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Neurotoxic shellfish poisoning:
A review

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Abstract

This review contains information on the neurotoxic shellfish poisoning (NSP) syndrome and the provoking toxins called brevetoxins, produced by the dinoflagellate *Gymnodinium breve*. Data on chemical structures and detection methods for brevetoxins, sources for brevetoxins, marine organisms associated with NSP, toxicity of brevetoxins for animals and man, possible preventive measures for NSP, case reports/outbreaks of NSP and regulations and monitoring for NSP are included. Finally some recommendations are given for a better control of the NSP problem in the future.

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Samenvatting

"Neurotoxic shellfish poisoning" (NSP) wordt veroorzaakt door brevetoxines welke worden geproduceerd door de dinoflagellaat *Gymnodinium breve*. De brevetoxines zijn depolariserende stoffen die potentiaal-afhankelijke natriumionkanalen in celwanden openen. Daardoor veranderen de membraan eigenschappen van exciteerbare celtypes zodanig dat de inkomende stroom van natriumionen in de cel wordt versterkt; dit effect kan worden geblokkeerd door externe toediening van tetrodotoxine. De brevetoxines werken op bindingsplaats 5 van de potentiaal-afhankelijke natriumionkanalen in een 1:1 stoichiometrie. De brevetoxines blijken hun sensorische effecten te veroorzaken door snelle natriumkanalen te veranderen in langzamere kanalen, hetgeen resulteert in langdurige activering en repetitieve activiteit. Na verloop van tijd zijn de exciteerbare cellen niet langer in staat actiepotentialen te geleiden hetgeen resulteert in een aantal symptomen zoals misselijkheid, braken, diarree, koude rillingen, transpireren, omgekeerde temperatuurwaarneming, lage bloeddruk, aritmie, tintelingen, verlammingen van lippen, gezicht en extremiteiten, krampen, bronchoconstrictie, toevallen en coma en in proefdieren hart- en ademhalings-stilstand en sterfte. In mensen werden geen chronische verschijnselen of sterfte waargenomen. De brevetoxines zijn toxisch voor vis, (mariene) zoogdieren, vogels en mensen, maar niet voor schelpdieren. Schelpdieren accumuleren de brevetoxines door "filter-feeding" van phytoplankton. Wanneer de gecontamineerde schelpdieren door de mens worden geconsumeerd, kan NSP optreden. De symptomen van NSP lijken op die van "paralytic shellfish poisoning" en ciguatera vergiftiging maar ze zijn minder ernstig. Brevetoxines verschillen van de meeste andere dinoflagellaat toxines door het feit dat ze door de lucht kunnen worden verspreid. Ten gevolge van de breekbaarheid van het *G. breve* organisme kan dit gemakkelijk worden open gebroken in de ruwe branding waarbij de toxines vrijkomen als aerosol. Wanneer mensen worden blootgesteld aan dit aerosol kan irritatie van ogen en ademhalingswegen optreden. Ook door direct contact tijdens het zwemmen kan irritatie van slijmvliezen van oog en neus optreden.

Naast *G. breve* blijken ook andere *Gymnodinium* soorten, namelijk *G. nakaniense* en een onbekende *Gymnodinium* soort, dezelfde irritatieeffecten in mensen na blootstelling aan het aerosol te veroorzaken als *G. breve*. Verder zijn er ook algensoorten behorende tot de klasse Raphidophyceae (raphidophieten), nl. *Chattonella antiqua*, *Chattonella marina*, *Fibrocapsa japonica* en *Heterosigma akashiwo*, die toxines produceren met vergelijkbare Rf waarden en retentietijden, in respectievelijk TLC en HPLC bepalingen, als de brevetoxines. De toxines die door de laatstgenoemde algensoorten worden geproduceerd zijn zeer toxisch voor vissen maar gevallen van vergiftingen van mensen door de toxines van deze algensoorten zijn tot nu toe nog niet gerapporteerd.

Tot enkele jaren geleden werden NSP uitbraken voornamelijk gezien aan de kusten van Florida en de Mexicaanse Golf in de USA. Later werd NSP ook waargenomen in North Carolina, USA. In de zomer van 1992-1993 trad een uitbraak van NSP op in Nieuw Zeeland.

De brevetoxines zijn methyl-gesubstitueerde cyclische polyethers en er worden twee types onderscheiden. Type 1 (ook genoemd type A) heeft een flexibele "backbone" van 10 gefuseerde polyether ringen en type 2 (ook genoemd type B) heeft een rigide "ladder" van 11 ringen. Type 2 brevetoxines zijn de meest voorkomende in de natuur. In schelpdieren zijn ook brevetoxine-analogen gevonden. Deze analogen zijn waarschijnlijk metabolieten gevormd door de schelpdieren zelf. Ook in knaagdieren en vissen vindt biotransformatie van brevetoxines plaats, maar er zijn geen metabolieten geidentificeerd. Verder zijn er nog fosfor-bevattende stoffen geisoleerd uit *G. breve*. Één van deze fosforhoudende verbindingen bleek een organothiofosfaat te zijn met een zeer hoge vistoxiciteit.

Voor de detectie van brevetoxines in schelpdierweefsel is thans de muizen “bioassay” de meest, door regelgevende instanties, geaccepteerde test. Er wordt getracht de niet-specifieke muizen “bioassay” te vervangen door meer diervriendelijke, specifieke testen. Voor brevetoxines zijn een *in vivo* vissen test, immunoassays (RIA en ELISA assays), een *in vitro* neuroblastoma assay, een *in vitro* synaptosoom bindingsassay en verscheidene chemische bepalingsmethodes (MEKC, LC-ESMS, HPLC/MS/MS) ontwikkeld.
Toxiciteitsgegevens voor brevetoxines zijn beperkt. Enkele acute studies in proefdieren laten een orale LD_{50} in vrouwelijke muizen van 6600 en 520 μg/kg lichaamsgewicht zien voor respectievelijk PbTx-2 en PbTx-3. De intraperitoneale LD_{50} waarden voor PbTx-2 en PbTx-3 zijn respectievelijk 200 en 170 μg/kg lichaamsgewicht. Acute studies met inhalatoire blootstelling zijn niet beschikbaar. Mensen die oraal zijn blootgesteld aan brevetoxines door het consumeren van gecontamineerde schelpdierproducten, vertonen acute effecten. Echter een NSP vergiftiging in zeekoeien, waargenomen in 1996, deed veronderstellen dat de waargenomen effecten in deze diersoort niet alleen acuut waren maar dat ook chronische orale zowel als inhalatoire blootstelling de effecten zou hebben kunnen veroorzaakt. Om de effecten ten gevolge van herhaalde orale en/of inhalatoire blootstelling aan lage doses brevetoxines te kunnen inschatten zijn derhalve (sub)chronische dierexperimentele studies nodig. Door het ontbreken van getallen voor blootstelling en door het gebrek aan relevante toxiciteitsstudies kan geen risicoberekening worden opgesteld.

Summary

"Neurotoxic shellfish poisoning (NSP) is caused by brevetoxins that are produced by the dinoflagellate *Gymnodinium breve*. Brevetoxins are neurotoxic. The 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 flow can be blocked by external application of tetrodotoxin. The brevetoxins act on binding site 5 in a 1:1 stoichiometry. The toxins appear to produce their sensory symptoms by transforming fast sodium channels into slower ones, resulting in persistent activation and repetitive firing. Over time, excitable cells are no longer able to conduct action potentials, causing a series of symptoms including nausea, vomiting, diarrhea, chills, sweats, reversal of temperature, hypotension, arrhythmias, numbness, tingling, paresthesias of lips, face and extremities, cramps, bronchoconstriction, seizures and coma and in animals, cardiac and respiratory arrest, leading to death. No chronic symptoms or mortality in humans were reported. The brevetoxins are toxic to fish, marine mammals, birds and humans, but not to shellfish. Shellfish can accumulate the brevetoxins by filter-feeding of phytoplankton. When the shellfish is eaten by humans NSP may occur. The symptoms of NSP are somewhat similar to paralytic shellfish poisoning (PSP) and ciguatera poisoning but are less severe. Brevetoxins differ from most other dinoflagellate toxins in that they can become aerosolized. Due to the relative fragility of the *G. breve* organism it is easily broken open in the rough surf releasing the toxins. When humans are exposed to brevetoxins via the aerosol eye and respiratory tract irritation may be experienced. In addition during swimming direct contact with the brevetoxins may take place and eye and nasal membrane irritation can occur.

