7 Cases and outbreaks of NSP

5.7.1 Europe

France

In France the presence of *Fibrocapsa japonica* was reported for the first time in October 1991 on the Channel coasts of Normandy (Van Apeldoorn *et al*., 2001). Video-recordings of *H. akashiwo* from the French coast showed a very high resemblance to specimens found in the Dutch North Sea in 1994 and German Wadden Sea in 1997 (Van Apeldoorn *et al*., 2001).

Germany

On 26 August and 15 December 1994, *H. akashiwo* was detected in the German Wadden Sea (Van Apeldoorn *et al*., 2001).

In Germany, *Fibrocapsa japonica* has been observed near Sylt in the summer of 1997. Since the summer of July 1995, *F. japonica* has been found in phytoplankton samples from the Wadden Sea near the harbour of Büsum on the west coast of Schleswig-Holstein. In 1996 and 1997, *F. japonica* was also listed in the messages of the German "Algenfrühwarnsystem" for the German Wadden Sea. At Büsum harbour, *F. japonica* concentrations increased from maximum numbers of 25 and 30 cells/cm^3 in 1995 and 1996 respectively, to 115 cells/cm^3 in 1997. The highest number of 327 cells/cm^3 was recorded on 24 July 1997 (Van Apeldoorn *et al*., 2001).

In German waters, *H. akashiwo* was also observed namely in Friedrichskoog in the summer of 1997. Cell concentrations were difficult to count (Rademaker *et al*., 1997).

Several extensive blooms caused by potentially toxic *Chattonella* sp. cells occurred from the German Bight to the almost North Skagen between late March and first half of May 1998, 2000 and 2001 and caused fish killing (Douding and Göbel, 2001).

Greece

A species similar to *G. breve* has been reported from the Aegean Sea but with no adverse effects (Smith *et al*., 1993).

The Netherlands

The *Raphidophyceae Fibrocapsa japonica*, *Chattonella antiqua* and *Chattonella marina* were detected for the first time in 1991 and thereafter in 1992 and 1993 in the Wadden Sea, the North Sea and/or the Delta area south of the Rhine estuary. Harmful events caused by the *Raphidophyceae* have not yet been recorded in the Netherlands, but an outbreak cannot be excluded because the species detected can potentially be present each year (Van Apeldoorn *et al*., 2001).
In the summer of 1997, *F. japonica* was found in almost all samples from the Dutch Algal Bloom Programme along the Dutch Coast from Noordwijk to Borkum. In the samples, cell densities were

2 cells/cm^3. The potentially toxic raphidophyte *Heterosigma akashiwo* was found for the first time in August 1994 in an algal bloom near Noordwijk with cell numbers of approximately 2 400 cells/cm^3 (Van Apeldoorn *et al*., 2001).

Portugal

A species similar to *G. breve* has been reported from the Atlantic coast of Portugal but with no adverse effects (Smith *et al*., 1993).

The Russian Federation

In September 1987, a red tide caused by *Chattonella* sp. caused fish mortality in Amurskii Bay (Orlova *et al*., 1998).

Spain

A species similar to *G. breve* has been reported from the Atlantic coast of Spain but with no adverse effects (Smith *et al.*, 1993).

The United Kingdom of Great Britain and Northern Ireland

Red tides of *Heterosigma akashiwo* have been reported from England and Bermuda causing mortality of cultured fish (Van Apeldoorn *et al*., 2001).

5.7.2 Africa

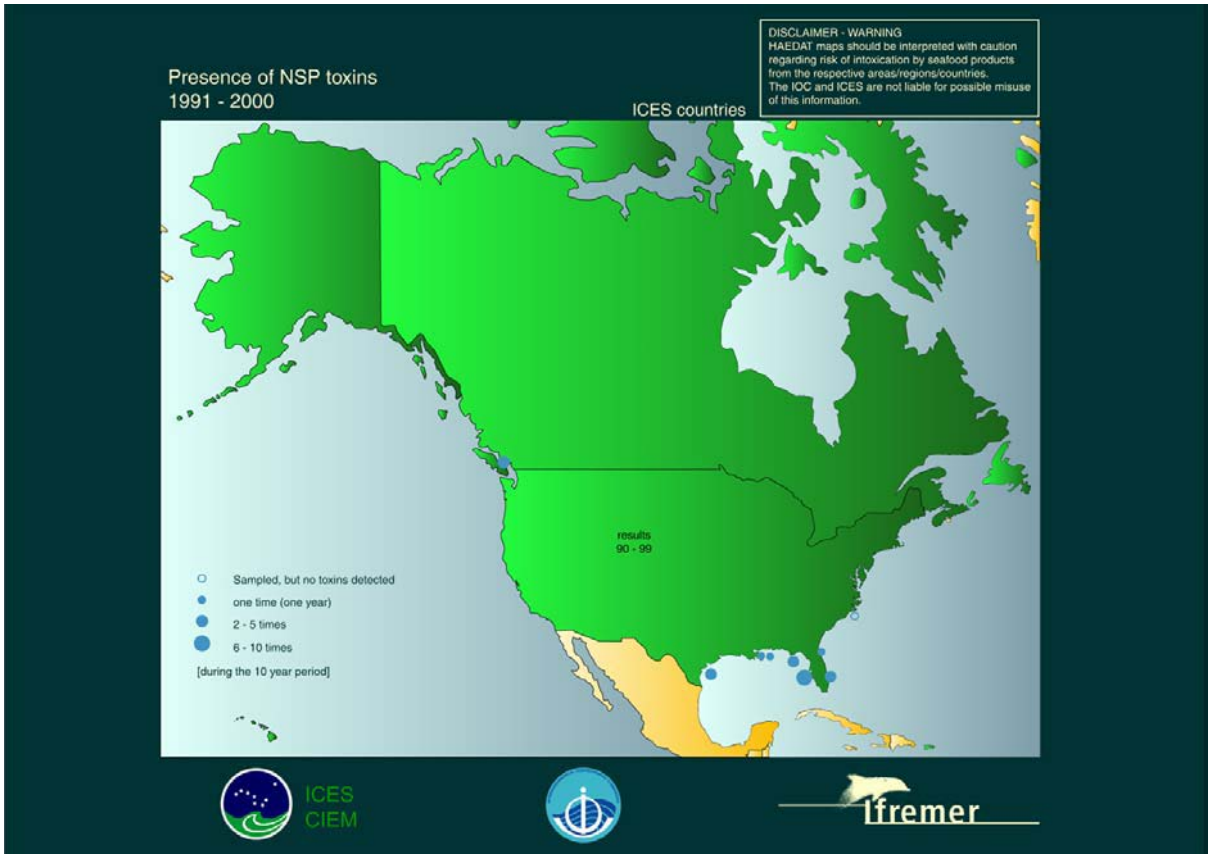
South Africa

In the summer of 1995 to 1996, a severe aerosol toxin problem was reported in False Bay, which later spread to the coastal resort of Hermanus in Walker Bay. Coughing, burning of the nasal passages, difficulty in breathing, stinging eyes and irritation of the skin were observed in beach goers and seaside residents. The aerosol toxin was linked to a bloom of a toxic dinoflagellate species *Gymnodinium*, first recorded in False Bay in 1988. Despite the species having bloomed on several occasions since then, the noxious effects in humans were never before as evident as in 1995-96. Faunal mortalities were however small, with the exception of larval mortalities experienced by several land-based abalone farmers in the Walker Bay area. Along the South African coast the dinoflagellate *Gymnodinium nagasakiense* is usually implicated in NSP. Most outbreaks have been reported from False Bay, where they are responsible for the olive-green discolouration of the seawater during autumn. Thirty tonnes of abalone were washed up in the HF Verwoerd Marine Reserve in 1989, following blooms of *Gymnodinium nagasakiense* (Van der Vyver *et al*., 2000).

5.7.3 North America

The presence of NSP toxins in North American ICES countries during the years 1991-2000 is illustrated in Figure 5.5.

Figure 5.5 Occurrence of NSP toxins in coastal waters of North American ICES countries from 1991 to 2000



Source: http://www.ifremer.fr/envlit/documentation/dossiers/ciem/aindex.htm

Canada

Red tides of *Heterosigma akashiwo* (belonging to the class Raphidophyceae) have been reported from embayments in Canada causing mortality of cultured fish (Van Apeldoorn *et al*., 2001).

The United States of America

East Coast

Brevetoxin-associated mortality was postulated in bottlenose dolphins (*Tursiops truncatus*) along the mid-Atlantic coast of the United States from 1987 to 1988 (Bossart *et al.*, 1998).

G. breve was identified (6 x 10^6 cells per litre) from water samples taken off the North Carolina coast on 2 November 1987. This was the first recorded occurrence of *G. breve* north of Florida and extended the range of this toxic, subtropical dinoflagellate over 800 km northward. Before the end of this bloom three and a half months later, there were 48 cases of NSP reported in humans and over 1 480 km^2 of shellfish (oyster and clam) harvesting waters were closed during prime harvesting season. In addition significant scallop mortalities were reported from some areas. It was suggested that the Florida Current Gulf Stream system transported *G. breve* northward to the coast of North Carolina in October 1987. During the bloom stages of *G. breve* in North Carolina total phytoplankton concentrations increased with time at all stations regardless of *G. breve* concentrations (up to 3.27x10^5 cells/litre) or the degree of bloom development. This is in contrast to blooms of *G. breve* in the Gulf of Mexico which were typically monospecific (Van Apeldoorn *et al*., 2001).

Red tides of *Heterosigma akashiwo* (belonging to the class *Raphidophyceae*) have been reported from embayments on the East Coast causing mortality of cultured fish (Van Apeldoorn *et al*., 2001).

Ten fish mortality events, involving primarily Atlantic menhaden, occurred from July through September 2000 in several bays and creeks in Delaware. Two events involved large mortalities estimated at 1.5 to 2 million fish in Bald Eagle Creek, Rehoboth Bay. The presence of *Chattonella cf. vericulosa* at a maximum density of 1.04 x 10^7 cells/litre was demonstrated. PbTx-2, PbTx-3 and PbTx-9 were detected (Bordelais *et al*., 2002).

Florida and the Gulf of Mexico

On 16 June 1996, three patients were diagnosed with NSP by Sarasota County Health Department, the Bureau of Environmental Epidemiology, the Florida Department of Environmental Protection and the FDA. All had eaten clams (*Chione cancellata*) and whelks (unidentified species) harvested from an area that had been closed to shellfish harvesting from 31 January 1996 through 8 June 1996 because of red tide of *G. breve*, and then closed again on 11 June. The clams had been cooked until they opened; cooking time for the whelks was unknown (Van Apeldoorn *et al*., 2001).

From early March to late April 1996, at least 149 manatees (*Trichechus manatus latirostris*) died in an unprecedented epizootic along approximately 80 miles of the southwest coast of Florida (Charlotte Harbour area). At about the same time, a significant red tide dinoflagellate bloom, largely composed of *G. breve*, producing brevetoxin, was present in the same geographic area as the manatee epizootic. Cell counts of *G. breve* were approximately 23.3 x 10^6 cells/litre. Autopsy showed neurointoxication facilitated by oral and inhalation exposure. There are three potential routes of intoxication: i) toxic aerosol inhalation; ii) toxic food ingestion; and iii) toxic seawater intake. Similar toxin-associated manatee mortality was speculated in southwest Florida in 1963 and 1982 (Bossart *et al.*, 1998).

In Florida, poisoning of manatees by brevetoxins contained in salps attached to sea grass has been reported (Hallegraeff, 1995). Brevetoxins have also been detected in menhaden and mullet from the coast of Florida (Quilliam, 1999).

Brevetoxin-associated mortality was postulated in bottlenose dolphins (*Tursiops truncatus*) in southwest Florida in 1946 and 1947 (Bossart *et al.*, 1998). This phenomenon was due to a bloom of *G. breve* which was identified in 1947 as the etiological agent and was considered the sole agent responsible for all the outbreaks described since 1844. In addition, brevetoxin-associated mortality in bottlenose dolphins was seen along the Atlantic coast in 1987 and 1988 (Bossart *et al.*, 1998).

All red tides in Florida have been associated with mass mortality in marine animals. These phenomena were observed 24 times from 1844 to 1971 and the fact that they occurred before the development of agriculture, towns, industries and tourism indicated their natural origin. Health problems caused by the consumption of toxin-infested shellfish and by inhalation of wind-sprayed cells have been noticed (Viviani, 1992).

Mortality among the double-crested cormorant (*Phalacrocorax auritus*) has been observed along the Florida gulf coast (Fleming and Baden, 1999).

In late October 1996 to December 1996, a bloom of *G. breve* occurred for the first time in the low salinity waters of the northern Gulf of Mexico. Salinities were considerably lower than is typically

for occurrences of *G. breve*. Oyster beds were closed from November 1996 to as late as April 1997 (Dortch *et al.*, 1998).

West Coast

Brevetoxin was the cause of summer mortality in common murres (*Uri aalge*) in California (Fleming and Baden, 1999).

Red tides of *Heterosigma akashiwo* (*Raphidophyceae*) have been reported from embayments on the West Coast causing mortality of cultured fish (Van Apeldoorn *et al*., 2001).

5.7.4 Central and South America

Brazil

Chattonella sp. and *Heterosigma akashiwo* represent a risk at intensive shrimp cultures and shellfish cultures in Santa Catalina (Ferrari, 2001).

Mexico

In the Gulf of Mexico, *G. breve* is the dominating species, developing huge blooms almost every year during autumn, causing fish kills along the coasts of Veracruz and Tamaulipas states and sometimes affecting other states within the Gulf of Mexico. Since 1994, the events increased in permanence (reaching more than 100 days during autumn 1997), as well as in consequences on the environment and human health, with huge fish kills and many individuals affected by exposure to sea sprays or immersion in the seawater (Sierra-Beltrán *et al.*, 1998).

5.7.5 Asia

China, Hong Kong Special Administrative Region

The first harmful bloom of raphidophytes in waters of the Hong Kong Special Administrative Region was caused by *Heterosigma akashiwo* in Yim Tim Tsai in March 1987. A bloom of *Chattonella marina* occurred in 1991. The blooms caused fish killings. *Chattonella antiqua* was also identified. These blooms of raphidophytes can pose a serious threat to finfish aquaculture (Songhui and Hodgkiss, 2001).

Japan

Red tides of *Heterosigma akashiwo* have been reported in embayments causing mortality of cultured fish (caged young yellowtail = *Seriola quinqueradiata*) (Van Apeldoorn *et al*., 2001).

Red tides of *Fibrocapsa japonica* were reported first from coastal areas of Ehime Prefecture in 1972 causing heavy mortalities of caged young yellowtail (*S. quinqueradiata*) and were later reported from Atsumi Bay (1973), the Seto Inland Sea (1987) and Harima Nada (1989) (Van Apeldoorn *et al*., 2001).

Chattonella antiqua formed heavy red tides in coastal regions of Japan killing large numbers of cultured fish (caged yellowtails) (Van Apeldoorn *et al*., 2001).

The Republic of Korea

Red tides of *Heterosigma akashiwo* have been reported in embayments causing mortality of cultured fish (Van Apeldoorn *et al*., 2001).

Malaysia

Red tides of *Heterosigma akashiwo* have been reported from embayments in Singapore causing mortality of cultured fish (Van Apeldoorn *et al*., 2001).

5.7.6 Oceania

Australia

In Boston Bay, Southern Australia, high levels of breve-like toxins (up to 142 σg/100 g) were found in the livers of farmed bluefin tuna (*Thunnus maccoyii*) sampled at different times at a main mortality episode. Plankton samples revealed a bloom of the raphidophyte *Chattonella marina* (up to 66 000 cells/L). Exposure to *C. marina* both before and, for at least a month after, the main mortality episode had occurred. Pathology of the tuna gills showed marked epithelial swelling, lifting of the epithelium and copious mucus production. Supporting evidence for the involvement of a toxic microalga was the typical pathology, the high gill area to bodyweight ratio and the extreme high ventilation volume of tuna which would maximize exposure to the toxic effects of *C. marina*. The fact that the farmed tuna received the highly-oxidized baitfish as feed would have been depleted endogenous antioxidants in the tuna fish and would have caused an exquisite sensitivity of the fish to activated oxygen radicals. *C. marina* is known to be toxic to fish by at least two mechanisms, the production of reactive oxygen radicals and production of ichthyotoxic brevetoxins (Van Apeldoorn *et al*., 2001). In January 1994, mussels from Tamboon Inlet on the Gippsland coast of Victoria contained a NSP toxin level of 27.5 MU/100 g in association with a *G. breve* type bloom (ANZFA, 2001).

New Zealand

Human and animal illnesses during the summer of 1992/1993 were associated with marine biotoxins in shellfish. Although the presence of four different types of toxin was demonstrated, only NSP and possibly DSP were associated with clinical illness. Algae similar but not identical to *G. breve* were considered to be responsible for typical NSP symptoms and for an acute respiratory irritation associated with aerosols of fragments of the alga. Throughout New Zealand 186 cases of NSP were recorded (Van Apeldoorn *et al*., 2001). During the 1993 shellfish poisoning outbreak, NSP toxin level reached 592 MU/100 g for edible shellfish (ANZFA, 2001). Over the period September 1994 to July 1996, 0.2 percent of samples of shellfish taken around the coastline of New Zealand on a weekly basis showed a NSP toxin level above the regulatory limit during a total of 10 NSP events (maximum level 26 MU/100 g (various shellfish species)). There was one widespread outbreak of human NSP poisoning involving 186 cases in the northeast of the North Island (see also above) (Sim and Wilson, 1997).

Fibrocapsa japonica was found on the east and west coasts of the North Island and on the east coast of the South Island in early 1993 (Van Apeldoorn *et al*., 2001).

Red tides of *Heterosigma akashiwo* have been reported from embayments in New Zealand causing mortality of cultured fish (Van Apeldoorn *et al*., 2001).

Immediately after a series of fish and marine fauna kill episodes and outbreaks of human respiratory illness being reported off Wairarapa coast and Hawke Bay on the North Island east coast, Wellington Harbour experienced a severe toxic outbreak that persisted from mid-February to April 1998. The outbreak decimated almost all marine life (including seaweeds) in the harbour. During this unusual outbreak, eels and flounders were first noticed as the major harbour kills, which then spread across to kills of other pelagic fish and marine invertebrates. Eighty seven people in Wellington Harbour reported suffering from respiratory illness; beach goers, swimmers, and wind-surfers all complained of a dry cough, a severe sore throat, running nose and skin and

eye irritations. Furthermore, hatchery workers and divers complained among other symptoms also of severe headaches and a facial sunburnt sensation. The unprecedented bloom was found to be dominated by a non-described *Gymnodinium* sp. (33.3 x 10^6 cells/litre). The morphological characters of this new species look like the Japanese *Gymnodinium mikimotoi*. The Wellington Harbour toxin was stable in both alkaline and acidic conditions, but was not stable in weak acid. This makes it less likely to pose any human healthy risk when it is eaten. When heated to 100 ∀C the toxin lost most of its toxicity. The toxin is also highly oxidisable and therefore can be destroyed by ozonation. One of the notable features of the 1998 Wellington Harbour bloom was the build-up of extensive sea-foam, persisting for several weeks. The impacts of this new *Gymnodinium* sp. on marine life certainly are more severe than those caused by *G. mikimotoi* from Japan, *G. breve* from the Atlantic coast of the United States, *G. cf. mikimotoi* from Western Europe and *G. galatheanum* from the North Sea. In terms of impacts of airborne and waterborne toxins on humans, this new *Gymnodinium* sp. is quite like those of *G. breve* from the Atlantic coast of the United States and *Gymnodinium* sp. recently reported for South Africa (Van Apeldoorn *et al*., 2001).

5.8 Regulations and monitoring

5.8.1 Europe

Denmark

A monitoring programme exists for several algal species a.o. *Gymnodinium* spp. At 5.10^5 cells per litre (depending on species) fishery product harvesting areas are closed (Van Egmond *et al*., 1992; Shumway, 1995)

Italy

NSP producing algae are monitored, and fishery product harvesting areas are closed at the simultaneous presence of algae in water and toxin in mussels. In Italy, the provision of the law is based on the mouse bioassay and established "not detectable" in shellfish (Van Egmond *et al.*, 1992; Viviani, 1992).

5.8.2 North America

The United States of America

A level of 80 σg PbTx-2/100 g of shellfish tissue (0.8 mg/kg or 20 MU/100 g or 4 σg/mouse) analysed by the mouse bioassay in shellfish triggers regulatory action by FDA (FDA, 2000). The regulatory application of information derived by using the mouse bioassay is based upon studies conducted in the 1960s that compared the incidence of human illness with the incidence of death in mice injected with crude extracts from shellfish in diethylether (Van Apeldoorn *et al*., 2001).

Florida and the Gulf of Mexico

The Florida Department of Natural Resources has run a general control programme since the mid-1970s. Only in 1984, *G. breve* blooms were specifically noted in control regulations. Closures of shellfish beds are made when *G. breve* concentrations exceed 5 000 cells/litre. Closures will take a few weeks up to six months. Two weeks after *G. breve* concentrations drop below 5 000 cells/litre, the first mouse bioassays of shellfish are carried out. When levels are below 20 MU/100 g the grounds are reopened. The bioassay system is slow; results take nearly one week. A field assay kit is under development (Viviani, 1992). The measures above should prevent cases of NSP related to consumption of contaminated shellfish in most of the Florida human population, but will not prevent the respiratory irritation associated with exposure to aerosolized red tide

toxins. Although other states like Texas have done otherwise, in Florida where the red tides are almost a yearly occurrence, beaches are not closed to recreational or occupational activities, even during very active near-shore blooms (Fleming and Baden, 1999).

5.8.3 Central and South America

Argentina

Argentina has a national monitoring programme of mussel toxicity in each coastal province involving regional laboratories and one fixed station in Mar del Plata (Ferrari, 2001).

Brazil

Brazil had a pilot monitoring initiative during one year but does not have a national monitoring programme (Ferrari, 2001).

Uruguay

Uruguay has a national monitoring programme on mussel toxicity and toxic phytoplankton (Ferrari, 2001).

5.8.4 Oceania

New Zealand

Since the detection of NSP in early 1993, New Zealand has rapidly evolved a management strategy. All commercial and non-commercial shellfish harvesting areas around the entire coastline are sampled on a weekly basis throughout the year. Most major commercial growing areas have weekly phytoplankton sampling programmes and a "library" system of harvest sampling for the purpose of addressing the temporal and spatial spread of toxic events has been initiated. A mouse bioassay (APHA method) is in force and 20 MU/100 g is employed as an acceptable level. This level corresponds to a survival time in mice of six hours (Trusewich *et al.*, 1996).

Currently shellfish testing involves mouse bioassay screen testing for NSP toxins with confirmatory testing (Busby and Seamer, 2001).

A new Biotoxin Monitoring Programme providing data that is highly accurate, in a shorter time and without the use of mouse bioassays is being developed. This new programme will implement test methods based on LC-MS providing chemical analytical data in place of bioassay screen test results. The development and implementation of new test methods are in discussion including funding, method validation, testing regulations, availability of analytical standards, comparison to existing tests, type of instrumentation and international cooperation (McNabb and Holland, 2001).

