NATIONAL INSTITUTE OF PUBLIC HEALTH AND THE ENVIRONMENT
BILTHOVEN, THE NETHERLANDS

Report 388802005

Paralytic shellfish poisoning; A review

M.N. Mons, H.P. van Egmond, G.J.A. Speijers

June 1998

This investigation has been performed by order and for the account of the Health Protection Inspectorate, within project 388802, 'natural toxins'

National Institute of Public Health and the Environment (RIVM), P.O. Box 1,
3720 BA Bilthoven, The Netherlands
Telephone: +31 - 30 - 274 91 11, telefax: +31 - 30 - 274 29 71

MAILING LIST

1	Director-General Public Health, VWS
2	Drs. F. Schuring, Chief Inspector Health Protection (IWV)
3	Director Public Health Department, The Netherlands
4	Dr. D. Groothuis, (Inspectorate for Health Protection, Commodities and Veterinary Public Health, The Netherlands
5	Dr. ir. W. de Koe, (Inspectorate for Health Protection, Commodities and Veterinary Public Health, The Netherlands
6	Dr. ir. N. Lucas Luijkx, Public Health Department, The Netherlands
7	Dr. J. Goebels, Public Health Department, The Netherlands
8	Dr. J. Nieuwenhuizen, Public Health Department, The Netherlands
9	Chairman of the Dutch Health Council
10-11	Regional IWV Goes: Dr. G. Roesink Dr. K. Jonker
12	Drs. H. Pieters (RIVO, The Netherlands)
13	Dr. H. Belvère (European Commision, DG24)
14	Directie van het Rijksinstituut voor Volksgezondheid en Milieu (RIVM, The Netherlands)
15	Dr. G. Elzinga
16	Depot van Nederlandse publikaties en Nederlandse bibliografie
17	Dr. ir. de Mik (director, dept. IV/RIVM)
18	Dr. R. Stephany (ARO/RIVM)
19	Prof. dr. J.G. Vos (LPI/RIVM)
20	Dr. W.H. Könemann (CSR/RIVM)
21	Prof. dr. C.L. van Leeuwen (CSR/RIVM)
22	Dr. A.B.T.J. Boink (LEO/RIVM)
23	Hr. P.J. Boekhof (LEO/RIVM)
24	Drs. Th. Ietswaart (LWD/RIVM)
25	Ir. A. Bresser (LWD/RIVM)
26	Dr. M. Fernandez (CRL Vigo, Spain)
27	Dr. C. Laso (CRL Vigo, Spain)
28	Dr. A. Boenke (Standard, Measuring and Testing, DG 12/EC)
29	Ing H. van den Top (ARO/RIVM)
30-32	Authors
33	Hoofd Bureau Voorlichting en Public Relations
34	Bureau Rapportenregistratie
35	Bibliotheek RIVM
36-37	Bibliotheek CSR
38-58	Bureau Rapportenbeheer

TABLE OF CONTENTS

Mailing list 2
Preface 4
List of Abbreviations 4
Samenvatting 5
Summary 6

1. Introduction 7
2. The PSP toxins 8
    2.1 Structures and properties 8
    2.2 Determination of PSP toxins 8
3. Source of PSP compounds 14
    3.1 Maine source organisms 14
    3.2 Other producers of PSP toxins 14
    3.3 Habitat 14
    3.4 Significance of blooms 15
    3.5 Induction of blooms: predisposing conditions 16
4. Shellfish associated with Paralytic Shellfish Poisoning 18
    4.1 In general 18
    4.2 Uptake, transformation and distribution 18
    4.3 Excretion 19
    4.4 Shellfish found to contain PSP toxins 20
    4.5 Toxicity in other marine organisms 20
5. Toxicity in mammalian animals and man 21
    5.1 Toxicity in animals 21
    5.2 Toxicity in man 23
    5.3 Theories on the toxic mechanism 24
    5.4 Treatment of intoxication 25
    5.5 Prevention of PSP intoxication 26
    5.6 Case-reports/outbreaks 27
6. Regulations for PSP 31
    6.1 Introduction 31
    6.2 Worlwide regulations 31
    6.3 Regulations in Europe 32
    6.4 Actions taken, when products contain unacceptable levels of toxins 33
7. Risk evaluation 34
8. Overall conclusions and recommendations 36
    8.1 Overall conclusions 36
    8.2 Conclusions considering The Netherlands 36
    8.3 Recommendations 36

References 38

PREFACE

This report has been written within the scope of the RIVM-project "Natural toxins" (project number 388802). The basis for this report has been made by Anneke Heeremans. She made, already in 1989, as a student her final thesis on this subject for her doctoral degree certificate. Her work is greatly appreciated.

List of abbreviations

SMT = European Commission's Standards, Measurements and Testing Programme
CE = capillary electrophoresis
DSP = diarrhoeic shellfish poisoning
ELISA = enzyme-linked immunosorbent assay
GTX = gonyautoxin
HPLC = high performance liquid chromatography
ILSI = International Life Sciences Institute
MS = mass spectrometry
MU = mouse unit
NEO = neosaxitoxin
PSP = paralytic shellfish poisoning
STX = saxitoxin
TTX = tetrodotoxin
WHO = World Health Organization

SAMENVATTING

Paralytic shellfish poisoning (PSP) wordt veroorzaakt door consumptie van schelpdieren die PSP toxinen bevatten. Er zijn 18 verschillende PSP toxinen, waarvan saxitoxine de meest bekende en de meest toxische is. PSP toxinen kunnen worden aangetoond met de muis bioassay, waarbij de dood van het dier als toxiciteitscriterium geldt. Deze methode wordt op dit moment nog veel toegepast, maar de internationale druk die wordt uitgeoefend door o.a. de dierenbeschermingsorganisaties stimuleert het onderzoek naar chemische analysemethoden om de muis bioassay te vervangen. Hoewel er verschillende methoden voorhanden zijn, heeft elke methode wel zwakke punten. Het grootste probleem is dat er weinig analytische standaarden en gecertificeerde referentiematerialen voorhanden zijn, alhoewel er wel veelbelovende ontwikkelingen op dit gebied zijn.
De PSP toxinen worden voornamelijk geproduceerd door zogeheten dinoflagellaten van het geslacht *Alexandrium*. Deze soort komt zowel in tropische gebieden als in de gematigde zones voor en wanneer schelpdieren zich voeden met deze algen, kunnen de toxinen accumuleren in de schelpdieren. De hoogste concentraties toxinen komen voor tijdens, of vlak na een algenbloei, maar zelf ondervinden de schelpdieren bijna geen effect van de toxinen. Sommige van de genoemde algensoorten produceren cysten die naar de bodem van de zee zakken en daar overwinteren. Vanwege hun hoge toxine-gehalte kunnen deze cysten ook een bron van verontreiniging voor de schelpdieren vormen.
Wanneer mensen de verontreinigde schelpdieren opeten kunnen ze vergiftigd raken met de PSP toxinen. De effecten zijn aanvankelijk het waarnemen van tintelingen en gevoelloosheid maar uiteindelijk kan verlamming optreden en in ernstige gevallen kan dit tot de dood leiden doordat de ademhalingsspieren verlamd raken.
Hoewel de meeste PSP toxinen hitteresistent zijn, zijn er aanwijzingen dat de processen tijdens inblikken (waaronder verhitten) tot een reductie van de concentraties aan PSP-toxinen kan leiden tot wel 90%.
De laatste jaren lijkt het alsof er een toename in het aantal intoxicaties, veroorzaakt door PSP, plaatsvindt. Het is echter niet geheel zeker of dit daadwerkelijk zo is, of dat dit beeld wordt veroorzaakt door verbeterde analysemethoden en toegenomen identificatie en registratie van de symptomen.
Op dit moment zijn er 25 landen met wetgeving op het gebied van PSP. De meeste landen hebben wetgeving voor PSP in het algemeen, maar sommige landen hebben ook specifieke wetgevingen voor een of meer PSP toxinen.

SUMMARY

Paralytic shellfish poisoning (PSP) is caused by ingestion of shellfish containing PSP toxins. The PSP toxins are a group of 18 closely related tetrahydropurines. The first PSP toxin chemically characterised was saxitoxin. The various PSP toxins significantly differ in toxicity, with saxitoxin being the most toxic. The presence of PSP toxins in shellfish can be demonstrated with the mouse bioassay, a non-selective testing procedure, with animal death as toxicity criterion. The test is currently used in many countries for checking the toxicity of shellfish but international pressure a.o. of animal welfare groups has led to stimulation of research to replace the mouse bioassay by chemical methods of analysis. It is expected, however, that several more years of intensive investigations will be needed, before a selective, sensitive and validated method of analysis becomes available and accepted. Although promising developments have been made, limited supplies of purified toxin standards are still delaying rapid progress.

The PSP toxins are produced mainly by dinoflagellates belonging to the genus *Alexandrium*, which may occur both in the tropical and moderate climate zones. Shellfish grazing on these algae can accumulate the toxins, but most of them are rather resistent to the harmful effects of these toxins. The highest concentrations in shellfish are found during or directly after an algal bloom. Some of the PSP producing algae may produce toxin-containing cysts. These immobile resting stages sink to the sediment-water interface, where they overwinter. Due to their high toxin concentration these cysts may be a source of contamination of shellfish with PSP.

People consuming the contaminated shellfish can become intoxicated. The primary mode of action of PSP toxins in mammals is their binding to sodium channels in nerve cell membranes, readily at nanomolar concentration. This leads to inhibition of impulses along the nerves, resulting in paralysis, respiratory depression and circulatory failure. Symptoms of human PSP intoxication vary from a slight tingling or numbness to complete respiratory paralysis. In fatal cases, respiratory paralysis occurs within 2-12 hours of consumption of the PSP containing food.

Although most PSP toxins are heat-stable under acid conditions, there are indications that canning of shellfish leads to a reduction of PSP concentrations of more than 90%.

During the last 20 years there seems to be an increase in intoxications, caused by PSP. As yet it is unclear whether the increase is real, whether it could be a consequence of improved identification, detection and medical registration, or whether it is due to expanded shellfish culture/consumption.

Currently, some 25 countries have regulations for PSP. Most regulations are set for paralytic shellfish poisoning as a group, but some countries indicate specific regulations for one of the PSP toxins, mostly saxitoxin.

INTRODUCTION

The microscopic planktonic algae of the world's oceans are critical food for filter-feeding bivalve shellfish (oysters, mussels, scallops, clams). Some species of the planktonic algae, called dinoflagellates, can produce algal toxins and bivalve shellfish feeding on these algae can accumulate the toxins and thus become very toxic. The consumption of these shellfish by humans can result in severe illness.

One of the first recorded fatal cases of human poisoning after eating shellfish contaminated with dinoflagellate toxins happened in 1793, when Captain George Vancouver and his crew landed in British Columbia in an area now known as Poison Cove. He noted that for local Indian tribes it was taboo to eat shellfish when the seawater became phosphorescent due to the dinoflagellate blooms.

On a global scale many cases of human poisoning through fish or shellfish consumption are reported each year. Many years the consumption of shellfish was confined largely to martime communities but with the advent of modern transportation and refrigeration systems, shellfish have as well in inland regions become an important food item. The increased international trade in fishery products and the possibility that exotic toxin producing algae are spread to "new" areas make it possible that problems with shellfish causing intoxication take on significant proportions in the field of global public health.

This report deals only with one of the most important categories of shellfish toxins, the paralytic shellfish poisoning (PSP) toxins. The purpose of this study is to summarize data to make a current update of the information on PSP available, and to find out whether there are enough quantitative toxicological data to enable a well funded toxicological evaluation and thus provide a world wide harmonisation of the limits to be used for PSP toxins.

2. THE PSP TOXINS

2.1 Structures and properties

The PSP toxins form a group of closely related tetrahydropurine compounds which make up four sub-groups: carbamate, N-sulfo-carbamoyl, decarbamoyl and deoxydecarbamoyl components. At least 18 PSP toxins have been identified, mainly from from marine dinoflagellates and shellfish that feed on toxic algae. Attempts to isolate PSP toxins began more than hundred years ago but their occurrence as mixtures of compounds with different ionizable functionalities complicated isolation procedures and early progress was slow. The development of ion-exchange chromatography, guided by mouse bioassays, eventually led to the isolation of a water-soluble basic toxin from the Alaska butterclam (*Saxidomus giganteus*). This compound was later given the trivial name saxitoxin (STX).
In 1975 the first crystalline derivative of saxitoxin was synthesised and the structure was studied (Bower *et al.*, 1981). By means of x-ray crystallographic and nuclear magnetic resonance (NMR) spectroscopic studies the structure of saxitoxin was elucidated (see figure 1 for the chemical strustures of saxitoxin and other PSP toxins). The dihydroxy or hydrated ketone group on the five ring is essential for its poisonous activity. Catalytic reduction of this group with hydrogen to a monohydroxy group eliminates the activity. Removal of the carbamoyl group side-chain on the six-membered ring, leaving a hydroxyl group in its place, produces a molecule with about 60% of the original toxic activity. The presence of this active hydroxyl group establishes a means for the preparation of various derivatives of STX (Halstead and Schantz, 1984). The PSP toxins are heat stable at acidic pH (with the exception of the N-sulfo-carbamoyl components), but unstable and easily oxidised under alkaline conditions (Krogh, 1988).