Besides *G. breve* some other *Gymnodinium* species viz. *G. nagakiense* and an unknown *G.* species, appeared to cause the same irritative complaints in man after exposure to the aerosol, as *G. breve*. Furthermore some algal species belonging to the class Raphidophyceae (raphidophytes) viz. *Chattonella antiqua*, *Chattonella marina*, *Fibrocapsa japonica* and *Heterosigma akashiwo*, produce toxins with similar Rf values and retention times on TLC and HPLC, respectively, as the brevetoxins. The toxins produced by the last mentioned algal species are severely ichthyotoxic but human cases of poisoning by the toxins of these algal species were until now, not yet reported.

Until several years ago outbreaks of NSP were reported exclusively on the coasts of Florida and the Gulf of Mexico in the USA. Later a case of NSP was reported from North Carolina, USA. In the summer of 1992-1993 a NSP outbreak occurred in New Zealand.

The brevetoxins are methylated cyclic polyethers and two types can be distinguished; type 1 (also called type A) containing a flexible backbone of 10 fused polyether rings and type 2 (also called type B) containing a rigid ladder of 11 rings. The type 2 brevetoxins are the most abundant in nature. Brevetoxin anologues have been detected in shellfish. These analogues are probably metabolites formed by the shellfish itself. In rodents and fish brevetoxins undergo also biotransformation but the metabolites were not identified. Furthermore some phosphorus containing toxic compounds were isolated from *G. breve*. One of these phosphorus containing compounds was identified and appeared to be an organothiophospate with a high ichthyotoxicity.

For the detection of brevetoxins in shellfish tissue currently the mouse bioassay is most widely accepted by regulatory authorities. Attemps are being made to replace the non-specific mouse bioassay with more humane, specific assays. For brevetoxins an *in vivo* fish

assay, immunoassays (a RIA and an ELISA assay), an *in vitro* neuroblastoma cell assay, an *in vitro* synaptosome binding assay and several chemical assays (MEKC, LC-ESMS, HPLC/MS/MS) have been developed.
Toxicity data with brevetoxins in mammals are limited. A few acute studies in experimental animals are available showing an oral LD_{50} value in female mice for PbTx-2 and PbTx-3 of 6600 and 520 μg/kg b.w., respectively, and an intraperitoneal LD_{50} value of 200 and 170 μg/kg b.w., respectively. Acute studies with inhalation exposure are not available.
Men exposed orally to brevetoxins by eating contaminated shellfish products, are reported to show acute effects. Effects in manatees, seen in 1996, did suggest that the effects might not necessarily be acute but might occur after chronic inhalation and/or ingestion. Therefore (sub)chronic oral and inhalation studies in experimental animals are needed to study effects after repeated exposure to low levels of brevetoxins.
Due to the lack of figures for exposure and relevant toxicity studies a risk assessment cannot be made.

1. Introduction

Microscopic planktonic algae are critical food for filter-feeding bivalve shellfish as well as for the larvae of commercially important crustaceans and finfish. In most cases, the proliferation of plankton algae (so-called "algal blooms"; up to millions cells per litre) therefore is beneficial for aquaculture and wild fish operations. However in some situations algal blooms can have a negative effect.

* There are algal species which produce basically harmless water discolourations; however, under exceptional conditions in sheltered bays, blooms can grow so dense that they cause indiscriminate kills of fish and invertebrates due to oxygen depletion.
* There are also algal species which produce toxins that can find their way through the food chain to humans, causing a variety of gastrointestinal and neurological illnesses, such as paralytic shellfish poisoning (PSP), diarrhoeic shellfish poisoning (DSP), amnesic shellfish poisoning (ASP), neurotoxic shellfish poisoning (NSP), ciguatera fish poisoning (CFP) and cyanobacterial fish poisoning.
* Furthermore, there are algal species, which are non-toxic to humans, but harmful to fish and invertebrates (especially in intensive aquaculture systems) by damaging or clogging their gills.

Unfortunately there is no clear cut correlation between algal concentrations and their harmful effects.
In the past two decades quantity and extent of large-scale growth occurrences or blooms were increased and many organisms not known to have bloomed in the past, or not indigenous to a particular area, were now blooming with unexpected frequency and at unusual locations.
Among the 5000 species of extant marine phytoplankton, some 300 species can at times occur in such high numbers that they obviously discolour the surface of the sea (so-called "red-tides", which are not only red but can be also brown, yellow, green or milky in color), while only 40 or so species have the capacity to produce potent toxins that can find their way through fish and shellfish to humans (as cited from Hallegraeff, 1995).
Mussels, clams and oysters particularly are target to accumulation of algal toxins because they feed by filtering particles, including phytoplankton, from the water. Toxic phytoplankton accumulates in the digestive system of these filter-feeders and subsequently causes illness or death to consumers such as birds, marine mammals and man (as cited in Van der Vyver *et al*., 2000).
Durborow (1999) reported that during the past 15 years, the number of dinoflagellates known to be toxic to fish, has increased to 55. It is estimated that worldwide there are about 2000 cases of shellfish poisoning in man caused by algal toxins each year and about 15% of these cases are fatal. Tibbets (1998) reported that about 60-80 algal species are toxic.
In this report a review is given on the knowledge of neurotoxic shellfish poisoning (NSP) toxins with respect to origin, occurrence, chemical structure, analysis, toxicity and existing regulations.
Neurotoxic shellfish poisoning (NSP) is caused by polyether brevetoxins produced by the unarmoured dinoflagellate *Gymnodinium breve* (also called *Ptychodiscus breve*). The brevetoxins are toxic to fish, marine mammals, birds and humans, but not to shellfish.. Until 1992/1993 neurotoxic shellfish poisoning (NSP)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 climatological conditions (higher than usual rainfall, lower than usual temperature) coinciding with an El Nino event (as cited from Hallegraeff, 1995).

2. The NSP Toxins

2.1 Chemical structures and properties

2.1.1 Brevetoxins

The NSP toxins, called brevetoxins, are tasteless, odorless, 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 10-11 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; as cited in Fleming and Baden, 1999) (see Figure 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*.

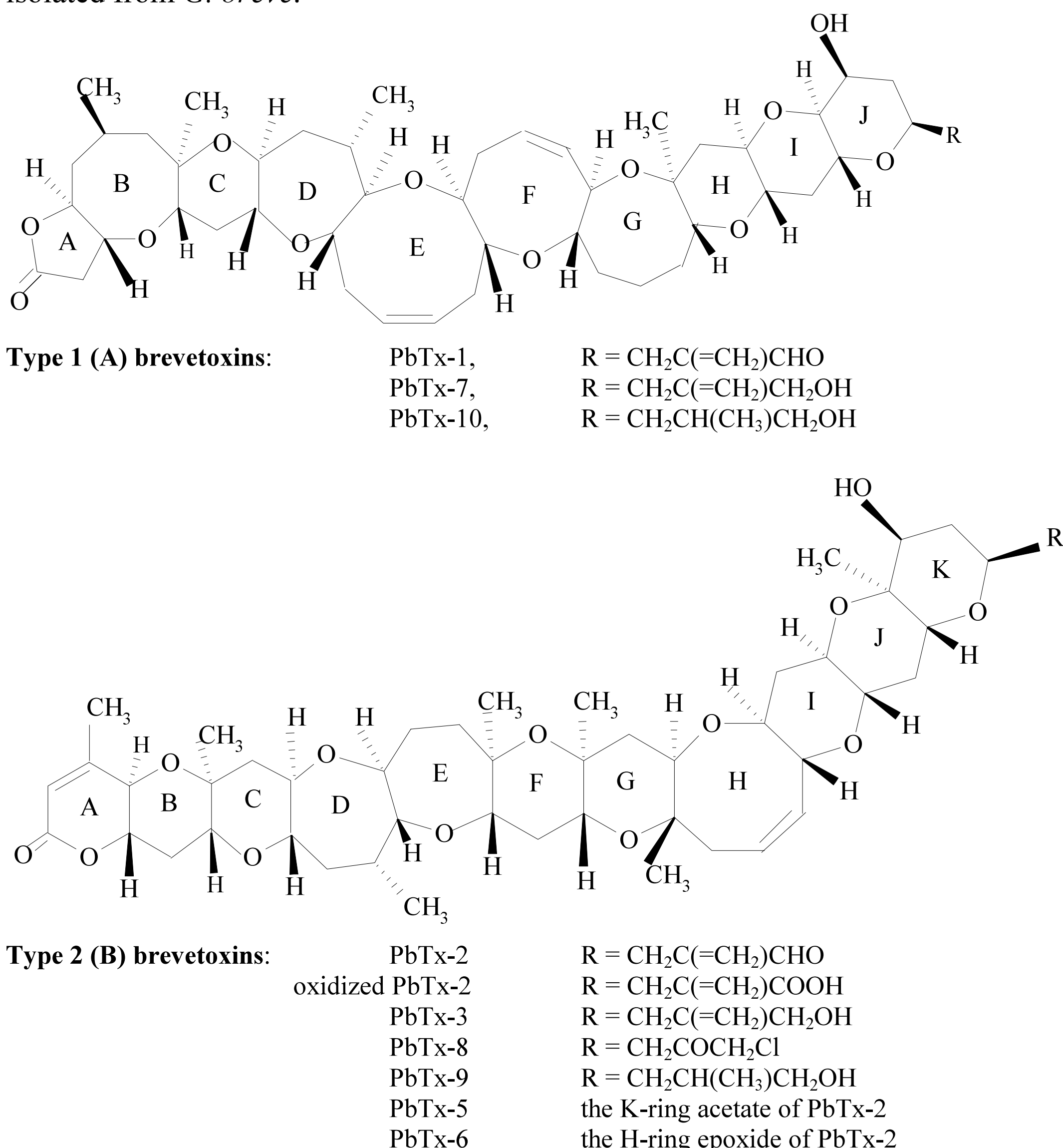

Figure 1. Chemical formulas of type A and B brevetoxins(from Hua et al., 1996)