6. Azaspiracid Shellfish Poisoning (AZP)

In November 1995, at least eight people in the Netherlands became ill after eating mussels (*Mytilus edulis*) cultivated at Killary Harbour, Ireland. Although the symptoms resembled those of diarrhoeic shellfish poisoning (DSP), concentrations of the major DSP toxins were very low (McMahon and Silke, 1996; Satake *et al*., 1998a). The known organisms producing DSP toxins were not observed in water samples collected at that time. In addition, a slowly progressing paralysis was observed in the mouse assay using the mussel extracts. These neurotoxic symptoms were quite different from typical DSP toxicity (Satake *et al*., 1998a). It was then that azaspiracid (formerly called Killary Toxin-3 or KT3) was identified and the new toxic syndrome was called azaspiracid poisoning (AZP).

6.1 Chemical structures and properties

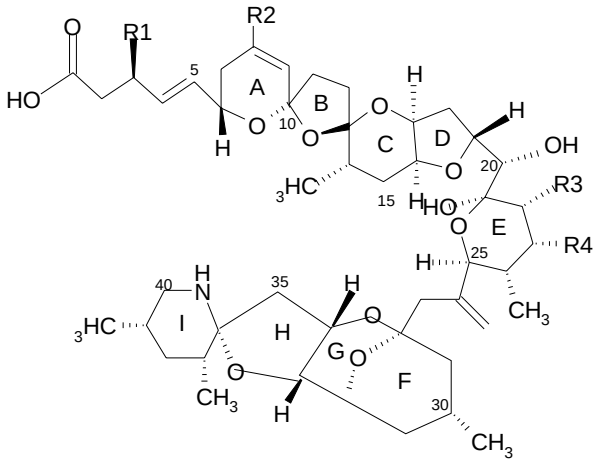
Satake *et al*. (1998b) elucidated the structure of azaspiracid after human intoxication due to the consumption of contaminated Irish mussels. Azaspiracid was extracted from contaminated whole mussel meat and appeared to be a colourless amorphous solid with no UV absorption maxima above 210 nm. In addition to azaspiracid (AZA), four analogues, AZA 2 to AZA5, were isolated and their chemical structures were elucidated (see Figure 6.1). Ofuji *et al*. (1999a) identified azaspiracid-2 (AZA-2) and azaspiracid-3 (AZA-3) and demonstrated that these compounds were 8-methylazaspiracid and 22-demethylazaspiracid, respectively. Ofuji *et al*. (2001) determined the structure of two further analogues of azaspiracid found in mussels namely azaspiracid-4 (AZA-4) and azaspiracid-5 (AZA-5) and showed that these compounds were 3-hydroxy-22-demethylazaspiracid and 23-hydroxy-22-demethylazaspiracid, respectively (thus hydroxylated analogues of AZA-3). No experimental data exist at present as to how and whether the toxins undergo structural modification in shellfish. By analogy with pectenotoxins and yessotoxins, that undergo structural modification by hydroxylation in mussels, it may be assumed that AZA-4 and AZA-5 are oxidized metabolites of AZA-3. Hence, AZA, AZA-2 and to AZA-3 are likely to be the genuine products of a causative marine organism. Azaspiracid was considered as the major causative agent (Satake *et al*, 1998b).

Azaspiracids differ from any of the previously known nitrogen-containing toxins found in shellfish or dinoflagellates (e.g. prorocentrolide, pinnatoxin, gymnodimine and the spirolides). Azaspiracids (AZAs) have unique spiro ring assemblies, a cyclic amine instead of a cyclic imine group and a carbocyclic or lactone ring is absent (Satake *et al*., 1998b).

Dounay and Forsyth (2001) performed synthetic studies toward the C5 – C20 domain of the azaspiracids to identify these sub-fragments of the azaspiracid.

Satake *et al*. (1998b) reported that mussel extracts did not show a significant decrease of toxicity when the extract was heated at 5 ^{o}C for 150 minutes in 1.0 N acetic acid/methanol or 1.0 N ammonium hydroxide solution and no significant change in toxicity occurred in solution during storage. Therefore AZAs are assumed to be relatively stable compounds.

Figure 6.1 Chemical structures of azaspiracids



	R_1	R_2	R_3	R_4
azaspiracid (AZA)	H	H	CH_3	H
azaspiracid-2 (AZA2)	H	CH_3	CH_3	H
azaspiracid-3 (AZA3)	H	H	H	H
azaspiracid-4 (AZA4)	OH	H	H	H
azaspiracid-5 (AZA5)	H	H	H	OH

6.2 Methods of analysis

6.2.1 In general

Attention should be paid to the possible co-occurrence in mussels of OAs, PTXs and YTXs. Coexistence of these toxins with AZA was noticed in mussels collected in Norway (Yasumoto and Aune, unpublished data in EU/SANCO, 2001).

Therefore it is recommended to test for these toxins by LC or LC/MS. During AZP outbreaks the occurrence of unknown toxin(s) was noted in mussels, although at low levels (about 12 percent of total toxicity). Mice injected with this unknown toxin showed immediately after injection agitation, paralysis and PSP-like convulsions before death. Besides these symptoms in mice, the unknown toxin was distinct from AZP toxins in chromatographic properties (probably the toxin has no acidic moiety). Mice injected with this toxin died within 30 minutes. In surviving mice recovery was quick. No data are available at present as to whether the unknown toxin(s) reach to a level high enough to interfere with the results of the mouse bioassay (EU/SANCO, 2001).

6.2.2 Bioassays

in vivo studies

<u>mouse bioassay</u>

Mussel extracts are injected intraperitoneally in mice like for the DSP mouse bioassay is done. The results suggest that azaspiracids can be extracted with acetone from raw meat due to increased solubility by the presence of water and lipids in the meat (EU/SANCO, 2001). The azaspiracid response is characterized by hopping, scratching and progressing paralysis which is atypical for DSP (Flanagan *et al.*, 2000; Satake *et al.*, 1998a). The shortest time for mouse death was 35 minutes (at six times of the lethal dose) and the longest was 30 hours and 46 minutes

(EU/SANCO, 2001). Usually, polyether toxins are concentrated in the digestive glands of shellfish, but this is not always the situation with azaspiracids. Azaspiracid and its analogues, AZA2 and AZA3, are distributed throughout shellfish tissues. Using conventional DSP mouse bioassay protocols (in which only the hepatopancreas is used for testing), only zero to 40 percent of the total azaspiracid content of the shellfish is measured, which can directly account for false-negative results (James *et al*., 2002a).

rat bioassay

This assay is based on diarrhoea induction in rats. The (starved) animals are fed with suspect shellfish tissue (mixed into the diet) and observed during 16 hours for signs of diarrhoea, consistency of the faeces and food refusal. The method is at best semi-quantitative (Hallegraeff *et al*., 1995 and Van Egmond *et al*., 1993). The test is still used routinely in the Netherlands and is an officially allowed procedure in EU legislation.

in general

The European Commission has recognised the needs of the analytical community to develop methods alternative to animal testing. A relevant call for proposals in the Commission's Sixth Framework Programme in Area 5: "Food Quality and Safety" is expected (EC, 2003), in which one of the objectives is to develop cost-effective tools for analysis and detection of hazards associated with seafood from coastal waters including also Azaspiracid Shellfish Poisons. If granted, this will mean that progress can be expected in the coming years.

in vitro studies

mammalian cell culture assay

The development of alternative diagnostic strategies for the detection of phycotoxin contamination in shellfish is driven by scientific, ethical and financial concerns. To address this, an assay has been developed based upon the cytopathological responses of cultured mammalian cells to phycotoxins. The primary response of these cells to any okadaic acid family of toxins is to "round-up" and lose their distinctive morphology, within three hours, yet they remain about 90 percent viable for up to 48 hours. Azaspiracid positive samples, when applied to this system do not cause the "rounding up" effect on cultured cells. Instead the cellular viability, as measured by an MTT assay, drops to less than 10 percent of the viability of control cells after 18 to 24 hours. Combination of cell morphology observation at three hours with 24-hour viability measurement enables the detection of both okadaic acid type toxins and azaspiracid in shellfish (Flanagan *et al*., 2000; 2001).

6.2.3 Chemical assays

mass spectrometry

The first LC-MS quantitative determination method reported for azaspiracids was based on selected ion monitoring (SIM) detection (Ofuji *et al*., 1999b), with one ion per compound and external calibration. Linearity was checked over a relatively wide concentration range (50 pg to 100 ng). The recovery data seemed correct, but it remained unclear how many different samples formed the basis for the recovery experiment(s). As only one ion per compound was monitored, no check on specificity was possible with ion intensity ratio. In short, the analytical basis of the method is not strong, which makes questionable its applicability in practice.

The start for application of LC-MS^n methods for AZAs – as published later on – was presented by James *et al*. (2001). A micro liquid chromatography-tandem mass spectrometry method (micro-LC-MS-MS) was developed for the determination of azaspiracids (Draisci *et al.*, 2000). The

method reported focused on the identification of azaspiracids, so in fact it had a qualitative accent. Eventually the aim was formulated as "…to investigate the suitability of LC-MS and LC-MS-MS in order to unambiguously detect azaspiracid in shellfish." By applying selected ion monitoring (SIM) on the ions corresponding to the protonated molecules only, the most sensitive form of detection was obtained (maximum intensities). Good sensitivity (defined as low detection limit) was also obtained by applying micro-LC (1.0 mm I.D. column), which is effective because Electrospray-MS is a concentration dependent detector. In this study a triple quadrupole MS (tripleQ) was used. In the area of marine biotoxins this instrument is more commonly used than the ion trap MS. Structural information was obtained by using the CID-MS-MS capabilities of the tripleQ, first in full scan mode to find appropriate daughter ions which could afterwards be used in SRM- mode. The optimized SRM mode resulted also in quantitative data. Good linearity (r^2>75[[<0#was observed for a small concentration range (0.1 –1 µg/ml), while the detection limit was approximately 20 ng of azaspiracid per gram of whole mussel. In conclusion, the developed method provided very selective and specific data. However, as stated by the authors, a "full validation was hampered by the lack of availability of the azaspiracid standard necessary for recovery experiments".

Lehane *et al*. (2002) reported the development of an LC-ESI-MS^n method for the determination of the three most prevalent AZA toxins (AZA1-3), as well as the isometric hydroxylated analogues (AZA4-5). They demonstrated that LC-multiple tandem MS resulted in more sensitive analysis than LC-single-MS, which suggests ".. that the reduction in background noise in MS^n is more dramatic than the decline in analyte signal." Notable is their use of WideBand activation, which allows them to reduce total elution time aiming at the determination of the five azaspiracids. Although the authors state to have developed a method that requires minimal sample preparation steps, total sample preparation will most probably require the major part of total analysis time.

Next to the article just mentioned, the same research group reported a comparison of solid-phase extraction methods for the determination of azaspiracids in shellfish by the LC-ESI-MS^n method of Lehane (Moroney *et al.*, 2002). Good recovery and reproducibility data were obtained for one diol SPE cartridge and two C_{18} SPE cartridge types. As they state: "…the efficient SPE methods presented here for sample preparation should prove more useful in the development of alternative analytical methods for AZP toxins in shellfish." This fits well to their earlier statement: "Sample preparation for the determination of phycotoxins in shellfish can be problematic due, in part, to an extensive variation in the toxic content."

The same group reported the same method development in a different journal (Furey *et al.*, 2002) "with the primary objective to produce a protocol that could be used for the regulatory control of azaspiracids in shellfish". Especially their extensive linearity studies for the determination in shellfish extracts are worth mentioning: rather good results were obtained for a concentration range over two decades. The data look convincing that regulatory control can be conducted with the methods reported. An application based on the just mentioned method was reported by the same group (James *et al*., 2002a; 2002b). The report shows LC-MS^3 spectra of AZA1-3 both as standards and as analytes in mussel extracts.

6.3 Source organism(s) and habitat

Since 1996, several AZP incidents have been identified in Ireland. In November 1997, cases of contamination recurred in the Avianmore Island region of Donegal, Northwest Ireland and caused human intoxication repeatedly (McMahon and Silke, 1998), also in other European countries (mainly by mussels cultivated in Ireland). The ultimate origin of azaspiracids is probably a dinoflagellate because of the highly oxygenated polyether structure and seasonal occurrence. However, none of the known toxic phytoplankton species was observed in water samples

collected at the time of the intoxication (James *et al*., 2000b; Satake *et al*., 1998b). Recent information (Peperzak *et al*., 2002) suggests that *Protoceratum crassipes* is the AZP producing dinoflagellate. McMahon (2000) reported that an organism belonging to the genus *Protoperidinium* has been suggested as the source organism.

6.4 Occurrence and accumulation in seafood

6.4.1 Uptake and elimination of AZP toxins in aquatic organisms

Typically, polyether toxins are concentrated in the digestive glands of shellfish but this is not always the situation with azaspiracids. Azaspiracid and its methyl- and demethyl-analogues, AZA2 and AZA3, respectively, are not confined to the hepatopancreas but are also distributed throughout shellfish tissues. The toxin profiles differed significantly in various mussel tissues with AZA as the predominant toxin in the digestive glands and AZA3 and an isomer of AZA predominant in the remaining tissues. Mussel digestive glands initially contained most of the azaspiracids due to grazing on toxic dinoflagellates. However, the transportation of these toxins to other shellfish tissues is unpredictable but, if this occurs, a prolonged period of shellfish intoxication is likely due to a low rate of natural depuration. Azaspiracids show an unusual solvent distribution during the extraction process of these toxins. This leads to the speculation that the polar amino acid and the non-polar polyether regions of azaspiracid impart detergent properties to this molecule. The ease with which azaspiracids can move through different polarities probably plays a significant role in the increased penetration of these toxins in shellfish and mammalian tissues. There can be a significant variation in the total level of AZAs in mussels from different sites in the same cultivation region. See Table 6.1 (James *et al*., 2002a).

Table 6.1 Distribution of AZP toxins through mussel tissues

Site No.	Meat[a] (total AZAs) σg/100g	HP (total AZAs) σg/100g	Total AZAs σg/100g	AZAs distribution (% meat/HP)[b]
1	14	0	12	100/ 0
2	14	34	17	67/33
3	6	10	7	75/25
4	7	18	9	67/33
5	84	12	72	96/4
6	37	100	48	64/36
7	48	33	45	88/12

[a] mussel meat without hepatopancreas
[b] average weight is 4.8 g; HP was 15-18% of total mussel tissue

6.4.2 Shellfish containing AZP toxins

Mussels and oysters were found to contain AZP toxins (James *et al*., 2000b).

6.5 Toxicity of AZP toxins

6.5.1 Mechanism of toxicity

No data

6.5.2 Pharmacokinetics

No data

6.5.3 Toxicity to laboratory animals

acute toxicity

oral studies

Acute oral studies with azaspiracid were performed in mice. Azaspiracid was extracted from mussels collected in Killary Harbour, Ireland in February 1996. During the course of toxin purification, the major toxin was concentrated in a lipid fraction coded KT3 (Ito *et al*., 2000). By oral administration (by gavage) of 60 µl of this KT3 fraction mice did not show any clinical changes during 24 hours. At autopsy after four hours, active secretion of fluid from the ileum and debris of necrotizing epithelial cells from upper portion of the villi were observed in the lumen (SEM) and after eight hours, erosion of the villi from the top resulted in the shortened villi, and prominent accumulation of fluid was observed accompanying edema in the lamina propria. Then after 24 hours, these changes were not observed but epithelial cells of adjacent villi were fused to each other (Ito *et al*., 1998).

Male ICR mice receiving orally by gavage a single dose of 500, 600 or 700 σg purified AZA/kg bw did not show any behavioural changes within four hours. The number of survivors after 24 hours were 0/2, 3/6 and 1/2 at 500 σg/kg bw (eight weeks old), 600 σg/kg bw (five weeks old) and 700 σg/kg bw (five weeks old) respectively. At 600 σg/kg bw and 700 σg/kg bw, diarrhoea and body weight decrease were observed within 24 hours. At single oral doses of 300 to 700 σg/kg bw, AZA caused dose-dependent changes in small intestines (necrotic atrophy in the lamina propria of the villi) and in lymphoid tissues such as thymus, spleen and Peyer's patches. In the spleen the number of non-granulocytes was reduced and damage to both T and B lymphocytes occurred. Additionally liver weight increased, the colour of the liver changed from dark red to pinkish red and fatty changes in the liver were observed. AZA did not cause prominent changes in the stomach mucosa but the appearance of many degenerating cells was observed in the large intestine. The pancreas appeared to loose zymogen granules locally, but cells were not injured. Histopathological damage to other organs (kidney, heart, and lung) was not observed. The acute morphological changes in the mouse, induced by AZA, were distinctly different from those of okadaic acid (Ito *et al*., 2000).

In the latest experiments from Ito *et al*. (2002), a total of 18 four-week old mice, five six-week old mice and two five-month old mice were used to produce severe injuries and then to observe recovery. Four dose levels (250, 300, 350 and 450 µg AZA (more purified extract from blue mussels at Killary Harbour and Arranmore Island in Ireland)/kg bw (dissolved in 50 percent ethanol) were given orally to five groups. Ten mice that survived the initial treatment received a second treatment on day three. Nine mice that survived the second treatment were killed between day seven and 90 after treatment. Thirteen control mice were used. The highest dose of 450 µg/kg bw caused death in 11/16 treated (four-week old) mice. Two out of two six-week old mice and another two out of two five-month old mice, receiving 300 and 250 µg/kg bw, respectively, also died. Of ten mice that survived the first treatment, one died after the second treatment with 350

µg/kg bw. Slow recoveries were revealed after oral administration of 300, 350 and 450 σg/kg bw. Erosions and shortened villi in the stomach and the small intestine persisted for more than three months, edema, bleeding, and infiltration of cells in the alveolar wall of the lung for 56 days, fatty changes in the liver for 20 days and necrosis of lymphocytes in the thymus and spleen for 10 days. Thus, the lowest oral dose of 250 µg AZA/kg bw appeared to be lethal to mice in this study.

It has to be noted that the partially purified KT3 toxin caused much more severe intestinal fluid accumulation and histological damage to the pancreas (Ito *et al*., 1998) than the more purified toxin used in the studies of Ito *et al*. (2002). May be several unknown analogues of azaspiracid are present in the crude fraction. It should also be mentioned that the difference between the mouse lethality by oral and intraperitoneal administration was much less significant with azaspiracid than with other phycotoxins (Ito *et al*., 2000).

intraperitoneal studies

Mice exposed to AZA by intraperitoneal injection react differently than those exposed to other shellfish toxins. After i.p. dosing of the partially purified KT3 to male ddY mice, the animals became sluggish, sat still in the corners and showed progressive paralysis and laboured breathing. No diarrhoea was observed. At low doses the animals died two to three days after dosing. The minimal lethal dose was reported to be 150 µg/kg bw (Satake *et al*., 1998a). Ito *et al*. (1998) injected 10 µl of the partially purified KT3 i.p. to 10 male ICR mice (age three-weeks). All animals showed inactivity and general weakness and died within 24 hours. Morphological changes caused by KT3 were distinctly different from those induced by DSP, PSP or ASP toxins. The main target organs of KT3 were liver, spleen, pancreas, thymus and digestive tract. In contrast, those of DSP toxins are the digestive tract, of PSP toxins the central nervous system and of ASP toxins the brain. The target site of KT3 was the small intestine, where villi degenerated from the top. At the histopathological level, parenchym cells of the pancreas and hepatocytes, which contain numerous rough endoplasmic reticula, are preferentially affected and it is probable that KT3 inhibits protein synthesis.

Satake *et al*. (1998b) reported an i.p. lethal dose of purified AZA to mice of 200 σg/kg bw. Intraperitoneal lethal doses for AZA-2 and –3 to mice were 110 and 140 µg/kg bw, respectively (Ofuji *et al.*, 1999a) and for AZA-4 and AZA-5 approximately 470 and less than 1000 σg/kg bw, respectively (Ofuji *et al*., 2001).

repeated dose toxicity

oral studies

Oral doses of 50, 20, 5 and 1 σg AZA/kg bw were given twice a week, up to 40 times, within 145 days, to four groups of 10, 10, 5 and 6 mice (four-weeks old), respectively. Nineteen control mice were used. Nine mice out of ten at 50 µg/kg bw and three out of ten at 20 µg/kg bw became so weak (inactivity and weight loss) that they were sacrificed before being treated 40 times (mainly after 30 treatments). Interstitial pneumonia and shortened small intestinal villi were observed. At 5 and 1 µg/kg bw no mortality was seen. The mice that survived 40 treatments were kept for up to three months after withdrawal. No fatty changes in the liver, previously seen at acute or lethal oral doses, were observed. At 50 σg/kg bw, a lung tumour was seen in 1/10 mice dosed 32 times. At 20 σg/kg bw a lung tumour was observed in 1/10 mice dosed 36 times and in two additional mice after withdrawal. In addition, hyperplasia of epithelial cells in the stomach was seen in 6/10 mice at 20 σg/kg bw. At 5 µg/kg all 5 mice showed erosion of small intestine (possibly attributed to unhealed injuries rather than late effects developed during withdrawal period). At 1 σg/kg, one out of six mice developed hyperplastic nodules in the liver and two mice out of six showed mitosis in liver (Ito *et al*., 2002).

reproduction teratogenicity
No data

mutagenicity
No data

in vitro toxicity
Azaspiracids were cytotoxic to P388 cells but to KB cells the potency was much less prominent (EU/SANCO, 2001). AZA did not inhibit protein phosphatase 2A. It was noted that *in vitro* studies performed in human cells from healthy donors suggest that the threshold for azaspiracid analogues to modify cellular function would be 24 σg/kg for a 60 kg person.

6.5.4 Toxicity to humans

In November 1995, at least eight people in the Netherlands became ill after eating mussels (*Mytilus edulis)* cultivated at Killary Harbour, Ireland. Although human symptoms such as nausea, vomiting, severe diarrhoea, and stomach cramps were similar of those of diarrhoeic shellfish poisoning (DSP), contaminations with the major DSP toxins okadaic acid (OA) and dinophysistoxins (DTXs) were very low. These observations prompted the investigators to explore the causative toxin in the mussels for structural studies. After chemical analytical research, the investigators identified and quantified AZA (Satake et al., 1998a; 1998b). Based on these results, the toxicity of the mussels was estimated to be 0.15 mouse units (MU)/g (equivalent to 0.6 σg AZA/g) (EU/SANCO, 2001). A higher toxin content of 1.4 σg AZAs/g of meat (0.4 MU/g of meat) was reported by Ofuji *et al*. (1999b). Human toxicity was seen between 6.7 (5 percent) and 24.8 (95 percent) σg/person with a mean value of 15 σg/person. However, new data on the heat stability of azaspiracid suggest that it is not appropriate to take into account a reduction in AZAs concentration due to heating. Therefore the recalculated range of the LOEL is 23 to 86 σg per person with a mean value of 51.7 σg/person (EU/SANCO, 2001).

6.5.5 Toxicity to aquatic organisms

No data

6.6 Prevention of AZP intoxication

6.6.1 Depuration

In the winter when shellfish are free of contamination by DSP toxins, AZP toxins may occur in mussels. The long duration of toxicity periods, which often extend to nearly six months, is troublesome (Ofuji *et al*., 2001). During the initial stages of intoxication, mussel digestive glands contain most of the AZP toxins. Migration of AZP toxins to other mussel tissues can occur leading to persistent intoxication. This unusual distribution of AZP toxins within the shellfish tissue can lead to the slow rates of natural depuration. In addition the DSP mouse bioassay protocol in which only the hepatopancreas is used at extraction, may fail to detect AZP toxins in mussels (James *et al*., 2002a).