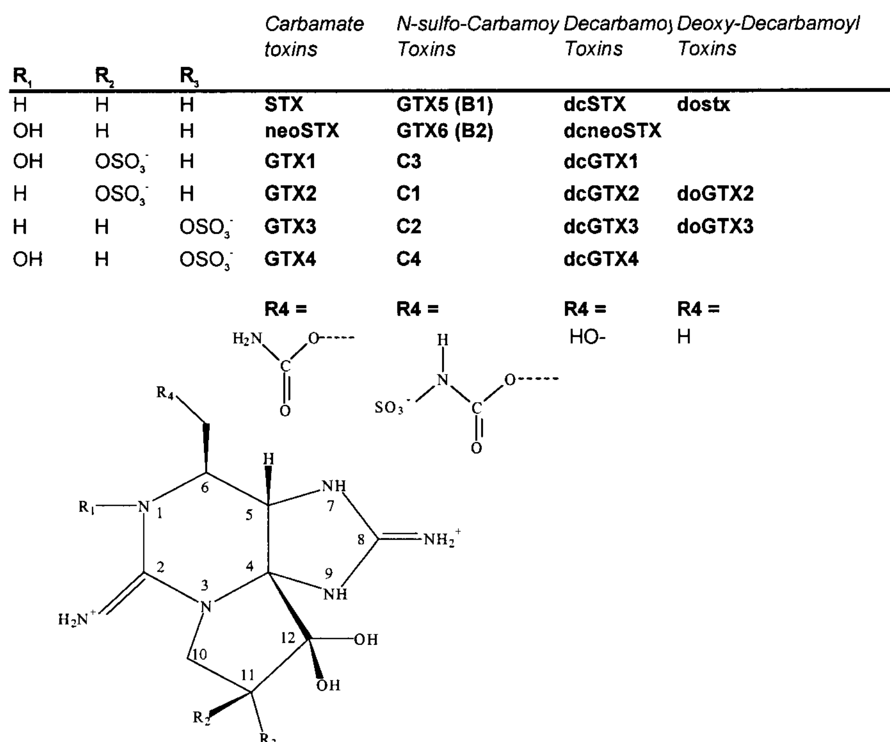
2.2 Determination of PSP toxins

2.2.1 In general

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

2.2.2 Bioassay

Presently the mouse bioassay still forms the basis of most shellfish toxicity monitoring programs. The procedure was developed more than half a century ago (Sommer and Mayer, 1937), and has been refined and standardised by the Association of Official Analytical Chemists (AOAC) to produce a rapid and reasonable accurate measurement of total PSP toxins (Hollingworth and Wekell, 1990). Twenty gram mice are injected with 1 ml of an acid extract of the shellfish, and the time taken for the animal to die is recorded. Highly toxic extracts are diluted to ensure that mortality occurs within 5 to 15 minutes. The toxicity of the



R1	R2	R3	Carbamate toxins	N-sulfo-Carbamoy Toxins	Decarbamoy Toxins	Deoxy-Decarbamoyl Toxins
H	H	H	**STX**	**GTX5 (B1)**	**dcSTX**	**dostx**
OH	H	H	**neoSTX**	**GTX6 (B2)**	**dcneoSTX**	
OH	OSO_3^-	H	**GTX1**	**C3**	**dcGTX1**	
H	OSO_3^-	H	**GTX2**	**C1**	**dcGTX2**	**doGTX2**
H	H	OSO_3^-	**GTX3**	**C2**	**dcGTX3**	**doGTX3**
OH	H	OSO_3^-	**GTX4**	**C4**	**dcGTX4**	
			R4 =	**R4 =**	**R4 =**	**R4 =**
			H_2N–C(=O)–O----	SO_3^-–N(H)–C(=O)–O-----	HO-	H

Figure 1: Structures of paralytic shellfish poisoning (PSP) toxins

sample is then calculated with reference to dose response curves established with saxitoxin standards and expressed in mouse units (MU). In most countries the action level for closure of the fishery is 400 MU/100g shellfish (1 MU is the amount injected toxin which would kill a 20 g mouse in 15 minutes, and is equivalent to 0.18 μg of saxitoxin). The limit of detection of the assay is approximately 40 μg saxitoxin/100 g of shellfish tissue with a precision of ± 15-20%. Known interferences include a high salt content of samples which suppress toxic effects (Schantz *et al.*, 1985), and highly toxic extracts may give extremely variable results (Park *et al.*, 1986). The practical drawbacks to using the method are:

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

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

2.2.3 Biochemical Assays

ELISA

Indirect enzyme-linked immunosorbent assays (ELISA) that exploit antibody-antigen binding are increasingly used as "dip-stick" assays for a variety of compounds. One method for production of PSP assay systems has been described by Chu and Fan (1985). A saxitoxin antigen is prepared using bovine serum albumin, and injected into rabbits. Antibodies raised by the rabbits are then collected and lyophilised. In the test system antigens are coated to microtitre plates, saxitoxin standards or mussel extracts and appropriate dilutions of antibodies are added, and the amount of bound antibody is determined using goat antirabbit IgG peroxidase conjugate, with measurement by a colorimetric substrate assay. Saxitoxin present in the mussel extract competes for binding with the saxitoxin antigen coated to the microtitre plates. Until recently ELISA test kits have only been available for saxitoxin. However, these are not totally specific for saxitoxin and some reaction is induced to decarbamoyl-saxitoxin (dc-saxitoxin) and neosaxitoxin. Cembella and Lamoreux (1991) described a polyclonal test kit which measures saxitoxin, neosaxitoxin, gonyautoxin 1 and gonyautoxin 3. Although the kit has not yet been fully evaluated, it appears to be more sensitive than HPLC and more specific than the mouse bioassay. However, the presence of cross reactions with lower binding specificity and the lack of response to other toxins within the PSP group have ensured that the application of ELISA systems are extremely limited.

Sodium Channel Blocking Assay

The mechanism by which the PSP neurotoxins disrupt cell function has been suggested as an alternative method of assay. The toxins bind to sodium channels in nerve cell membranes disrupting normal depolarisation. The amount of binding is proportional to toxicity. One method of assay has been described by Davio and Fontelo (1984) in which the amount of radiolabeled saxitoxin displaced from a rat brain preparation is measured. An alternative approach has been developed in mouse neuroblastoma cells by Kogure *et al.* (1988) and Gallacher and Birkbeck (as cited by Van Egmond *et al.*, 1993). Mouse neuroblastoma cells swell and eventually lyse upon exposure to veratridine, which, when added together with ouabain, enhances sodium ion influx. In the presence of saxitoxin, which blocks the sodium channels, the action of the other two compounds is inhibited and the cells remain morphologically normal. The tissue culture bioassay using mouse neuroblastoma cells, takes advantage of these principles. In this bioassay the fraction of the cells protected from the actions of ouabain and veratridine is in direct proportion to the concentration of STX and its analogues. Jellett *et al.* (1992) have modified this bioassay, improving its speed and convenience by eliminating the need to count individual cells to determine the saxitoxin equivalents, and instead have employed a microplate reader for automated determinations of absorbances of crystal violet from stained neuroblastoma cells. When these changes and other minor technical modifications were tested in the tissue culture bioassay systematically, the lower detection limit was found to be around 10 ng STX equivalents per ml of extract (= 2.0 µg STX eq/100 g shellfish tissue). This version of the tissue culture bioassay was compared with the standard mouse bioassay using 10 acid extract of dinoflagellates (*Alexandrium excavata* and *Alexandrium fundyense*) and 47 extracts of shellfish tissues, prepared according to the AOAC procedure. The tissue culture bioassay provided results virtually identical to those obtained with the mouse bioassay ($r > 0.96$), and moreover, was considerably more sensitive. The results obtained from high performance liquid chromatography (HPLC) analysis of a subset of 12 extracts were less consistent when compared with the results from both bioassay methods (Jellett *et al.*, 1992).

Although it would require an extended period of parallel testing before this test could partially or totally replace the mouse bioassay, it might be a good alternative to live animals testing for paralytic shellfish poisoning.

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

2.2.4 Chemical Assays

Fluorimetric/Colorimetric Techniques
The alkaline oxidation of PSP toxins yields fluorescent products, allowing simple determination using fluorimetric techniques (Bates and Rapoport, 1975; Bates *et al.*, 1978). However, such techniques are subject to several sources of variability. The adjustment of pH during extraction and before oxidation is critical, ion exchange column cleanup is necessary to remove interfering co-extractants, and the presence of a variety of metals can affect oxidation and subsequent fluorescent yield. Moreover, the toxins do not fluoresce equally, and for several of the carbamate toxins fluorescence is very weak. One way of circumventing the latter problem is to apply multiple fluorescence and colorimetric assays on the same samples. The fluorescence assay was reported to be an order of magnitude more sensitive, and the colorimetric assay slightly more sensitive, than the mouse assay (Mosley *et al.*, 1985).
Hungerford *et al.* (1991) have automated a fluorescence method by using flow injection analysis. The method allows automatic correction for background fluorescence and rapid screening of shellfish samples for the presence of PSP toxins.

Chromatographic Techniques
Techniques based on high performance liquid chromatography (HPLC) are the most widely used nonbioassay methods for determination of PSP compounds. During the last decade considerable effort has been applied to the development of an automated HPLC method for routine analysis of PSP toxins. The assays are generally based on separation of the toxins by ion-interaction chromatography and use a post-column reactor that oxidises the column effluent to produce readily detectable derivatives. The methodology developed by the U.S. Food and Drug Administration was reported to be capable of resolving 12 carbamate and sulfocarbamoyl PSP toxins (Sullivan, 1988). The methodology has been validated against the mouse bioassay and correlations between the techniques are generally good ($r^2 > 0.9$) (Sullivan, 1988). Detection limits are generally an order of magnitude lower than with the mouse assay. In practice, the method of Sullivan (1988) has shown difficulties in separating saxitoxin from dc-saxitoxin (Van Egmond *et al.*, 1994) and has therefore gone out of use in most European laboratories involved in PSP-analysis.

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

separation (Lawrence and Menard, 1991; Lawrence *et al.*, 1995), but as yet not all of the known PSP toxins have been separated by this method because some of the known toxins (e.g. GTX2 and GTX3) lead to the same oxidation products.
Furthermore, for accurate quantitation, it is essential to calibrate the system continually using PSP toxins standards. This is because of differences in the chemistry of each PSP toxin that result in different oxidation rates for each compound in the post-column reactor. Until recently only a saxitoxin standard was commercially available, and accurate estimation of the amounts of the other PSP toxins in the mixture was impossible. Now certified standards of saxitoxin, neosaxitoxin, GTX 1-4 are commercially available (Laycock *et al.*, 1994), and their availability significantly improves the quality of the data that is obtained by the HPLC-method (Wright, 1995).
In addition, a project was carried out from 1993-1997 within the framework of the European Commission's Standards, Measurements and Testing Programme (SMT), to develop shellfish reference materials with certified mass fractions of some PSP toxins. The work was carried out by a consortium of 13 public laboratories and 6 universities, representing the 5 main shellfish producing countries in the Community and some other EC-countries that had an interest in the area of PSP-determinations. A preliminary interlaboratory study in the EC had already shown that there was a basis for the development of reference materials and that there existed a large interest for this in the EU.
The research programme involved:
- studies on the improvement and evaluation of the chemical methodology;
- identification and determination of purity of PSP standards, and their stability in solution;
- two intercomparison studies of analytical methods;
- preparation of reference materials, including homogeneity and stability studies;
- a certification exercise.

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

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

2. Capillary Electrophoresis
Capillary electrophoresis (CE) is a relatively new technique, and to date there have been few applications in the field of toxin analysis, but the flexibility of CE systems suggests that it is a promising area for research. In essence, the technique employs a narrow (~100 μm id) fused

silica capillary in place of the electrophoretic gel, and nanolitre amounts of the sample are introduced to the end of the column before it is used to bridge two buffer reservoirs. The toxins migrate through the column when high voltage is applied and may be detected as they pass through a UV or fluorescence cell. The technique is applicable to broad classes of compounds with electrophoretic mobility, and even where no net charge occurs it is possible to trap compounds in micelles which will then migrate.
Wright *et al.* (1989) applied a CE system coupled to a laser fluorescence detector for the determination of saxitoxin standards. The technique allowed detection of saxitoxin at the µg/kg level. Even though the injection volume is necessarily small (1 to 10 nl), the theoretical detection limits for samples are in the µg/kg range. The present drawbacks to the technique are that the same separation has not been demonstrated for biota with mixed toxins, the equipment is not commercially available and is expensive, and the methodology suffers from the same problems as HPLC in that a fluorescent derivative must be prepared before separation or detection.
Thibault *et al.* (1991) have applied a CE method to samples of marine biota. Separations of neosaxitoxin and saxitoxin were achieved and using UV spectrometry a detection limit of 5 µM (approx. 1.5 µg/ml) was demonstrated. The authors suggest that the CE-UV technique holds considerable promise for the routine screening of these toxins in natural extracts, but presently detection limits appear to be too high to be of use in monitoring programs.

Mass Spectrometry
Purified samples of algal toxins have been analysed directly by fast atom bombardment (FAB) (Maruyama *et al.*, 1984; White *et al.*, 1986). However, mass spectrometry is generally used as an alternative detection system to fluorescence or UV spectrometers and has been coupled to HPLC and CE. There is no doubt that HPLC/MS can be made to work. For example, Quilliam *et al.* (1989), have demonstrated the determination of saxitoxin by HPLC interfaced to MS using the ion-spray technique. In positive ion-spray mode, the detection limit was found to be 0.1 µM (1 µl injection), which is about five times more sensitive than with the mouse assay. However, the apparatus is presently very expensive; commercially-produced electro/ion-spray interfaces for HPLC/MS are only available for the top range products, and complete HPLC/MS systems cost more than $200,000. Interfaces for bench top mass spectrometers are generally based on thermospray, and it is doubtful that the required detection limits would be met. Thibault *et al.* (1991) have used a similar interface for CE/MS. Once again, the technique was demonstrated to be effective for saxitoxin and neosaxitoxin detection (0.5 and 1.3 ng, respectively) but suffers from the same drawback of expense.