2.1.2 Brevetoxin analogues

Four brevetoxin analogues (see Figures 2a and 2b) were isolated only from contaminated shellfish and not from field blooms or *G. breve* cultures. The shellfish was derived from NSP incidents in New Zealand. The brevetoxin analogues were analyzed 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. 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).

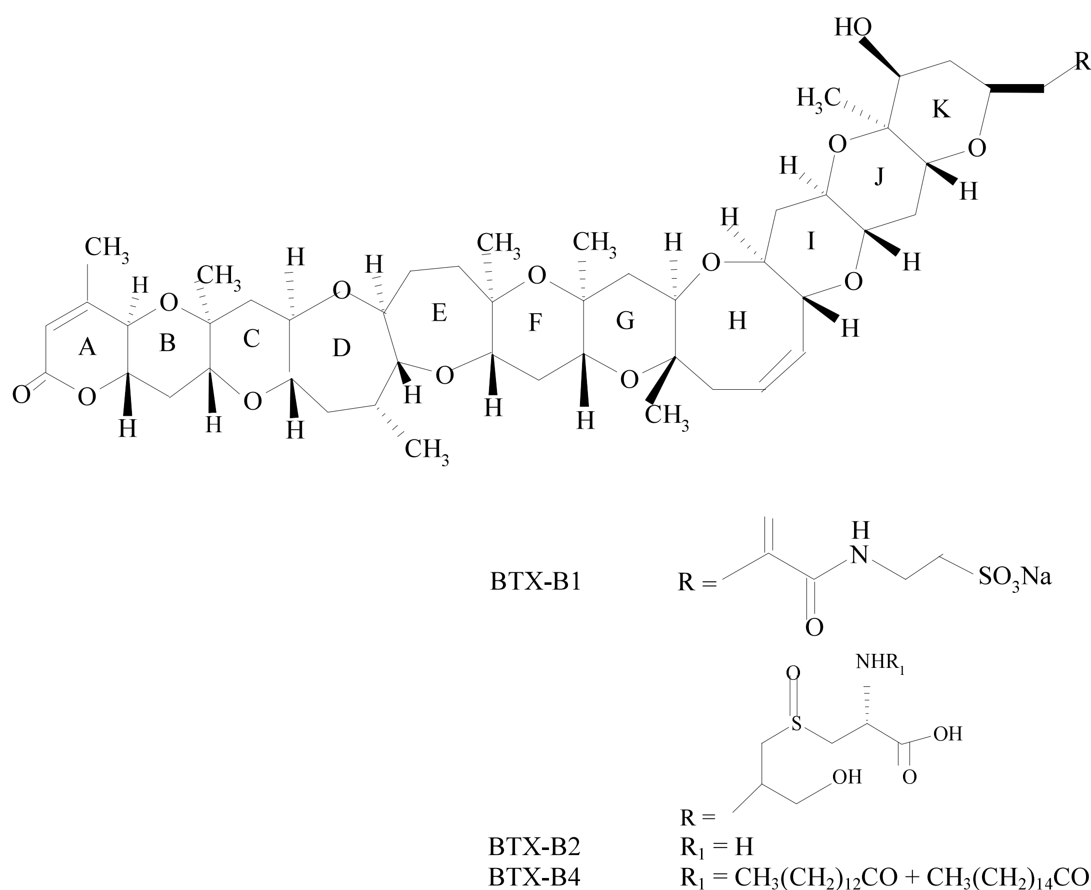

Figure 2a. Chemical formulas of brevetoxin analogues BTX-B1, -B2 and –B4 isolated from contaminated shellfish

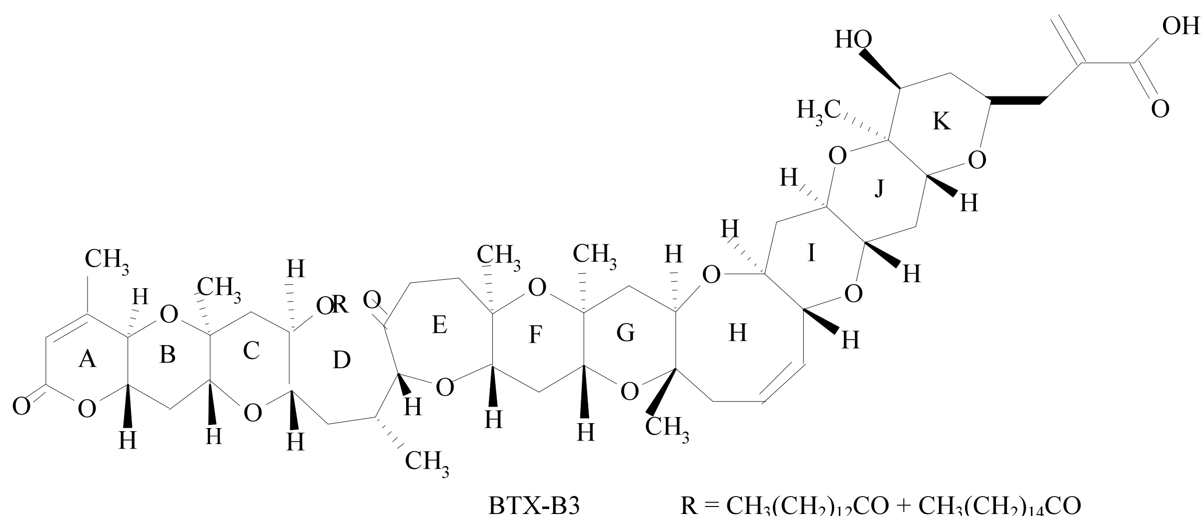

Figure 2b. Chemical formula of brevetoxin analogue BTX-B3 isolated from contaminated shellfish

2.1.3 Other toxins isolated from *G. breve*

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 (as cited in Mazumder *et al*., 1997).

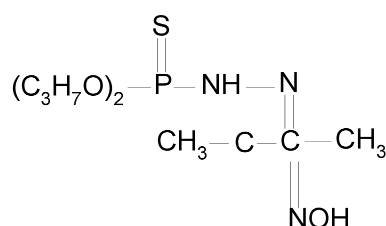

Figure 3. Phosphorus containing ichthyotoxic toxin isolated from G. breve.

2.2 Determination of NSP toxins

2.2.1 Bioassays

In vivo 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) (as cited in Hokama, 1993). One MU is defined as the amount of crude toxic residue that, on average, will kill 50% of the test aninals (20 g mice) in 930 minutes (as cited in 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, the discovery of a novel bioactive compound (gymnodimine), produced by the dinoflagellate *Gymnodinium mikimotoi*, a common species in New Zealand waters during neurotoxic events, did the authorities return 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 program now employs diethylether extraction as a means of discriminating gymnodimine activity from NSP toxicity (as cited in 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 (as cited in 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 (as cited in Hokama, 1993).

In vivo fish bioassay
Mosquito fish (*Gambusia affinis*) bioassays are conducted in 20 ml seawater (3.5% 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 (as cited in 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 (as cited in Viviani, 1992).

2.2.2 In vitro 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 (as cited in 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 μl tissue extract. PbTx can be detected within 4-6 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-colored 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 4-6 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 μl sample in neuroblastoma cells (as cited in 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 (as cited in 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).

2.2.3 In vitro 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 2.2.5), 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 seen in the immunoassay (as cited in 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 3 hours, has a detection limit of less than 1 ng and can analyze dozens of samples simultaneously. The assay is 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 (as cited in Quilliam, 1999).