6.7 Cases and outbreaks of AZP

6.7.1 Europe

The presence of azaspiracids in European ICES countries is illustrated in Figure 6.2.

Ireland

In November 1995, at least eight people in the Netherlands became ill after eating mussels (*Mytilus edulis*) cultivated at Killary Harbour, Ireland (McMahon and Silke, 1996; Satake *et al.*, 1998a). A toxin then called Killary Toxin-3 or KT3 was detected. Satake *et al*. (1998b) elucidated the structure of KT3 and called the toxin azaspiracid. Mussels collected in February 1996, showed a toxin content of 0.15 MU/g (=0.6 µg AZA/g) (EU/SANCO, 2001).

Since 1996 several AZP incidents have been identified in Ireland. Cases of contamination recurred in 1997 and repeatedly caused human intoxication in Ireland – in the Arranmore Island region of Donegal, Northwest Ireland (McMahon and Silke, 1998) – and other European countries. Although no known toxic phytoplankton were observed in cultivation areas after these intoxications, it is probable that AZP toxins were produced by marine dinoflagellates (James *et al*., 2002a).

Mussels collected at Killary Harbour on 23 April 1996 (five months after the incident) contained 1.14 µg AZA/g of meat, 0.23 µg AZA2/g of meat and 0.06 µg AZA3/g of meat (total AZAs 1.4 µg/g of meat). Mussels collected at Arranmore Island on 3 November 1997 (one to two months after the incident) contained 0.865 µg AZA/g of whole mussel meat (including hepatopancreas), 0.25 µg AZA2/g and 0.24 µg AZA3/g (total AZAs 1.36 µg/g). Results of the mouse bioassay revealed 0.4 MU/g of meat (Ofuji *et al*., 1999b). McMahon (2000) reported that the maximum AZA content in shellfish during the Arranmore Island incident was 10.7 µg/g of hepatopancreas. In November 1997, James and Furey (2000) detected 2.21 µg AZAs/g in raw whole meat of mussels.

After the initial intoxication in Arranmore Island and Killary Harbour, the toxin persisted for a further seven to eight months. Oysters seem to be just as susceptible as mussels to intoxication by AZP toxins as illustrated in Table 6.2 below (James *et al*., 2000).

Table 6.2 Levels of AZAs in mussels and oysters from Ireland

Location in Ireland	Date	Total AZAs σg/100 g (mussel)	Total AZAs σg/100 g (oyster)
County Cork	Nov. 1998	70	70
County Cork	Feb. 2000	10	20
Bruckless, Co. Donegal	Nov. 1999	10	30

In Ireland during 1999, some 1800 samples were tested for DSP/AZP toxins using the mouse bioassay of Yasumoto *et al.* (1978). Approximately 5 percent of the samples were positive. Azaspiracid was detected in several production areas and harvesting of all bivalves has been prohibited in Bruckless Bay, Northwest Ireland since August 1999 due to detection of AZP toxins in samples tested weekly (EU-NRL, 2000).

Norway

Azaspiracids have been identified in mussels (James *et al*., 2000b).

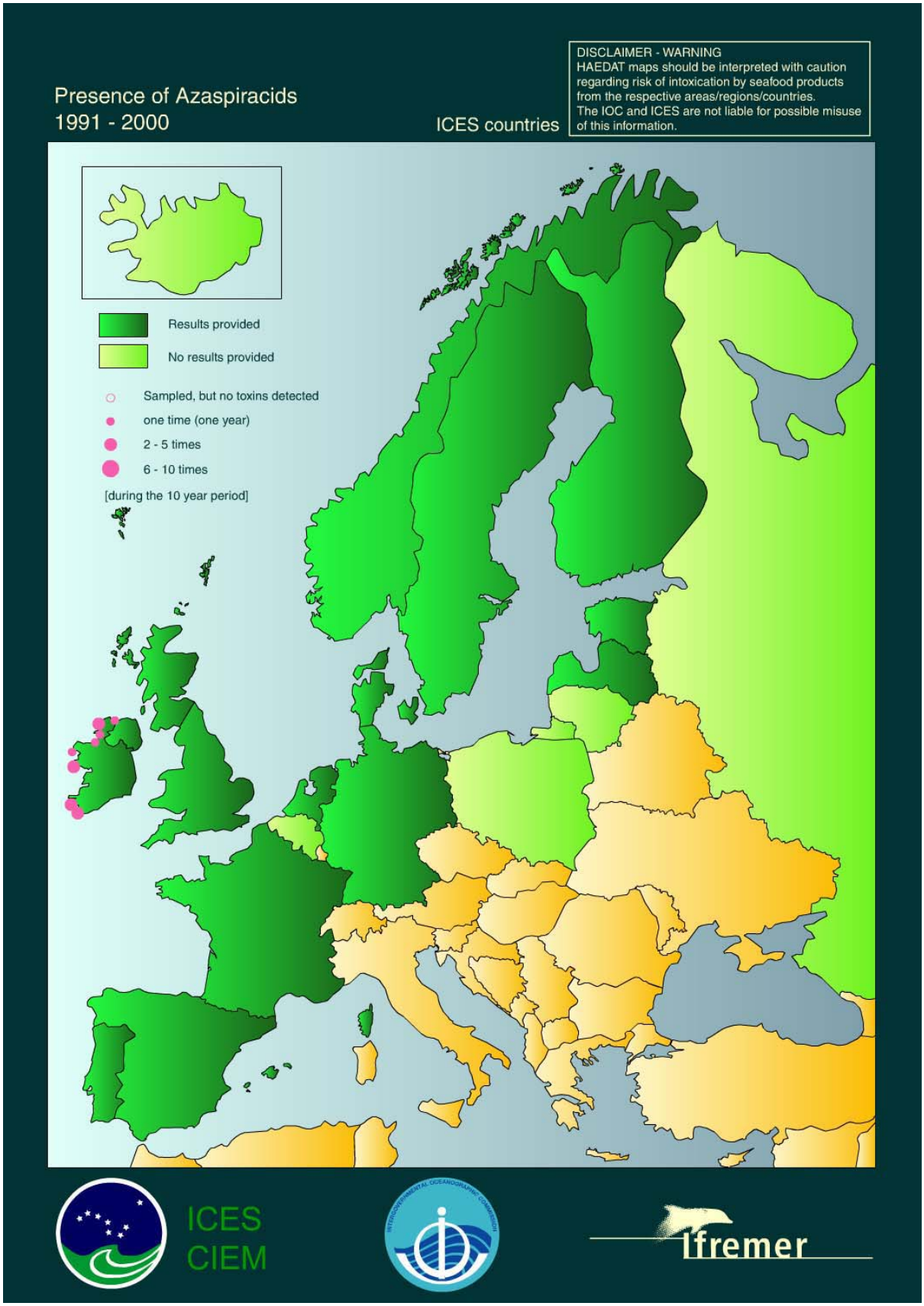
Portugal

A strange toxicity in cockles (*Cerastoderma edule*) similar to AZP and not found in mussels was reported (EU-NRL, 1998).

United Kingdom

Azaspiracids have been identified in mussels (James *et al*., 2000b).

Figure 6.2 Occurrence of AZP toxins in coastal waters of European ICES countries from 1991 to 2000



Source: http://www.ifremer.fr/envlit/documentation/dossiers/ciem/aindex.htm

6.8 Regulations and monitoring

It was the opinion of the Irish experts who carried out the risk assessment that, because of the lack of data on AZP toxins and the uncertainty outlined in the risk assessment, the prevailing tolerable limit of 8 σg/100 g of shellfish (see Chapter 8.5 Risk Assessment for Azaspiracid Shellfish Poisoning (AZP) and Chapter 9.1.5 Conclusions related to AZP) should be reviewed prior to being adopted into legislation.

Attention should be paid to the possible co-occurrence of okadaic acid, pectenotoxins and yessotoxins in the shellfish. Coexistence of these toxins with AZP toxins was noticed in mussels collected in Norway (Yasumoto and Aune in EU/SANCO, 2001).

6.8.1 Europe

In March 2002 the European Commission laid down the following rules (EU, 2002a):

- Maximum levels of AZP toxins in bivalve molluscs, echinoderms, tunicates and marine gastropods (whole body or any part edible separately) shall be 160 µg/kg.
- The mouse or the rat bioassay is the preferred methods of analysis. A series of analytical methods such as LC with fluorimetric detection, LC-MS and immunoassays can be used as alternative or complementary methods to the biological testing methods, provided that either alone or combined they can detect at least the following analogues, that they are not less effective than the biological methods and that their implementation provides an equivalent level of public health protection: AZA, AZA2 and AZA3
- When results of analyses demonstrate discrepancies between the different methods, the mouse bioassay should be considered as the reference method.

Ireland

The Biotoxin Monitoring Programme in Ireland began in 1984 and was initially based on the screening of samples for the presence of DSP toxins by bioassays. In recent years, the detection of additional toxins, including DA and in particular the azaspiracids, has led to an increase in monitoring effort and the programme now includes weekly shellfish testing using DSP mouse bioassay, LC-MS (okadaic acid, DTX2, azaspiracids) and LC (DA) as well as phytoplankton analysis. Regular reports of the results of sample analysis are sent to the regulatory authorities, health officials as well as the shellfish producers and processors. A Web-based information system is being developed to increase access to information (McMahon *et al*., 2001).

McMahon (2000) reported that the Food Safety Authority Ireland proposed an interim threshold concentration of 0.1 µg AZA/g of whole mussel. It was proposed to review and, if necessary, to revise this value as new data become available.

7. Ciguatera Fish Poisoning (CFP)

Ciguatera fish poisoning (CFP) has been known for centuries. It was reported in the West Indies by Peter Martyr de Anghera in 1511, in the islands of Indian Ocean by Harmansen in 1601 and in the various archipelagos of the Pacific Ocean by De Quiros in 1606. Endemic areas are mainly the tropical and subtropical Pacific and Indian Ocean insular regions and the tropical Caribbean, but continental reef areas are also affected (Legrand, 1998). The name ciguatera was given by Don Antonio Parra in Cuba in 1787 to intoxication following ingestion of the "*cigua*", the Spanish trivial name of an univalve mollusc, *Turbo pica*, reputed to cause indigestion. The term "*cigua*" was somehow transferred to an intoxication caused by the ingestion of coral reef fishes (De Fouw *et al*., 2001). The causative toxins, the ciguatoxins, accumulate through the food chain, from small herbivorous fish grazing on the coral reefs into organs of bigger carnivorous fish that feed on them (Angibaud and Rambaud, 1998; Lehane, 2000).

In the past, the ciguatera food poisoning in humans was highly localized to coastal, often island communities of indigenous peoples. However, with the increases in seafood trade, increased worldwide seafood consumption and international tourism, the target populations have become international. At present, ciguatera is the most common type of marine food poisoning worldwide and, with an estimated 10 000 to 50 000 people worldwide suffering from the disease annually, it constitutes a global health problem (De Fouw *et al*., 2001; Lehane, 2000).

No indicator such as the highly visible surface phenomenon, the so-called "red tide" as seen by shellfish poisonings, has ever been associated with ciguatera. It is this lack of warning signal that has contributed to the dread of ciguatera poisoning (De Fouw *et al*., 2001).

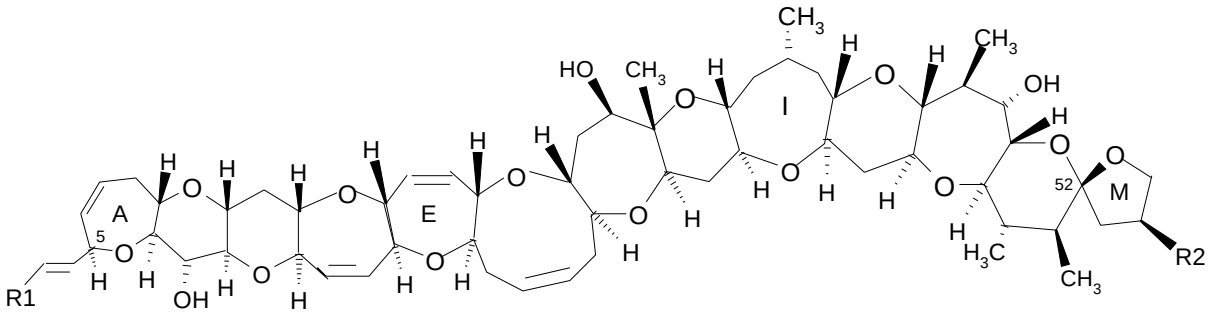
7.1 Chemical structures and properties of ciguatoxins

Ciguatoxins are lipid-soluble polyether compounds consisting of 13 to 14 rings fused by ether linkages into a most rigid ladder-like structure (see Figure 7.1). They are relatively heat-stable molecules that remain toxic after cooking and exposure to mild acidic and basic conditions. Ciguatoxins arise from biotransformation in the fish of precursor gambiertoxins (Lehane and Lewis, 2000; Lehane, 2000).

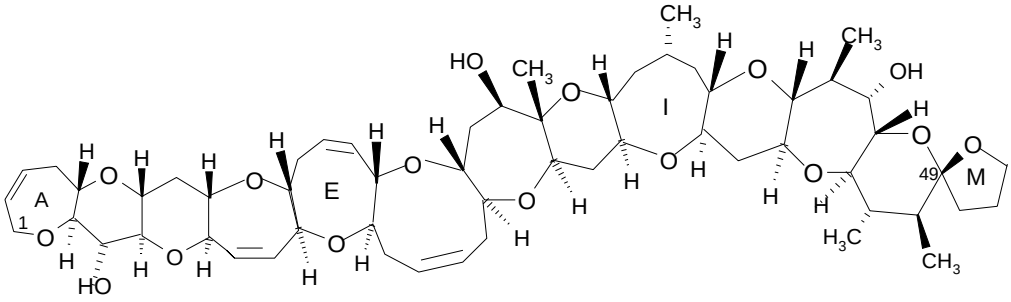
In areas in the Pacific, the principal and most potent ciguatoxin is Pacific ciguatoxin-1 (P-CTX-1, mol. wt. 1112). Its likely precursor is gambiertoxin-4B (GTX-4B). The main ciguatoxins in the Pacific, P-CTX-1, P-CTX-2 and P-CTX-3, are present in fish in different relative amounts (Lehane and Lewis, 2000; Lehane, 2000). The structures of more than 20 congeners of ciguatoxin were elucidated. Structural modifications were mainly seen in the both termini of the toxin molecules and mostly by oxidation (Naoki *et al.*, 2001; Yasumoto *et al*., 2000). Caribbean (and Indian Ocean) ciguatoxins differ from Pacific ciguatoxins. Caribbean CTX-1 (C-CTX-1) is less polar than P-CTX-1. Structures of two Caribbean ciguatoxins (C-CTX-1 and C-CTX-2) were elucidated in 1998. Multiple forms of ciguatoxin with minor molecular differences and pathogenicity were described. CTX-1 is the major toxin found in carnivorous fish and poses a human health risk at levels above 0.1 µg/kg fish (de Fouw *et al.*, 2001).

Various species of parrotfish have previously been reported to contain a toxin less polar than CTX-1, named scaritoxin. Judging from the reported chromatographic properties, scaritoxin seems to correspond to a mixture of CTX-4A and CTX-4B (De Fouw *et al*., 2001).

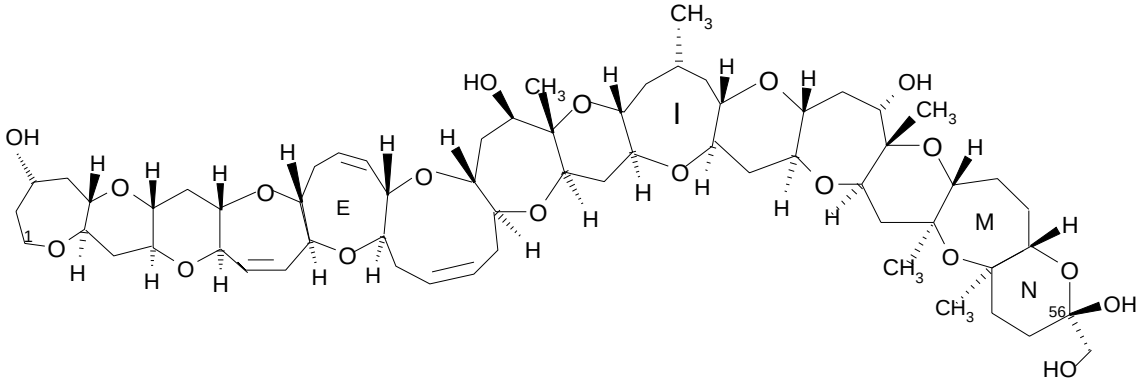
Figure 7.1 Structure of Pacific (P) and Caribbean (C) ciguatoxins (CTXs)



	R1	R2
P-CTX-1:	$^{1}CH_2OHCHOH$	OH
P-CTX-3 (P-CTX-2):	$^{1}CH_2OHCHOH$	H
P-CTX-4B (P-CTX-4A);	$^{1}CH_2CH$	H



P-CTX-3C



C-CTX-1 (C-CTX-2)

The energetically less favoured epimers, P-CTX-2 (52-epi P-CTX-3), P-CTX-4A (52-epi P-CTX-4B) and C-CTX-2 (56-epi C-CTX-1) are indicated in parenthesis. 2,3-Dihydroxy P-CTX-3C and 51-hydroxy P-CTX-3C have also been isolated from Pacific fish (Lewis, 2001).

7.2 Methods of analysis

7.2.1 In general

Ciguatoxins are odourless, tasteless and generally undetectable by any simple test. Therefore, bioassays have traditionally been used to monitor suspected fish. Many native tests for toxicity of fish have been examined including the discolouration of silver coins or copper wire, or the repulsion of flies and ants, but all of these were rejected as invalid (Park, 1994).

Feeding tests to cat or mongoose are simple and relatively sensitive but they are cumbersome and non-quantitative. The mouse bioassay requires purification of fish extracts since the mouse is not very sensitive to ciguatoxin. The alternative mosquito bioassay correlates well with cat and mouse bioassay. Other bioassays that have been developed have used chicken, brine shrimp and guinea pig atrium. All traditional bioassays have one common disadvantage, the lack of specificity for individual toxins. Recent studies have also focused on the development of chemical methods, such as TLC and LC for the detection and quantification of ciguatera-related toxins. Alternative assays based on immunochemical technology have been developed and show greatest promise for use in seafood safety monitoring programmes (Park, 1994).

7.2.2 Bioassays

All the mentioned bioassays have the limited chemical specificity for individual toxins in common (Juranovic and Park, 1991), although for a broad screening this property can be advantageous detecting a poisoning. The bioassays are semi-quantitative and sensitive. Ciguatoxin induces characteristic signs of toxicity but the use of some animal species can be problematic in terms of cost and ethical difficulties.

in vivo assays

mouse bioassay

The mouse bioassay, based on the method described by Banner *et al.* (1960) is presently the most widely used assay for the detection of ciguatoxins in fish. The method consists of injecting i.p. (intraperitoneal) serially diluted semi-purified or crude toxic extracts into mice and observing the symptoms for 24 hours. The procedure of the assay is described in detail by Yasumoto *et al.* (1984b). This assay has been described for the detection of ciguatoxins in up to 20 mg of ether extract from the flesh of fish. The diethyl ether fraction containing ciguatoxin is suspended in 0.5 ml 1-5% Tween 60/0.9% saline solution and injected intraperitoneally into mice (20 ∂ 2 g) of either sex. Mice are observed continuously for the first two hours, after that regularly checks are performed. Two mice are tested for each fraction. Mice are housed at 23 ∂ 2 ^{o}C and observed over seven days and signs and times to death recorded. Rectal body temperature is intermittently measured. The relationship between dose and time to death is used to quantify each fraction. Total lethality is expressed in mouse units (MU). For the mix of ciguatoxins found in carnivorous fish (Lewis and Sellin, 1992; Lewis *et al.* 1991) this relationship is approximated by log MU = 2.3 log $(1 + T^{-1})$, where MU is the number of mouse units of ciguatoxin injected and T is time to death in hours (see also Table 7.2). One MU is the LD_{50} dose for a 20 g mouse which is equivalent to 5 ng, 48 ng and 18 ng of CTX-1, CTX-2 en CTX-3, respectively. (Lewis and Sellin, 1992; Lewis *et al.* 1991). It is recommended that additional purification is undertaken to separate the various toxins, especially the maitotoxins (see Chapter 7.3.1) from ciguatoxins since maitotoxins induce effects in mice, often mistaken for effects of ciguatoxins despite the clear differences (see Table 7.2). Therefore modified extraction procedures have been reported that may improve separation of these two types of toxins (Yokayama *et al.*, 1988; Holmes *et al.*, 1991; Holmes and Lewis, 1994; Legrand *et al.*, 1992).

The mouse assay has been traditionally used but it is unsuitable as a market test. There are other disadvantages such as the variation in mouse weight, that must be limited involving a large breeding colony of the mice, and the death time relationship to dose is non-linear.

chicken assay

This assay provides a rapid means of assaying the toxicity of fish liver by administering small portions of liver directly into the crop of young chickens at 10 percent of their body weight. Administration of fish flesh is physically more difficult but can be accomplished (Vernoux *et al*., 1985).

mongoose and cat assay

For the mongoose (Banner *et al*., 1960) and cat assay (Lewis, 1987; Bagnis *et al*., 1985) the same procedure is followed as with chicken, only flesh of fish is fed and also in large quantity (5 to 15 percent of the test animal weight was fed). The cat is less satisfactory as test model because it often regurgitates part of the test meal. Test animals are observed for 48 hours. Although the tests are simple in screening fish for toxicity, they are cumbersome and not quantitative (Bagnis *et al*., 1987).

brine shrimp assay

The brine shrimp assay was the first non-vertebrate assay developed. However, false positive results were caused by the toxic effects on brine shrimp of the Tween 80 recommended to emulsify the extract and no toxic effect attributable to ciguatoxin could be detected (Granade *et al*., 1976; Hungerford, 1993).

mosquito assay

A bioassay using mosquitoes has also been developed. Only a few laboratories perform this assay, perhaps because of difficulties in obtaining and housing mosquitoes and a lack of familiarity in handling and recognising signs characteristic of intoxication by ciguatoxins. This procedure involves intrathoracic injection of the mosquitoes of serially diluted fish extract, and the toxicity is expressed in mosquito LD_{50}. It is a rapid assay, depending on a simple extraction requiring a small amount of fish. However, the assay is non-specific and non-quantitative (Bagnis *et al*., 1985, 1987).

diptera larvae assay

The diptera larvae assay could replace the mouse bioassay in the absence of alternative *in vitro* tests. However, the assay is not validated yet (Labrousse *et al*., 1992). In this assay the diptera larva (*Parasarcophaga argyrostoma*) is used to detect ciguatoxin in fish flesh. These larvae are selected for their simple breeding and easy handling, their ability to consume spontaneously large quantities of fresh meat, and their very high sensitivity to ciguatoxin. For the growth test the larvae were fed about 5 g of the test sample. Larvae grown overnight on meat can easily be seen with the naked eye. After 24 hours, the larvae are weighted. Weight loss or a smaller increase of weight compared to healthy samples indicates the degree of toxicity of the sample. The limit of detection for ciguatoxin expressed as CTX-1 was determined either by weighing the larvae or examination with the naked eye, and fluctuated around 0.15 ng/g flesh. Samples containing more than 1 ng of CTX/g flesh (moray eel) killed the larvae in three hours, samples with lower concentrations inhibited larval growth. The reading with the naked eye seems to be satisfactory down to 0.2 ng of CTX/g, while that by weighing, a more objective method, was acceptable down to 0.10 to 0.15 ng CTX/g. The test is very sensitive, simple and inexpensive, but it would be useful to establish a standard growth curve. Another element to improve the test is the response

time. The response is acceptable for toxic fish, but more time is needed for low-toxicity samples (comparable to the response in the mouse bioassay) (Labrousse and Matile, 1996).

in vitro assays

sodium channel binding assays for ciguatoxins

Ciguatoxins bind to sodium channels causing them to open at normal cell resting membrane potentials. This results in an influx of sodium ions, cell depolarization and the appearance of spontaneous action potentials in excitable cells. This sodium influx can be enhanced by the addition of sodium channel activator toxins through an allosteric mechanism. The reported cell based assay for the ciguatoxins (Manger *et al*., 1993, 1994, 1995) takes advantage of this phenomenon to produce an assay that is highly sensitive to ciguatoxins and other sodium channel activator toxins. This assay is 10 000 times more sensitive than the mouse assay for ciguatoxins.