3. SOURCES OF PSP COMPOUNDS

3.1 Main source organisms

The PSP compounds are present in 3 genera of dinoflagellates and one species of blue-green algae. The dinoflagellates, with their ability to photosynthesise, play an important role in the productivity of the ocean and, along with other microalgae, constitute the basis of the marine food chain. The diameter of these cells varies between 40 - 50 μm. Their motility is based on two flagels. Some dinoflagellates are bioluminescent (Krogh, 1983; Halstead and Schantz, 1984). There are only a few known toxic dinoflagellates among the 400 or more described species. This ratio of toxic to nontoxic organisms is no greater than among higher plants.
Several species of the genus *Alexandrium* (formerly named *Gonyaulax* or *Protogonyaulax*) are identified as contaminators in the shellfish. These are *Alexandrium tamarensis* (syn. *A. minitum, A. excavata), A. acetenella, A. fraterculus,* and *A. cohorticula*. Other, clearly distinct dinoflagellates have also been recognised as sources of the saxitoxins. These are *Pyrodinium bahamense* var. *compressa* and *Gymnodinium catenatum* (Bower *et al.*, 1981; Bryan, 1980; Schulze, 1985). The toxicity of the dinoflagellates is due to a mixture of STX derivatives of which the composition depends on the producing species.
There is also an immobile form of some dinoflagellates, the resting cyst or the hypnozygote. The cyst has a diameter of about 25 μm, is eggformed, has a weak calcified skeleton and an orange-red or brown-blue colour with radial striping (Schulze, 1985; Krogh, 1983; White and Lewis, 1982). The cysts sink to the bottom of the sea and accumulate at the borderline of water and sediment, where they overwinter (Krogh, 1983). When favorable growth conditions return, the cysts may germinate and reinoculate the water with swimming cells that can then bloom. In this way the survival of certain dinoflagellates from one season to the other season is assured. The cysts are itself also toxic, however the exact toxicity is not clear. Some investigators claim a toxicity of the same order as the dinoflagellate itself, but other mention a ten to thousand fold higher concentration of PSP toxins in the cysts than in the mobile cells (Krogh, 1983; White and Lewis, 1982).

3.2 Other producers of PSP toxins

Apart from the protista the freshwater cyanophyte *Aphanazomenon flos-aquae* has also been shown to contain saxitoxin and neosaxitoxin (Shimizu, 1984; Hall *et al.*, 1990).
Other investigators indicated the presence of PSP components in shellfish and crabs without any sign of the appearance of toxic protista. These species were e.g. *Spondylus butleri* and *Zosimeus aenus* (Bower *et al.*, 1981). It is not clear to what content the consumptions of coral reef algae were responsible for this effect.

3.3 Habitat

The areas in which dinoflagellates and their cysts occur are in particular the waters near North America, Europe and Japan but occurrences in Asia are being reported increasingly (Hallegraef, 1993).
Alexandrium acatenella has been observed particularly along the coast of North America, Southern Japan and Venezuela whereas *Alexandrium tamarensis* is found in North America, northern Japan, southern Europe, Turkey and Australia (Hall *et al.*, 1990, Hallegraef, 1993).

A. excavata (syn. *A. minutum, A. excavata*) has been reported from the north-east coast of North America, Egypt, Australia in the North Sea (Denmark, Germany, Netherlands, Norway and Great Britain) and from the Mediterranean coast (Hallegraef, 1993; Halstead and Schantz, 1984).
Outbreaks of PSP in Japan, the north-west coast of North America, southern Ireland, Spain, Mexico, Argentina and Tasmania have also been caused by blooms of *Gymnodinium catenatum*. The present day distribution of *Gymnodinium catenatum* includes the Gulf of California, Gulf of Mexico, Argentina, Venezuela, Japan, the Philippines, Palau, Tasmania, the Mediterranean and the Atlantic coast of Spain and Portugal (Hall, 1990; Hallegraef, 1993).
The first harmful implications of *Pyrodinium* bloom became evident in 1972 in Papua New Guinea. Since then toxic *Pyrodinium* blooms have apparently spread to Brunei and Sabah and the central and northern Philippines. Most unexpectedly, during a *Pyrodinium* bloom in 1987 on the Pacific coast of Guatemala 187 people had to be hospitalised and 26 people died. In 1989 another bloom swept northward along the Pacific coast of Central America, again causing illness and death (Hallegraef, 1993).

3.4 Significance of blooms

Dinoflagellates are capable of a fast, asexual reproduction. If also the cysts are involved, which may decyst under favourable conditions, the concentration of dinoflagellates may rapidly increase. When the concentration exceeds the value of 20.000 cells/ml seawater one deals with bloom. The colour of the bloom depends on the responsible algal species, the concentration and the depth at which the bloom occurs. Generally the term "red tide" is used, but this name can be misleading as the bloom may also be yellow, green, blue or brown (Bower *et al.*, 1981) and an accumulation of nontoxic, harmless algae can also change the colour of ocean water. In addition, many toxic events are called red tides, even when the water shows no discoloration and the catch of toxic mussels is not always associated with algal bloom, as shellfish can remain toxic for a long time after a bloom.
The presence of a bloom should make the responsible authority aware of the possible danger of shellfish intoxication. The population density of algae can not be used as a parameter for the intoxications of shellfish as concentrations starting from only 200 dinoflagellates per ml seawater were located to cause already mussel intoxication (Halstead and Schantz, 1984; Schulze, 1985). Older dinoflagellate cells contain more toxin than young cells and the possible role of the cysts is also important (Kodama *et al.*, 1982; Halstead and Schantz, 1984).
While harmful algal blooms, in a strict sense, are completely natural phenomena which have occurred throughout recorded history, in the past two decades the public health and economic impact of such events appear to have increased in frequency, intensity and geographic distribution. Reports of harmful algal blooms, associated human illnesses or damage to aquaculture operations are receiving increased attention in newspapers, electronic media and the scientific literature. Consequently, more and more researchers are now surveying their local waters for the causative organisms. Increased reports on the occurrence of dinoflagellates of the genus *Alexandrium* are a good example. Until 1988, the type species *A. minutum* was only known from Egypt; it has now been reported from Australia, France, Spain, Portugal, Italy, Turkey and the east coast from North America (Hallegraef, 1993).
In addition, with increased problems of overfishing of coastal waters, more and more countries are looking towards aquaculture as an alternative. Aquaculture operations act as

sensitive 'bioassay systems' for harmful algal species and can bring to light the presence in water bodies of problem organisms not known to exist before (Hallegraef, 1993).

3.5 Induction of blooms; predisposing conditions.

3.5.1 Distribution of algae/cysts

It is not predictable when a bloom of dinoflagellates will develop, neither is the population density a predictable factor. Within a short period a high concentration of dinoflagellates may be developed, which may result in red tide and/or shellfish intoxication. Not much is known about conditions which favour the explosive reproduction of dinoflagellates. However there are some factors of which it is believed that they may be of influence.

The presence of resting cysts seems to be a condition for the development of algal blooms. A bloom begins as a small population of toxic dinoflagellate cells in the lag phase or in the form of resting cysts residing in the bottom sediment and the timing and location of a bloom depends on when the cysts germinate and where they were deposited. Climatological and environmental conditions such as changes in salinity, rising water temperature, and increased nutrients and sunlight trigger cyst germination to a vegetative stage that enables rapid reproduction. Once the dinoflagellate bloom begins, an exponential growth phase causes a tremendous increase in their population. In time, depletion of nutrients and carbon dioxide in the water and degraded environmental conditions caused by the bloom decrease population growth. A stationary phase ensures leveling off the population. At this high level of the bloom, the water can have a fluorescent reddish colour referred to as red tide. Continued environmental degradation increases cell death and ultimately leads to a population crash. At this phase of the bloom many dinoflagellate species form resting cysts that sink to the bottom, ready for the next bloom. Within this bloom cycle, the most toxic cells occur generally during the middle of the exponential growth phase (Anderson, 1990).

Cyst production facilitates species dispersal as well; blooms carried into new waters by currents or other means can deposit "seed" populations to colonise previously unaffected areas. The (tide)stream of the water is necessary for the transportation of the cysts (after the winterseason) and decysting has to take place, requiring a temperature of 5 °C or more (White and Lewis, 1982).

In addition, dinoflagellate species that are not endemic to a region can be inadvertently introduced when their resistant resting stages are discharged with the ballast-tank water and sediments of bulk cargo vessels. A survey of 343 cargo vessels entering 18 Australian ports showed that 65% of ships were carrying significant amounts of sediment on the bottom of their ballast tanks. Of considerable concern was the detection in 16 ships of cysts of the dinoflagellates *Alexandrium catenella*, *Alexandrium tamarense* and *Gymnodinium catenatum*. One single ballast tank was estimated to contain > 300 million viable *A. tamarense* cysts, some of which were successfully germinated in the laboratory to produce toxic cultures (Hallegraef & Bolch, 1992).

Another vector for the dispersal of algae (especially their resting cysts) is with the transfer of shellfish stocks from one area to another. The faeces and digestive tracts of bivalves can be loaded with viable *Alexandrium* cells and sometimes can also contain resistant resting cysts (Hallegraef & Bolch, 1992; Hallegraef, 1993).

3.5.2 Climatological conditions

There seems to be season dependency, which is mainly determined by the temperature of the water and the sunlight. Dinoflagellates develop at relative high temperatures and abundant sunlight. In Europe and South Africa cases of intoxications and mortality thus occurred mainly between May and November, whereas in North America the intoxications were reported between July and September (Halstead and Schantz, 1984).
There are no reasons to assume that shellfish intoxication can be predicted by the properties of the regional area. The type of habitat in which PSP intoxications have been observed varies considerably. Probably hydrographic conditions play a important role, especially the presence of a thermocline is very important (an upperlayer of seawater which does not mix with the underlying water). Indirectly windforce and turbulency in the water may influence the existence of this thermocline (Halstead and Schantz, 1984, Van Egmond *et al.*, 1993).
Maclean (1989) presented evidence for a coincidence between *Pyrodinium* blooms and El Niño-Southern Oscillation (ENSO) climatological events. El Niño is caused by an imbalance in atmospheric pressure and sea temperature between the eastern and western parts of the Pacific Ocean and results in a shoaling of the thermocline (Maclean, 1989 as cited by Hallegraef, 1993).

3.5.3 Nutrients

The amount of nutrients in the seawater has to be adequate to fulfil the needs of the organisms, especially the concentration of trace elements, chelators, vitamins and organic material in general. There are many uncertainties in the determination of the exact role of nutrients in the development of red tides. For example, the development of red tides is sometimes stimulated by low salt concentrations, whereas in other cases high concentrations of salt seem to induce the bloom (Acres and Gray, 1978; Halstead and Schantz, 1984).
In general, red tides often occur when heating or freshwater runoff creates a stratified surface layer above colder, nutrient-rich waters. Fast-growing algae quickly strip away nutrients in the upper layer, leaving nitrogen and phosphorus only below the interface of the layers, called the pycnocline. Nonmotile phytoplankton cannot easily get to this layer, whereas motile algae, such as the dinoflagellates, can thrive. Many swim at speeds in excess of 10 meters a day, and some undergo daily vertical migration; they reside in surface water like sunbathers, then swim down to the pycnocline to take up nutrients at night. As a result, blooms can suddenly appear in surface waters that are devoid of nutrients and would seem incapable of supporting such prolific growth (Anderson, 1994).
Evidence is increasing from diverse areas such as Hong Kong Harbour, the Seto Inland Sea in Japan and North European coastal waters that 'cultural eutrophication' from domestic, industrial and agricultural wastes can stimulate harmful algal blooms. It is even possible that algal species which are normally not toxic may be rendered toxic when exposed to atypical nutrient regimes (e.g. phosphate deficiencies) resulting from cultural eutrophication. Changed patters 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 (Hallegraef, 1993).

4. SHELLFISH ASSOCIATED WITH PARALYTIC SHELLFISH POISONING

4.1 In general

Bivalve shellfish feed by filtering food particles suspended in the water and then transferring such food particles from the gills to the digestive organs. If a bloom of toxic dinoflagellates would occur, the shellfish in that area are likely to serve as unwitting concentrators of the toxins and can remain toxic for a considerable period afterwards. Almost any bivalve is susceptible, regardless of whether it is submerged at low tide or not (Kao, 1993).
Most of these filter-feeders are relatively insensitive for the PSP toxins, because many of them have nerves and muscles operated mainly by voltage-gated calcium channels and saxitoxin and other PSP toxins block only the voltage-gated sodium channel with great potency (high affinity) (Kao, 1993). This enables them to continue feeding whereafter they become highly toxic. Their high tolerance to saxitoxins and continued feeding on toxic algae can result in initially toxin-free blue mussels exceeding the 80 μg saxitoxin level in less than 1 hr (Bricelj *et al.*, 1990; Beitler and Liston, 1990).
Although most filter-feeders are relatively insensitive to the saxitoxins there are, however, differences among the various species of bivalves in the way they deal with and respond to the saxitoxins. Mussels, for instance, appear in general to accumulate much higher levels of PSP toxins than oysters under similar circumstances. Subsequent laboratory feeding studies showed that mussels readily consumed concentrations of *Alexandrium* equal to or greater than those that caused oysters to cease pumping and close up. Electrophysiological investigations of isolated nerves from Atlantic coast bivalves demonstrated that those from oysters were sensitive to the toxins, while those from the mussels were relatively insensitive (Hall *et al.*, 1990).
There are even some species of bivalves known to avoid toxic dinoflagellates. One species of particular interest is the northern quahog or hard clam, *Mercenaria mercenaria*. In a laboratory study in the presence of *A. tamarense* the quahog first retracts its siphons and then completely isolates itself from the external environment by means of shell valve closure. The animals did not re-open their valves until the addition of clean sea water (Shumway *et al.*, 1995).