Whitney *et al.* (1997) reported the complex behavior 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 cooperative, noncompetitive inhibition of ^{3}H-PbTx-3 specific binding and increased nonspecific binding to synaptosomes. Whitney *et al.* (1997) developed a correction method for these problems.

2.2.4 In vitro 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 saxitoxin, brevetoxin and domoic acid 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.

2.2.5 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 (as cited in 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 (as cited in 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 horse radish 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 (as cited in 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 haemocyanin (KLH) was used instead of BSA, more efficient antibody production occurred (as cited in 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 induced as well. Normal tissue extraction will not release bound toxin in fish tissue. The ELISA was entirely satisfactory for detecting and quantitating brevetoxins in dinoflagellate cells, requiring as few as 10-50 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.

Garthwaite *et al*. (2001) developed a group ELISA for ASP, NSP and DSP toxins and for 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.

2.2.6 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 coeluting 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, USA. PbTx-2, PbTx-1 and PbTx-3 were detected at 60, 10 and 5.7 μg/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 2 min in some cases) and can be totally automated (as cited in Quilliam, 1998).

HPLC/MS/MS

A fish tissue procedure based on gradient reversed-phase HPLC/tandem mass spectrometry (HPLC/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).

3. Sources of NSP toxins

3.1 Global increase of harmful algal blooms

In the last twenty years algal blooms appear to have increased in frequency, intensity and geographic distribution. A number of explanations for this apparent increase of algal blooms can be given (as cited from Hallegraeff, 1995).

- Increased scientific awareness of toxic species.
  Reports of human illnesses or damage to aquaculture operations are receiving increased attention in newspapers, the electronic media and the scientific literature. Consequently more and more surveys in local waters take place to detect the causative organisms.
- Increased utilization of coastal waters for aquaculture.
  More and more countries are looking towards aquaculture as an alternative for increased problems of overfishing of coastal waters. Some fisheries scientists predict that within the next 10-20 years, the increasing production of world aquaculture may well approach the decreasing total catch of wild fish and shellfish. Aquaculture operations act as sensitive ‘bioassay systems’ for harmful algal species and can trace the presence in water bodies of problem organisms not known to exist there before.
- Stimulation of plankton blooms by cultural eutrophication and/or unusual climatological conditions.
  While some organisms among which the dinoflagellate *G. breve*, appeared to be unaffected by coastal nutrient enrichments, many other algal bloom species appeared to be stimulated by 'cultural eutrophication' from domestic, industrial and agricultural wastes. The nutrient composition of treated wastewater is never the same as that of the coastal waters in which it is being discharged. Algal species which are normally not toxic may be rendered toxic when exposed to atypical nutrient regimes 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 run-off. Acid precipitation can further increase the mobility of humic substances and trace metals in soils. Furthermore global climate change studies need to consider possible impacts on algal bloom events. Until recently NSP caused by brevetoxins produced by *G. breve* 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. 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. Unexpectedly, in early 1993 more than 180 human shellfish poisonings were reported from New Zealand, caused by an organism similar (but not identical) 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 climatological conditions (higher than usual rainfall, lower than usual temperature).
- Transportation of dinoflagellate cysts in ship's ballast water or associated with translocation of shellfish stocks.
  The problem of ballast water transport of plankton species gained considerable interest when evidence was brought forward that non-indigenous toxic dinoflagellate species had been introduced into Australian waters into sensitive aquaculture areas with disastrous consequences for commercial shellfish farm operations. While the planktonic stages of dinoflagellates show only limited survival during the voyage in the dark ballast tanks, their resistant resting spores are quite well suited to survive these conditions. More than 300 million toxic dinoflagellate cysts were estimated to be transported with a single ballast tank.

Another vector for the dispersal of algae (especially their resting cysts) is with the translocation of shellfish stocks from one area to another. The faeces and digestive tracts of bivalves can be loaded with viable dinoflagellate cells and sometimes can also contain resistant resting cysts.

3.2 Source organisms for NSP toxins

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 1) (Benson *et al*., 1999). However four brevetoxin analogues (see Figures 2a and 2b) which were isolated from contaminated shellfish only and not from field blooms or *G. breve* cultures, were considered to be metabolites formed in the shellfish from the brevetoxins shown in figure 1 (Ishida *et al*., 1995; Morohashi *et al*., 1995, 1999; Murata *et al*., 1998). Besides the neurotoxic brevetoxins *G. breve* produce also, 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; this can be carried in land depending on wind and other environmental conditions (as cited in Fleming and Baden, 1999).

Furthermore brevetoxin-like toxins were producec 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 (Khan *et al*., 1996a).
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 (Khan *et al*., 1996b).
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 (Khan *et al*., 1997).
Four neurotoxic components were isolated from *Chattonella marina* and were identified to be PbTx-2, PbTx-3, PbTx-9 and oxidized PbTx-2 (Khan *et al*., 1995a).

3.3 Distribution of source organisms

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 they 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). Brevetoxins have been quantitatively detected in Muir birds from the coast of California, in some tuna samples from Australia, in menhaden and mullet from the coast of Florida and in shellfish from New Zealand (Quilliam, 1999).
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 70000 per liter) and in Bream Bay (cell counts up to 100000 per liter) in January 1993. Cell counts declined during February and March to less than 300 cells per liter in April in Coromandel (Smith *et al*., 1993).
In the summer of 1995-96, a severe aerosol toxin problem in South Africa viz. in False Bay, which later spread to the coastal resort of Hermanus in Walker Bay was reported. 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 so 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 usully 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 *et al.*, 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 (Tomas, 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, (as cited in Khan *et al*., 1996b). 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, Korea, Singapore, Canada, New Zealand, England, eastern and western areas of North America and Bermuda (as cited in Khan *et al*., 1997).
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 (as cited in Khan *et al.*, 1996a). Also in South-East 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 rich in organic material from India, Australia and Japan.

3.4 Habitat of species producing NSP toxins

G. breve blooms on the west coast of Florida occur from summer to winter, and most frequently in the fall. Changes in bloom occurrence correlate with wind and sea surface temperature. Blooms typically initiate offshore in the summer when the winds are weakest. However, they appear at the coast and continue during fall, 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 man-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 0.5-20.0 meters. 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 3500 square miles to 7000 square miles. In recent years transport of the dinoflagellate from the Gulf has documented. In 1987-88 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 also brevetoxins, occurred in brackish coastal areas rich in organic material (Hallegraeff and Hara, 1995). Optimal growth was seen at temperatures of 20-25 °C, salinities of 20-30 ‰, light intensities of 60-140 $\mu E\ m^{-2}\ s^{-1}$ and at pH 7.5-8.5. Growth did not occur at temperatures below 15 °C or above 30 °C, and at salinities below 10 ‰ (Khan *et al*., 1998).
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}. Run-off, 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 5 divisions/day) causing the production of red tides in a short period (Honjo, 1993). 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 S=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 S=25 to 28 was therefore not expected (as cited in Vrieling *et al*., 1995). 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.
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, may be for lack of an adequate sampling scheme (as cited in Vrieling *et al*., 1995). *C. antiqua* growed 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 (7.6-8.3) no significant effects on growth of *C. antiqua* were seen and maximal growth was observed (Nakamura and Watanabe, 1983a).
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 $\mu E/m^2/s$) whereas good motility was seen at 60-180 $\mu E/m^2/s$ (Khan *et al*., 1995b). 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 (Nakamura and Watanabe, 1983b).

4. Marine organisms associated with NSP

4.1 Uptake and elimination of NSP toxins in marine organisms

There are little quantitative data on rates of accumulation and depuration of brevetoxins in bivalves. Oysters accumulate the toxins in less than 4 hours in the presence of 5000 cells/ml and depurate (60%) the accumulated toxins in 36 hours. Potency of depuration is species-specific and highly variable, even under controlled laboratory conditions (as cited in Viviani, 1992).
Crassostrea virginica depurated brevetoxins 2-8 weeks after the bloom. Biotransformation is species-specific and may lead to more potent derivatives (Steidinger *et al*., 1998).

Gulf toad fish (*Opsanus beta*) received orally ^{14}C- PbTx-3 in a fishmeal slurry and were killed 72 hours later. The hepatobiliary system contained 40% of body burden confirming the key role of this system in detoxification and elimination of brevetoxin. Muscle tissue contained 27% of body burden, followed by gastrointestinal tract with 25% (Washburn *et al*., 1994).