An assay for ciguateric fish based on the ability of ciguatoxins to selectively inhibit the binding of ^{3}[H]-brevetoxin to sodium channels in rat brain synaptosomes was reported by Legrand and Lotte (1994).

Both in vitro sodium channel assays mentioned are more sensitive than the mouse bioassay and have considerable potential to replace this assay for the detection of ciguatoxins in crude fish extracts. However, in their current format, these assays are unlikely to be cost-effective for routine screening of individual fish.

alternative bioassays

Several assays have been developed such as the guinea pig ileum assay (Dickey *et al*., 1982), the guinea pig atrium assay (Lewis, 1988; Lewis and Endean, 1986), the isolated frog nerve fibre assay (Benoit *et al*., 1986), and assays with human and mouse hemolytic blood cells (Escalona De Motta *et al*., 1986), and the bioassay that measures the mouse body temperature depression following intraperitoneal injections of toxic fish extracts (Gamboa *et al*., 1990; Sawyer *et al*., 1984). With the guinea pig atrium assay, the tissue extract is used to bath the atrium after removal from the guinea pig. Observations are made then for the characteristic inotropic effects indicative of ciguatoxin (DeFusco *et al*., 1993).

7.2.3 Biochemical assays

immunoassays

An ideal assay for the detection of marine toxins should be simple, highly sensitive and specific. Therefore the evaluation of marine toxin detection assays has moved in the direction of immunologic analysis (Hokama and Smith, 1990). Immunochemical methods such as a radioimmunoassay (RIA) (Hokama *et al*., 1977), a competitive enzyme immunoassay (EIA) (Hokama *et al*., 1983, 1984, 1986), and a rapid enzyme immunoassay stick test (Hokama, 1985; Hokama *et al*., 1985, 1987) have been developed. Problems with these immunochemical methods are their cross-reactivity with other polyether compounds and the limited antibody supply.

The presence of another family of ciguatoxins in the Caribbean region has important implications for the detection of ciguateric fish. Antibody detection methods, which are being developed based on antibodies raised against P-CTX-1 or P-CTX-1 fragments, may not be suitable for detecting Caribbean ciguatoxins (Vernoux and Lewis, 1997).

radioimmunoassay

In 1977, a radioimmunoassay (RIA) was developed for the detection of ciguatoxin directly in contaminated fish (Hokama *et al*., 1977). In this assay, CTX conjugated to human serum albumin was injected into sheep and rabbits, thereby producing antibodies. The sheep antibody to CTX was used in the RIA after being purified and coupled to ^{125}I as a label. In practice, some false positives were reported. This method could not be used for analyses of large numbers of fishes.

enzyme-linked immunosorbent assay (ELISA)

The practicality of detection improved when Hokama *et al*. (1983) developed an enzyme immunoassay (EIA) for the detection of CTX. The procedure incorporated a sheep anti-ciguatoxin horseradish peroxidase conjugate and colorimetric determination of absorbance following the enzymatic reaction. The assay was shown to be similar in efficacy to the earlier RIA developed, but less expensive and more practical. However, it was still tedious and therefore abandoned as detection method.

stick tests

The speed of detection improved when Hokama (1985) further simplified the enzymatic procedure by incorporating correction-fluid coated skewered bamboo sticks as test tools which meant that fish tissue need only be poked with the bamboo stick and the stick with the adherent tissue fluid mixed with reagents. This method proved to be successful in separating toxic from non-toxic fish. However, six tests per fish appeared necessary for accurate determination of ciguateric fish that were tested close to the borderline level.

The final goal, a rapid visual colour test, was achieved by coating a bamboo stick that had been inserted into fish flesh with sheep anti-ciguatoxin coupled to horseradish peroxidase. After a ten minute incubation the colour of the stick is evaluated visually, ranging from colourless (non-toxic) to intense bluish purple (highly toxic) (Hokama *et al*., 1987).

Later, a rapid (within 15 minutes) stick-enzyme immunoassay using horseradish peroxidase-labelled sheep anti-ciguatera toxin antibody has been developed by Hawaii Chemtect International (Ciguatect⊇) for detecting ciguatera toxins and toxins associated with diarrhoeic shellfish poisoning. The Ciguatect⊇ test can only be used as a general screening method to select samples for further analysis because the lack of CTX standards has hampered the determination of relative cross reactivity with various derivatives. The rate of false positive responses has not yet been determined (Park, 1995). The Ciguatect⊇ test was planned to be studied in a formal AOAC International Collaborative Study. To date, the study has not yet been carried out because the antibodies used were not monoclonal, which questioned the long-term availability and quality necessary for this type of methodology development and validation. The study coordinators are developing new hybridoma cell lines for the production of anti-ciguatoxin monoclonal antibodies (Quilliam, 1998a, 1999).

immunoassays based on monoclonal antibodies

Early studies all employed a polyclonal antibody raised to ciguatoxin in sheep. A disadvantage of such an approach is that for long-term antibody production a continuous supply of antigen is required for booster injections. Monoclonal antibodies on the other hand can provide a continuous supply of a selected antibody. Hokama *et al.* (1985, 1989a) and Hokama (1990) reported production of monoclonal antibodies to a related polyether toxin, as well as to ciguatoxin (likely CTX-1).

Speed, practicality and specificity were all combined when the technology of monoclonal antibodies was incorporated into the stick test procedure (Hokama *et al*., 1989b). With this assay,

CTX was conjugated to human serum albumin with carbonimide, and BALB/c mice were injected with the conjugate. The non-immunoglobulin synthesising mouse myeloma cells used for fusion were those designated PBX63-Ag8·65B as used in other studies (Hokama *et al*., 1989a). The stick enzyme immunoassay than remains essentially the same as the original design (Hokama *et al*., 1987), except that the horseradish peroxidase was now conjugated to the anti-CTX monoclonal antibody (MAB-CTX). This method has been used extensively for surveys and for clinical confirmation.

solid-phase immunobead assay

In 1990, a solid-phase immunobead assay (SPIA), with coloured polystyrene particles coated with MAB-CTX began to be used for direct detection of CTX adsorbed on bamboo paddles coated with organic correction fluid (Hokama, 1990; Hokama *et al*., 1993). The membrane immunobead assay (MIA) presented by Hokama *et al*. (1998) is based on the immunological principles used to develop the SPIA. It uses a monoclonal antibody prepared against purified moray eel (MAB-CTX) coated onto coloured polystyrene beads. The polyether toxins extracted from a piece of fish tissue bind to the hydrophobic polyvinylidene membrane on a plastic support (membrane stick) and can be detected with the MAB-CTX coated onto the coloured polystyrene beads. The intensity of the colour on the membrane portion of the membrane stick is related to the concentration of CTX in the methanolic extracts. Overall, the MIA showed a reasonable limit of detection for CTX (approx. 0.032 ng CTX/g tissue). During development of the MIA, several factors critical to obtaining accurate and repeatable results were noted: i) the membrane portion of the membrane stick must not be touched, because touching may cause false-positive reactions; ii) the membrane stick must be soaked in the methanol/fish sample suspension for at least 20 minutes for optimal results; iii) the stick and the test tube must be completely dry before the latex immunobead suspension is added to the test tube; and iv) the membrane stick should not be soaked in the latex immunobead suspension for more than 10 minutes. The method of Hokama *et al*. (1998) was subjected to a semi-quantitative collaborative study of AOAC International in 1999 (Hokama and Ebesu, 2000). The study collaborators received dried fish samples, non-spiked or spiked with standard extract containing CTX. The study is still in the evaluation process with AOAC's Methods Committee on Natural Toxins, but a first assessment of the results has shown a sensitivity (defined as percent of truly (known) positive samples that are found by the method to be positive) and a specificity (defined as percent of truly (known) negative samples that are found by the method to be negative) of 91 percent and 87 percent respectively.

7.2.4 Chemical assays

chromatographic detection

Ciguatoxins do not possess a useful chromophore for selective spectroscopic detection but contain a relatively reactive primary hydroxyl group through which (after appropriate clean-up) a label could be attached prior to detection. High performance liquid chromatography (LC) coupled to fluorescence detection provides a highly sensitive method that has the potential to detect natural levels of ciguatoxins in crude extracts from fish flesh. Dickey *et al*. (1992b) and Yasumoto *et al*. (1993) have reported encouraging results by labelling ciguatoxin with novel coumarin-based fluorescent reagents or the fluorescent 1-anthroylnitrile, respectively, prior to LC separation and fluorescence detection. LC coupled to selective-ion monitoring ionspray mass spectrometry (MS) is an alternative to fluorescence detection of ciguatoxin in LC eluants. This approach has shown considerable potential for the detection of labelled diarrhoeic shellfish toxins (Pleasance *et al*., 1992b). Preliminary studies with CTX-1 indicate that such an approach could form the basis of a confirmatory analytical assay for ciguatoxins in fish (Lewis *et al*., 1994).

nuclear magnetic resonance (NMR)/mass spectrometry (MS)

NMR and/or MS techniques have been used to characterize ciguatoxins present in fish viscera (Murata *et al.*, 1990; Lewis *et al.*, 1991) and flesh (Lewis and Sellin, 1992) and to characterize gambiertoxins in wild and cultured *G. toxicus* extracts (Murata *et al*., 1990; Satake *et al*., 1993).

Present analytical methods used to characterize ciguatoxins (NMR and MS) require large-scale extraction of ciguatoxins present in low concentrations in highly toxic fish and in most instances the characterization of ciguatoxins present at levels below 0.1 nmol/kg has not been possible. Lewis and Jones (1997) described gradient reverse-phase liquid chromatography/mass spectrometry (LC/MS) methods to identify the ciguatoxins accumulated by fish. The analysis was performed on 5 σg samples of partially purified highly toxic moray eels from the Pacific Ocean. P-CTX-1, the major toxin in the flesh and viscera of carnivorous ciguateric fish of the Pacific, was used as the reference ciguatoxin in this study. The method appears to be more sensitive and selective than the mouse bioassay, identifying 11 new P-CTX congeners in an enriched fraction from the viscera of moray eels. The potency and origin of these congeners remain to be established.

mass spectrometry

A state-of-the-art LC-ESI-MS/MS (ESI= ElectroSpray Ionisation) application paper with very practical notes on the detection and determination of ciguatoxins was reported by Lewis *et al.* (1999). Levels equivalent to 40 ng/kg P-CTX-1, and 100 ng/kg C-CTX-1, in fish flesh could be detected. Several real-life samples were analysed.

capillary zone electrophoresis

A method applying capillary zone electrophoresis (CZE) with UV detection was developed to detect maitotoxin (MTX) (see Chapter 7.3.1), a toxin associated with ciguatera fish poisoning (Bouaïcha *et al*., 1997b). The authors demonstrated the applicability of CZE in the rapid and high-resolution separation of MTX in a solution of a commercial standard (which was not pure). They reported that an amount as low as 50 pg was visible in the electropherogram, by UV absorption at 195 nm. They concluded that CZE is a promising alternative compared to existing techniques such as LC/MS, to the determination of MTX in food, although solid-phase extraction would be a necessary technique for the extraction of the toxin from fish, as it is normally present in ng/kg amounts in ciguateric fish.

7.3 Source organism(s), habitat and distribution

7.3.1 Source organism(s)

Gambierdiscus toxicus is the source of two types of marine toxins, i.e. the water-soluble maitotoxins (MTXs) and the fat-soluble ciguatoxins. MTXs are produced by all strains of *G. toxicus* examined to date, with each strain apparently producing only one type of MTX. MTXs are principally found in the gut of herbivorous fishes and have no proven role in CFP. On the other hand, ciguatoxins are produced only by certain strains of *G. toxicus*, are found in the liver, muscles, skin and bones of large carnivorous fishes, and are regarded as the principal cause of CFP in humans (Chinain *et al.*, 1999; Lehane and Lewis, 2000).

The dinoflagellate *Gambierdiscus toxicus* was identified in the late 1970s near the Gambier Islands. This dinoflagellate lives in epiphytic association with bushy red, brown and green seaweeds and also occurs free in sediments and coral rubble (Hallegraeff *et al.*, 1995). The dead coral and marine algae thriving in tropical and subtropical reef systems are eaten by herbivorous fish; these fish accumulate and concentrate the toxins produced by the dinoflagellate. The

herbivorous fish are eaten by larger carnivorous fish. During the passing through the food chain there is an oxidative biotransformation of the less oxidized gambiertoxins to the more oxidized and more toxic ciguatoxins (Durborow, 1999; Lehane and Lewis, 2000). In the stomach of herbivorous fish, incomplete biotransformation of gambiertoxins to ciguatoxins could be seen. After accumulation in herbivores the toxins are transferred to carnivorous fish. Carnivorous fish have been shown to contain ciguatoxins and no gambiertoxins, indicating that any remaining gambiertoxins present in the herbivorous prey is completely biotransformed in the carnivorous fish (Burgess and Shaw, 2001). In the Puerto Rico area, the benthic dinoflagellate *Ostreopsis lenticularis* was shown to be a vector of CFP (Tosteson *et al*., 1998). In the literature, other dinoflagellates were also mentioned, which may play a role in the production of toxins associated with ciguatera poisoning such as *Prorocentrum concavum*, *P. mexicanum*, *P. rhathytum, Gymnodinium sangieneum* and *Gonyaulax polyedra* (Aseada, 2001).

The Caribbean (C-CTXs) and Pacific toxins (P-CTXs) possess closely related structures but are chromatographically distinguishable from each other, indicating that the ciguatoxins from the Caribbean Sea are members of another family of ciguatoxins. The presence of different families of toxins may underlie the differences in ciguatera symptoms found between the Pacific and Caribbean region. It is likely that the Caribbean ciguatoxins arise from a small number of precursor toxins, similar to ciguatera in the Pacific where one gambiertoxin (GTX-4A) can give rise to at least four ciguatoxins which accumulate in fish. Probably different strains of *G. toxicus* are able to produce different arrays of polyether toxins and a Caribbean strain of *G. toxicus* is suggested to be a source of C-CTX-1 and –2 (De Fouw *et al*., 2001).

7.3.2 Predisposing conditions for growth

G. toxicus is slowly growing and distributed circumtropically between 32∀N and 32∀S. It appears to be most prolific in the shallower waters away from terrestrial influences, with most ciguateric endemic areas being characterized by oceanic salinity waters (De Fouw *et al*., 2001). Low salinity and high light intensities adversely affected *G. toxicus* growth. Research on *G. toxicus* populations in the Florida Keys showed that *G. toxicus* preferred depths of 1 to 4 m, grew best at 11 percent of full sunlight and that maximum abundance occurred at a water temperature of about 30∀ C (Lehane and Lewis, 2000). *G. toxicus* is commonly found growing epiphytically on macroalgae colonizing damaged coral reefs, such as *Turbinaria ornata*, *Amphiroa* spp., *Halimeda opuntia* and *Jania* spp. (De Fouw *et al*., 2001).

Environmental studies suggested that the development of *G. toxicus* increased with insolation (exposure to sunlight), with the presence of silicates and oxides from land lateral soils, and with algal detritus which results in the development of peculiar algal turfs *Turbinaria, Jania* and *Amphiroa* species. Population densities of *G. toxicus* are patchy and can increase or decrease rapidly. Such growth patterns presumably underlie the spatial and temporal variability of ciguatera outbreaks. However, little is known of the precise environmental conditions that result in increased gambiertoxin production in nature (De Fouw *et al*., 2001). In the Puerto Rico area maximum toxicity of the benthic dinoflagellate *Ostreopsis lenticularis* was seen in October to December preceded by several months (August to September) of exposure to sustained elevated sea surface temperatures lasting to an average of 20 days. *Spyraenea barracuda* caught in this area in October to December showed maximum toxicity following 24 days of exposure to elevated sea surface temperatures during the preceding months (August to October). Several factors may account for the correlation between increased sea surface temperatures and ciguatoxicity in fish. Changes of two or three degrees in ambient temperatures would be expected to produce marked responses in respiration and metabolic rates, circulating hormones and predatory activity in a variety of fishes (Tosteson *et al*., 1998).

From February 1993 to December 1997, *Gambierdiscus* spp. population densities were monitored weekly in the French Polynesian Papara area in relation to temperature and salinity. A total of 58 blooms were recorded of which 65 percent occurred in 1995 and 1996 alone. Seasonality in cell densities was found from February 1993 to May 1995. During this period *Gambierdiscus* spp. populations tended to reach maximum abundance at the beginning and the end of the hot season. In contrast, salinity did not appear to be a determining factor in the seasonal abundance of this dinoflagellate. The noticeable increase in both peak densities and frequency of blooms further noticed in 1995 and 1996 was preceded by unusually high water temperatures in January to April 1994, concomitant with a severe coral-bleaching episode. Toxicity screening revealed that toxin production was maximum from October 1994 through December 1996 and no correlation was found between toxicity of the blooms and their biomass, nor the seasonal pattern of temperatures (Chinain *et al*., 1999).

Lehane (2000) stated that the presence of *G. toxicus* is unpredictable and its abundance does not necessarily reflect the potential to produce gambiertoxins. Some research indicates that certain bacteria are found symbiotically associated with dinoflagellates and play a role in the elaboration of toxins by the symbiont dinoflagellates. It was suggested that bacteria might produce nutrients that were assimilated by dinoflagellates and were necessary for producing toxins. Another suggestion was the synthesis by bacteria of toxins which are then phagocytosed by dinoflagellates (Lehane, 2000).

Over the last decades, evidence has been accumulating that reef disturbance by military and tourist developments increase the risk of ciguatera by increasing benthic substrate for dinoflagellate growth (Hallegraeff *et al.*, 1995). Although there seems no seasonal variation in the occurrence of ciguatera intoxication, according to some authors the frequency of ciguatoxic barracuda caught, varied seasonally, with peak values (60 to 70 percent toxic fish) in the late winter-early spring (January to May) and autumn (August to November). Minimal frequencies (0 to 10 percent toxic fish) were observed in summer (June and July) and December. The seasonal variations in barracuda ciguatoxicity may reflect variability in the toxicity of their immediate prey, as well as the capacity of their detoxification system (the detoxification mechanism is inhibited by hormones produced in the reproductive cycle, and at reduced temperatures) (De Fouw *et al*., 2001).

7.3.3 Habitat

G. toxicus is distributed circumtropically between 32∀N and 32∀S and consequently ciguatera is mostly confined to discrete regions in the Pacific Ocean, western Indian Ocean and the Caribbean Sea (Lewis, 2001).

7.4 Occurrence and accumulation in seafood

7.4.1 Uptake and elimination of CFP toxins in aquatic organisms

The uptake and distribution of ciguatoxins was determined in Caribbean fish caught from 1980 to 1983 on the island of St. Barthelemy (French Caribbean). Extracted lipids from several parts of these fish were analysed by mouse bioassays. The fish species belonged to the families of *Muraenidae, Serranidae, Scombridae, Carangidae,* and *Sphyraedinae*. The ciguatoxin concentration was highest in the viscera, particularly in the liver, spleen, and kidney, and lowest in the bones. The ratios of the toxin concentrations in the liver or viscera to that in the flesh were high and varied with the species suggesting that the toxin is distributed in different ways in different fish. The fact that highly vascularized organs such as liver, spleen, and kidney retained

the highest quantity of ciguatoxin per unit weight suggests that blood is involved in the distribution of ciguatoxin to other tissues (De Fouw *et al*., 2001; Pottier *et al*., 2001).

Ciguatoxin becomes more concentrated as it moves up the food chain and its level is up to 50 to 100 times more concentrated in the viscera, liver and gonads of affected fish than in other tissues. It is not known why the fish are asymptomatic after toxin ingestion and how affected fish can remain toxic for years (De Fouw *et al*., 2001).

Toxins in tissues from the herbivorous surgeonfish (*Ctenochaetus striatus*) collected in the Great Barrier Reef were characterized by mouse bioassay and chromatography. The biodetritus (on turf algae) on which the fish feeds, were collected and the toxins present were compared with those found in *C. striatus*. It appeared that levels of gambiertoxins entering the fish were typically higher than levels found later in the liver. Consequently, the gambiertoxins and biotransformed products (ciguatoxins) do not appear to be accumulated in a simple, additive manner, suggesting that depuration of ciguatoxins and/or gambiertoxins may be significant in *C. striatus* (De Fouw *et al*., 2001).

7.4.2 Fish containing ciguatoxins

Many species and many families of reef fishes are involved in ciguatera globally. These include the herbivorous *Acanthuridae* and corallivorous *Scaridae* (parrot fish), which are considered key vectors in the transfer of ciguatoxins to carnivorous fish. Many more species of carnivorous fish cause ciguatera. These include *Muraenidae* (moray eels) and *Lutjanidae* (snappers such as red bass) which are notorious in the Pacific, *Serranidae* (groupers) including coral trout from the Great Barrier Reef, *Epinephelidae*, *Lethrinidae*, *Scombridae* (mackerel), *Carrangidae* (jacks) and *Sphyraenidae* (barracudas). The latter two families are a particular problem in the Caribbean (Crump *et al.,* 1999b; Lewis, 2001). More than 400 species of bony fish have been reported in the literature to have caused ciguatera poisoning. The larger carnivores such as moray eels, snappers, groupers, carrangs, Spanish mackerels, emperors, certain inshore tunas and barracuda are the most toxic (IPCS, 1984).

Along the southwest coast of Puerto Rico, the caught barracuda is involved in ciguatera poisoning. Head, viscera and flesh tissue components of 219 barracudas (528 tissue samples) were screened for their toxicity during the period March 1985 through May 1987. Twenty nine percent of these fish yielded toxic preparations in at least one of their tissue components (De Fouw *et al*., 2001).