4.2 Uptake, transformation and distribution

Rates of intoxication and detoxification (or: contamination and decontamination) of filter-feeding shellfish by toxic algae are species-specific and are, in most cases, directly related to the number of cells available to the animals. Toxicity of individual shellfish in any given area is highly variable.
During the process of filtration the dinoflagellate cells and cysts are transported to the oesophagus and the stomach of the bivalve molluscs. The digestion takes place in the stomach and the diverticulae whereby the PSP toxins are released and enter the digestive organs. The particular toxin mixture retained in soft tissues of the shellfish varies in concentration and over time, and is determined by the species and strains of the dinoflagellates and shellfish as well as by other factors like environmental conditions (Martin *et al.*, 1990). In mussels it was found that the viscera, which constitute only 30% of the total tissue weight, contribute 96% of total toxicity (Bricelj *et al.*, 1990). In clams the toxins rapidly concentrate in the viscera and gradually decrease afterwards. After a lag period for 4 or more weeks the toxins are mainly

detected in the siphon (Bower *et al.*, 1981, Beitler and Liston, 1990). The composition is not consistent but varies with the time and location in the animals.
After uptake and distribution the toxins may undergo transformation. In feeding experiments nontoxic butter clams were fed *Alexandrium catenella* containing GTX I-IV and NEO but no STX. After a period of 83 days STX was detected as well, making the authors conclude that some type of synthesis or biotransformation of GTX I-IV and/or NEO to STX occurs *in vivo* (Beitler and Liston, 1990). Similar findings were reported by Martin *et al.* (1990).
One common transformation, termed epimerization, occurs when a portion of the original saxitoxin molecule rearranges. Scallop and mussel, for example, can perform epimerization of saxitoxin they receive from the toxic algae when the H and OSO_3^- switch locations on the number 11 position of the saxitoxin molecule (Oshima *et al.* 1990). Such a transformation can decrease the toxicity by 11 times.
On the contrary there are also transformations that increase toxicity. For example, a six-fold increase in toxicity occurs when the SO_3^- group is separated from position 21 on the saxitoxin molecules by acid hydrolysis (Hall *et al.*, 1990).
The butter clam has a distinctive ability to chemically bind the highly toxic STX in its siphon tissue and can retain PSP toxins for up to 2 yr. after initial ingestion. The little neck clam *Prothotaca staminea* can become toxic as well, but less toxic than the butter clam. The lower toxicity of the little neck clam is due partially to its ability to perform transformations that change highly toxic saxitoxins to the moderately toxic forms (Sullivan *et al.*, 1983). The combined effect of the littleneck clam's capability to transform saxitoxins to less toxic forms, and the ability of butter clams to concentrate and retain highly toxic forms can result in a wide difference in toxicity between these two species. This toxicity difference is particularly significant since butter clams and littleneck clams can coexist on the same beach, and are, to the unskilled harvester, similar in appearance.

4.3 Excretion

In general the toxic properties of the shellfish are reversible as they clean themselves of saxitoxins through depuration. The time required for elimination in mussel tissue varies considerably (Schulze, 1985). If the contaminating dinoflagellates have disappeared from the surrounding water there is a gradual decline in the amount of toxin (Shimizu, 1984). The toxicity of the mussel *Mytilus edulis* can decrease by 50 % in 12 days in dinoflagellate free, salt water with a temperature of 15-20 °C (Halstead and Schantz, 1984; Schulze, 1985). The elimination time of PSP toxins from the clam *Saxidomus giganteus* however is much longer. It takes a year or longer after the exposure to the toxic dinoflagellates to loose the toxins (Schulze, 1985; Halstead and Schantz, 1984).
The rate of loss varies with season and low water temperatures apparently retard toxin loss; however the degree to which temperature affects the uptake and release of toxins is not clearly understood. Further, the rate of detoxification is highly dependent on the site of toxin storage within the animal (toxins in the gastointestinal tract are eliminated much more readily than toxins bound in tissues), and on initial or peak level of toxicity (Shumway *et al.*, 1995).
Mussels are known to accumulate PSP toxins faster than most other species of shellfish and also eliminate the poisons quickly. While oysters do not accumulate the toxic species as readily as mussels, they take considerably longer to detoxify (Shumway, 1990; as cited by Shumway *et al.*, 1995).

4.4 Shellfish found to contain PSP toxins

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

4.5 Toxicity in other marine organisms

Algal toxins can also cause mortalities as they move through the marine food web. Some years ago tons of herring died in the Bay of Fundy after consuming small planktonic snails that had been feeding on *Alexandrium*. From the human health point of view, it is fortunate that herring, cod, salmon and other commercial fish are sensitive to PSP toxins and, unlike shellfish, die before the toxins reach dangerous levels in their flesh. Some toxins, however, accumulate in the liver and other organs of the fish, and so animals such as other fish, marine mammals and birds that consume whole fish, including the viscera, are at risk (Anderson, 1994). In 1987 14 humpback whales died suddenly from exposure to a bloom of *Alexandrium tamarense* in Cape Cod Bay (Massachusetts). Researchers later learned that the whales had eaten mackerel whose organs contained high concentrations of saxitoxin (Anderson, 1994).
By secondary toxin transfer in the foodchain also certain fish and crab species can accumulate toxic doses of PSP toxins. In these cases mortality of fish has been reported (Koyama, *et al.*, 1983; Shimizu, Y; 1984). Human intoxication occurs however (mostly) by direct consumption of the contaminated shellfish. Of the crabs involved in human paralytic shellfish poisoning in Japan and Fiji most are xanthid crabs (*Lophozozymus pictor*)(Tsai *et al.*, 1995), though some other species are also involved. The common feature they have is that they live in coral reefs, and feed by surface grazing. (Kao, 1993).

5. Toxicity in mammalian animals and man

"People who eat them think they're going to die, and else they wish they would......"

5.1 Toxicity in animals

5.1.1 In general

The toxicity of the PSP toxins is almost always expressed as saxitoxin, or saxitoxin equivalents. When the specific toxicity of saxitoxin (STX) is estimated by means of the mouse bioassay the value is 2045 mouse units (MU)/mol = 5500 MU/mg (Shimizu, 1984). The mouse is very sensitive for the PSP toxins when compared to e.g. fish, amphibians, reptiles, and animals of a low order (Bower *et al.*, 1981). The LD50's for the different routes of administration are shown in table 1. The acute toxicity was also established in other animal species. The oral LD50-values are shown in table 2. Toxicological experiments other than acute toxicity studies with PSP toxins have not been reported.

Table 1. Acute toxicity of STX in mice after oral, intravenous and intraperitoneal administration (Bower *et al.*, 1981; Halstead and Schantz, 1984; Krogh, 1983; Davio, 1985).

Route of administration	LD50 in µg/kg body weight
Oral	260-263
Intravenous	2.4-3.4
Intraperitoneal	9.0-11.6

Table 2. The oral LD50 values established in different animal species (Krogh, 1983; Shimizu, 1978).

Animal species	LD50 in µg/kg body weight
Rat	192-212
Monkey	277-800
Cat	254-280
Rabbit	181-200
Dog	180-200
Guinea pig	128-135
Pigeon	91-100

Aside from mouse lethality bioassays which are used to determine the relative potency of all analogues (see table 3), the full biological actions have been studied for only half of the natural analogues. However, from those that have been studied, the cellular mechanism of action seems to be basically the same. The N-sulfocarbamoyl compounds are appreciably less toxic than their counterparts of the carbamoyl series, but they are readily converted to the corresponding carbamoyl compounds under acidic conditions with increases in toxicity of up to 40-fold. Such conversion has some potential clinical and public health significance, because weakly toxic shellfish containing N-sulfocarbamoyl toxins could cause disproportional severe poisoning once ingested. Experimentally, however, it has been found that the conversion occurs in artificial gastric juice of the mouse and rat at a pH of 1.1, but

not in genuine gastric juice remaining at a buffered pH of 2.2 (Harada *et al.*, 1984; as cited by Kao, 1993).

Table 3. Relative toxicity of some PSP toxins in the mouse bioassay (Usleber *et al.*, 1997).

Toxin	Relative toxicity
STX	1
NEO	0.5 - 1.1
$GTX_{2/3}$ [a]	0.39/1.09 - 0.48/0.76
$GTX_{1/4}$ [a]	0.8/0.33 - 0.9/0.9
dc-STX	0.43
dc-NEO	0.43
B1	0.07 - 0.17
B2	0.07 - 0.09
C1 to C4	< 0.01 - 0.14
dc-GTX1 to dc-GTX4	0.18 - 0.45

a = α/β epimeric mixture

5.1.2 Type of toxic effects

In animal experiments effects of saxitoxin on the respiratory system, myocard, muscle and nervous tissue (both peripheral and central) have been studied.

Effects on the respiratory system
If PSP intoxication occurs, the effects on the respiratory system are responsible for the fatal ending. The cause of death is asphyxiation due to progressive respiratory muscle paralysis (Halstead and Schantz, 1984). In animals (cat, rabbit) doses of 1 - 2 µg STX/ kg body weight administered intravenously caused a decreased respiratory activity reflected in both a decline in the amplitudo and velocity. When the dose was raised to 4 - 5 µg STX/kg b.w. a strong depression of the respiration occurred. By artificial respiration death can be avoided. If the dose is not too excessive the respiration may return spontaneously. In animal experiments only peripheral paralysis has been noticed by direct effects on the muscle of the respiratory system. The respiratory centre of the nervous system is not inhibited, action potentials are sent off to the midriff and the middle rib muscles (Evans, 1969). Other investigators however suggest a central influence (Acres and Gray, 1978). The possibility of central effects on the respiratory neurones may therefore not be excluded. The occurrence of paraesthesia and feeling of lightness are often connected with a central effect, however the peripheral effects on the nervous system may be the cause of these symptoms.

Cardiovascular effects
In anaesthetised animals doses above 1 µg STX/kg b.w. (i.v.) can already provoke hypotension (paralysis of muscles is already observed at lower dose levels). This cardiovascular effect is seldomly observed in human cases of intoxications and is more likely the reflection of peripheral effects, although the central nervous system might be involved to a certain extent (Bower *et al.*, 1982; Evans, 1969). About the peripheral action there are uncertainties. Apart from a direct effect on the muscle tissue the possibility of an axonal

blockade of the sympathic nervous system can not be excluded (Evans, 1969a). Most investigators agree on the fact that no or hardly no direct cardial effects occur. As an exception a direct disturbance of the atrio-ventricular sinus conduction is mentioned (Acres and Gray, 1978).

Neuromuscular effects
An intravenous dose of 1 - 2 µg STX causes a fast weakening of muscle contractions; both contractions by direct stimulation as contractions by indirect motoneuron stimulation are affected. The effects include all skeletal muscle tissues. This dose level induces also a decrease of the action potential-amplitudo and a longer latency time in the peripheral nervous tissue. Both motoric and sensory neurones are influenced, but the sensory neurones are already inhibited at lower dose levels. By this influence on the sensory system the numbness and the proprioceptive loss may be explained, but not the paraesthesia (Evans, 1969 a,b). About the possible theory on the toxic mechanism no clarity has been achieved, and many scientific debates reflect this.

Effects on the central nervous system
There are uncertainties about the existence of an effect of PSP toxins on the central nervous system. Most symptoms can be attributed to peripheral effects. However central effects can not be excluded. For example investigators reported the influence of STX on the hamstring reflex (Murtha, 1960; as cited by Evans, 1969).

5.2 Toxicity in man

5.2.1 Acute toxicity

The level at which PSP-intoxications occurred in man varies considerably. This variation is mainly due to individual difference in sensitivity and the fluctuation in method of determination (Krogh, 1983). Oral intake causing intoxication varied from 144 to 1660 µg STX eq./person. Fatal intoxications were reported after a calculated consumption of 456-12400 µg STX eq./person (Acres and Gray, 1978; Krogh, 1983; Gessner and Middaugh, 1995). These values are only reconstructed from what was left over of the toxic mussels and again varies greatly. An oral consumption of 300 µg PSP toxin per person was in some cases reported as fatal, whereas others noted the absence of toxic symptoms after an oral dose of 320 µg toxin per person (Bower *et al*, 1981). In Alaska paralytic shellfish poisoning was fatal to one fisherman, while two others eventually recovered. The patients stomach contents contained 370µg PSP toxin (saxitoxin eq.)/100g (U.S. Department of Health and Human Services, 1991; as cited by Van Egmond *et al.*, 1993).
The mortality rate of paralytic shellfish poisoning varies considerably. In recent outbreaks in North America and Western Europe involving over 200 people, no deaths occurred, but in similar outbreaks in south-east Asia and Latin America, death rates of 2-14% have been recorded. A large part of the difference is due to the fact that in the former cases, intoxication often occurred in urban areas where victims have already access to hospital care, whereas in the latter cases, it occurred in rural areas where the local population and health professionals have never before encountered such poisonings (Kao, 1993).