Gulf toad fish (*Opsanus beta*) received intravenously (via an implanted indwelling cannula in caudal vein) 0.5 µg ^{3}H-labelled PbTx-3/kg b.w. Radioactivity in blood was determined at several times during 3 hours after dosing. Fish (1-3) were killed 1, 3, 6, 24, 72 and 96 hours after dosing and radioactivity in tissues was determined. 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% of body burden). Through 96 hours radioactivity in liver remained constant (7.8%), while levels in bile, kidney and skin increased (34.5, 13.8 and 6.7%, respectively) and levels in all other tissues decreased, particularly in muscle (15.9%). Approximately 24% 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% of radioactivity in bile). No metabolites have been identified (Kennedy *et al*., 1992).

Immature red fish (*Scianops ocellatus*) were given orally 1.5 or 2.5 µg PbTx-3/100 g b.w. in a fishmeal slurry by gavage. At the high dose PbTx-3 significantly increased the activity of the hepatic P450 enzyme ethoxyresorufin *O*-deethylase (EROD). 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 (Washburn *et al*., 1994).

The effects of PbTx-2 on xenobiotic metabolizing enzymes and the possible identification of potential biomarkers of exposure, were examined in striped bass (*Morone saxatilis*). Seven striped bass were exposed orally by gavage for 4 days to a 0.5 g/100 g body weight of a toxin laden slurry (~50 µg/100 g b.w.). A negative control group received control slurry and a positive control group received intraperitoneally β-naphthoflavone (5 mg/100 g b.w.). 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 hepato-somatic index was seen and both microsomal and cytosolic proteins in the liver were significantly lower. PbTx-2 caused a 3-fold increase in EROD activity whereas β-naphthoflavone caused a 30-fold increase. PbTx-2 caused a 35 and 50% increase in the activity of two gluthathione S-transferase (GST) isozymes. These increases seen in GST isozymes make them potentially useful biomarkers (Washburn *et al*., 1996).

In fish (striped bass *(Morone saxatilis)*) 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 (Washburn *et al*., 1997).

4.2 Shellfish containing NSP toxins

Major seafoods containing brevetoxins are shellfish (Viviani, 1992). Several species (a.o. 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 (as cited in 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 2a and 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). The shellfish was derived from NSP incidents in New Zealand. 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). BTBX-3 did not kill mice at intraperitoneal injection of 300 μg/kg b.w. (Morahashi *et al*., 1995). No data on the ichthyotoxicity of BTBX-3 are available.
Whelks (*Busycon contrarium*) and clams (*Chione cancellata* and *Mercenaria* spp.) collected from Sarasota Bay, Florida, USA, an area in which NSP occurred in three people in 1996, were analyzed for brevetoxins by radioimmunoassay and by 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).

4.3 Other marine 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% transfer from copepods to fish (viscera) over a 2 hours digestion time was found.
Also in experiments under b) transfer of the brevetoxins from copepod to fish (viscera) was observed within 2 hours (after 40-50 minutes of feeding with copepods). Toxin level in viscera decreased up to 8 hours, but 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 2 hours, while after 2-6 hours to 25 hours toxin transferred to fish muscle (Tester *et al*., 2000).

Brevetoxins have been identified in manatees, tuna fish, finfish, menhaden, mullet and Muir birds (Bossart *et al*., 1998; Quilliam, 1999).

5. Toxicity of NSP toxins to animals and man

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 (as cited in Fleming and Baden, 1999). The brevetoxins act on binding site 5 in a 1:1 stoichiometry (as cited in 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 (as cited inWatters, 1995).
Conformational analysis revealed that the unsaturated H-ring of brevetoxin B (see figure 1) favors the boat-chair conformation as does the saturated G-ring of brevetoxin A (see figure 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 (as cited in 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 recent work doubts on the cathepsin mechanism (as cited in Fleming and Baden, 1999).

In vitro studies
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 (Rodriguez *et al*., 1994).

5.2 Toxicity to laboratory animals

5.2.1 Uptake, distribution, biotransformation and excretion

Oral administration

Rats

Male F344 rats received a single oral dose of ^{3}H-labeled PbTx-3 and were killed after 6, 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 feces were collected at 24 hour intervals for a period of 7 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 studies of Poli *et al*. (1990a, b) (see next paragraph) 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% of the dose was excreted via urine and feces, with equivalent amounts in each. However, during the first 48 hours, more PbTx-3 was cleared through the feces, whereas afterwards, most toxin was cleared through urine (Cattet and Geraci, 1993).

Intravenous administration

Rats

Intravenous studies in male Sprague-Dawley rats with ^{3}H-labeled PbTx-3 showed a rapid clearance of PbTx-3 from bloodstream (<10% remained after 1 minute) and distribution to the liver (18% of the dose after 30 minutes), skeletal muscle (70% of the dose after 30 minutes) and gastrointestinal tract (8% 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% of the dose. By 24 hours radioactivity in skeletal muscle decreased to 20% of the dose while radioactivity in liver remained constant and radioactivity in stomach, intestines and feces increased suggesting biliary excretion as an important route of elimination. By day 6, 14.4% of radioactivity had been excreted in urine and 75.1% in feces, with 9.0% remaining in carcass. Thin layer chromatography of urine and feces 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 (Poli *et al*., 1990a, b).

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% 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% 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 >80% 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 μg/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% of the administered dose during 48 hours of topical application (calculated by dividing % of dose excreted following topical administration by % 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% (based on accumulation of radioactivity in receptor fluid) or 9.9% (based on receptor fluid and dermis). Penetration through the epidermis into the dermis was rapid; maximal dermal accumulation was seen at 4 hours (9.1% *in vivo* and 18% *in vitro*). At 24 hours the amount in the dermis decreased to 2.3 and 15% *in vivo* and *in vitro*, respectively. In the in vitro study >95% 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, feces and tissues were collected. Over 80% 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%), intestines (32%) and liver (8%); only 6% was found in the lung after 0.5 hour. Blood, brain and fat contained the lowest levels. About 20% of the intial level in tissues was retained for 7 days. The majority of PbTx-3 was excreted within 48 hours in feces and urine with approximately twice as much in feces (60%) as in urine (30%). 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 seaspray during red tides (Benson *et al*., 1999).

5.2.2 Acute toxicity

Table 1. Acute toxicity of brevetoxins in female Swiss mice (Baden and Mende, 1982)

brevetoxins	**route**	**obser-vation time**	**LD_{50} value μg/kg b.w.**	**vehicle**
PbTx-3	oral	24 hours	520	0.9% saline
PbTx-3	intraperitoneal	24 hours	170	0.9% saline
PbTx-3	intravenous	24 hours	94	0.9% saline
PbTx-2	oral	24 hours	6600	0.9% saline
PbTx-2	intraperitoneal	24 hours	200	0.9% saline
PbTx-2	intravenous	24 hours	200	0.9% saline

Table 2. Acute toxicity of brevetoxin analogues in mice

brevetoxin analogues	route	survi-val time	mini-mum lethal dose µg/kg b.w.	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 (Poli *et al.*, 1990b; Templeton *et al.*, 1989b).
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 (Poli *et al.*, 1990b; Templeton *et al.*, 1989b). In anesthetized cats a triad of bradycardia, hypotension and bradypnea that correlates both with reflex and central actions inasmuch at it is abolished by either vagotomy or the administration of atropine (as cited in Berman and Murray, 1999).

Brevetoxin analogues BTX-B2 and BTX-B4 caused paralysis of hind limbs, diarrhea, dyspnea, and convulsion after intraperitoneal injection (Morohashi *et al.*, 1999). BTX-B1 caused irritability after intraperitoneal injection, 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 µg/kg bw=sublethal dose) over 1 hour. Rats pretreated 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 μg/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 6 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 8 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 (Templeton *et al*., 1989b).

Intravenous dosing
Mice
The intravenous LD_{50} in mice of the hemagglutinative fraction separated from red tides of *Chattonella marina*, appeared to be 2-4 mg/kg b.w. The mice showed repiratory paralysis (Onoue and Nozawa, 1989).

Rats
Groups of 4 male rats received after surgical preparation and a 24 hour recovery, an intravenous infusion during 1 hour with vehicle only or with 12.5, 25, 50 or 100 μg PbTx-2/kg b.w. and were monitored for 6 hours or until death. All animals at the 100 μg/kg b.w. dose level died within 2 hours. One out of 4 animals at 50 μg/kg b.w. died during the 6 hours study; the remainder of the animals survived. Within 90 minutes the respiratory rates at 12.5 μg/kg b.w. fell to near 60% of baseline value and at 25, 50 and 100 μg/kg b.w. to 20% of baseline value. Recovery to normal respiratory rates occurred 6 hours after exposure except in the 50 μg/kg b.w. group which recovered to only 60% of baseline value. During the first 2 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 μg/kg b.w. group. An average decrease in peripheral body temperature of 0.5 °C was seen in the 12.5 μg/kg b.w. group. Blood gas values remained normal, except terminally. Electrocardiography showed at doses ≥25 μg/kg b.w. heart block, premature ventricular contractions and idioventricular rhythms (Templeton *et al*., 1989a).