In the continental United States, the grouper, red snapper, jack, and barracuda are the most commonly reported fish species associated with ciguatera poisoning (De Fouw *et al*., 2001). In Florida, in the majority of cases, the great barracuda has been involved in ciguatera poisonings between 1954 and 1992. Apart from the barracuda, other commonly reported species are snapper, hogfish, jack, and grouper (De Fouw *et al*., 2001).

In Hawaii, jack, black snapper and surgeonfish are most frequently involved with ciguatera toxin (De Fouw *et al*., 2001). In the Mascareignes archipelago, 34 fish species have been identified to be involved in ciguatera poisoning. Large predators such as grouper (*Serranidae* 53 percent, *Carangidae* 10 percent, *Lethrinidae* 15 percent) are mostly involved in CFP. Most toxic fish were caught by fishing offshore on coral banks located north of Mauritius (De Fouw *et al*., 2001).

An incomplete list of fish species associated with ciguatera is presented in Table 7.1. A complete list would be nearly impossible because in some areas hundreds of fish species may be involved in CFP.

CTX-1, CTX-2 and CTX-3 are the major ciguatoxins (determined by LC/MS and mouse bioassay) present in the flesh of ciguateric fish (*Scomberomorus commersoni*, *Plectropomus* spp. and *Pomadasys maculatus*) caught at Australian coasts. Two minor toxins, which may be further oxidized analogues of CTX-1 and CTX-2, were also identified (De Fouw *et al*., 2001).

Table 7.1 Examples of fish associated with ciguatera

Species	Distribution
Lined surgeonfish (*Acanthurus linearis*)	Indo-Pacific
Bonefish (*Albula vulpes*)	Worldwide in warm seas
Gray triggerfish (*Balistes carolinensis*)	Atlantic, Gulf of Mexico
Gaucereye porgy (*Calamus calamus*)	Western Atlantic
Horse-eye jack (*Caranx latus*)	Atlantic
Whitetip shark (*Carcharinus longimanus*)	Worldwide
Humphead wrasse (*Cheilinus undulatus*)	Indo-Pacific
Heavybeak parrotfish (*Chlorurs gibbus*)	Indo-Pacific
Red groupper (*Epinephelus morio*)	Western-Atlantic
Giant moray (*Gymnothorax javanicus*)	Indo-Pacific
Hogfish (*Lachnolaimus maximus*)	Western Atlantic
Northern red snapper (*Lutjanus campechanus*) Tarpon (*Megalops atlanticus*)	Western Atlantic, Gulf of Mexico Eastern Atlantic
Narrowhead gray mullet (*Mugil capurri*)	East Central Atlantic
Yellowtail snapper (*Ocyurus chrysurus*)	Western Atlantic
Spotted coral grouper (*Plectropomus maculatus*)	Western Pacific
Blue parrotfish (*Sparus coeruleus*)	Western Atlantic
Spanish mackerel (*Scomberomorus maculatus*)	Western Atlantic
Lesser amberjack (*Seriola fasciata*)	Western Atlantic
Great barracuda (*Sphyraena barracuda*)	Indo- Pacific, Western Atlantic
Chinamanfish (*Symphorus nematophorus*)	Western Pacific
Swordfish (*Xiphias gladius*)	Atlantic, Indo-Pacific, Mediterranean

Source: Farstad and Chow, 2001

7.4.3 Other aquatic organisms containing ciguatoxins

Although the vast majority of ciguatera fish poisoning is seen after ingestion of carnivorous fish, other marine species are suspect in human ciguatera intoxication. Notably ciguatoxin was found in the viscera of a turban shell (*Turbo argyrostoma*, a marine snail). This snail has occasionally caused ciguatera-like intoxication in humans (IPCS, 1984).

Invertebrates (small shrimps and crabs) may also be a vector in the transfer of gambiertoxins to carnivorous fish. This suggestion was made based on a study with the often ciguateric blotched javelin fish (*Pomadasys maculatus*) which was found to feed predominantly on small shrimps and crabs in Platypus Bay, Queensland. Only shrimps contained detectable levels of ciguatoxin-like toxins (detected by mouse bioassay). It remains to be established if shrimps are capable of

biotransformation of the gambiertoxins to ciguatoxins or if this capacity is exclusive for fish (De Fouw *et al.*, 2001).

In Platypus Bay, inside Fraser Island, Queensland (Australia), *Alpheidae* shrimps appeared to be an important vector transferring ciguatoxins to the small carnivore *Pomadasys maculatus*. *P. maculatus* probably passes these toxins to the large mackerel (*Scomberomerus commersoni*) which is notorious in this region. Given the diversity of prey preferences among the families of carnivores, it seems likely that additional herbivore vectors of ciguatoxins will be identified in the future (Lewis, 2001)

7.5 Toxicity of CFP toxins

7.5.1 Mechanism of action

The mechanism of action of ciguatoxins is related to its direct effect on excitable membranes. Such membranes are critical to the function of nerve and muscle, mainly in their ability to generate and propagate action potentials. Ciguatoxins are characterized by their affinity binding to voltage sensitive sodium channels, causing them to open at normal cell resting membrane potentials. This results in an influx of Na^+ ions, cell depolarization and the appearance of spontaneous action potentials in excitable cells. As a consequence of the increased Na^+ permeability, the plasma membrane is unable to maintain the internal environment of cells and volume control. This results in alteration of bioenergetic mechanisms, cell and mitochondrial swelling and bleb formation on cell surfaces. Ciguatoxin acts at the same receptor site (site 5) of the Na^+ channel as brevetoxin, but the affinity of CTX-1 for voltage-dependent Na^+ channels was around 30 times higher than that of brevetoxin, while CTX-4B had about the same affinity as brevetoxin. CTX-1 and CTX-4B were shown to competitively inhibit the binding of brevetoxin to the voltage-dependent Na^+ channel of rat membranes. Ciguatoxin exerted a significant slowing of nerve conduction velocity and prolongation of the absolute refractory and supernormal periods indicating an abnormally prolonged Na^+ channel opening in nerve membranes (Lehane and Lewis, 2000 and De Fouw *et al.*, 2001).

Cardiovascular effects of ciguatoxins were thought to result from a positive inotropic effect on the myocardium. When ciguatoxin affects voltage-dependent Na^+ channels causing Na^+ to move intracellularly, normal cellular mechanisms begin to extrude sodium and take up calcium. Calcium is the intracellular trigger for muscle contraction. Although much of the increased calcium is buffered by the sarcoplasmic reticulum, it is likely that locally increased calcium concentrations increase the force of cardiac muscle contraction as is observed at ciguatoxin poisoning.

A similar mechanism of ciguatoxin-induced intracellular transport of calcium occurs in intestinal epithelial cells. The increased concentration of intracellular calcium caused by ciguatoxin acts as a second messenger in the cell, as it disrupts important ion-exchange systems. This results in fluid secretion, which presents itself as diarrhoea (Lehane and Lewis, 2000).

7.5.2 Other toxins mentioned to play a role in ciguatera

Maitotoxins are also produced by *G. toxicus* and are, via the intraperitoneal route, more toxic than ciguatoxin. However, maitotoxins are approximately 100 times less potent by the oral route compared with the intraperitoneal route, whereas the ciguatoxins are equipotent (De Fouw *et al.*, 2001).

While ciguatoxins act on Na^+ channels in nerves and muscles, maitotoxin stimulates the movement of Ca^{2+} ions across biomembranes and is a potent activator of changes in the intracellular Ca^{2+} concentrations of cells from a wide variety of organisms. As a consequence of an influx of Ca^{2+}, maitotoxins can produce several effects: hormone and neurotransmitter secretion; phosphoinositides breakdown and activation of voltage gated Ca^{2+} channels due to membrane depolarization. No specific blocker has been identified for this maitotoxin-induced channel. However, the primary target of MTXs remains still undefined. It is strongly suggested that these toxins have no ionophoretic activity. Among natural products, maitotoxins have the largest molecular weight (3422 Da) compared with any natural product known, besides biopolymers like proteins or polysaccharides. Molecular mechanic studies suggested that rather than being a flat accumulation of linked rings, the molecule might represent a molecular 'wire' (Escobar *et al.,* 1998). Maitotoxins also accumulate in the viscera of herbivorous fish, but obviously are not accumulated at sufficiently high doses in carnivorous fish to cause problems at human consumption. If maitotoxins were involved in CFP, qualitative differences in symptomatology might be expected, given that the pharmacology of maitotoxins is quite different from that of ciguatoxins (Lewis, 2001).

Various species of parrot fish have previously been reported to contain a toxin less polar than CTX-1, named scaritoxin. Judging from the reported chromatographic properties, scaritoxin seems to correspond to a mixture of CTX-4A and CTX-4B. Poisoning with scaritoxin is not well described. The name is derived from the poisonous fish *Scarus gibus*. Poisonings have two phases of symptoms, the first set of symptoms resembling typical ciguatera poisoning, the other, developing five to ten days after onset with failure of equilibration and marked locomotor ataxia (De Fouw *et al.,* 2001).

7.5.3 Pharmacokinetics

Ciguatoxins are fat soluble and absorption from the gut is rapid and substantial, although an early onset of vomiting and diarrhoea may exist in expelling some of the toxins before they are absorbed. Since cleaning ciguateric fish can cause tingling of the hands and eating them can cause altered sensation in the oral cavity and dysphagia, it would appear that ciguatoxins can penetrate the skin and mucous membranes. The related brevetoxins also have this property. Ciguatoxins are carried in the blood bound to human serum albumin and moderate (unspecified) levels of ciguatoxin in serum of a patient were reported 22 weeks after consuming ciguatoxic fish. Ciguatoxins are also transmitted in breast milk and are able to cross the placenta and affect the foetus (Lehane and Lewis, 2000).

Sexual transmission of ciguatera from female to male (penile pain after intercourse) and vice versa (pelvic and abdominal pain after intercourse) has been described (De Fouw *et al.*, 2001).

Dysuria, or painful urination, suggest that ciguatoxins are excreted at least in part and possibly unchanged in urine. However, such excretion could be neither rapid nor complete given the serum levels 22 weeks after poisoning. As ciguatoxins accumulate in the body, they may reactivate clinical symptoms from time to time. If stored in adipose tissue, ciguatoxins are probably not a problem unless the tissue is rapidly broken down for example at rapid weight loss (Lehane and Lewis, 2000). Because of their similar structure, ciguatoxins are supposed to behave in a similar pharmacokinetic manner to brevetoxins. This means that the biliary/faecal route is the major route of elimination for ciguatoxins as was demonstrated for brevetoxins (Lehane and Lewis, 2000).

7.5.4 Toxicity to experimental animals

acute toxicity

To determine the origin of watery secretion and type of diarrhoea seen at ciguatoxin poisoning a study with male mice was carried out. Semi-pure ciguatoxin (85.7 percent) was extracted from the viscera of the moray eel. The CTX amounts are expressed by MU (mouse unit). MU was defined here as the amount of CTX to kill a mouse (15 g) in 24 hours, and corresponds to 7 ng of pure CTX. This definition deviates from the definition given below and in Table 7.2. To estimate the potency of CTX causing diarrhoea, it was compared with diarrhoea caused by the cholera toxin. CTX was administered by gastric tube and intraperitoneal route at different doses. Diarrhoea and morphological influences on digestive tracts caused by CTX were observed microscopically. The results of the study revealed that:

- Diarrhoea occurred by intraperitoneal treatment but not by *per os* treatment. It is likely that CTX given per oral route was absorbed and metabolised in a slightly different manner from that of intraperitoneal route, and therefore did not cause diarrhoea.
- There was an effective dose range to cause diarrhoea of 0.14 to 1 MU.
- Diarrhoea probably resulted from hypersecretion of mucus in the colon and accelerated excretion at the rectum, so only the lower portion of the intestine was affected.
- Diarrhoea stopped within one hour, the mucus secretion was stimulated even after 24 hours accompanied by an abnormal increase in the number of goblet cells.
- The type of diarrhoea was similar to that seen at choleratoxicosis. The potency of CTX to cause diarrhoea was suggested to be about 1 300 to 8 500 times stronger than that of cholera toxin (De Fouw *et al.*, 2001).

In mice, symptoms are well defined and hypothermia is a characteristic response. However, whether ciguatoxin has direct effects on the central nervous system and what its targets in the brain may be are not known. The action of intraperitoneally administered ciguatoxin (0.5 MU) (1 MU = LD_{50} dose for a 20 g mouse) isolated from the *G. toxicus* MQ2 Caribbean strain, in ICR female mice was investigated in order to identify discrete central nervous system targets for ciguatoxin. As a marker for neuroexcitability c-*fos* was used. The effect of CTX on c-*fos* mRNA was investigated to establish a time course of action on the brain and its effect on the c-*fos* translation product was examined to identify specific neuronal pathways activated by this toxin. A pronounced decrease in body temperature was seen between 10 and 20 minutes after administration. Ciguatoxin causes a rapid induction of c-*fos* mRNA in the brain that corresponds with the decrease in body temperature. The primary targets of CTX appeared to be the hypothalamus and brain stem. The results indicate that CTX has neuroexcitatory actions on brain stem regions receiving vagal afferents and ascending pathways associated with visceral and thermoregulatory responses (De Fouw *et al.*, 2001).

Table 7.2 [(1)] Effects of ciguatoxins (CTXs), gambiertoxins (GTXs) and maitotoxins (MTXs) administered intraperitoneally (i.p.) to (18-)20 g mice

Toxin	ip. LD_{50} (σg/kg bw)	MU[(2] (ng)	Signs of intoxication	Min. / max. time to death [(3)]
P-CTX-1	0.25	5	hypothermia below 33∀C, piloerection, diarrhoea, lachrymation, hypersalivation, dyspnoea, wobbly upright gait, gasping, terminal convulsions with tail arching, death from respiratory failure	37 min./– 24 h
CTX-1B	0.33			mean survival time 10-20 h [6]
P-CTX-2	2.3	9	as for P-CTX-1, plus progressive hind limb paralysis	53 min./ –100 h
CTX-2A2	1.9			mean survival time 10-20 h [6]
CTX-2A1	3.5			mean survival time 3.5-4.5 h [6]
P-CTX-3	0.9	18	as for P-CTX-1, plus progressive hind limb paralysis	60 min./ – 26 h
CTX-3C	2.5			mean survival time 10-20 h [6]
GTX-3C	1.3	26		
CTX-4B	10			mean survival time 3.5-4.5 h [6]
GTX-4B	4.0	80	as for P-CTX-1, plus hind limb paralysis	
MTX-1 [(4)]	0.05	1	hypothermia, piloerection, dyspnoea, progressive paralysis from hind extending to fore limbs, mild gasping, mild convulsions preceding death > 30 seconds	72 min./– 72 h
MTX-2 [(4)]	0.08	1.6	as for MTX-1	41 min./–72 h
MTX-3 [(4)]	– 0.1	– 2	as for MTX-1	72 min./– 72 h
C-CTX-1[(5)]	3.6			
C-CTX-2[(7)]	1			

[(1)] Hallegraeff *et al.* (1995).

[(2)] Mouse unit is 1/50 x LD_{50} (~1 MU is the LD_{50} dose for a 20 g mouse) (Lewis and Sellin, 1992)

[(3)] Minimum time to death estimated; maximum time to death estimated from effects of doses near the LD_{50} dose (De Fouw *et al.*, 2001).

[(4)] From *Gambierdiscus toxicus* but are unlikely to accumulate in flesh of fish to levels toxic for humans via the oral route. MTXs can induce slight watery anal secretion, but do not cause diarrhoea.

[(5)] Fouw *et al.* (2001).

[(6)] Dechraoui *et al*. (1999).

[(7)] Lehane and Lewis (2000).

From Table 7.2, it appears that maitotoxins are more lethal to mice after i.p. injection than ciguatoxins. However the maitotoxins are about 100-fold less toxic by the oral route than by i.p. route (Lehane and Lewis, 2000).

Male ICR mice were given ciguatoxin or ciguatoxin-4c (at a dose level of 0.7 σg/kg body weight) by the oral or intraperitoneal route. Ciguatoxin-4c was not specified. Histopathological and ultrastructural changes of various organs and the modifying effects of several antagonists on the membrane permeability of sodium were examined. The heart, medulla of adrenal glands, autonomic nerves and the penis appeared to be the target organs. There were no differences in clinical signs or histopathological changes in mice receiving ciguatoxin or ciguatoxin-4c. Ultrastructural changes in the heart after the administration were characteristic. Marked edema between myofibrils and other organelles was prominent. It is of interest that antagonists to cholinergic and adrenergic autonerves used in this experiment had no effect on cardiac injuries. Therefore, the effect of ciguatoxin on cardiac muscle may be based on its direct activity on cardiac muscles. Despite the severe diarrhoea, there were no morphological changes in the mucosal layer of the small intestine but the autonomic nerve system in muscle layers of the small intestine was sensitive to the toxins. Pre-treatment with atropine prevented the diarrhoea caused by the toxins and therefore it was suggested that the diarrhoea is probably induced by a direct action of these toxins on the autonomic system in the small intestine. No changes were seen in the cortical layer of the adrenal glands but degeneration of the medulla of the adrenal glands was prominent. Erect penises of treated mice were observed even after death. The precise mechanism is unknown, but direct or indirect effects of the toxins on penile cavernous bodies via autonomic endings as well as the formation of thrombi in the cavernous bodies may play a role (De Fouw *et al.*, 2001).

The morphologic response of the mouse heart was examined after repeated (15 days) low dose (0.1 σg/kg body weight) exposures to ciguatoxin or ciguatoxin-4c after oral and intraperitoneal administration. Furthermore the sequential changes of the heart injuries up to 14 months after either repeated low doses or after a single high dose (0.7 σg/kg body weight) was investigated for both exposure routes and for both toxins. A single dose of 0.1 σg/kg body weight caused no discernible morphological changes in hearts of mice, in contrast to repeated administration which resulted in severe morphological changes such as marked swelling of the myocardial and the endothelial lining cells of blood capillaries. The effects seen after repeated exposure are similar to those observed after the administration of one single high dose. The prominent swelling of the endothelial lining cells is likely to cause serious alteration of the permeability, which may result in plasma migration from the degenerated endothelial lining cells into the interstitial space. Within one month after the administration, myocytes and capillaries appeared to be normal. The effusion in the interstitial spaces resulted in bundles of dense collagen, which persisted for 14 months. The results indicate that ciguatoxin and ciguatoxin-4c have a cumulative effect on the cardiac tissue. This means that if there are repeated exposures to low doses of ciguateric fish, even the ingestion of fish slightly contaminated by ciguatoxin may play a role in the development of the heart disease (De Fouw *et al.*, 2001).

repeated administration

No data

reproduction/teratogenicity

No data

mutagenicity

No data

in vitro studies

Experiments were carried out on nodes of Ranvier of myelinated nerve fibres isolated from the sciatic nerve of adult frogs. CTX-1b, the major toxin involved in ciguatera fish poisoning, was extracted and highly purified from moray-eel liver and viscera. The authors did not explain why they defined the ciguatoxin as CTX-1b. CTX-1b produced swelling of the nodes of Ranvier. The swelling was prevented by the Na^+ channel blocker tetrodotoxin, indicating that the swelling originated in Na^+ entry through voltage –dependent Na^+ channels. D-mannitol caused shrinkage of nodes of Ranvier previously swollen by CTX-1b. CTX-1b induced spontaneous action potentials and caused a persistent activation of a fraction of Na^+ current, D-mannitol suppressed these spontaneous action potentials (De Fouw *et al.,* 2001).

The results of a study with ciguatoxin on guinea pig atria and papillary muscles suggested that the toxic effects of ciguatoxin stem from its direct action of opening myocardial Na^+ channels. Extrasystoles developed in atria and papillary muscles within 45 minutes of addition of ciguatoxin (> 0.15 MU/ml) and appeared to result mainly from its effect on neural Na^+ channels causing an increased release of noradrenaline from the nerves associated with the myocardium. The papillary muscles were less sensitive to the toxic effects of ciguatoxin than those of the atrium. This corresponded to a 10-fold difference in their sensitivity to positive inotropic doses of ciguatoxin (De Fouw *et al.,* 2001).

7.5.5 Toxicity to humans

clinical symptoms

After consumption of ciguatoxin contaminated fish, the onset of the first symptoms can be as short as 30 minutes for severe intoxications, while in milder cases onset may be delayed for up to 24 hours to occasionally 48 hours. The first symptoms can be either gastrointestinal or neurological in nature (e.g. circumoral tingling). Gastrointestinal symptoms usually last only a few days, while some neurological symptoms can take several days to develop. Ciguatera symptoms typically last for several weeks to several months. In a small percentage of cases (less than 5 percent), certain symptoms may persist for a number of years.

A combination of a few to more than 30 gastrointestinal, neurological and/or generalized disturbances have been reported. Gastrointestinal symptoms involving vomiting, diarrhoea, nausea and abdominal pain (>~50% of cases) typically occur early in the course of the disease and often, but not always, accompany the neurological disturbances. Neurological disturbances invariably occur in ciguatera and include tingling of the lips, hands and feet, unusual temperature perception disturbances where cold objects give a dry-ice sensation, and a severe localized itch of the skin (>~70 percent of cases). These symptoms and a profound feeling of fatigue (90 percent of cases) can occur throughout the illness. Muscle (>80 percent), joint (>70 percent) and teeth aches (>30 percent) occur to varying extents, and mood disorders including depression and anxiety (50 percent) occur less frequently. Severe cases can involve hypotension with bradycardia, respiratory difficulties and paralysis but deaths are uncommon (less than 1 percent according to Lehane, 2000). The low fatality rate (2 percent) appears to arise because fish rarely accumulate sufficient levels of ciguatoxin to be lethal at a single meal, perhaps because fish succumb to the lethal effects of higher ciguatoxin levels (Lewis, 2001).

Lehane and Lewis (2000) noted that most cases of CFP in the Pacific involved the consumption of fish containing 0.1-5 nmol P-CTX-1/kg, which is equivalent to about 0.1-5 µg/kg of fish flesh.

persistence and recurrence of symptoms

Neurological disturbances usually resolve within weeks of onset, although some symptoms may persist for months or even years. Symptoms such as pruritus, arthralgia and fatigue can also persist for months or years. Analysis of ciguatoxins in blood samples suggests that the toxin can be stored in adipose tissue and that symptoms may recur during periods of stress, such as exercise, weight loss, or excessive alcohol consumption. Sensitivity to alcohol may also persist for years after the first attack (Lehane, 2000).

factors influencing clinical symptoms

sensitization

The phenomenon of sensitization has been observed where persons who previously were intoxicated with ciguatoxin may suffer a recurrence of typical ciguatera symptoms after eating fish that do not cause symptoms in other persons. Such sensitization can occur many months or even years after an attack of CFP (De Fouw *et al.,* 2001).