5.2.2 Kinetics

Clinicians have observed that, if patients with paralytic shellfish poisoning survive 24 hr, either with or without mechanical ventilation, chances for a rapid and full recovery are excellent. Such observations suggest that toxin(s) responsible for PSP undergo either rapid excretion, metabolism or both. In spite of the fact that most PSP toxins are positively charged, they are readily absorbed through the gastrointestinal mucosa. Depending on the severity of poisoning, the symptoms vary somewhat. The determinants of the severity are the specific toxicity of the PSP toxin in the ingested food, the amount ingested, and the rate of elimination of the PSP toxin(s) from the body. If the amount of the toxic food is high enough the first symptoms occur within a few minutes (Kao, 1993).
Rapid excretion in urine was observed in rats after i.v. administration of saxitoxin in a sublethal dose (ca. 2 μg/kg) (Hines, 1993; Stafford and Hines, 1995). No radioactivity was detectable in faeces at any time. Four hours after injection, approximately 19% of the saxitoxin dose was excreted in urine. By 24 hours, approximately 58% of the administered dose was excreted. Average total urinary excretion of administered saxitoxin was approximately 68% for the full study period. The authors concluded that these results demonstrate that small quantities of unmetabolised saxitoxin can be detected in rat urine up to 144 hr after i.v. administration (Stafford and Hines, 1995).

5.2.3 Toxic symptoms

Clinical symptoms of paralytic shellfish poisoning are in mild cases a tingling sensation or numbness around lips, which appear mostly within 30 min. These effects are clearly due to local absorption of the PSP toxins through the buccal mucous membranes. These sensations then spread gradually to face and neck. Prickly sensation in fingertips and toes is frequent as are headache, dizziness, nausea, vomiting, and diarrhoea. Sometimes, temporary blindness is observed as well. Most symptoms have a quick onset (hours) but may last for days and are virtually invariant in all cases of paralytic shellfish poisoning. They precede distinct muscular weakness, because sensory nerves, being thinner and having shorter internodes than motor nerves, are always affected first by any axonal blocking agents.
In moderately severe poisoning, paresthesia progresses to the arms and the legs, which also exhibit motor weakness. Giddiness and incoherent speech are apparent. Cerebellar manifestations such as ataxia, motor incoordination and dysmetria are frequent. Respiratory difficulties begin to appear as a tightness around the throat. In severe poisoning, muscular paralysis spreads and becomes deeper. Experimentally, in awake guinea-pigs, saxitoxin causes a continuous decline of the frequency and the tidal volume of ventilation. The pulse usually shows no alarming abnormality. Pronounced respiratory difficulty appears and death through respiratory paralysis may occur within 2-24 h of ingestion (Hallegraef, 1993., Kao, 1993).
Since saxitoxins are charged, water-soluble molecules, it is probable that they do not penetrate the blood-brain barrier well and most of their effects are on peripheral nerves (Laverty, 1993).

5.3 Theories on the toxic mechanism

The pharmacological action of the PSP toxins strongly resemble that of tetrodotoxin (TTX), another potent marine toxin present in fin-fish (Caterall, 1980; Shimizu, 1978). Due to the almost identical action of STX and TTX it was assumed that both molecules had the same interaction with the receptor (Kao and Levinson, 1986). Much attention has been given to the

elucidation of the mechanism via which the blockade of the channel is achieved as STX and TTX are the only agents which block the voltage-gated sodium channel in a selective manner and with high affinity.
The voltage-gated sodium channel is a protein of ca. 250,000 Da, which traverses the plasma membrane of many excitable cells and is characterised by uniform conduction, potential dependency and ion selectivity (Guo *et al.*, 1987; Kao, 1993). Among these are all mammalian nerves, skeletal muscle fibres, and most cardiac muscle fibres. Upon appropriate depolarisation of the cell, a conformational change occurs in the sodium channel molecule such that an aqueous path opens to permit movement of Na^+ from the extracellular phase into the cell under the existing electrochemical driving forces. The inward sodium current is responsible for the rising phase of the action potential. Voltage-gated potassium channels are also present in the membrane, and when open, they permit outward passage of intracellular K^+ and consequent repolarisation. Saxitoxin and several other PSP toxins block the voltage-gated sodium channel with great potency, thus slowing or abolishing the propagation of the action potential. However, they leave the potassium channel unaffected.
The 7,8,9-guanidine function has been identified as being involved in the channel blockade. The C12-OH (as hydrated ketone) is important, whereas the carbamoyl side chain contributes but is not vital to channel blockade. Several hydrogen bonds between the toxin molecule and the binding side add to the binding energy (Kao, 1993). There is a general agreement among the investigators on the kinetic aspects of the toxin binding (French *et al.*, 1984; Green *et al.*, 1987; Guo *et al.*, 1987; Modzydlowski *et al.*, 1984). The averaged blocking time of the channel is not dependent on the toxin concentration, but on the dissociation velocity. The life-time of the open channel however is reversibly correlated with the toxin concentration and depends on the association constant.

5.4 Treatment of intoxication

There is no specific antidote for paralytic shellfish poisoning. The clinical management of poisoned victims is entirely supportive. If no vomiting has occurred spontaneously, induced emesis or gastric lavage should be used to remove sources of unabsorbed toxins. As the PSP toxins are strongly charged at the gastric pH, they would be effectively adsorbed by activated charcoal. These steps are especially important in the management of child victims of poisoning, as the severity of the intoxication is directly dependent on the concentration of the toxins in the body. In the 1987 Guatemala epidemic, the mortality rate in children up to 6 years of age was 50% while for adults it was 7% (Rodrigue *et al.*, 1990).
In moderate severe cases, maintenance of adequate ventilation is the primary concern. In uncomplicated paralytic shellfish poisoning the airway is not obstructed by excessive excretion. As ventilatory failure is due to varying degrees of paralysis of the respiratory nerves and muscles, positive pressure assisted ventilation, when indicated, is desirable. Fluid therapy is essential to correct any possible acidosis. Additionally, it will facilitate the renal excretion of the toxins.
Time-honoured conservatively supportive management has proven effective. If the patient survives 18 hours, the prognosis is good, with complete and rapid recovery (Bower *et al.*, 1981). Some say that even 9h should be adequate for a physiological reduction of the toxins concentration to relatively harmless levels (Kao, 1993), except in those cases where the toxin concentration began at exceptionally high levels, or in victims with impaired renal function.

5.5 Prevention of PSP intoxication

5.5.1 Introduction

Various attempts have been made at detoxifying shellfish contaminated with paralytic shellfish poisons in an effort to reduce the duration of off market times. The most obvious method is to transfer shellfish to waters free of toxic organisms and allow them to self-depurate. While this may appear to be a satisfactory method for many species of shellfish, rates of detoxification vary considerably between species, and some species remain toxic for extended periods of time e.g. up to several months for *Crassostrea*, *Plactopecten*, *Spisula* and others (Shumway *et al.*, 1995). Further, transferring large quantities of shellfish is very labour-intensive and costly.

5.5.2 Effects of processing

Shumway *et al.* (1995) has put various methods together and evaluated these, based on numerous articles. The following information has been derived from his evaluation, without naming the original authors.
Detoxification of PSP-toxins using temperature or salinity stress has been tried with marginal success. Instantaneous electrical shock treatments were found to accelerate toxin excretion in scallops. Reduced pH has been tried as a means of detoxifying butter clams, but with no success. Chlorination has been used in France; however, this process alters the flavour of the shellfish and thus decreases marketability. Ozonised seawater however, can be of value in detoxification of shellfish contaminated recently by the vegetative cell phase of toxic dinoflagellates. In a study during a red tide outbreak, it was shown that ozone treatment of the seawater does prevent shellfish from accumulating PSP toxins. This activation could be achieved in a marketable species such as Mya within a economically feasible time frame. On the contrary, ozone is useless in detoxifying cysts or in bivalves that have ingested cysts or have the toxins bound in their tissue over long periods of time. Further, detoxification of algal toxins from shellfish, especially paralytic shellfish poisons, over long periods of time is not economically feasible. Ozone is not recommended as a practical or safe means of eliminating algal toxins from shellfish.
Cooking has also been touted as a possible means of detoxifying shellfish contaminated with paralytic shellfish poisons. Cooking does not eliminate the danger of intoxication; however, it may reduce levels of toxins. If the initial level of toxicity is low, cooking may effectively reduce toxicity to safe levels. Pan frying seems to be more effective than other methods of cooking. When clams or mussels are steamed or boiled, toxins lost from the tissues are contained in the cooking liquid rendering the fluids extremely toxic.
Berenguer *et al.* (1993) studied the effects of operations carried out during the industrial canning process on the contamination of *Acanthocardia tuberculatum* (Mediterranean cockle) by paralytic shellfish poisons. The observed effects of boiling and sterilising averaged over 70.6 - 77.9% and 81.8 - 90.9% reduction of toxicity, respectively.
Takata *et al.* (1994) investigated the reduction in toxicity of PSP-infected oysters (*Crassostrea gigas*) by heat treatment. The methods of heat treatment were boiling (98 °C, 5-60 min) and retorting (120 °C, 5-60 min.). Boiling at 98 °C resulted in 53-88.3% detoxification, retorting at 120 °C resulted in 57.4-100% detoxification. Boiling and retorting for 60 min. resulted in more detoxification than boiling and retorting for 5 min.
Less detoxification after boiling and autoclaving of PSP-infected oysters was observed by Mizuta *et al.* (1995). Oysters having 17.4 or 29.8 MU of toxicity were boiled, canned and autoclaved. The toxicity was reduced by about 20% after boiling and by less then 10% after autoclaving. The effectiveness of canning as a means of reducing PSP -toxicity levels below

quarantine levels is dependent upon the initial levels of toxicity and should be approached with great caution.

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

5.5.3 Effects of alcohol consumption and sex

The effect of alcohol consumption on paralytic shellfish poisoning is still unclear. Some say that alcohol might be a protective factor against the adverse effects of paralytic shellfish poison but the mechanism by which alcohol might reduce the risk is unknown. Since the elimination of paralytic shellfish poison occurs at least in part through the urine, alcohol may influence illness by a diuretic effect; alternatively, alcohol may cause hepatic enzyme induction (Gessner & Middaugh 1995). In a case-control study in Alaska 47 outbreaks were studied for which the consumption histories of all persons were known. Alcohol consumption and eating cooked rather than raw shellfish were associated with a reduced risk of paralytic shellfish poisoning, while sex was not related to illness. An association between illness and either the toxin level or dose ingested was not found (Gessner & Middaugh, 1995).

5.5.4 Preventive measurements

At present the economic feasibility of efficiently detoxifying shellfish on a large scale in artificial systems is not promising. In areas prone to regular outbreaks of toxic algal species, culturists and commercial fishermen alike must still depend on monitoring systems to warn of toxic shellfish and plan their activities accordingly. Through combined efforts of an intensive monitoring program and culture of 'rapid release' species (e.g. *Mytilus edulis*), species known to avoid toxic dinoflagellates (e.g. *Mercenaria*, most oysters) or scallops, economic loss can be kept to a minimum.
Preventive measurements include regular inspection of seawater bodies in which the shellfish are grown on the possible appearance of toxic dinoflagellates especially in the season that blooms may occur. Also the presence of cysts of the dinoflagellates should be looked for and the shellfish itself has to be inspected routinely as well. In case of contaminated shellfish measurements have to be taken to prevent consumption and cases of human PSP intoxications should be reported to the health authorities as soon as possible. More information on regulations and monitoring will be presented in chapter 6.

5.6 Case-reports/Outbreaks

5.6.1 Introduction

The increased global distribution of paralytic shellfish poisoning is illustrated in Hallegraef, 1993. Until 1970, PSP-producing dinoflagellates blooms of *Alexandrium tamarense* and

Alexandrium catenella were only known from temperate waters of Europe, North America and Japan. By 1990, PSP was well documented from throughout the Southern Hemisphere, in South Africa, Australia, India, Thailand, Brunei, Sabah, the Philippines and Papua New Guinea. Of course, the increasing interest in utilising coastal waters for aquaculture is leading to an increased awareness of toxic algal species. But whether this is the only explanation or not still remains unsure.
In the following the attention will be focused on outbreaks in European countries.

5.6.2 Occurrence in Europe

Denmark
At the east coast of Jutland PSP was found in mussels in 1987 and in 1990 (and maybe also in 1988). The causing algae were *Alexandrium tamarense* and *Alexandrium ostenfeldii* (CRL, 1995).

France
Most of the toxic episodes related to marine biotoxins were related to diarrhoeic phycotoxins; but some minor toxic episodes were related to paralytic phycotoxins. These episodes occurred only at the northern Brittany coast and were always caused by *Alexandrium minutum.* Maximal toxicity in mussels and oysters was 400 µg STX eq./100g meat. Late 1992 paralytic toxins were found in mussels from Atlantic coast without any occurrence of toxigenic algae in water (CRL, 1995).