Guinea-pigs
Catheterized male Hartley guinea pigs received an intravenous infusion with PbTx-3 at a rate of 0.63 μg/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 μg/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 (Franz and LeClaire, 1989).

Intraperitoneal dosing
Mice
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 (Onoue and Nozawa, 1989).

5.2.3 Studies with a phosphorus containing *G. breve* component

Besides potent brevetoxins, some phosporus containing toxic components have also been isolated and characterized from *G. breve* (as cited in Koley *et al*., 1995). One ichthyotoxic component of *G. breve* toxin 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 is similar in chemical structure to an organothiophosphate (as cited in Husain *et al*., 1996).

Mice

In the study of Husain *et al*. (1996) acute intraperitoneal toxicity (i.p.) of the synthetic *O*,*O*-dipropyl(*E*)-2-(1-methyl-2-oxopropylidene)phosphorohydrazidothioate-(*E*)oxime and its analogues was studied with special attention to acetylcholinesterase (AChE) inhibition (IC_{50}) *in vitro* as well as in cerebral and peripheral tissue in mice *in vivo*. The intraperitoneal LD_{50} value in mice is highest voor the parent toxin (=dipropoxy (P=S) compound) (55.36 mg/kg b.w.) whereas it is lowest for the diisopropoxy(P=O) analogue (27.75 mg/kg b.w.). LD50 values increased in the following order, diisopropoxy(P=O)< diethoxy(P=O)< diethoxy(P=S) <diisopropoxy(P=S) <dipropoxy(P=S) (=parent toxin).
IC_{50} value for diisopropoxy analogue(P=O) is 4.0 mM, whereas it is 2.3 mM for diethoxy(P=O) analogue. Other analogues (P=S) are direct inhibitors of AChE and inhibit AChE only *in vivo*.
Single lethal intraperitoneal doses (LD_{50}'s) in mice caused a maximum inhibition of AChE in blood (61%) and brain (36.6%) with the parent toxin [=dipropoxy (P=S)], whereas minimum AChE inhibition was seen in blood (30.1%) with diethoxy(P=S) analogue and in brain (14.9%) with diisopropoxy(P=S) analogue.
AChE inhibition pattern in blood showed the following order diethoxy (P=O)< diisopropoxy (P=S)< diisopropoxy (P=O)< diethoxy (P=O)< dipropoxy (P=S) (=parent toxin).
AChE inhibition pattern in brain showed the following order diisopropoxy(P=S)< diethoxy (P=S)< diisopropoxy (P=O)< diethoxy(P=O)< dipropoxy (P=S) (=parent toxin).
In conclusion the parent toxin showed the highest AChE inhibition in vivo whereas the acute toxicity was the lowest of all analogues suggesting involvement of other factors than AChE inhibition, affecting the toxicity. The mice exposed to the parent toxin 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% inhibition of brain AChE is observed. In this study brain AChE activity was inhibited by 36.6% after intraperitoneal dosing of the parent toxin. Such inhibition might cause only mild symptoms (Husain *et al*., 1996).

Rats

Anaesthetized conscious male Wistar rats received a single intravenous injection with 16, 24, 48 or 72 μg/kg b.w. of *O*,*O*-dipropyl(*E*)-2-(1-methyl-2-oxopropylidene)phosphorohydrazidothioate-(*E*)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 μg/kg b.w.). At higher doses the toxin caused irreversible hypotension and bradycardia. Noticeably, the bradycardiac effect did not fully recover even after administration of a lower dose. The animals died of cardiac arrest immediately after intravenous administration of 72 μg/kg b.w. 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 α-adrenergic and ganglionic blockade accompanied by modulation of K^+ channel activity (Mazumder *et al*., 1997).

Cats

An intravenous study with of *O*,*O*-dipropyl(*E*)-2-(1-methyl-2-oxopropylidene)-phosphorohydrazido-thioate-(*E*)oxime in anaesthetized cats was performed to study the effects on mean arterial blood pressure, ECG pattern, unit discharge of baroreceptors and respiratory activity. Intravenous doses of 0.25-1.5 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 (Koley *et al*., 1995).

5.2.4 Repeated dose toxicity

No studies with repeated dosing of brevetoxins are available.

5.2.5 Teratogenicity

No teratogenicity studies with brevetoxins are available.

5.2.6 Mutagenicity

No mutagenicity data on brevetoxins. are available

5.3 In vitro studies with brevetoxins

The non-neural effects of PbTx-3, in particular the effects on hepatic cell structure, were studied 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. Ultrastructurally, 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 account for the increased basophilic reaction of the cells, specially 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 (Rodriguez-Rodriguez and Maldonado, 1996)

Rodgers *et al*. (1984) reported positive inotropic and arrhythmogenic effects on isolated rat and guinea pig cardiac preparations 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.

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 μg/ml), or the purified fractions PbTx-2 and PbTx-3 (0.01-0.07 μg/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 more potency than the crude brevetoxins. In all cases depolarizations stabilised within 4 min. 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 min. 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 (Richards *et al*., 1990).

5.4 Toxicity to man

5.4.1 Oral exposure

When brevetoxins are accumulated in shellfish, consumption of the raw or cooked shellfish can cause neurotoxic shellfish poisoning (NSP), a toxic syndrome somewhat similar to PSP (paralytic shellfish poisoning) and ciguatera intoxication but less severe. The symptoms of NSP occur within 30 minutes to 3 hours, last a few days and include nausea, vomiting, diarrhea, 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; Novak, 1998; Tibbets, 1998; as cited in Berman and Murray, 1999). Treatment is primarily supportive (as cited in Fleming and Baden, 1999).

5.4.2 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; Novak, 1998; Tibbets, 1998).

5.4.3 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; Novak, 1998; Tibbets, 1998).
G. *breve* toxins stimulate post-ganglionic cholinergic fibers 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 (as cited in Fleming and Baden, 1999 and in Watters, 1995).
PtBx-3 was indicated to be the primary toxin responsible for respiratory discomfort in man (as cited in Benson *et al*., 1999).

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* (Oda *et al*., 1992).

Incubation of the sea urchin (*Lytechinus variegatus*) and the seatrout (*Cynoscion nebulosis*) in the sea-surface microlayer collected off the Florida Keys, particularly when taken from slicked areas, can adversely affect 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, they did tend to 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 (Rumbold and Snedaker, 1999).

According to Viviani (1992) fish usually start to die when *G. breve* counts reach the 250000 cells/L range. However, other authors report that fish kills will occur at counts of ≥100000 cells/L (as cited in 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 (as cited in Kennedy *et al*., 1992).

Toxicity tests with 5-6 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 $1.95x10^3$ cells/ml. In low density cultures (on 2nd day) fish did not die, but showed abnormal movements for about 30-45 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-logarithmic growth phase. In the late logarithmic growth phase, there was a gradual decrease in toxicity. 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 2 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* (Khan *et al*., 1996a).
No toxicity of *F. japonica* cultures to the red sea bream was detected until cell density reached $4.1x10^3$ cells/ml. Toxicity also appeared to vary with the growth phases and increased with age; it was the highest on the 8th day. Thereafter toxicity began to decline to low levels as the cells entered the early stationary phase (Khan *et al*., 1996b).
In *H. akashiwo* cultures the red sea bream showed no abnormal behaviour at a cell density of 34000 cells/ml but exhibited a violent paralysis, leading to death, when the cell density surpassed 120000 cells/ml. When exposed to a red tide of *H. akashiwo* at a cell density of 30000 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 >100000 cells/ml (Khan *et al*., 1997).

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) (Khan *et al*., 1997).

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 seabream (*Pagrus major*) were exposed to the three fractions (0.02%) in beakers of seawater. The fish died within 7-9 minutes at exposure to the neurotoxic fraction showing conspicious edema on their second lamellae. At exposure to the hemolytic and hemagglutinative fractions fish died within 20-50 minutes with a marked mucous release on their gill filaments (Onoue and Nozawa, 1989).

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 (Endo *et al*., 1992).

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 occurrred via inhalation and oral ingestion (Bossart *et al*., 1998). One of the likely vectors for the toxin is being filter-feeding sea squirts (as cited in Marsden, 1993). Grossly, severe nasopharyngeal, pulmonary, hepatic, renal, and cerebral congestion was present in all cases. Nasopharyngeal and pulmonary edema and hemorrhage were also seen. Consistent macroscopic lesions were catarrhal rhinitis, pulmonary hemorrhage and edema, multiorgan hemosiderosis, and nonsuppurative 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 reportedfrom 4 manatees rescued alive from the epizootic. Immunohistochemical staining with interleukin-1-β-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).