It was also noted that individuals who had suffered from CFP, often have symptoms after eating any seafood and often nuts, nut oils and alcoholic beverages as well. Therefore patients suffering from CFP are recommended to avoid these food products. Eating fish with low levels of toxin over several years in the absence of symptoms could eventually result in sensitization to the toxin. This may be a matter of accumulation of ciguatoxin in the host or possibly an induction of an immunological reaction (De Fouw *et al.,* 2001).

fish species involved

Large variations are noted in the frequency and severity of the symptoms after ciguatera poisoning. Ciguatera case reports from the Hawaii State Department of Health were examined for patterns of symptomatology in relation to the types of fish consumed. While individuality and variability of human's response to particular toxin cannot be ruled out as the cause of the wide variations, the data presented would suggest that there are also differences in symptoms which are fish-specific or toxin-specific. It may be postulated that the carnivores feed on different herbivores or metabolise the toxins from the same prey to more or less active forms (De Fouw *et al.,* 2001).

ethnic variation

Though variation in symptomatology is possibly the result of inconsistent reporting, it has also been speculated that it relates to differences in toxins within the same contaminated fish. Some authors reported that the symptoms correlated with ethnic groups. It appeared that Melanesians more commonly had pruritis, ataxia, abdominal pain and weakness, that Europeans experienced more neck stiffness, lachrymation, arthralgia and reversal of temperature sensation, and that Asians had more diarrhoea and abdominal pain (De Fouw *et al.,* 2001).

geographic variation

In the Pacific Ocean, neurological symptoms predominate, while in the Caribbean Sea, gastrointestinal symptoms are a dominant feature of the disease. These differences in symptoms provide clear evidence that different ciguatoxins may underlie ciguatera in Pacific and Caribbean waters. A third class of ciguatoxins is likely to underlie the different pattern of symptoms observed in the Indian Ocean where ciguateric fish cause a cluster of symptoms reminiscent of hallucinatory poisoning including lack of coordination, loss of equilibrium, hallucinations, mental depression and nightmares, in addition to symptoms typical of ciguatera. Ciguateric fish in the Indian Ocean are also more frequently contaminated by lethal levels of toxin (Lewis, 2001).

Percentages given for symptoms in different regions are:

- Neurological symptoms: paresthesia is found in 36 percent of cases in US Virgin Islands, 70 to 76 percent in Australia and Miami, and in 87 to 89 percent of cases in French Polynesia, Fiji and the Caribbean area (De Fouw *et al.,* 2001).
- Gastrointestinal symptoms: diarrhoea appears to be common in 32 percent of cases in Fiji to 86 percent in other regions (De Fouw *et al.,* 2001).
- Cardiac manifestations: Bradycardia and hypotension are reported in French Polynesia (16 percent) and Fiji (9 percent) (De Fouw *et al.,* 2001).

The toxin responsible for ciguatera in the Gove region of Northern Australia is the same as the major toxin responsible for poisoning from carnivorous fishes in the Pacific Ocean but differs from the toxins involved in the Indian Ocean and the Caribbean Sea (De Fouw *et al.,* 2001).

sexual transmission of intoxication

Four men became ill after the ingestion of freshly caught trevally and coral trout a few hours before the characteristic symptoms of ciguatera poisoning developed. In addition to these symptoms, two men complained of intense penile pain and one of these patient's female partner, who had not eaten any fish, complained of circumoral dysesthesiae, pruritus, arthralgia, nausea and lethargy within 24 hours of having unprotected sexual intercourse with him (De Fouw *et al.,* 2001).

effects during pregnancy

Ciguatoxin is transferred across the placenta from mother to foetus. It does not affect foetal development but has been attributed to accelerated foetal movements. It can also pass from mother to infant via breast milk. Mothers who breast fed their babies had reported excessive pain of their nipples. The babies showed diarrhoea. Women who had chronic symptoms with ciguatera occasionally reported worsening of symptoms during their menses (Beadle, 1997).

A family of four in Queensland (Australia), two children, father and mother who was 11 weeks pregnant, was diagnosed with ciguatera poisoning after eating a coral trout. The poisoning was confirmed clinically and by mouse bioassay. The concentration of ciguatoxin in the trout eaten, being 1.3 ng/g, is considered relatively high. The father and mother, showing more severe intoxication, were intravenously treated with 20 percent mannitol (250 ml over 30 minutes). The mother recovered quickly after mannitol infusion, in the father a second mannitol infusion a week after the poisoning had beneficial effects. Twenty-eight weeks later, the mother gave birth to a 3.4 kg male. The newborn showed respiratory problems at birth and was treated for persistent pulmonary hypertension which was not attributed to ciguatoxin exposure *in utero*. No residual symptoms were seen after two months (De Fouw *et al.,* 2001).

A pregnant woman in San Francisco (USA) showed symptoms characteristic of ciguatera poisoning, four hours after she had eaten a large portion of a barracuda fish. Many of the symptoms lasted for several weeks. The woman, who was in her second trimester, experienced an increase of foetal movements one hour after the poisonous meal, which lasted for a few hours. The presence of ciguatoxin was confirmed in two bioassays (guinea pig atrium stimulation test and a mouse bioassay) and a stick enzyme immunoassay. The newborn at term was normal and follow-up visits revealed no abnormalities in the first 10 months (De Fouw *et al.,* 2001).

Two days before the expected birth of a child, a woman had eaten ciguateric coral trout. Within four hours she experienced the characteristic gastrointestinal and neurological symptoms of CFP. Tumultuous foetal movements were experienced and an intermittent peculiar foetal “shivering”,

which began simultaneously with her own systemic symptoms. The bizarre foetal movements continued strongly for 18 hours and gradually decreased over the next 24 hours. A 3.8 kg male was delivered by Caesarean section two days later. He exhibited left-sided facial palsy (possibly myotonia of the small muscles of the hands) and respiratory distress syndrome but recovered within six weeks (Lehane and Lewis, 2000).

treatment

A real antidote therapy is not known. If the patient presents symptoms of ciguatera intoxication soon after ingestion of the fish, gastric lavage followed by treatment with activated charcoal might help. The biggest breakthrough in the treatment of ciguatera came with the use of mannitol. It does not seem to affect the cardiovascular or gastrointestinal symptoms but does reduce the severity and duration of neurological symptoms. Ideally mannitol should be administered in the acute phase to be effective. Clinical research shows that mannitol is not effective if administered more than 48 hours after symptoms appear (De Fouw *et al.,* 2001).

Only one single blind controlled trial with mannitol (patients were unaware of the treatment received) has been reported. This trial showed that 250 ml of 20 percent mannitol given intravenously in one hour was slightly more effective than a combination of vitamins and calcium also given intravenously in one hour. Treatment with 20 percent mannitol solution in water intravenously at a dose of 1 g/kg bw at an initial rate of 500 ml/hour caused an improvement in the symptoms (De Fouw *et al.,* 2001).

The mechanism of mannitol treatment is not completely understood. One theory is that mannitol actually competes with sodium channels. A second theory is that mannitol's effectiveness is in its ability to act as an osmotic agent at the cellular level to reduce fluid excess in the cytoplasm of nerve cells or to prevent an influx of sodium through sodium channels to stabilise the cell membrane. A third theory suggests that mannitol may react directly with the toxin to neutralise it or displace it from its binding site on the cell (De Fouw *et al.,* 2001).

It has also been suggested that the presence of mannitol in the extra-cellular fluid sterically inhibits the movement of sodium ions through channels which have been blocked by the ciguatoxin molecule. Another suggestion is that mannitol may act as a scavenger for hydroxyl radicals in ciguatoxic systems (De Fouw *et al.,* 2001).

In the case of dehydration and hypotension, intravenous crystalloid infusion and vasoactive agents may be required. Atropine sulphate for bradycardia and dopamine infusion for severe hypotension may be life-saving. In cases of respiratory depression, mechanical ventilation may be necessary (De Fouw *et al.,* 2001).

Two patients in a hospital in Santiago, Chile who had CFP after eating a dusky grouper in the Dominican Republic were successfully treated with gabapentin (400 mg orally three times a day) (Perez *et al.,* 2001).

Amitryptiline may be useful for treating dysesthesia which may be chronic (Crump *et al.,* 1999b).

experimental data

Five CFP patients still experiencing intense paresthesia were selected to perform temperature studies. It appeared that temperature perception covering a range from very cold to hot was normal in these patients. The cut-off point of the peculiar symptoms described as reversal of temperature perception (such as tingling, burning, smarting and electric) was recorded around 24 to 26 ∀C and this temperature appears to correlate very closely to the cold threshold from C-

polymodal nociceptors (23∀C). This finding suggests that the paradoxical sensory discomfort experienced is, most likely, a result of an exaggerated and intense nerve depolarization occurring in small peripheral nerve tissue such as A-delta myelinated fibres and in particular the unmyelinated C-polymodal nociceptor fibres. These kind of cutaneous unmyelinated fibres respond to mechanical, heat, cold, and chemical stimuli in the painful intensity range. By the same mechanism, the intense sensation of itch experienced in a large percentage of ciguatera patients is characteristic of lower frequency discharges in some C-polymodal nociceptor fibres (De Fouw *et al.*, 2001).

7.5.6 Toxicity to aquatic organisms

fish

Individual tropical fish can carry sufficient ciguatoxin in their tissues to poison several humans, without showing obvious pathology. However, ciguatoxin has been shown to be lethal to freshwater fish and marine fish. Na^+ channels of marine fish are susceptible to ciguatoxin, and ciguatoxin exerts similar effects on fish and mammalian Na^+ channels. It can be concluded that:

- fish are susceptible to ciguatoxin but at doses higher than those required to cause death in mammals
- Na^+ channels and/or Na^+ gates of both ciguatoxin-carrier and ciguatoxic-non-carrier fish were sensitive to being opened by ciguatoxin; and
- sensitivity of fish nerves to ciguatoxin and the lack of overt pathology in toxic fish suggested that carrier fish have a partitioning or detoxification mechanism to keep the toxin away from target sites.

It was suggested that the presence of a ciguatoxin-induced soluble protein-ciguatoxin association in the muscle of toxic species of narrow-barred Spanish mackerel may be the basis of a sequestration mechanism that diminishes the binding of ciguatoxin to the target sites of the Na^+ channels of excitable membranes in fish (Lehane and Lewis, 2000).

The adverse effects of ciguatoxin on medaka (*Oryzias latipes*) embryos were quantified by microinjection into the egg yolk of the embryos. Embryos microinjected with 0.1-0.9 pg/egg showed tachycardia but no reduction in hatching success; however 22 percent of the fish which hatch at this dose range have lethal spinal defects. At higher levels (1.0-9.0 pg/egg) a direct decrease in success was seen together with a 93 percent incidence of lethal spinal defects. Embryos exposed to 10-20 pg/egg ciguatoxin have 0 percent hatching success. The results of this study indicated that maternal transfer of low levels of ciguatoxin may represent an unrecognized threat to the reproductive success of reef fish and a previously undetected ecological consequence of proliferation of ciguatoxin-producing algae in reef systems increasingly impacted by human perturbations (Edmunds *et al.*, 1999).

7.6 Prevention of CFP intoxication

7.6.1 Depuration

Ciguatoxin cannot be identified by odour, taste or appearance. It is also temperature stable so cooking or freezing will not destroy it. Ciguatoxin can also not be eliminated by salting, drying, smoking or marinating. The contaminated fish can remain toxic for years, even on a nontoxic diet (Beadle, 1997). Apart from the avoidance of consumption of large predatory fish, the use of animal screening tests is the only tools presently available to prevent intoxication (De Fouw *et al.*, 2001).

7.6.2 Preventive measures

The major source of ciguatera cases has been the fish caught by sport fishing (79 percent). If people could be educated to avoid consuming heads, viscera and roe of reef fish, and avoid fish caught in the areas known for frequent occurrence of ciguatoxin intoxication, the incidences of ciguatera probably would decrease dramatically (De Fouw *et al.*, 2001).

Large predatory reef fish are most likely to be affected; the larger the fish, the greater the risk. Some authorities advocate avoiding fish that weigh more than 1.35 to 2.25 kg but this is only a relative precaution. However, there is no way of knowing the size of fish from which the steak or filet was cut. Organ meats, including the roe, appear to contain higher concentrations of toxins and should be avoided. Consuming small portions from several fish per meal instead of a large portion of any suspect fish will reduce the risk too (De Fouw *et al.*, 2001).

7.7 Cases and outbreaks of CFP

7.7.1 General

As many as 50 000 cases of CFP worldwide are reported annually; the condition is endemic in tropical and subtropical regions of the Pacific Basin, Indian Ocean and Caribbean. Isolated outbreaks occur sporadically but with increasing frequency in temperate areas such as Europe and North America. Increase in travel between temperate countries and endemic areas, and importation of susceptible fish has led to the encroachment of CFP into regions of the world where CFP has previously been rarely encountered (Ting and Brown, 2001). In the primary endemic areas including the Caribbean and South Pacific Islands the incidence is between 50 and 500 cases per 10 000 people (Perez *et al.*, 2001). In the developed world, CFP poses a public health threat due to delayed or missed diagnosis. Without treatment, distinctive neurologic symptoms persist, occasionally being mistaken for multiple sclerosis. Constitutional symptoms may be misdiagnosed as chronic fatigue syndrome (Ting and Brown, 2001). It was supposed that the incidence figures were likely to represent only 10 to 20 percent of actual cases, with the extent of under-reporting likely to vary between countries and over time (De Fouw *et al.*, 2001).

7.7.2 Europe

France

Two people showed signs of CFP after eating frozen fish (not specified) from China (Province of Taiwan) (IPCS, 1984).

After eating pieces of various fish, a 60 year old man developed CFP with diarrhoea, facial paresthesia, myalgia, cramps and weakness. Physical examination revealed a motor distal deficit of the four limbs, myokimia and ataxia. EMG testing was in favour of an axonal neuropathy. Neurological symptoms persisted for two months. This case illustrates a new pathophysiological mechanism of neuropathy: “axonal channelopathy” (Derouiche *et al.*, 2000).

A few days after eating a shellfish meal (trocas=*Tectus pyramis*), one patient suffered ataxia and stupor. The patient was confused with cerebellar signs and ocular disturbances (hypotropia). Blood results, cerebrospinal fluid and brain CT scan were unremarkable. The patient developed a septic shock and died four weeks after admission. No necropsy was performed. The clinical picture strongly suggested a seafood poisoning, namely ciguatera. However, no toxicological assay was performed. CFP has never been reported with trocas (Angibaud *et al.*, 2000). A confirmed case of CFP was reported in 2002 (EU-NRL, 2002).

Germany

A case of ciguatera poisoning in a 40 year old man in Germany following a travel to the Dominican Republic, has been described. The man showed the characteristic ciguatera symptoms after having eaten a meal of grouper. On return to Germany, he was admitted to the hospital. Due to the typical history and clinical findings, ciguatera toxin ingestion was diagnosed. All symptoms were finally resolved after 16 weeks (De Fouw *et al.*, 2001).

After cutting short their holidays in the Dominican republic, four people from a travel group presented, on return to Germany, complex neurological symptoms including paresthesia, nervousness, inverse temperature perception, muscle cramps, headache and dizziness. Dinner at the holiday location existing of "peak bass and lemon sauce" led to the diagnosis of ciguatera poisoning. The first symptoms in all members of the travel group (26 persons) were diarrhoea, sickness and sweating (Blume *et al.*, 1999).

A 45 year old woman showed signs of CFP on return to Germany after a journey to the Red Sea. She appeared to have consumed a fish meal during her vacation. The usual treatment with mannitol etc. three weeks after the onset of the symptoms proved inefficient. However, during the 21 months of follow-up, a marked spontaneous clinical and electrophysiological reversal of symptoms occurred (Ruprecht *et al.*, 2001).

Italy

CFP has begun to appear in Italian travellers to the Caribbean islands (Bavastrelli *et al.*, 2000).

The Netherlands

Five patients with symptoms of ciguatera poisoning were seen in the outpatients department of Tropical Medicine in an Amsterdam hospital. The patients had eaten fish in Curaçao and Isla de Margarita (Venezuela). Ciguatera could only be diagnosed based on the clinical symptoms and the fact that a fish was eaten in the Caribbean area (De Fouw *et al.*, 2001).

7.7.3 Africa

Madagascar

A very severe outbreak of ciguatera poisoning, presumably caused by a shark, occurred in Manakara, a city on the east coast of Madagascar, on 28 November 1993. The mortality rate was 20 percent (98 out of 500 poisoned people died). When the medical team arrived five days after the tragedy, most of the serious cases had already died. One hundred and fifty patients were still in hospital (35 in a critical state, of whom 15 died within a few days). The symptomatology presented by the patients in critical state were not indicative for CFP as a consequence of their severity and included coma, body rigidity, myosis, mydriasis, convulsions, respiratory distress and pulmonary oedema, cardiovascular collapses, bradycardia, gengivorrhagia and dehydration. The symptoms in the moderately poisoned persons (115 cases) were typical for CFP. Unfortunately, no remains of the shark were available for chemical investigations (Boisier *et al*., 1995).

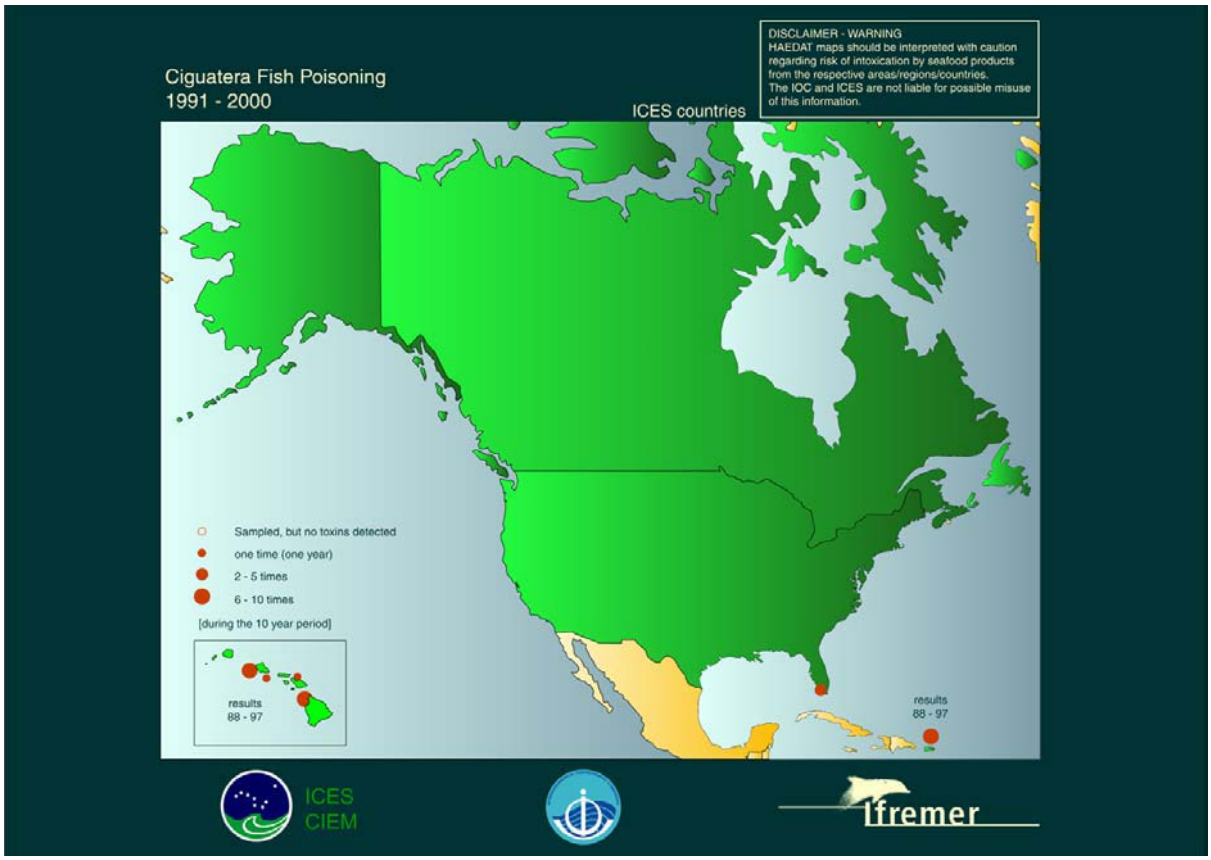
Epidemiological data concerning the same outbreak in Manakara in November 1993 as described above, were reported. The attack rate was about 100 percent. Records of 188 hospitalized patients were reviewed. The first clinical signs appeared within five to 10 hours after ingestion. The overall mortality was close to 30 percent, perhaps because of the inadequacy of local life-support technology. The patients suffered almost exclusively from neurological symptoms, the most prominent being a constant, severe ataxia. Rare cases also manifested digestive or cardiovascular signs. Gastrointestinal troubles, like diarrhoea and vomiting, were rare. Two liposoluble toxins

were isolated from the liver and tentatively named carchtoxin-A and –B, respectively. They were distinct from ciguatoxin in their chromatographic properties. The mouse lethality of the shark liver was about 30 mouse units (MU) per g of liver (1 MU was defined as the amount of toxin required to kill a mouse weighing 16 g within 24 hours). This figure exceeded highest ciguatoxin level reported from moray eel liver (20 MU/g liver). Both toxins caused diarrhoea, laboured breathing, paralysis of limbs, and convulsions before death in mice, as does ciguatoxin. However, a distinction was noted between the shark toxins and ciguatoxin in dose-survival time response. Mice given the shark toxins died within 4 hours, or otherwise survived. In contrast, mice given ciguatoxin died even after 24 hours (De Fouw *et al.*, 2001).

7.7.4 North America

The presence of CFP toxins in North American ICES countries is illustrated in Figure 7.2.

Figure 7.2 Occurrence of CFP toxins in North American ICES countries from 1991 to 2000



Source: http://www.ifremer.fr/envlit/documentation/dossiers/ciem/aindex.htm

Canada

Canadians have been affected by CFP through the consumption of tropical fish, mainly when travelling in the north Caribbean region or occasionally through imported fish. The second group of individuals who are exposed to ciguatoxins are those who buy tropical fish from local fish markets or who eat such fish in restaurants. During the years from 1983 to 1997, 22 cases of CFP were reported in Canada, mostly from imported fish (Todd, 1997). In 1998, a CFP incident was reported from hospitals in Montreal, Quebec, involving seven cases. The cases concerned members of three families each of whom had consumed barracuda. The patients revealed

gastrointestinal and neurological symptoms. One of the cases had left over fish. At toxicological testing in the mouse assay mortality occurred. An ELISA assay was inconclusive (Anonymous, 2000b). Recently Health Canada was notified of thirty Canadians who developed ciguatera fish poisoning as a result of consuming cooked coral reef fish that had been brought back from Fiji (Anonymous, 2002b).

The United States of America

From 1983 through 1992, 129 outbreaks of ciguatera poisoning involving 508 persons were reported in the United States, however, no ciguatera-related deaths were reported. Most outbreaks were reported from Hawaii (111) and Florida (10). The other outbreaks in different parts of the country have been associated with consumption of imported fish (De Fouw *et al.,* 2001).

California

A 34 year old woman and a 40 year old man became ill within six hours of eating barracuda they caught in turbid water near Cancun, Mexico. Five weeks after the onset of the symptoms, the diagnosis CFP was made. Ten weeks after eating barracuda the patients were free of the symptoms (Farstad and Chow, 2001).

An outbreak of cases in Southern California was tracked to grouper harvested off the coast of the Baja peninsula during an El Niño year (Farstad and Chow, 2001).