Germany
Since the beginning of the century, no poisonings related to PSP were reported after the consumption of mussels collected in German waters. However, in 1976, several persons showed PSP symptoms after consumption of mussels originating from Vigo, Spain. Since then, mussels are regularly monitored for PSP toxins. Causative organisms like *A. tamarensis*, *A. minutum* and *A. ostenfeldii* occur, but only in limited numbers. In 1987, three cases of PSP occurred in Lower Saxonia which could, again, be attributed to the consumption of canned Spanish mussels (CRL, 1995).
Since March 1992, viable cysts of a species very similar or identical to *Gymnodinium catenatum* have been found in German coastal waters of the North and Baltic Seas. The toxicity of the strain has not (yet) been reported. In freshwater, PSP producing blue green algae have been isolated. Other species known to produce PSP toxins have not yet been reported in German waters (IOC, 1995).

Ireland
In Ireland to date only 1 PSP event has been noted. This occurred in Cork Harbour in July 1992 and persisted for 1 week only. The phytoplankton associated with this event was *Alexandrium tamarensis* (CRL, 1995).

Italy
In the Adriatic Sea dinoflagellate red tides are a recurring phenomenon and, despite the presence of potentially toxic species such as *Alexandrium* spp. and *Dinophysis* spp., no cases of paralytic shellfish poisoning in humans have been reported until now. Recently some shellfish samples were found to be contaminated by PSP along the Emilia Romagna coasts. The phenomenon seems to be related to the presence of *Alexandrium minutum* in seawater (CRL, 1995).

Netherlands

PSP-producing algae have, until now, hardly been observed in The Netherlands. *Alexandrium* species have been found in the North Sea in 1989 but shellfish containing PSP toxins have not been found until now (Peperzak, 1994). Once, scallops with PSP have been reported to be found, but this was only a side-catch from the North Sea (CRL, 1995).
In 1990 symptoms of paralysis were reported after consumption of shellfish from the Wadden Sea but later it was found out that this was not related to PSP (Peperzak, 1994).

Norway

PSP contamination events in Norway are among the earliest recorded in Europe. PSP has been detected nearly every year in many localities and cases of human intoxication have been reported 7 times (1901, 1939, 1959, 1979, 1981, 1991, 1992), with a total of 32 victims, including 2 fatalities (Van Egmond *et al.,* 1993).

Portugal

Since 1986 and until 1990 there were occurrences of PSP outbreaks at the Portuguese coasts north from Roca Cape. In 1991 the problem did not appear. In 1992 it appeared again but occurred also off the south coast of Lisbon and at the Algarve coast in concentrations of 100-500 μg (saxitoxin eq.)/100g. In 1993 PSP was found almost all year round, covering the entire coast. Observed concentrations ranged from 113 μg (saxitoxin eq.)/100 g in December at the Northern coast up to 9145 μg (saxitoxin eq.)/100 g at the Lisbon coast in September. In 1994 PSP outbreaks have occurred off the south coast of Portugal and off the Algarve coast. The main causative organism was *Gymnodinium catenatum. Alexandrium lusitanicum* was also found to be responsible for PSP outbreaks at the Obidos Lagoon (IOC, 1995).

Spain

Mussel aquaculture is an important industry for the Galician Rias, located in the north-western Atlantic coast of Spain. Since 1976 this region has been seriously affected by incidents of PSP. Spanish mussels contaminated with PSP have caused food poisoning not only in Spain, but also in several other European countries supplied at the same time. This happened in 1976, when mussels imported from Spain caused paralytic shellfish poisoning in countries like Germany, France, Switzerland and Italy (Van Egmond *et al.,* 1993).
In January 1989, routine toxicity testing of bivalve warty Venus (*Venus verrucosa*) from the Mediterranean coast of southern Spain revealed rising levels of PSP, probably related to the presence of high levels of naked dinoflagellate cells (*Gymnodinium catenatum* Graham) in sea-water samples. PSP toxins were detected in different mollusc species (*Venus verrucosa, Venerupis rhomboides, Callista chione, Acanthocardia tuberculatum*) from the affected areas, in concentrations higher than 80 μg PSP (saxitoxin equivalent)/100 g meat, which caused the collection and sale of molluscs to be banned by the regional health authorities. This incident affected the southern Mediterranean coast of Spain between Malaga and Algeciras, a span of approximately 150 km, probably reaching the coast of Morocco, which would explain the toxicity found in *A. tuberculatum* imported from Morocco in February 1989 (Berenguer *et al.*, 1993).
A particularly bad episode occurred in 1993, when the toxic events lasted for an unusually long period. Many people became ill with unusual symptoms. The chemical analysis of the mussel samples taken revealed a complex toxin profile, with both DSP and PSP toxins present. The observed PSP toxins were B1 and the decarbamoylated derivatives of saxitoxin, GTX2 and GTX3. Small amounts of saxitoxin and other (unidentified) PSP toxins were also

observed. The contamination of the mussels was probably due to the dinoflagellate *A. catenatum* (Gago-Martinez *et al.*, 1996).

Sweden
Contamination with PSP in Swedish mussel farming occurs at the end of spring and beginning of summer. PSP toxins have been detected in mollusc meat between 1985 and 1988, with the highest level of toxicity being reached in 1986 and 1987, i.e. 1000 MU/100g meat. The species involved was *Alexandrium excavatum.*

United Kingdom
The first cases of PSP intoxication occurred in 1968 (78 people admitted at hospital). From then on it has been detected very often with only in 1972 and 1973 levels below 400 MU/100g shellfish. In 1977, toxicity reached 1792 MU/100g and from 1978 to 1981 PSP events were annual occurrence on the north-east coasts, spreading toward Scotland. However, during this entire period the monitoring network prevented human intoxication from taking place (Van Egmond *et al.*, 1993).
In 1990, the first cases of PSP were noted along the west coast of Scotland, with maximum toxin concentrations in the order of 16480 MU/100g in mussels and scallops (Van Egmond *et al.*, 1993).

6. Regulations for PSP

6.1 Introduction

There appears to be general, world-wide agreement on the need for measurements to control shellfish toxins in seafood and many countries have taken legal action to ensure that phycotoxin-contaminated shellfish do not reach the consumer.
In establishing regulatory criteria and limits for phycotoxins, various factors play a role. These include:
- the availability of survey data;
- the availability of toxicological data;
- the distribution of phycotoxins throughout sampled lots and the stability of the toxins in the samples;
- the availability of methods of analysis of toxins;
- regulations in force in other countries.

Data on the occurrence of toxic algal species may indicate which toxins may be expected during periods of algal blooms and which seafood products should be considered for analytical monitoring. A problem is that certain algal species, which have never occurred in a certain area, may suddenly appear and then rapidly cause problems (Van Egmond *et al.*, 1992). Weighing the various factors that play a role in the decision-making process of establishing shellfish toxin tolerances may not be easy. Despite these dilemmas, there are a number of countries that have established limits and regulations for shellfish toxins.

6.2 Worldwide regulations

The number of countries known to have in-force or proposed regulations are 25 at this moment (see table 4). Most regulations are set for paralytic shellfish poisons as a group. Some countries indicate specific regulations for one of the PSP toxins, mostly saxitoxin. In most cases the regulations concern shellfish, but some countries mention more generally molluscs, or more specifically bivalves, as the types of products for which the maximum permissible levels of PSP toxins are set.
Many countries use the standard mouse bioassay of the AOAC as the method of analysis. A (non-selective) spectrophotometric method is applied in Austria and in Germany. A few other countries apply a HPLC-method, sometimes in addition to the mouse bioassay (Shumway *et al.*, 1995). In the EU in January 1993 a new directive came into force, stating that the total PSP content in molluscs has to be determined according to the "biological testing method in association, if necessary, with a chemical method for detection of saxitoxin. If the results are challenged, the reference method shall be a biological method".
Different concentration units are used to express the tolerance level: mouse units/g (MU/g) and µg/g (incidentally µg/ml). The latter unit seems to be less appropriate in the countries that use the mouse bioassay, because they actually test for toxicity in the mouse. Expression of a tolerance level for PSP in µg/g would be valuable, if the various PSP toxins exhibit the same toxicity, which is not the case (see table 2). If the unit µg/g still would be preferred above MU/g, one might consider application of a toxic equivalence factor and expression of the concentration of the various PSP (if these can be selectively measured) in concentration units of saxitoxin.

Table 4. Regulations for paralytic shellfish poisoning toxins in various countries (Shumway *et al.*, 1995)

Country	Product	Toxin	Tolerable level	Method of analysis
Australia	shellfish	saxitoxin	80 µg/100g	mouse bioassay
Canada	molluscs	PSP	< 80 µg/100g [1)]	mouse bioassay
European Union	bivalve molluscs	PSP	80 µg/100g [2)]	(Mouse)bioassay, if necessary in association with a chemical method for detection
Guatemala	molluscs	saxitoxin	400 MU/100g	mouse bioassay
Hong Kong	shellfish	PSP	400 MU/100g	mouse bioassay
Japan	bivalves	PSP	400 MU/100g	mouse bioassay
South- Korea	bivalves	gonyautoxins	400 MU/100g	mouse bioassay HPLC method
New Zealand	shellfish	PSP, NSP, DSP, ASP		regulations being developed
Norway	all types of mussels	PSP	40-80 µg/100g	mouse bioassay
Panama	bivalves	PSP	400 MU/100g	mouse bioassay
Singapore	bivalves	saxitoxin	80 µg/100g	mouse bioassay
United States	bivalves	PSP	80 µg/100g	mouse bioassay

1) Products having levels between 80-160 µg/100g may be canned.
2) If the results are challenged, the reference method is the biological method.

As legislation calls for methods of control, accurate methods of analysis have to be available. Currently, the application of analytical-chemical methodology (HPLC) for regulatory purposes is hampered by the lack of validated analytical methodology, pure analytical standards of the various PSP toxins, and reference samples for analytical purposes (Van Egmond *et al.*, 1993). However, the development of HPLC still shows promise in selective toxin measurements. Recent advances by the National Research Council of Canada have made available certified solutions for PSP toxins (Laycock, 1994). In addition, the work carried out with SMT resulted in the development of mussel reference materials, certified for the contents of some PSP toxins (see section 2.2.4.) (Van Egmond *et al.*, 1998). At the time of writing, the AOAC mouse bioassay still remains the only method of detection, generally accepted by the various controlling authorities. A main disadvantage of the bioassay is the ethical aspect of the test, which has led to growing resistance from animal welfare groups.
All countries that have a *limit for toxicity* apply a level of 400 MU/100 g (which correspond to approximately 80 µg STX eq./100 g). Most countries that apply *tolerance levels* expressed in physico-chemical concentration units, have 80 µg PSP (or specifically STX)/100g as the limit.

6.3 Regulations in Europe

In the European Union, where originally divergent tolerances for PSP toxins were administrated, the limits were harmonised in 1991 (harmonized levels in force since 1993) at 80 µg PSP/100g mollusc flesh (Council of the European Communities, 1991). This limit has

been derived on the basis that levels up to 80 µg total PSP per 100 g have not been shown toxic for consumers and a tolerance of 80 µg total PSP per 100 g shellfish would ensure that no single component, such as saxitoxin, exceed 30 µg/100g shellfish. Despite the preference of some EU-countries to go as low as 40 µg/100g with their limit (Germany, Italy, The Netherlands), in view of the fact that most toxicological data relate solely to saxitoxin, and a toxicological evaluation of the whole PSP-mixture was not available, they follow the common EU-directive.
It might be appropriate for international authoritative bodies such as the WHO and ILSI to re-evaluate current knowledge about toxicity of PSP and give guidance as to safe intake levels for PSP (Van Egmond *et al.*, 1993).

6.4 Actions taken, when products contain unacceptable levels of toxins

Most countries do not allow entry of consignments of shellfish products containing an inadmissible amount of toxin. A few countries (Austria, United Kingdom) destroy condemned imported goods. In the case of domestic produce, several countries (Canada, Guatemala, Ireland, Norway, Korea, Sweden, UK and USA) stop harvest of fishery products if levels of toxins exceed the limits and a waiting period is established until the concentrations of toxins are below acceptable limits. Harvested products containing too much toxin are usually destroyed (Shumway *et al.*, 1995).

7. Risk evaluation

The human health concern for paralytic shellfish poisoning is mainly restricted to the occurrence of STX and its derivatives in edible shellfish. For this reason there is a strong need for rapid, reliable and sensitive methods to determine STX and its derivatives. The present mouse bioassay is not sensitive enough, shows a considerable variation, is time consuming and is unethical in terms of animal welfare. It is possible to measure PSP compounds by a number of analytical-chemical methods, but they all have some limitations, and they often cannot easily be operated because of the lack of reference materials, although recently some progress has been made in this area (see section 2.2.4.)