Trainer and Baden (1999) demonstrated that brevetoxin (PbTx-3) was 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 dependance (decreased toxic-receptor affinity and lower measured percentages of specific binding as temperature increases from 0 to 37 °C).

The brevetoxin anologues (or metabolites) found in New Zealand cockles (*Austrovenus stutchburyi*) (BTX-B1) and in New Zealand green shell 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).

An organophosphate isolated from *G. breve*, namely *O*,*O*-dipropyl(*E*)-2-(1-methyl-2-oxopropylidene)phosphorohydrazidothioate(*E*)oxime, appeared to be very ichthyotoxic (0.9 mg/L against *Lebistes reticulatus*) (as cited in Mazumder *et al*., 1997).

5.6 Toxicity to water fowl

Mortality among the double-crested cormorant (*Phalacrocorax auritus*) caused by brevetoxins, was observed along the Florida gulf coast (as cited in Fleming and Baden, 1999).

Brevetoxin was the cause of a summer mortality in common murres (*Uri aalge*) in California (as cited in Fleming and Baden, 1999).

6. Prevention of NSP intoxication

6.1 Depuration

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 (as cited in Marsden, 1993).

The most usual way of depurating bivalves is selfdepuration, 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 (as cited in Marsden, 1993). Depuration of brevetoxins in *Crassostrea virginica* is typically 2-8 weeks after a bloom had dissipated (as cited in Steidinger *et al*., 1998).

Cell cultures of *G. breve* in artificial seawater were subjected to microwave irradiation at 2450 MHz. Irradiation was for four 60 seconds intervals separated by 5 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%. A pronounced decrease or threshold effect was evident at energy levels above 0.08 kJ/ml of culture (Mahram *et al*., 1998).

Using a half-factorial experimental design *G. breve* cells were cultured and fed to Pacific oysters (*Crassostrea gigas*) at rates of between 10.45 and 24.5 million cells per oyster over 24 hour periods. Thereafter the oysters were detoxified in various laboratory tanks over 5-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 120 hours (5 x 5.0 or 5 x 3.5 million cells per oyster) with those fed for 24 hours (10.79 or 24.5 million cells per oyster). With the exception of one (4 tanks), all treatment combinations resulted in an initial decline of the brevetoxin level reaching a minimum <20 MU per 100 g by day 3 regardless of the initial toxin level or whether the toxin had accumulated over 1 or 5 days. The 3-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. The presence of final brevetoxin levels just above 20 MU per 100 g (20.6 MU per 100 g) in some samples means, that the process is not yet commercially viable (Fletcher *et al*., 1998).

The effect of ozonated artificial sea water on *G. breve* cells and toxins was studied by Schneider and Rodrick (1995). When artificial sea water which was ozonated for 60 seconds,

was added to *G. breve* cultures the number of surviving cells decreased approximately 80% ($t_{1/2}$=10 seconds). When cultures of *G. breve* in artificial sea water were directly ozonated for 60 seconds, non intact cells were found ($t_{1/2}$=2.5 seconds). Experiments carried out in artificial sea water demonstrated that extracted *G. breve* toxins (PbTx-1, -2, -3, -5, -7 and -9) reintroduced into artificial sea water as well as toxins in whole cell cultures of *G. breve* in artificial sea water 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% 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 1 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 5 minutes total amounts of all toxins were reduced.

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. Reactivitiy of brevetoxins to acid attack shows the following order: PtBx-1>PtBx-2>PtBx-9. Under basic conditions, head-side lactone ring opening initiated by hydroxide ion attack proceeds to completion in 120 and 50 minutes for PtBx-2 and PtBx-9, respectively, while that for PtBx-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).

Oysters accumulate brevetoxins in less than 4 hours in the presence of 5000 cells/ml, and depurate 60% 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 1-2 months after the termination of any single bloom episode. Canning cannot be a way to decrease brevetoxin concentration in bivalves (as cited in Viviani, 1992).

In *Crassostrea virginica* depuration of brevetoxins occurs 2-8 weeks after the bloom has dissipated. However, the metabolic fate of toxins in shellfish is species-specific (as cited in Steidinger *et al*., 1998).

6.2 Preventive measures

Toxic blooms of *G. breve* are generally detected by visual confirmation (water discoloration 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 mouse bioassays (as cited in Millie *et al*., 1997). The exact environmental conditions leading 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 programs 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 (as cited in Millie *et al*., 1995).

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 (Millie *et al*., 1997).

Lohrenz *et al*. (1999) compared microphotometric methods with conventional spectrophotometric methods for the assessment of spectral absorption of monospecies cultures and demonstrated the feasability of using microphotometry as a means of characterizing spectral absorption coefficients of a.o. *G. breve*. 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 airborn 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 5000 cells/L 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/L, 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 discolored surface water detectable by color sensors onboard satellites. Tester *et al*. (1998) reported a minimum detection level of approximately 100000 cells/L by remote sensing; 10 times more sensitive than visual detection. In this case there could be a minimum of 3-6 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.

7. Case reports/outbreaks and surveys

7.1 North America

7.1.1 Canada

Red tides of *Heterosigma akashiwo* (belonging to the class Raphidophyceae) have been reported from embayments in Canada causing mortality of cultured fish (Khan *et al*., 1997).

7.1.2 USA

East coast
Brevetoxin-associated mortality was postulated in bottlenose dolphins (*Tursiops truncatus*) along the mid- Atlantic coast of the U.S.A. in 1987-1988 (Bossart *et al*., 1998). However, this hypothesis that brevetoxins poisoned the dolphins, remained controversial (Brody, 1989).

G. breve was identified (6 x 10^6 cells per liter) 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 3.5 months later, there were 48 cases of NSP reported in humans and over 1480 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 (Fowler and Tester, 1989; Tester and Fowler, 1989).
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 $3.27 x 10^5$ cells/L) or the degree of bloom development. This is in contrast to blooms of *G. breve* in the Gulf of Mexico which were typically monospecific (West *et al*., 1996).

Red tides of *Heterosigma akashiwo* (belonging to the class Raphidophyceae) have been reported from embayments on the east coast causing mortality of cultured fish (Khan *et al*., 1997).

Florida and the Gulf of Mexico
On June 16, 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 U.S. FDA. All had eaten clams (*Chione cancellata*) and whelks (unidentified species) harvested from an area that had been closed to shellfish harvesting from January 31 1996 through June 8 1996 because of red tide of *G. breve*, and then closed again June 11 because of heavy rainfall. The clams had been cooked until they opened; cooking time for the whelks was unknown (Hopkins *et al*., 1997)(Steidinger *et al*., 1998).

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 Harbor 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/L. Autopsy showed neurointoxication facilitated by oral and inhalation exposure. There are three potential routes of intoxication: 1) toxic aerosol inhalation, 2) toxic food ingestion, and 3) 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 seagrass, was reported (Hallegraeff, 1995).

Also in menhaden and mullet from the coast of Florida brevetoxins have been detected (Quilliam, 1999).

Brevetoxin-associated mortality was postulated in bottlenose dolphins (*Tursiops truncatus*) in southwestern Florida in 1946-1947 (Bossart *et al*., 1998). This phenomenon was due to a bloom of *G. breve* which was identified in 1947 as the aetiological 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 U.S. Atlantic coast in 1987-1988. Similar toxin-associated manatee mortality was speculated in southwest Florida in 1963 and 1982 (as cited in Bossart *et al*., 1998).

All red tides in Florida were associated with mass mortality in marine animals. These phonomena 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 were noticed (Viviani, 1992).

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 sea water (Sierra-Beltrán *et al*., 1998).

Mortality among the double-crested cormorant (*Phalacrocorax auritus*) was observed along th Florida gulf coast (as cited in Fleming and Baden, 1999).

In late October 1996 to December 1996 for the first time a bloom of *G. breve* occurred 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 a summer mortality in common murres (*Uri aalge*) in California (as cited in Fleming and Baden, 1999).

Red tides of *Heterosigma akashiwo* (Raphidophyceae) have been reported from embayments on the west coast causing mortality of cultured fish (Khan *et al*., 1997).

7.2 Europe

7.2.1 France

In France the presence of *Fibrocapsa japonica* was reported for the first time in October 1991 on the Channel coasts of Normandy (Billard, 1992).

Videorecordings 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 (Rademaker *et al*., 1997).

7.2.2 Germany

On 26 August and 15 December 1994 *H. akashiwo* was detected in the German Waddensea (Rademaker *et al*., 1995).