Florida

In Miami, the estimated annual incidence rate is 50 per 100 000 population (De Fouw *et al.,* 2001). In 1972, 34 cases of CFP were reported during an outbreak after eating barracuda (Pottier *et al*., 2001). In 1980, 129 people exhibited CFP symptoms after eating local grouper and snapper. No mortality was seen (IPCS, 1984). Twenty cases of CFP following consumption of amberjack were reported to the Florida Department of Health and Rehabilitative Services in August and September 1991. Forty percent of samples from amberjacks originating from a dealer in Key West and from restaurants and grocery stores in Florida and Alabama were positive in the mouse bioassay (Anonymous, 1993). The estimated rate of CFP in south Florida is 1 300 cases per year, among which 10 percent are caused by fish caught in Florida waters. Many of the ciguateric fish come from the Bahamas. An annual incidence of five CFP cases per 10 000 inhabitants in Dade County (Miami) is reported (Pottier *et al*., 2001).

Maryland

In 1980, twelve people were reported to show CFP symptoms after eating grouper from Florida (IPCS, 1984).

New York

A 36 year old man was presented to the Emergency Department of a hospital in New York six hours after a late night flight from Aruba. The patient suffered from nausea and vomiting (five episodes), diaphoresis, abdominal pain and loose, watery stools (three episodes). The symptoms began about three hours after returning from vacation. The patient had eaten an unknown local fish stew just before departure home. After four hours in hospital the patient was sent home. Six hours later the patient returned to the hospital with continued gastrointestinal problems together with pruritus, a "numb" feeling around the mouth and mild difficulty in walking caused by myalgia. The patient had taken alcohol at home. Neurological evaluation showed sensory reversal dysesthesia and generalized paresthesia. The patient responded well to supportive therapy and was discharged home after two days (Aseada, 2001).

North Carolina

In 1987, 10 cases of CFP were reported during an outbreak after eating barracuda, dolphin fish and yellow fin tuna (Pottier *et al*., 2001).

Rhode Island

A male patient in Rhode Island suffered from CFP after ingestion of a fish soup. The patient developed gastrointestinal and neurological symptoms, respiratory distress and cyanosis, progressing to stupor and coma. Coma is unusual but it has been reported. It might be that the patient had consumed a large amount of toxin. It is also possible that the alcohol consumption, the ingestion of non-seafood-related toxins or genetic susceptibility caused a more severe response to ciguatera toxin. A sample of the fish soup was tested and the stick immune assay resulted in "non-edible toxic". The mouse bioassay resulted in death of the mouse within 48 hours, but the mouse response did not show all ciguatera-like symptoms. The guinea pig atrium assay was negative; both atria did not show the typical inotropic response to ciguatera toxin (De Fouw *et al.,* 2001).

Vermont

In 1986, two persons in Vermont showed CFP symptoms after eating barracuda originating from Florida's coastal waters. Portions of a single barracuda frozen by one restaurant were positive for ciguatoxin by the enzyme immune assay (Anonymous, 1986).

Hawaii

Based on the epidemiological records for CFP cases of the Hawaii State Department of Health, over a five year period (January 1984 through December 1988) a total of 150 outbreaks were reported involving 652 exposed individuals, resulting in 462 cases showing symptoms of ciguatera intoxication (overall attack rate 70.9 percent). The Kona coast of the Island of Hawaii was responsible for most incidents (De Fouw *et al.,* 2001).

The South Point of the Island of Hawaii and the Napali coast of the island of Kauai were frequently implicated areas. An annual incidence rate in Hawaii of 8.7 per 100 000 from 1984 to 1989 was reported by Gollop and Pon (1992) as compared to 2.5 per 100 000 from 1975 to 1981 (De Fouw *et al.,* 2001).

A confirmed (in left-over fish by immunoassay EIA) ciguatera poisoning was reported in 1985 in which 15 persons of various ages became ill after eating an amberjack caught off the western shore of the island of Kauai (Hawaii). All individuals developed characteristic gastrointestinal and neurological symptoms within 1.5 to six days. Furthermore 10 of the 15 persons demonstrated cardiovascular symptoms, such as bradycardia and hypotension. Duration of the illness ranged from two to 132 days. Bradycardia was associated with increasing age and body weight as well as the amount of fish consumed. An increased duration of the illness (but not an increased severity) was correlated with both increasing age and weight, and was independent of amount and components of toxic fish consumed (De Fouw *et al.,* 2001).

7.7.5 Central and South America

Anguilla

The CFP incidence is reported to vary between two to five cases per 1 000 inhabitants per year (Pottier *et al*., 2001).

The Bahamas

In March 1982, 14 members of a crew of an Italian freighter showed CFP poisoning after eating a local barracuda. No mortalities were reported (Anonymous, 1982).

After eating a contaminated barracuda caught from the Cay Sal Bank of the Bahamas on 12 October 1997, 17 crew members of a Norwegian cargo ship showed symptoms of ciguatera poisoning (nausea, vomiting, diarrhoea, and muscle weakness) two to 16 hours later. Three samples of left-over raw barracuda and red snapper, caught simultaneously with the consumed barracuda, were tested for ciguatoxin using an experimental membrane immunobead assay. The samples from both fish tested positive for ciguatoxin (Smith *et al*., 1998)

Chile

A farm-raised salmon, possibly imported from Chile, was suspected of causing CFP in 1992. The affected woman became seriously ill 1.5 hours after eating the fish (Durborow, 1999).

Cuba

Ten cases per year are generally recorded officially except for in 1974 when 174 cases were reported (Pottier *et al*., 2001). In 1978, 100 cases of CFP were reported after eating local moray eel and Spanish mackerel. No mortality occurred (IPCS, 1984).

In 1987, an outbreak involving 57 cases of CFP was reported (Pottier *et al*., 2001). Three out of four people who ate barracuda on vacation in Cuba developed frequent watery diarrhoea and vomiting within five hours. The fourth patient developed similar but less severe symptoms within 12 hours. Gastrointestinal symptoms gradually subsided over 24 to 48 hours during which time weakness, generalized pruritus, and peri-oral and distal extremity paresthesias developed (Butera *et al.*, 2000).

Dominican Republic

In 1989, 81 CFP cases were reported. Six out of these 81 were isolated cases while the remaining 75 cases were seen in 13 outbreaks (Pottier *et al*., 2001).

Guadeloupe

In Saintes Islands (southern Caribbean islands), a study over 20 years estimated an average incidence of three cases per 10 000 inhabitant per year. During the first six months of 1970, several outbreaks occurred in many localities. From 1980 to 1985, 255 cases were reported with five requiring resuscitation. Since 1992, a CFP incidence of 0.7 per 10 000 inhabitants per year was reported. However, this appears to be a gross underestimate because the reporting was done by only 32 out of 300 physicians in the archipelago. Medical supervision reported an estimate of 100 cases per year for Guadeloupe (Pottier *et al*., 2001).

Haiti

In 1985, two cases of CFP were reported (Pottier *et al*., 2001). In February 1995, six US soldiers in Haiti became ill after eating a locally caught fish, the so called greater amberjack (*Seriola dumerili*). The symptoms presented were characteristic for ciguatera with gastrointestinal and neurological symptoms. Three patients developed bradycardia and hypotension. All patients recovered fully in one to three months (gastrointestinal and cardiovascular symptoms abated within 72 hours). Analysis of a portion of the cooked fish showed indeed approximately 20 ng Caribbean ciguatoxin-1 (C-CTX-1)/g flesh. Additionally a less and a more polar minor toxin were detected (De Fouw *et al.*, 2001).

Jamaica

In 1978, 250 people showed CFP symptoms after eating local grouper and barracuda. No mortality occurred (IPCS, 1984). Reports on CFP are rare in Jamaica with most outbreaks involving five to 18 persons (Pottier *et al*., 2001).

Martinique

Eighty intoxications were reported in 1982. In 1983, the annual incidence was estimated at 41 per 100 000 inhabitants per year (Pottier *et al*., 2001).

Mexico

CFP is present at both coasts of Mexico. There are poisoning cases every spring and summer season, both on the Pacific as well as Caribbean coasts (Sierra-Beltrán *et al*., 1998).

In 1974, 24 people on board of a ship were reported to show CFP symptoms after eating barracuda from the Gulf of Mexico. No mortality occurred (IPCS, 1984). In 1984, a total of 200 cases of ciguatera intoxication occurred in La Paz, Baja California Sur in Mexico after consuming contaminated snapper fish (*Lutjanus* sp.) (Ochoa *et al.,* 1998). In May 1993, the entire crew of a fishing boat became ill with symptoms resembling ciguatera after eating fish (*Serranidae* and *Labridae*) that were caught in the Alijos Rocks (west coast of the Baja California Peninsula) at depths fluctuating between 9 and 36 metres. After analysis of the suspected fish using the mouse bioassay, the presence of ciguatera-like toxins was confirmed (De Fouw *et al.,* 2001).

In July 1994, 10 cases of CFP occurred on the Isla de Mujeres after consuming barracuda. Between 20 minutes to 12 hours after eating the contaminated fish poisoning symptoms were reported. All suffered gastrointestinal disturbances as the main manifestation. Watery diarrhoea was the earliest complaint. Cold-to-hot temperature reversal dysesthesia occurred in all but there were differences in the occurrence and severity of other symptoms. No associations between the amount of toxic fish ingested with the latency period and the severity and duration of the symptoms were found (Arcila-Herrera *et al.,* 1998).

Twenty-five cases of ciguatera poisoning on the Pacific Coast of the USA as discovered by the Department of Health Services in San Diego (California) over a four-month period, were reported. All persons had eaten a fish called flag cabrilla captured at the coast of Baja California (Mexico). The persons suffered primarily from gastrointestinal symptoms (diarrhoea, vomiting, nausea) and neurological symptoms (extremity paresthesias, pruritis, paresis, dizziness, headache), one woman had bradycardia and hypotension (De Fouw *et al.*, 2001).

In the period from 1993 to 1996, in El Pardito, a small island complex in the Gulf of California, human CFP cases occurred after eating viscera of *Serranidae* and *Lutjanidae* fish (Sierra-Beltrán *et al*., 1998). In 1997, an outbreak of CFP involving 30 French tourists was reported (Pottier *et al*., 1997). Sierra-Beltrán *et al*. (1998) reported that the last outbreak in Mexico caused two deaths. Since the coasts of the country are frequently struck by hurricanes, it is possible that these conditions favour the spreading of the toxin producers *G. toxicus*, *O. ovata* or *P. mexicanum*.

Puerto Rico

CFP mostly involves the smaller islands. Between 1980 and 1982, 100 outbreaks involving 215 persons were recorded. An annual incidence of 90 per 10 000 inhabitants was estimated (Pottier *et al*., 2001).

Saint Barthelemy

About 30 patients per year are treated by physicians. The patients are mainly tourists or fishermen who have eaten groupers, mackerels, jacks or snappers. However, with avoidance of local risk fish and an increased import of fish into Saint Barthelemy, the incidence of CFP will be reduced (Pottier *et al*., 2001).

Saint Martin

The incidence of CFP is estimated to be two to five cases per 1 000 inhabitants per year (Pottier *et al.*, 2001).

Saint Vincent

In 1985, an outbreak of CFP with 105 patients after eating barracuda was reported (Pottier *et al*., 2001).

Venezuela

Two hundred cases of CFP on a cruise ship resulted in several fatalities (Farstad and Chow, 2001).

Virgin Islands

A household survey in the United States Virgin Islands showed an annual incidence rate of 730 per 100 000 population (De Fouw *et al.*, 2001). In 1981, 14 outbreaks of CFP involving 65 patients were reported after eating black fin snapper (Pottier *et al*., 2001). In 1982 and 1983, 33 and 51 people, respectively showed CFP symptoms after eating local carrang and/or snapper. No mortality occurred (IPCS, 1984).

A CFP incidence of 940 cases per 60 000 persons on St. Thomas and St. John was estimated in 1980, while in 1982 estimates varying from 73 per 10 000 to 360 per 100 00 inhabitants per year were reported (Pottier *et al*., 2001).

7.7.6 Middle East

Israel

Two families complained of a sensation of “electric currents”, tremors, muscle cramps, nightmares, hallucinations, agitation, anxiety and nausea of varying severity. The symptoms lasted for 12 to 30 hours and resolved completely. All patients had eaten rabbit fish ("aras"). The typical clinical manifestations along with the known feeding pattern of the rabbit fish suggested CFP (Raikhlin-Eisenkraft and Bentur, 2002).

7.7.7 Asia

China

In Hong Kong Special Administrative Region, 47 outbreaks of CFP involving 397 people were reported to the Department of Health from 1988 to 1992. Snapper had accounted for most (59.6 percent) of these outbreaks (Chan and Kwok, 2001).

A CFP incidence in Hong Kong Special Administrative Region occurred in humans a short time after consumption of a mangrove snapper caught in the South China Sea. All four persons became ill, showing the gastrointestinal and neurological features (nausea, abdominal pain, diarrhoea,

paresthesia and numbness of extremities) typical for CFP. One patient showed also a life-threatening bradycardia and hypotension (De Fouw *et al.,* 2001).

Eight family members showed signs of CFP after consuming a grouper. One out of these eight patients was treated in the hospital with mannitol and improvement of the clinical symptoms occurred initially. After one week some of the symptoms (mainly neurological) recurred and stayed on for 45 days after consumption of the toxic fish (Chan and Kwok, 2001).

Fiji

In 1984, 925 cases of CFP were reported after eating local snapper, barracuda, grouper and emperor. One person died (IPCS, 1984).

French Polynesia – New Caledonia (South Pacific)

Although a rare disease two centuries ago, ciguatera now has reached epidemic proportions in French Polynesia. In the period from 1960 to 1984, more than 24 000 patients were reported from this area (Hallegraeff *et al.,* 1995).

Four adult tourists developed CFP after eating contaminated fish in Vanuatu (Ting and Brown, 2001). In 1979, 3 009 people were affected by CFP by eating local fish (surgeon fish, parrot fish, grouper, snapper, carrang, emperor and barracuda). Three people died (IPCS, 1984).

Indian Ocean

Very little information is available on incidence in the islands in the Indian Ocean (Comores, the Seychelles, Mauritius and Rodrigues) but the annual incidence rate was estimated to be 0.78 per 10 000 residents (De Fouw *et al.,* 2001).

La Reunion (Indian Ocean)

After eating snapper from Salya de Malha, 367 people were affected by CFP in 1978. No mortality occurred (IPCS, 1984).

South Pacific Islands

The mean reported incidence rate of CFP for the South Pacific islands during a five-year period (1979 to 1983) was 97 per 100 000. The South Pacific Commission reported a mean annual incidence of 217 per 100 000 population in 1987. During the years from 1985 to 1990 the Pacific Islands of Kiribati, Tokelau and Tuvalu reported 90 to 100 cases per 10 000 population per year. In French Polynesia, Vanuatu, Marshall Islands, and Cook Islands the reported cases varied from approximately 35 to 50 per 10 000 population per year. Less than 20 cases per 10 000 population per year were reported for Fiji, Northern Marianas, New Caledonia, Wallis and Futuna, American and Western Samoa, Niue, Guam, Nauru, Fed. St. of Micronesia, Palau, Tonga and Papua New Guinea. Data are from the South Pacific Epidemiological and Health Information Service (De Fouw *et al.,* 2001).

7.7.8 Oceania

Australia

In Australia an annual incidence of 30 per 100 000 was estimated. The annual incidence in Queensland is reported to be about 1.6 cases per 100 000 population (De Fouw *et al.,* 2001). Each year, outbreaks of CFP occur from consumption of fish caught along the tropical coast of eastern Australia. In 1988, clinical details from a Queensland database of 617 cases from 225 outbreaks

collected over 23 years were published. Major outbreaks occurred in Sydney in 1987 (63 people affected) and 1994 (43 people affected), after the consumption of Spanish mackerel from Queensland (Lehane, 2000 and Lehane and Lewis, 2000).

An outbreak of CFP was reported after eating a single fish (coral cod) captured from the Arafura Sea (Northern Australia) causing 20 poisoning events. When a 230 g sample of the fish was analysed by mouse bioassay and LC/MS the presence of Pacific ciguatoxin-1 (P-CTX-1) was found. This was the first time that the toxin contributing to ciguatera in the Arafura Sea has been identified (De Fouw *et al.,* 2001).

More recently from July 1997 to August 1998, there were three small outbreaks of CFP in the inner Sydney area caused by reef fish. In all three incidents, diagnosis was based on clinical grounds. The first outbreak (six cases) was caused by coral trout from Fiji; the second outbreak (10 cases) by coral trout from Queensland and the third outbreak (10 cases) by spotted cod from Queensland. The third outbreak included two exclusively breastfed infants who exhibited symptoms two days after onset of their mother's symptoms (Karakis *et al.,* 2000).

In September 1997, an outbreak of CFP in outer Melbourne was traced to a 16.2 kg Maori Wrasse fish imported in Victoria from Trunk Reef in Queensland. Thirty individuals who attended a banquet at an Asian restaurant consumed at least one of four different dishes prepared from the flesh and viscera of the fish. All 30 reported one or more symptoms, mainly gastrointestinal symptoms and/or in 18 cases neurological symptoms. Seventeen cases were seen in four different hospitals and nine were treated with parenteral mannitol therapy. Nine out of eighteen cases were still symptomatic 10 weeks after the episode (Ng and Gregory, 2000).

Two male patients were admitted to a hospital in Herston, Queensland in 1998 with CFP symptoms including cardiac toxicity. In one patient, the cardiac symptoms resolved over three days and the non-cardiac-symptoms over the subsequent 14 days. In the second patient, all symptoms normalised within six weeks (Miller *et al.,* 1999).

From 1990 to 2000, in total 132 CFP cases in 10 outbreaks were registered. Not included in this total is an average of 48 annual cases of CFP estimated in Queensland each year, which will increase the ten-year total to 612 cases (Sumner and Ross, 2002).

New Zealand

Three imported cases were notified in 1997 (Crump *et al.,* 1999a). A 42 year old man was presented at a hospital in Christchurch with CFP symptoms three weeks after returning from Fiji. In Fiji, he developed CFP symptoms within three hours of eating barbecued fish. The patient required a period of respiratory supportive therapy. Dysesthesia of the hands and feet persisted for weeks but resolved after five days on amitriptyline (Crump *et al.,* 1999a).

Tonga

A CFP case associated with cindarian (jellyfish and related invertebrates) ingestion was reported. Cindaria have not previously been associated with direct ciguatera intoxication in humans. A 12 year old Tongan girl had eaten jellyfish about two hours prior to the presentation of gastrointestinal and neurologic symptoms characteristic for CFP. All other persons who had eaten the jellyfish were without symptoms, which might suggest that the girl had prior ciguatoxin intoxication with sensitization and re-emergence of symptoms with new exposure. Serum samples of the girl were drawn and examined for ciguatera toxins. Following discharge, serum ciguatera toxin assay result was 3.5 (on a 1 to 5 scale), strongly positive, and comparable with values previously obtained from acute CFP victims. Attempts to obtain a portion of the ciguatoxic

jellyfish served at meal, and to further specify the source or species of the jellyfish to evaluate ciguatoxin contamination were unsuccessful (De Fouw *et al*., 2001).

7.8 Regulations and monitoring

Very few specific regulations exist for ciguatera toxins (Van Egmond, *et al.*, 1992). In some areas, public health measures have been taken that include bans on the sale of high risk fish from known toxic locations. Such bans have been used in American Samoa, Queensland, French Polynesia, Fiji, Hawaii and Miami. The bans were apparently with some success but with attendant economic loss (De Fouw *et al*., 2001).

7.8.1 Europe

In the EU, Council Directive 91/493/EEC (EC, 1991b) is in force, laying down the health conditions for the production and the placing on the market of fishery products. This directive states: "The placing on the market of the following products shall be forbidden; fishery products containing biotoxins such as ciguatera toxins", without further specific details about the analytical methodology.

In France, this directive is incorporated in French legislation and it is applicable for products imported from outside the EU. The regulation permits the import of certain marine fish species, for which a positive list exists (De Fouw *et al*., 2001).

7.8.2 North America

The United States of America

Hawaii, Puerto Rico and Florida are the principal locations affected. There are neither standards, nor an official method. For this reason, there are no effective testing programmes for CFP, and the most widespread sanitary measure applied for its prevention is the prohibition of the sale of fish species known to be potentially toxic, or for which some CFP outbreaks have been reported (Fernández, 1998; Van Egmond *et al*, 1992)

In Hawaii a limited programme has been instituted using an immunoassay. Fish testing positive are considered unsafe and removed from the market (Van Egmond *et al.*, 1992).

7.8.3 Oceania

Australia

In Platypus Bay, Queensland, a ban has been imposed on the capture of the ciguateric fish species Spanish mackerel (*Scomberomorus commersoni*) and barracuda (*Spyraena jello*) to reduce the adverse impacts of ciguatera. Reef carnivores such as the moray eel, chinaman, red bass and paddletail fish have long been considered regular ciguatera carriers and are now not sold by marketing authorities in Australia (De Fouw *et al*., 2001).

8. Risk Assessment

The allowance levels currently valid for phycotoxins are based mainly on data derived from poisoning incidents. However, these data are seldom accurate and complete, and mainly restricted to acute toxicity. In some cases, the allowance level is also adapted to the limitations of the detection method. For risk assessment purposes, human intake levels of (shell)fish should be standardized.

8.1 Risk Assessment for Paralytic Shellfish Poisoning (PSP)

Currently the toxicological risk evaluation for PSP toxins can only be based on acute toxicity data. Sub-chronic and chronic data for animals as well as humans are not available. Lowest doses causing mild symptoms of PSP in humans vary between 120 and 304 µg/person and lowest doses associated with severe intoxications/fatalities vary between 456 and 576 µg STX/person. In order to protect more susceptible persons (children, elderly, unhealthy) usually an uncertainty factor of 10 is applied for calculation of TDI values for contaminants, based on human data. However, for PSP the calculations are complicated by the following factors: at what levels should the effects be considered as "adverse", and what level is the actual NOAEL and LOAEL? On the other hand, since the data on PSP represent many individuals, displaying large differences in susceptibility, an uncertainty factor of 10 may not be needed (Aune, 2001). Most countries apply a tolerance level of 80 µg STX eq/100 g mussel meat. If the consumption of shellfish is estimated to be between 100 and 300 g/meal, a margin of safety of about < 1 to 3.8 toward mild symptoms is present and, more important, a margin of safety of only 1.9 to 7.2 toward serious intoxications or death. These margins are quite small or there is no margin at all.

However, it is neither practical nor realistic to establish a very low tolerance level because the mouse bioassay is currently the most widely used method to determine PSP toxins and the present detection limit of this assay is approximately 40 σg PSP (STX eq)/100 g shellfish. Once more sensitive (and reliable) analytical chemical methods are available, the toxicity figures of STX and derivatives after acute and (sub)chronic exposure should be re-evaluated.

8.2 Risk Assessment for Diarrhoeic Shellfish Poisoning (DSP)

The various toxins in the DSP complex can be divided into three groups namely okadaic acid and the structurally related DTXs, the PTXs and the YTXs.

An EU Working Group on Toxicology of DSP and AZP has recommended allowance levels for these three groups of DSP toxins (EU/SANCO, 2001).