The difficult analytical situation, and the lack of pure PSP compounds are important factors preventing the development of reliable risk assessment. The tolerance levels set for PSP toxins thus far are largely pragmatic decisions based on intoxication events, and although there are many reported cases of human intoxications due to shellfish toxins, it is difficult to obtain *reliable* human toxicity data. For example, variations in observed toxicity of PSP toxins to humans may be due not only to variable sensitivity between people, but also to the composition of individual toxins in the samples. Toxin profiles can vary according to the species of shellfish consumed and the area of harvest. In addition, toxic doses are often estimated from left-over toxic seafood. This is not necessarily representative of the ingested food, because PSP toxins may be unevenly distributed throughout lots and within individual shellfish, and not all PSP toxins are stable.
At this moment only data on the acute oral toxicity both in experimental animals and man are available. Once it is known that repeated exposure to lower sublethal dose level is a common feature, the toxic effects of sub-acute or sub-chronic exposure could also become of interest. Again, a sensitive method of determination and detailed descriptions of food consumption patterns will be determining factors.
At this moment the toxicological risk evaluation can only be based on the acute toxicity data in man. Because the acute toxic effects concern a fatal effect the lowest published lethal dose level should be used.
The lowest reported dose with human mortality (see section 5.2.1) is 300 μg PSP toxin. Assuming this would be a reliable figure, the following calculation can be made for a 60 kg adult:

$$300\ \mu g / 60\ kg = 5\ \mu g/ kg \text{ body weight.}$$

A margin of safety of at least 10 has to be applied as this feature concerns mortality in an adult. The safe dose level would in that case be 0.5 μg PSP toxin/kg body weight.
The tolerance level for PSP toxins in shellfish can then be calculated as follows:
Assuming that an adult person consumes 250 g shellfish (soft tissue), a content of
$(0.5 \times 60) / 2.5 = 12$ μg PSP toxin /100 g shellfish (soft) tissue can be considered as safe. These data concern observations in adults, but do not cover mortality in children, who are claimed to be more vulnerable (Rodrigue *et al.*, 1990).
The present detection limit with the mouse bioassay is approximately. 40 μg PSP (STX equivalent) /100 g shellfish (Van Egmond *et al.*, 1993). As the mouse bioassay is currently the most widely used method to determine PSP toxins, it is neither practicable nor realistic to establish a lower tolerance level. Until more reliable and sensitive analytical methods for both saxitoxin and derivatives have been developed and validated, lowering the tolerance level for the total PSP compounds makes no sense. Once more sensitive (and reliable) analytical chemical methods are available, the toxicity figures of saxitoxin and derivatives after acute

and (sub)chronic exposure should be re-evaluated. If more accurate figures on the acute human toxicity would become available, establishment of a tolerance level for PSP toxins in shellfish could be based on the results of scientific research, rather then empirically derived. This might lead to an adjustment of the desired tolerance level to either a higher or a lower value than the present values established in the regulations.

8. Overall conclusions and recommendations

8.1 Overall conclusions

PSP is causing problems in many countries around the world and development of risk assessment would be highly desirable. A pressing problem hindering the risk evaluation is the poor availability of pure PSP compounds and certified reference samples, although there has been some development in this respect. Saxitoxin, neosaxitoxin and gonyautoxin 1-4 are now commercially available, and their availability significantly improves the quality of the data that are obtained by the HPLC-methodology. The efforts undertaken by the European Commission's SMT Programme have led to shellfish reference materials with certified mass fractions of some of the toxicologically most significant PSP toxins.

8.2 Conclusions considering The Netherlands

Until now no PSP events have occurred in the Netherlands. However, in surrounding countries like Denmark, the United Kingdom and France, PSP producing algae and shellfish containing PSP toxins have been observed, sometimes accompanied by human intoxication after consumption of contaminated shellfish. Until now, PSP producing algae have hardly been observed in the Netherlands (Peperzak, 1994). However, considering the increasing global distribution of paralytic shellfish poisoning, it cannot be excluded that such events could also occur in The Netherlands. Factors of influence on the induction of algal blooms are the distribution of cysts, climatological conditions and nutrients. New algal species could be introduced with the ballast-tank water of ships and the transfer of shellfish from one area to another. The Netherlands, being a country with a lot of import and export, has a lot of shipping and can, in this way, become exposed to "new" algal species. In addition, the cultural eutrophication from domestic, industrial and agricultural wastes could stimulate the blooming of these species.
Although it is still unclear why no PSP events have occurred in The Netherlands, it cannot be excluded that such events could occur in the (near) future.

8.3 Recommendations

Risk evaluation should be the scientific basis for setting tolerance levels for PSP toxins.
Risk evaluation may be considered in two stages. Firstly, the risk of exposure to the toxins, and, secondly, the hazard to health associated with exposure.

1. With regard to exposure, some data exist for the current spatial distribution of PSP producing algae, but more data are required in the following areas:
- Baseline data for potential sites for mariculture development
- Information on the relationship of resting cysts and motile cells with bloom development
- Data for temporal and positional production of toxins within each species
- Examination of bloom development with respect to hydrographic and climatic conditions, and nutritional status of the water column.
- Further information on the spatial distribution of bloom events from other countries.

2. Tolerance levels suggested for intake of PSP toxins are presently largely pragmatic decisions derived from some intoxication events and detection limits of assay systems. To derive better funded limits further information is needed on:
- The oral toxicity of the individual PSP toxins and mixtures of PSP-toxins
- The kinetics of uptake, metabolism and excretion of the compounds
- Effects after sub-acute and sub-chronic exposure to the PSP toxins

Further work on risk of exposure and risk to human health can only be facilitated by well-defined methods of identification and quantitation of the PSP toxins. As no universally acceptable methods of analysis for the toxins exist, attention should be given to:
- The further development of analytical techniques capable of separating, identifying and quantifying individual toxins;
- The further development and validation of biochemical/cellular techniques which offer the way forward for development of rapid screening/low cost analysis.
- The development of bioassays as alternatives to rodent assays, to be used when uncharacterised bloom events occur. Emphasis on the use of *in vitro* techniques where blooms have been characterised should reduce the use of test systems with live animals.

REFERENCES

Anderson, D.M.
Red Tides.
Scientific American, **271**, pp. 62-68, 1994.

Acres, J.; Gray, J.
Paralytic shellfish poisoning.
CMA Journal, **119**, pp. 1195-1197, 1978.

Bates, H.A.; Kostriken, R.; Rapoport, H.
A chemical assay for saxitoxin. Improvements and modifications.
J. Agric. Food Chem., **26**, pp. 252-254, 1978.

Bates, H.A. and Rapoport, H.
A chemical assay for saxitoxin, the paralytic shellfish poison.
J. Agric. Food Chem., **23**, pp. 237-239, 1975.

Beitler, M.K.; Liston, J.
Uptake and tissue distribution of PSP toxins in butter clams
In: Toxic Marine Phytoplankton. Graneli, E.; Sundström, B.; Edler, L., Anderson, D.M. (eds), pp. 257-262, 1990.

Berenguer, J.A.; Gonzalez ,L.; Jimenez, I.; Legarda, T.M.; Olmedo, B.; Burdaspal, A.
The effect of commercial processing on the paralytic shellfish poison (PSP) contant of naturally contaminated *Acanthocardia tuberculatum L.*
Food Addit. Contam., **10**, pp. 217-230, 1993.

Bower, D.J.; Hart, R.J.; Matthews, P.A.; Howden, M.E.
Nonprotein neurotoxins.
Clin. Tox., **18**, pp. 813-843, 1981.

Botana, L.; Rodriguez Vieytes, M.; Alfonso, A.; Louzao, C.
Phycotoxins: Paralytic shellfish poisoning and diarrhetic poisoning.
Food Sci. Technol.; **77**: Handbook of Food Analysis, **2**, pp. 1147-1169, 1996.

Boyer, G.L.; Fix-Wichmann, C.; Mosser, J.; Schantz, E.J.; Schnoes, H.K.
In: Toxic dinoflagellates, Anderson, D.M.; White A.W.; Baden D.G.(eds.), pp. 281-286.
Elsevier Science Publishing, New York, 1979.
(Cited in: Van Egmond, H.P.; Aune, T.; Lassus, P.; Speijers, G.J.A.; Waldock, M. Paralytic and diarrhoeic shellfish poisons: occurrence in Europe, toxicity, analysis and regulation. Journal of Natural Toxins, **2**, pp. 41-83, 1993).

Bricelj, V.M.; Lee, J.H.; Cembella, A.D.; Anderson, D.M.
Uptake of *Alexandrium fundyense* by *Mytilus edulis* and *Mercenario mercenaria* under controlled conditions.
In: Toxic Marine Phytoplankton. Graneli, E.; Sundström, B.; Edler, L.; Anderson, D.M.(eds), pp. 269-274, 1990.

Bryan, F.L.
Epidemiology of foodborne diseases transmitted by fish, shellfish and marine crustaceans in the United States, 1970-1978.
J. Food Prot., **43**, pp. 859-863, 1980.

Caterall, W.A.
Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes.
Ann. Rev. Pharmacol. Toxicol., **20**, pp. 15-24, 1980.

Cembella, A; Lamoreux, G.
Rev. Int. Oceanogr. Med., 1991.
(Cited in: Van Egmond, H.P.; Aune, T.; Lassus, P.; Speijers, G.J.A.; Waldock, M. Paralytic and diarrhoeic shellfish poisons: occurrence in Europe, toxicity, analysis and regulation. Journal of Natural Toxins, **2**, pp. 41-83, 1993).

Chu, F.S.; Fan, T.S.L.
Indirect enzyme-linked immunosorbent assay for saxitoxin in shellfish.
J. Assoc. Off. Anal. Chem, **68**, pp. 13-16, 1985.

Council of the European Communities
Council decision on reference laboratories for the monitoring of marine biotoxins. (93/383/EEG). Off. J. Europ. Comm. L.166/31-33.

CRL
"State of the art " NRLs on Marine Biotoxins.
CRL rep 01/95, European Communities Reference Laboratory on Marine Biotoxins, Vigo, Spain, 1995.

Davio, S.R.; Fontelo, P.A.
A competitive displacement assay to detect saxitoxin and tetrodotoxin.
Anal. Biochem., **141**, pp. 199-204, 1984.

Evans, M.H.
Differences between the effects of saxitoxin paralytic shellfish poison and tetrodotoxin on the frog neuromusculair junction.
Brit. J. Pharmacol., **36**, pp. 426-536, 1969.

Evans, M.H.
Mechanisms of saxitoxin and tetrodotoxin poisoning.
Br. Med. Bull., **25**, pp. 263-267, 1969.

Frace, A.M.; Hall, S.; Brodwick, M.S; Eaton, D.C.
Effects of saxitoxin analogues and ligand competition on sodium currents of squid axons.
Am. J. Physiol., **251**, pp. 159-166, 1978.

French, R.J.; Worley, J.F.; Krueger, B.K.
Voltage dependent block by saxitoxin of sodium channels incorporated into planar lipid bilayers.
Biophys. J., **45**, 1984.

Fun, S.C.; Kuo-Hui, H.; Xuan, H.; Barret R.; Allison C.
Screening of paralytic shellfish poisoning toxins in naturally occuring samples with three different direct competitive enzyme-linked immunosorbent assays.
J. Agric. Food Chem., **44**, pp. 4043-4047, 1996.

Gago-Martinez, A. ; Rodrigues-Vazquez, J.A.; Thibault, P.; Quilliam, M.A.
Simultaneous occurrence of diarrhetic and paralytic shellfish poisoning toxins in Spanish mussels in 1993.
Natural toxins, **4**, pp. 72-79, 1996.

Gessner B.D.; Middaugh J.P..
Paralytic shellfish poisoning in Alaska: a 20 year retrospective analysis.
Am. J. Epidemiol., **141**, pp. 766-770, 1995.

Green, W.N.; Weiss, L.B.; Andersen, O.S.
Bactrachotoxin-modified sodium channels in planar lipid bilayers.
J. Gen. Physiol., **89**, pp. 873-903, 1987.

Guo, X.; Uehara, A.; Ravindran, A.; Bryant, S.H.; Hall, S.; Moczydlowski, E.
Kinetic basis for insensitivity to tetrodotoxin and saxitoxin in sodium channels of canine heart and rat skeletal muscle.
Biochem., **26**, pp. 7546-7556, 1987.

Hald, B; Bjergskov, T.; Emsholm, H.
Monitoring and analytical programmes on phycotoxins in Denmark.
In: Proceedings of Symposium on Marine Biotoxins, Paris, January 1991. Frémy, J.M. (ed.), pp.181-187. Centre National d'Etudes Vétérinaires et Alimentaires, Paris, 1991.

Hall S.; Strichartz, G.; Moczydolowski, E.; Ravindran, A; Reichardt, P.
The saxitoxins: sources, chemistry and pharmacology.
In: Marine toxins: origin, structure and molecular pharmacology.
American Chemical Society Symposium Series, Washington DC,
Hall S., Strichartz G. (eds.), pp. 29-65, 1990.

Hallegraef G.M.
A review of harmful algae blooms and their apparent global increase.
Phycologia, **32**, pp. 79-99, 1993.

Hallegraef, G.M.
Harmful agal blooms: A global overview.
In: IOC, Manual on harmful marine microalgae, pp. 1-22, UNESCO, 1995.

Halstead B.W.; Schantz, E.J.
Paralytic shellfish poisoning.
WHO Offset Publication 79, 1984.

Hines, H.B.; Naseem, S.M.; Wannemacher, R.W.
(3H)-saxitoxinol metabolism and elimination in the rat.
Toxicon, **31**, pp. 905-908, 1993.

Hollingworth, T.; Wekell, M.M.
Fish and other Marine Products 959.08. Paralytic shellfish poisoning. Biological Method, Final Action. In: K. Hellrich (ed.). Official Methods of Analysis of the Association of Official Analytical Chemists, 15th edition, pp. 881-882. Arlington, Virginia, U.S.A., 1990.

Hungerford, J.M.; Lee, S.; Hall, S.
In: Fifth International Conference on Toxic Marine Phytoplankton, Newport (USA). October-November 1991.
(Cited in: Van Egmond, H.P.; Aune, T.; Lassus, P.; Speijers, G.J.A.; Waldock, M. Paralytic and diarrhoeic shellfish poisons: occurrence in Europe, toxicity, analysis and regulation. Journal of Natural Toxins, **2**, pp. 41-83, 1993).