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 July 24 1997 (Rademaker *et al*., 1997).
In German waters also *H. akashiwo* was observed, namely in Friedrichskoog in the summer of 1997. Cell concentrations were difficult to count (Rademaker *et al*., 1997).

7.2.3 Greece

A species similar to *G. breve* has been reported from the Aegean Sea, but with no adverse effects (Smith *et al.*, 1993).

7.2.4 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 raphidophycean have not yet been recorded in the Netherlands, but an outbreak cannot be excluded because the species detected can potentially be present each year (Vrieling *et al*., 1995).

In the summer of 1997 *F. japonica* was found in almost all samples from the Dutch Agal 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 2400 cells/cm^3 (Rademaker *et al*., 1997).

7.2.5 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).

7.2.6 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).

7.2.7 United Kingdom

Red tides of *Heterosigma akashiwo* have been reported from England and Bermuda causing mortality of cultured fish (as cited in Khan *et al*., 1997).

7.3 Asia

7.3.1 Japan

Red tides of *Heterosigma akashiwo* have been reported in embayments causing mortality of cultured fish (caged young yellowtail=*Seriola quinqueradiata*) (as cited in Khan *et al*., 1997).

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) (as cited in Khan *et al*., 1996b).

Chattonella antiqua formed heavy red tides in coastal regions of Japan killing large numbers of cultured fish (caged yellowtails) (as cited in Khan *et al*., 1996a).

7.3.2 Korea

Red tides of *Heterosigma akashiwo* have been reported in embayments in Korea causing mortality of cultured fish (as cited in Khan *et al*., 1997).

7.3.3 Malaysia

Red tides of *Heterosigma akashiwo* have been reported from embayments in Singapore causing mortality of cultured fish (as cited in Khan *et al*., 1997).

7.4 Africa

7.4.1 South-Africa

In the summer of 1995-96, a severe aerosol toxin problem in False Bay, which later spread to the coastal resort of Hermanus in Walker Bay was reported. Coughing, burning of the nasal passages, difficulty in breathing, stinging eyes and irritation of the skin were observed in beachgoers 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 so 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 usully implicated in NSP. Most outbreaks have been reported from False Bay, where they are responsible for the olive-green discolouration of the sewater during autumn. Thirty tons of abalone were washed up in the HF Verwoerd Marine Reserve in 1989, following blooms of *Gymnodinium nagasakiense* (Van der Vyver *et al.*, 2000).

7.5 Oceania

7.5.1 Australia

In Boston Bay, Southern Australia, high levels of breve-like toxins (up to 142 µg/100g) 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 66000 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-oxidised 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 (Hallegraeff et al., 1998; Munday and Hallegraeff, 1998).

7.5.2 New Zealand

Human and animal illness in 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 (Bates *et al*., 1993; O'Hara, 1993).

Fibrocapsa japonica was found at the east and west coasts of the North Island and at the east coast of the South Island in early 1993 (as cited in Khan *et al*., 1996).

Red tides of *Heterosigma akashiwo* have been reported from embayments in New Zealand causing mortality of cultured fish (as cited in Khan *et al*., 1997).

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, and then spread across to kills of other pelagic fish and marine invertebrates. Eighty seven people in Wellington Harbour reported suffering from respiratory illness; beachgoers, 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 sun-burnt sensation. The unprecedented bloom was found to be dominated by an undescribed *Gymnodinium* sp. (33.3×10^6 cells/L). 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 USA, *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 pretty much like those of *G. breve* from the Atlantic coast of the USA and *Gymnodinium* sp. recently reported for South Africa (Chang, 1999).

8. Regulations and monitoring

8.1 USA

A level of 80 µg PbTx-2 /100 g of shellfish tissue (0.8 mg/kg or 20 MU/100 g or 4 µg/mouse) analyzed 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 (as cited in Dickey *et al*., 1999).

8.1.1 Florida and the Gulf of Mexico

The Florida Department of Natural Resources has run a general control program since the mid 1970s. Only in 1984 were *G. breve* blooms specifically noted in control regulations. Closures of shellfish beds are made when *G. breve* concentrations exceed 5000 cells/L. Closures have lasted between a few weeks and six months. Two weeks after *G. breve* concentrations drop below 5000 cells/L, 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 in 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 not the respiratory iritation 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 (as cited in Fleming and Baden, 1999).

8.2 Europe

8.2.1 Denmark

A monitoring program exists for several algal species a.o. *Gymnodinium* spp. At 5.10^5 cells per liter (depending on species) fishery product harvesting areas are closed (Van Egmond *et al*., 1992; Shumway *et al.,* 1995)

8.2.2 Italy

NSP producing algae are monitored and at simultaneous presence of algae in water and toxin in mussels, fishery product harvesting areas are closed. In Italy provision of law is based on the mouse bioassay and established "not detectable " in shellfish (Van Egmond *et* al., 1992; Viviani, 1992).

8.3 Oceania

8.3.1 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 year around. 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 of 6 hours (Trusewich *et al*., 1996). Garthwaite *et al*. (2001) developed a group ELISA for ASP, NSP and DSP toxins and for 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.

9. Risk assessment

A risk assessment cannot be performed due to the lack of sufficient data on toxicity and exposure.

10. Discussion and recommendations

Blooms of the organism *G. breve* (red tides) are most prevalent along the coasts of Florida and the Gulf of Mexico in the USA, but *G. breve* or *G. breve*-like species occur also in other parts of the world (South-Africa, Japan, Europe, Oceania). Furthermore some other algal species belonging to the class Raphidophyceae (raphidophytes), appeared to produce brevetoxin-like toxins. These raphidophytes are known as major red tide organisms in coastal areas in Japan where they caused massive fish kills. However also in the Florida coastal waters, in Australia and European coastal waters these raphidophytes have been found. Although no harmful effects on mariculture in European waters were seen yet and no cases of human poisoning by the toxins of these raphidophytes have been reported, continuous monitoring for these raphidophytes may be recommendable in order to follow the development of these algal species and to prevent NSP outbreaks in the future.
Toxic blooms of *G. breve* are generally detected by visual confirmation (water discoloration and fish kills), illness to shellfish consumers and/or human respiratory irritation with actual toxicity verified through time-consuming mouse bioassays and chemical analyses for brevetoxins in shellfish samples (as cited in Millie *et al*., 1997). 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 programs 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 (as cited in Millie *et al*., 1995).
Therefore alternative evaluation systems for predicting bloom occurrences are highly desirable. Variations in spectral signature of light absorption by phytoplankton can be used to provide information about major pigment groups and as a diagnostic tool for detecting phytoplankton taxa prior to bloom status (as cited in Lohrenz *et al*., 1999). A number of these evaluation systems have been reported. One study reported a minimum detection level of approximately 100000 cells/L by remote sensing; (100 times above background levels) 10 times more sensitive than visual detection. In this case there could be a minimum of 3-6 days between bloom biomass detection and population growth to levels known to cause massive fish kills. In the meantime the presence of *G. breve* can be verified (Tester *et al*., 1998).
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. Reliable hazard assessment is therefore not possible.
Furthermore exposure assessment is not possible, due to difficulties with the determination of the brevetoxins as summarized below:

* The brevetoxins are a multicomponent family of toxins. In addition to 10 brevetoxins, 4 metabolites have been identified, occurring in cockles and greenshell mussels in the New Zealand case. Whereas these metabolites are not ichthyotoxic, they exhibit a potency to activate Na channels. Depending on the type of analytical method employed, they therefore may have a siginificant 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, also the presence of metabolites was 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). E.g. 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: the presence and temporal distribution of metabolites in shellfish; the efficiencies of the different extraction solvents; and 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 (as cited from 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 and 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.
* Reference materials (both calibrants and matrices) for the brevetoxins and their metabolites are chronically lacking.

Based on the lack of toxicity data and the analytical difficulties in determining brevetoxin exposure scientifically based risk assessment is not possible.

Pure toxins and toxin metabolites would be needed to be able to carry out toxicity studies. As well analytical reference materials would be needed to further develop and improve the analytical methodology and to allow analytical quality assurrance of monitoring laborotories. Currently the various obstacles on the way to reliable assessment of brevetoxin occurrence and exposure further hamper risk assesment and thus the establishment of meaningful regulations.

Despite these problems regulations for NSP toxins in shellfish are in force in a few countries viz. the USA, Italy and New Zealand based on the mouse bioassay. The action level is 20 MU/100 g shellfish flesh (~80 µg PbTx-2/100 g shellfish flesh).

The scientific basis for this action level seems rather slender, in view of the above-given conclusion that scientifically based risk assessment is not possible (see also paragraph 8.1 on page 45).

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