OA and DTXs

In animal experiments, cancer promoting and genotoxic effects of OA and DTXs are seen at relatively high doses and long exposure periods compared with the levels causing diarrhoea in humans shortly after consumption of contaminated shellfish. Consequently it is unlikely that a substantial risk of cancer exists in consumers of shellfish due to these toxins. Therefore, human risk assessment is based on a N(L)OAEL from animal or human data with the use of an uncertainty factor. Human data are preferred when available.

Taking into account all human exposure figures, it can be concluded that the lowest levels causing diarrhoeic effects in humans vary from 32 to 55 µg OA and/or DTX1. These figures have been

derived from Japanese and Norwegian human data. The effects seem to be restricted to diarrhoea, vomiting, headache and general discomfort. No serious and irreversible adverse health effects have been seen at these levels (EU/SANCO, 2001). Current European Regulations allow maximum levels of OA, DTXs and PTXs together of 160 µg OA eq/kg edible tissue. If the consumption of shellfish is estimated to be between 100 and 300 g/meal, there is a margin of safety of about < 1 to 3.4 toward the diarrhoeic effects. These margins are quite small or there is no margin at all. EU/SANCO (2001) stated that, if the level of OA and DTXs in shellfish is not higher than 16 µg/100 g shellfish meat, there is no appreciable health risk at a consumption of 100 g mussel meat/day.

PTXs

Concerning the PTXs, human toxicity data are not available. Therefore a safe level for humans is based on animal toxicity data. For toxins in the PTX group, data on animal toxicity are only available for PTX2. Effects such as tumour induction and tumour promoting are not known. The LOAEL for PTX2 by oral administration to mice was reported to be 0.25 mg/kg bw based on diarrhoeic effects and effects on the liver. The NOAEL should be estimated by applying a factor of 10 to the LOAEL. To extrapolate the animal data to human risks, a factor of 100 is applied. Thus, by applying an uncertainty factor of 1 000, a safe level of 0.25 µg/kg bw can be calculated for humans ~ 15 µg for an adult weighing 60 kg. EU/SANCO (2001) has recommended an allowance level of 15 µg/100 g shellfish meat. However, if the consumption of shellfish is estimated to be between 100 and 300 g per meal, the allowance level has to be between 5 and 15 µg/100 g edible shellfish tissue.

For PTX2 seco acid (PTX2-SA), human exposure data are available from a pipi shellfish poisoning event (56 cases of hospitalisation) in New South Wales (Australia) in December 1997 (ANZFA, 2001). According to Quilliam *et al*. (2000), PTX2-SA may have contributed to the gastrointestinal symptoms, vomiting or diarrhoea in humans (Aune, 2001). Burgess and Shaw (2001) reported that the patients consumed approximately 500 g of pipis containing 300 µg PTX-2SA/kg (~150 µg PTX-2SA/person ~2.5 µg/kg bw for a 60 kg weighing person). A safe level for humans of 0.025 µg/kg bw for PTX-2SA can be calculated by applying an uncertainty factor of 100 (10 for intraspecies differences and 10 for extrapolation from LOAEL to NOAEL) (~1.5 µg/person weighing 60 kg). This means that for PTX2-SA, the allowance level has to be between 0.5 and 1.5 µg/100 g edible tissue at consumption between 100 and 300 g per meal.

YTXs

For the YTXs, no human data are available. Therefore, a safe level in humans is based on animal data. The NOAEL in mice by acute oral administration was estimated to be 1.0 mg/kg bw based on cardiac effects. A safe level for humans towards acute toxic effects of YTX is calculated to be 10 µg/kg bw by applying an uncertainty factor of 100. For an adult weighing 60 kg, this would mean a safe level of 600 µg YTX. In view of the lack of data on repeated administration and a high uncertainty factor recommended by WHO for a substance that injures cardiac muscles, the calculated safe level for humans given above could be lowered by a factor 6 to 100 µg (EU/SANCO, 2001). EU/SANCO (2001) recommended an allowance level of 100 µg YTXs/100 g shellfish meat. However, if the consumption of shellfish is estimated to be between 100 and 300 g per meal, the allowance level has to be between 33 and 100 µg/100 g edible shellfish tissue.

8.3 Risk Assessment for Amnesic Shellfish Poisoning (ASP)

The generally applied guideline value of 20 mg DA/kg mussels is derived from an ASP incident in Canada (Prince Edward Island) and is taken on by several other countries. The guideline level of 20 mg DA/kg is equal to an intake of 0.03 to 0.1 mg DA/kg bw per person with a body weight of 60 kg assuming that consumption of mussels is between 100 and 300 g/meal. The epidemiological data used to derive the guideline value, revealed mild gastrointestinal effects in humans at 1 mg DA/kg bw. Afterwards the guideline value was supported by acute studies in animals. However, when doses required to cause overt toxicity in animal species were compared, mice and rats appeared to be relatively insensitive compared with monkeys and oral dosing required more toxin (more than 10 times in rodents) to achieve the same effects as i.p. dosing. Rats showed overt effects of DA poisoning at single oral doses of about 80 mg/kg bw, whereas monkeys showed vomiting, gagging and yawning already at 1 mg/kg bw. A single oral dose of 0.75 mg DA/kg bw in monkeys did not induce overt effects. This apparent decreased sensitivity in rodents may be the result of their inability to vomit and/or the finding that the plasma half-lifetime of DA in the rat is about 6 times less than that in the monkey. Comparing the guideline value of 20 mg DA/kg of mussel tissue (~ 0.1 mg/kg bw for humans assuming a consumption of 300 g mussels per meal) with the no-effect dose (0.75 mg/kg bw) in acute oral studies in monkeys, a factor smaller than 10 is between these figures. There is no knowledge of the effects of long-term exposure to low levels of DA. However, short-term animal studies with repeated exposure do not point to altered DA clearance from serum or greater neurotoxic responses than after single exposures.

Reasonable good dose-response data were determined for 10 persons involved in the Canadian incident (elderly people, aged from 60 to 84 years). According to these data the NOAEL is 0.2-0.3 mg DA/kg bw, while the LOAEL was 0.9-2.0 mg DA/kg bw and serious intoxications were recorded at 1.9 to 4.2 mg DA/kg bw. Interestingly, the intake estimates showed surprisingly large consumption of blue mussels, 120 to 400 g mussel meat per person per meal (Aune, 2001). This means that there is a factor two between the NOAEL and the regulatory limit of 20 mg DA/kg mussel meat which is equivalent to 0.1 mg/kg bw for a 60 kg weighing person with a mussel meat consumption of 300 g per meal. Between the LOAEL and the regulatory limit there is a margin of 9 to 20 and between the level of serious effects and the regulatory limit there is a margin of 19 to 42.

8.4 Risk Assessment for Neurologic Shellfish Poisoning (NSP)

Based on the lack of sufficient data on toxicity and the analytical difficulties in determining brevetoxin exposure, risk assessment is not possible. Current risk management (in states on coasts of the Gulf of Mexico) is based on shellfish bed closures at 5 000 *G. breve* cells/litre with reopening based on determination of PbTx in shellfish at <80 µg/100 g.

8.5 Risk Assessment for Azaspiracid Shellfish Poisoning (AZP)

EU/SANCO (2001) stated that based on poisoning incidents in Ireland, levels of AZAs causing human intoxication were calculated to be between 6.7 and 24.9 µg. These figures included a reduction in AZA content due to heating of the mussels. New data on heat stability revealed that this reduction of the toxin content due to heating was not justified. Therefore the recalculated range of the lowest observed adverse effect level (LOAEL) appeared to be between 23 and 86 σg per person assuming a maximum consumption of 100 g shellfish per meal. EU/SANCO (2001) applied a safety factor of three to convert the LOAEL to a NAOEL. Based on an intake level of a maximum of 100 g shellfish meat/meal, and the lowest LOAEL divided by three, EU/SANCO (2001) stated that an allowance level of 8 σg AZAs/100 g of shellfish should result in no

appreciable risk for human health. To allow for detection by the mouse bioassay a level of 16 µg/100 g was proposed. However at a shellfish consumption of 300 g per meal, a person will consume already an amount of AZAs equal to the LOAEL in humans.

Ofuji *et al*. (1999b) reported a level for total AZAs in raw mussel meat in poisoning incidents of 1.4 µg/g of meat. At a consumption of 100 to 300 g per meal this means an intake of 140 to 420 µg AZAs/person. As these figures represent an effect level (LOAEL) usually a factor 10 is used for calculation of a NOAEL. This means that the NOAEL is 14 to 42 µg per person assuming a consumption of 100 to 300 g shellfish meat/meal. As a consequence the allowance level in shellfish meat has to be 14 µg/100 g. It has to be noted that no factor of 10 was applied to the NOAEL for intraspecies differences (variation in the human population).

8.6 Risk Assessment for Ciguatera Fish Poisoning (CFP)

The available animal data on ciguatoxin are not suitable for risk assessment. Therefore, human data derived from poisoning incidents should be used.

Mild CFP symptoms in some persons can be already expected after consuming fish containing the main Pacific ciguatoxin (P-CTX-1) at a level of 0.1 µg/kg. The main Caribbean ciguatoxin (C-CTX-1) is less polar and 10-fold less toxic than P-CTX-1. Assuming a fish consumption of 500 g per meal and a human body weight of 50 kg, this corresponds to 0.001 µg/kg bw (=LOAEL). These figures are derived from a large serving of the least toxic fish causing effects in some people. A level of 0.01 µg/kg bw, which is ten times the level causing mild symptoms in some persons, would be expected to be toxic in most people. By applying an uncertainty factor of 10 (for intraspecies differences) to the lowest level causing mild symptoms in humans (=LOAEL), a "safe" level of 0.01 µg/kg of fish flesh can be calculated (Lehane, 2000; Lehane and Lewis, 2000). It has to be noted that the usual application of an uncertainty factor of 10 to the LOAEL for calculation of a NOAEL was not performed.

8.7 Concluding remarks

At present the risk assessment of phycotoxins has not been performed in a straightforward way. Risk management and risk assessment have been mixed in the process complicating the procedure. In general, there is a lack of toxicological data particularly on repeated exposure. Epidemiological data mainly existed of poisoning incidents with their inherent limitations. This all cumulated in provisional risk assessments of certain phycotoxins which were not always logic and consistent. For some phycotoxins, even the lack of minimal data has prohibited risk assessment.

If adequate scientific (toxicological, epidemiological and occurrence) data are available, a risk assessment can be performed by applying generally accepted safety or uncertainty factors. An adequate set of animal data will allow the derivation of a no-observed adverse effect level (NOAEL). A safe level for humans can be calculated by applying an uncertainty factor of 100 (10 for interspecies differences and 10 for intraspecies differences) to the NOAEL. If an adequate set of human data is available, a safe level for all humans can be calculated by applying an uncertainty factor of 10 (for intraspecies differences) to the NOAEL, derived from those human data.

9. Conclusions and Recommendations

9.1 Conclusions

Consumption of a variety of shellfish and fish causes an increasing number of human intoxications around the world. Diagnosis depends mainly on recognition of specific signs and symptoms and on identification of marine toxins present in remains of the seafood involved. Indicators for effects and exposure are usually not available due to inadequate analytical methods for the sometimes complex algal toxin mixtures. The effects of algal toxins are generally observed as acute intoxications. Health effects of episodic exposure and chronic exposure to low levels of algal toxins are hardly known. The latter effects may go unreported by the affected individual(s) or may be misdiagnosed by physicians.

Monitoring seafood for toxicity is essential to manage the risks. However, there are several limitations in monitoring for toxicity such as the variation in toxin content between individual shellfish, different detection and even extraction methods for the various toxins requiring a decision which toxins one is testing for, and the frequency of sampling to ensure that toxicity does not rise to dangerous levels in temporal or spatial gap between sampling times or locations. Furthermore, the growing harvest of non-traditional shellfish (such as moon snails, whelks, barnacles, etc.) may increase human health problems and management responsibilities.

Monitoring for toxic plankton may possibly overcome some of these problems. However, plankton populations are patchy and ephemeral, it is difficult to make a quantitative correlation between numbers of toxic plankton and levels of toxins in seafood and the amount of toxin per cell can vary widely. Data on the occurrence of toxic algal species may indicate which toxins may be expected during periods of algal blooms and which seafood products should be considered for analytical monitoring. A problem is that certain algal species, which have never occurred in a certain area, may suddenly appear and then rapidly cause problems. The plankton observations are used to focus toxicity testing, but are not in themselves used for regulatory decisions. Moreover, most monitoring and regulatory programmes often are not adequate to meet the expanding threat of new harmful algal blooms. As a result, when new outbreaks occur, the response is often uncoordinated and slow. Harmful algal blooms cannot be predicted and there is little information on bloom initiation.

Toxic blooms are mostly detected by visual confirmation (water discolouration and fish kills), illness to shellfish consumers and/or human respiratory irritation with actual toxicity verified through time-consuming mouse bioassays and chemical analyses in shellfish samples. This “after-the-fact” strategy is the consequence of the extremely difficult prediction of the occurrence and magnitude of a bloom. To prevent human intoxication, monitoring programmes relying on enumeration and microscopic identification of harmful taxa in water samples generally suffice. However microscopic based monitoring requires a high level of taxonomic skill, usually takes considerable time, and can be highly variable among personnel.

One of the most serious problems is the lack of information on the biology of harmful algae. For example, little is known about the abundance, distribution, population dynamics and physiology of most of the harmful species, both in local waters and elsewhere. Long-term, routine monitoring of phytoplankton and the environment is essential to obtain data necessary to determine even the most elementary ecology of harmful species. Moreover, because bloom dynamics are complex, the factors that determine bloom dynamics of a species in one geographic area may not affect that

species in another area, even though the areas are not widely separated. Therefore alternative evaluation systems for predicting bloom occurrences are highly desirable.

In establishing regulatory criteria and limits for marine toxins, various factors play a role such as the availability of survey data, the availability of toxicological data, the distribution of the toxins throughout sampled lots and the stability in the samples, the availability of analytical methods and regulations already in force in several countries. With respect to toxicity, until now only data on the acute oral toxicity both in experimental animals and humans are available for the majority of the marine toxins. However, repeated exposure to lower sublethal dose levels may be a common feature.

Concerning detection methods, there is a general, worldwide need for rapid, reliable and sensitive methods to determine marine toxins in (shell)fish. The present mouse bioassay is not sensitive enough, shows a considerable variation, is time consuming, is vulnerable to interferences and is unethical in terms of animal welfare. Quilliam (1998b) argues for LC-MS as a universal detection method for all marine toxins. This technique has a low limit of detection, high selectivity and the ability to deal with the structural diversity and labile nature of the toxins. In addition, separation of complex mixtures, accurate and precise quantitation, automation and high throughput, legal acceptability for confirmation and structural information of new toxins are possible with this method. Another new approach that seems promising is the development of biosensors with which multiple toxins can be determined simultaneously.

The development and introduction of adequate and efficient analytical methods can be accelerated by providing information in a fast and proper way, for instance by setting up an Internet accessible database. The database should include parameters such as (chemical) names, physical/chemical properties, classification(s), toxic effect(s), sources, habitat, regulatory limits and literature references.

9.1.1 Conclusions related to Paralytic Shellfish Poisoning (PSP)

The tolerance levels set for PSP toxins thus far are largely pragmatic decisions based on intoxication events, and although there are many reported cases of human intoxications due to shellfish toxins, it is difficult to obtain *reliable* human toxicity data. For example, variations in observed toxicity of PSP toxins to humans may be due not only to variable sensitivity between people, but also to the composition of individual toxins in the samples. Toxin profiles can vary according to the species of shellfish consumed and the area of harvest. In addition, toxic doses are often estimated from left-over toxic seafood. This is not necessarily representative of the ingested food because PSP toxins may be unevenly distributed throughout lots and within individual shellfish, and not all PSP toxins are stable.

It is possible to measure PSP compounds by a number of analytical-chemical methods but they all have some limitations, and they often cannot easily be operated because of the lack of reference materials, although recently some progress has been made in this area. In 2003, certified standards of STX, neoSTX, dc-STX, GNTX 1-4, GNTX 2/3 and GNTX 5 are commercially available. However, they are expensive and mainly available from one source. Yet, their availability significantly improves the quality of the data that are obtained by LC-methodology. The efforts undertaken by the European Commission's SMT Programme have led to shellfish reference materials with certified mass fractions of some of the toxicologically most significant PSP toxins. Despite these positive developments, the analytical situation remains difficult and the lack of pure PSP compounds in sufficient quantities for repeated dose toxicity studies is a limiting factor in the development of reliable risk assessment.

9.1.2 Conclusions related to Diarrhoeic Shellfish Poisoning (DSP)

The variety in biological activities of the DSP toxins may cause some problems. Although PTXs and YTXs are acutely toxic to mice after i.p. injection, their oral toxicity to humans is unknown. Therefore, more toxicological data on PTXs and YTXs have to become available. Furthermore OA and DTX possess tumour promoting activity and OA shows also genotoxic and immunotoxic activity. These effects raise questions as to the human health risks of (sub)chronic exposure to low levels of these compounds. A pressing problem is the lack of sufficient quantities of DSP toxins to perform (sub)chronic animal toxicity studies.

Although mammalian bioassays for DSP toxicity are applied worldwide, there are large differences in performance of, for instance, the mouse bioassay (toxicity endpoint is animal death; no consensus on appropriate observation time) among different countries, resulting in differences in specificity and detectability. A major problem is the fact that the mouse bioassay detects all DSP components and probably also other toxins. However, it is not possible to distinguish between the various toxins whereas specific legal limits for the toxin groups have been established (for instance in the EU). On the other hand, the rat bioassay detects only OA and DTXs because the endpoints in this assay are soft stool, diarrhoea and feed refusal which effects are known to be caused by OA and DTXs only (and AZAs).

Chemical methods (LC) are useful for identification and quantification of selected diarrhoeic toxins (usually OA or DTXs). Recently an LC method for the detection of YTXs was developed, but until now no method for PTXs is available except an LC-MS method; however its performance is not yet satisfactory. Chemical methods are applied as a regulatory tool primarily for confirmation of the results obtained in a bioassay.

None of the many approaches to determine DSP toxins in shellfish has been evaluated in a formal collaborative study according to ISO/IUPAC/AOAC so that the performance characteristics are not fully known. The further development, evaluation and comparison of the various techniques would become significantly easier if reliable reference standards and reference materials (such as lyophilized mussel samples with certified contents of several DSP toxins) could be developed and made available to the scientific community.

9.1.3 Conclusions related to Amnesic Shellfish Poisoning (ASP)

Compared to the paralytic and diarrhoeic shellfish poisons, problems with amnesic shellfish poisons seem to be of a lesser magnitude. Only one confirmed outbreak of ASP causing severe illness in exposed people was reported worldwide, specifically in Prince Edward Island, Canada in 1987. After the first outbreak in Canada, human illnesses (mild and short lived) were only observed in one outbreak, specifically after consumption of contaminated razor clams (from the West Coast of the United States). However, health authorities were not able to confirm that the illnesses were caused by DA. In two outbreaks, the death of cormorants and/or brown pelicans due to the consumption of contaminated anchovies or mackerel was reported indicating that herbivorous fish can act as vectors for DA. In the last few years (1999 to 2002), DA was detected also in shellfish from some European countries.

Methods of analysis for DA are rather straightforward and less complex than those for paralytic and diarrhoeic shellfish poisons. One chemical method for DA in mussels (LC with UV detection) has been successfully validated in a formal collaborative study, whereas another (improved) method is currently subject to a collaborative study. Certified reference materials and calibrants are readily available.

9.1.4 Conclusions related to Neurologic Shellfish Poisoning (NSP)

When humans are exposed to brevetoxins, different exposure routes are possible; the oral route via consumption of contaminated shellfish, the inhalatory route via exposure to aerosolised brevetoxins, and the dermal route via direct contact with contaminated seawater. The effects of the various exposure routes on humans are difficult to assess because toxicity data for brevetoxins are limited. Some acute studies in mice and data from poisoning cases in humans and (marine) mammals are available but acute dermal and inhalation studies are lacking, as well as oral, dermal and inhalation studies with repeated exposure of laboratory animals. Therefore reliable hazard assessment is not possible.

Pure toxins and toxin metabolites would be needed to be able to carry out toxicity studies. In addition, analytical reference materials would be needed to further develop and improve the analytical methodology and to allow analytical quality assurance of monitoring laboratories. Currently the various obstacles on the way to reliable assessment of brevetoxin occurrence and exposure further hamper risk assessment and thus the establishment of meaningful regulations.

Despite these problems, regulations for NSP toxins in shellfish are in force in a few countries, specifically the USA, Italy and New Zealand based on the mouse bioassay. The action level is 20 MU/100 g shellfish flesh (~80 σg PbTx-2/100 g shellfish flesh).

9.1.5 Conclusions related to Azaspiracid Shellfish Poisoning (AZP)

One cause for concern is the lung tumours found in mice after repeated doses of 20 µg AZA/kg bw and higher. This finding should be confirmed in experiments with larger numbers of mice and longer exposure periods (Ito *et al*., 2002).

The current allowance level has to be revised as new data become available. However, the lack of supply of pure toxins is a serious obstacle to all kinds of studies. The production of pure toxins, in turn, depends on the availability of large amounts of toxic mussels. Development of rapid detection methods such as LC-FLD, ELISA and functional assays should be explored.

9.1.6 Conclusions related to Ciguatera Fish Poisoning (CFP)

Ciguatera poisoning mainly occurs in tropical regions throughout the world and is sporadic in Europe, particularly in the Northern European countries. Therefore, a regular analytical check on the presence of ciguatoxins in imported large predatory fish from endemic areas is considered adequate in countries which are not an endemic area for CFP.

A few specific regulations exist for ciguatoxins. A positive finding in a fish would remove that fish from sale. In some cases, restrictions are placed on the sale of fish of certain species or size from a given area, with no testing of the toxin. The larger a fish is, the older it probably is, and the more toxin it has probably accumulated. Reef carnivores considered being regular ciguatoxin carriers are often banned from sale as a matter of principle. The hazard is linked to the accumulation in the food chain of a toxin, which is impossible to link with any algal bloom. Cell counting of plankton will not predict when a fish has accumulated ciguatoxins or not (Boutrif and Bessey, 2001).

9.2 Recommendations

Based on the preceding conclusions, the following recommendations are presented:

1. Data on bloom development with respect to hydrographic and climatic conditions, and nutritional status of the water column are needed.
2. Toxicity studies on effects after repeated exposure to marine toxins should be performed.
3. Chemical analytical techniques capable of separating, identifying and quantifying individual marine toxins should be further developed
4. As alternatives to rodent assays, assays have to be developed to be used when uncharacterized bloom events occur. Emphasis on the use of in vitro techniques where blooms have been characterized should reduce the use of test systems with live animals.
5. To facilitate fast application of adequate analytical methods for marine biotoxins, a database should be developed including basic data on marine biotoxins such as chemical structures, physical/chemical properties and analytical methods.
6. Both for the submission of toxicity data and for the development and validation of analytical techniques, the production of pure toxin standards and certified reference material are required.
7. Formal risk assessments of the marine biotoxins should be performed by recognized international bodies – such as the Joint FAO/WHO Committee on Food Additives (JECFA) and the European Food Safety Authority (EFSA) – and should be based on sound scientific data of toxicity and exposure. In the absence of sufficient data, an expert consultation could be considered in order to explore the possibilities for adequate risk assessment which should be the basis for meaningful regulations.

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