Ikawa, M.; Auger, K,; Mosley, S.P.; Sasner, J.J.; Noguchi, T.; Hashimoto, K.
In: Toxic Dinoflagellates , Anderson, D.M.; White, A.W.; Baden, D.G. (eds.), pp. 299-304. Elsevier Science Publishing, 1985.
(Cited in: Van Egmond, H.P.; Aune, T.; Lassus, P.; Speijers, G.J.A.; Waldock, M. Paralytic and diarrhoeic shellfish poisons: occurrence in Europe, toxicity, analysis and regulation.
Journal of Natural Toxins, **2**, pp. 41-83, 1993).

IOC-UNEP-WHO-FAO training course on qualitative and quantitative determination of algal toxins, Friedrich-Schiller University of Jena, Jena, Germany, 18-28 oct 1994.
Training Course Report, IOC Conference Monograph, 29,1995.

Jellett J.F.; Marks, L.J.; Stewart, J.E.; Dorey, M.L.; Watson-Wright, W.
Paralytic shellfish poison (saxitoxin family) bioassays: automated endpoint determination and standardization of the *in vitro* tissue culture bioassay, and comparison with the standard mouse bioassay.
Toxicon, **30**, pp. 1143-56, 1992.

Kao, D.Y.
Paralytic Shellfish Poisoning.
In: Algal toxins in seafood and drinking water. Falconer IR (ed.), pp. 75-86, Academic Press, New York, 1993.

Kao, D.Y.; Levinson, S.R.
Tetrodotoxin, saxitoxin and the molecular biology of the sodium channel.
Annal. N.Y. Ac. of Sciences, **479**, pp. 52-67, 1986.

Kodama, M.; Ogata, T.; Sato, S.
Bacterial production of saxitoxin.
Agric. Biol. Chem., **52**, pp. 1075-1078, 1988.

Kogure, K.; Tamplin, M.L.; Simidu, U.; Colwell, R.R.
A tissue culture assay for the tetrodotoxin, saxitoxin and related toxins..
Toxicon, **26**, pp. 191-197, 1988

Koyama, K.; Noguchi, T.; Uzu, A.; Hashimoto, K.
Resistibility of toxic and nontoxic crabs against paralytic shellfish poison and tetrodotoxin.
Bull. Jpn. Soc. Sci. Fish., **49**, pp. 485-490, 1983.

Krogh, P.
Algal toxins in seafood and drinking water.
Chemistry International, **5**, pp. 45-48, 1983.

Krogh, P.
Report of the Scientific Veterinary Committee (Section Public Health) on Paralytic Shellfish Poisons. Document VI/6492/88-EN/Rev.1, Commisson of The European Communities, Directorate General for Agriculture VI/B/II.2. Brussels, 1988.

Laverty, R.
Modes of action of shellfish toxins.
Marine toxins and New Zealand shellfish. Proceedings of a workshop on research issues, 10-11 June 1993. The Royal Society of New Zealand, miscellaneous series 24. Jasperse, J.A. (Ed), 1993.

Lawrence, J.F.; Ménard, C.
Liquid chromatographic determination of paralytic shellfish poisons in shellfish after prechromatographic oxidation.
J. Assoc. Off. Anal. Chem., **74**, pp. 1006-1012, 1991.

Lawrence, J.F.; Ménard, C.; Charbonneau, C.F.; Hall, S.
A study of ten toxins associated with paralytic shellfish poison using prechromatographic oxidation and liquid chromatography with fluorescence detection.
J. Assoc. Off. Anal. Chem., **74** , pp. 404-409, 1991.

Laycock, M.V.; Thibault, P.; Ayer, S.W.; Walter, J.A.
Isolation and purification procedures for the preparation of paralytic shellfish poisoning toxin standards.
Natural toxins, **2**, pp. 175-83, 1994.

Ledoux, M.; Bardouil, M.; Nezan, E.; Erard, E.
Field and experimental studies of shellfish contaminated by an *Alexandrium minutum* strain.
In: Proceedings of Symposium on Marine Biotoxins, Paris, January, 1991. Frémy, J.M. (ed.), pp.43-51. Centre National d'Etudes Vétérinaires et Alimentaires, Paris, 1991.

Thielert, G.; Kaiser, I.; Luckas, B.
HPLC methods for the determination of PSP toxins.
In: Proceedings of Symposium on Marine Biotoxins, Paris, January, 1991. Frémy, J.M. (ed.), pp.121-125. Centre National d'Etudes Vétérinaires et Alimentaires, Paris, 1991.

Martin, J.L.; White, A.W.; Sullivan, J.J.
Anatomical distribution of paralytic shellfish toxins in soft-shell clams.
In: Toxic Marine Phytoplankton. Graneli, E. ; Sundström, B.; Edler, L.; Anderson, D.M. (eds),
pp. 379-384, 1990.

Maruyama, J.; Noguchi, T.; Matsunaga, S.; Hasimoto, K.
Fast atom bombardment- and secondary ion-mass spectrometry of paralytic shellfish poisons and tetrodotoxin.
Agric. Biol. Chem., **48**, pp. 2783-2788, 1984.

Mizuta, M.; Takata, K.; Monden, T.; Yamauchi, S.
Reduction in toxicity of PSP infected oysters during canning process.
J. Food Hyg. Soc. Japan, **36**, pp. 423-427, 1995.

Moczydlowski, E.; Hall, S.; Garber, S.S., Strichartz, G.S., Miller, C.
Voltage-dependent blockade of muscle Na^+ channels by guanidinium toxins.
J. Gen. Physiol., **84**, pp. 687-704, 1984.

Mosley, S; Ikawa, M, Sasner, JJ
Toxicon, 23, p. 375, 1985.
(Cited in: Van Egmond, H.P.; Aune, T.; Lassus, P.; Speijers, G.J.A.; Waldock, M. Paralytic and diarrhoeic shellfish poisons: occurrence in Europe, toxicity, analysis and regulation. Journal of Natural Toxins, **2**, pp. 41-83, 1993).

Onoue, Y; Noguchi, T; Maruyama, J; Haishimoto, K; Seto, H.
J. Agric. Food Chem., 31, pp. 420-423, 1983.
(Cited in: Van Egmond, H.P.; Aune, T.; Lassus, P.; Speijers, G.J.A.; Waldock, M. Paralytic and diarrhoeic shellfish poisons: occurrence in Europe, toxicity, analysis and regulation. Journal of Natural Toxins, **2**, pp. 41-83, 1993).

Oshima, Y.; Sugino, K.; Hirota, H.; Yasumoto, T.
Comparative studies on paralyic shellfish toxin profile of dinoflagellates and bivalves.
In: Toxic Marine Phytoplankton. Graneli, E. ; Sundström, B.; Edler, L.; Anderson, D.M. (Eds), pp. 391-396, 1990.

Park, D.L.; Adams, W.N.; Graham, S.L.; Jackson, R.C.
Variability of mouse bioassay for determination of paralytic shellfish poisoning toxins.
J. Assoc. Off. Anal. Chem., **69**, pp. 547-550, 1986.

Peperzak, L.
Plaagalgen in de Noordzee.
Rijksinstituut voor Kust en Zee/RIKZ, report nr. DGW-93.053, 1994.

Quilliam, M.A.; Thomson, B.A.; Scott, G.J.; Siu, K.W.M.
Ion-spray mass spectrometry of marine neurotoxins.
Rapid Commun. Mass Spectrom., **3**, pp. 145-150, 1989

Rodrigue, D.C.; Etzel, R.A., Hall, S.; De Porras, E.; Velasquez, O.H.; Tauxe, R.V.; Kilbourne, E.M.; Blake, P.A.
Lethal paralytic shellfish poisoning in Guatemala.
Am. J. Trop. Med. Hyg., **42**, pp. 267-271, 1990.

Schantz, E.J.; McFarren, E.F.; Schafer, M.L., Lewis, K.H.
Purified shellfish poison for bioassay standardization.
J. Assoc. Off. Anal. Chem., **41**, pp. 160-168, 1985

Shimizu, Y.
Dinoflagellate toxins.
Marine Natural Products, **1**, pp. 1-42, 1978.

Shimizu, Y.
Paralytic shellfish poisons.
Fortschr. Chem. Org. Naturst., **45**, pp.235-264, 1984.

Schulze, K.
Musschelvergiftungen durch Algentoxine.
Berl. Munch. Tierarztl. Wschr., **98**, pp. 383-387, 1985.

Shumway, S.E.; Van Egmond, H.P.; Hurst, J.W.; Bean, L.L.
Management of shellfish resources.
In: IOC, Manual on harmful marine microalgae, pp. 433-461 UNESCO, 1995.

Sommer, H.; Meyer, K.F.
Paralytic shellfish poisoning.
Arch. Path., **24**, pp. 560-598, 1937.

Stafford, R.G.; Hines, H.B.
Urinary elimination of saxitoxin after intravenous injection.
Toxicon, **33**, pp. 1501-1510, 1995.

Sullivan, J.; Iwaka, W.T.; Liston, J.
Enzyme transformation of PSP toxins in the little neck clams, Protothaca staminea.
Biochemical and biophysiological research communications, **114**, pp. 465-472, 1983.

Sullivan, J.J.
Methods of analysis for DSP and PSP toxins in shellfish: a review.
J. Shellfish Res., **7**, pp. 587-595, 1988.

Takata K.; Mizuta, M.; Monden, T.
Reduction in toxicity of PSP-infected oysters by heat treatment.
J. Food Hyg. Soc. Japan, **35**, pp. 624-630, 1994.

Thibault, T.; Pleasance, S.; Laycock, M.V.
Analysis of paralytic shellfish poisons by capillary electrophoresis.
J. Chromatogr., **542**, pp. 483-501, 1991.

Trainer, V.L.; Baden, D.G.; Catterall, W.A.
Detection of marin toxins using reconstituted sodium channels.
Journal of AOAC International, **78**, pp. 570-573, 1995.

Tsai, Y.H.; Hwang, D.F.; Chai, T.J.; Jeng, S.S.
Occurrence of tetrodotoxin and paralytic shellfish poison in the Taiwanese crab *Lophozozymus pictor*.
Toxicon, **33**, pp. 1669-1673, 1995.

Van Egmond, H.P.; Speijers, G.J.A.; Wouters, R.B.M.
Naturally occurring toxicants in foodstuffs. 3. Phycotoxins.
Microb. Hyg. Ali., **3**, pp. 21-25, 1991.

Van Egmond, H.P.; Speijers, G.J.A.; Van den Top, H.J.
Current situation on worldwide regulations for marine phycotoxins.
J. Nat. Toxins, **1**, pp. 67-85, 1992.

Van Egmond, H.P.; Aune, T.; Lassus, P.; Speijers, G.J.A.; Waldock, M.
Paralytic and diarrhoeic shellfish poisons: occurrence in Europe, toxicity, analysis and regulation.
Journal of Natural Toxins, **2**, pp. 41-83, 1993.

Van Egmond, H.P.; Van den Top, H.J.; Paulsch W.E.; Goenaga, X.; Vieytes, M.R.
Paralytic shellfish poison reference materials: An intercomparison of methods for the determination of saxitoxin.
Food Addit. Contam., **11**, pp. 39-56, 1994.

Van Egmond, H.P.; Mouriño, A.; Burdaspal, P.A.; Bustos, J.; Legarda, T.; Mesego, A.; Paulsch, W.E.; Salgado, C.; Van den Top, H.J.; Boenke, A.
The certification of the mass fractions of saxitoxin and dc-saxitoxin in two mussel reference materials (CRM 542 & 543) including the identification of other PSP toxins, and a spiking procedure based on an enrichment solution (CRM 663) with a certified mass concentration of saxitoxin.
Publication of the European Communities. Directorate-General Science, Research and Development, 1998. EUR Report (in press).

Waldock, M.J.; Evans, K.M.; Law, R.J.; Fileman, T.W.
An assessment of the suitability of HPLC techniques for monitoring of PSP and DSP on the east coast of England. In: Proceedings of Symposium on Marine Biotoxins, Paris, January, 1991. Frémy, J.M. (Ed.), pp.137-147. Centre National d'Etudes Vétérinaires et Alimentaires, Paris, 1991.

White, K.; Sphon, J.A.; Hall, S.
Fast atom bombardment mass spectrometry of 12 marine toxins isolated from *Protogonyaulax*.
Analyt. Chem., **58**, pp. 562-565, 1986.

White, A.W.; Lewis, C.M.
Resting cysts of the toxic, red tide dinoflagellate *Gonyaulax excavata* in Bay of Fundi sediments.
Can. J. Fish. Aq. Sci., **39**, pp.1185-1194, 1982.

Wright, J.L.C.; Boyd, R.K.; De Freitas, A.S.W.; Falk, M.; Foxall, R.A.; Jamieson, W.D.; Laycock, M.V.; McCulloch, A.W.; McInnes, A.G.; Odense, P.; Pathak, V.P.; Quilliam, M.A.; Ragon, M.A.; Sim, P.G.; Thibault, P.; Walter, J.A.
Identification of domoic acid, a neuroexcitatory amino acid, in toxic mussels from eastern Prince Edward Island.
Can. J. Chem., **67**, pp. 481-490, 1989.

Wright, J.L.C.
Dealing with seafood toxins: present approaches and future options.
Food Res. Int., **28** , pp. 347-358, 19

Annex I

Shellfish found to contain PSP toxins, with literature references.

Type	Common name	Scientific name	Reference
clams	purple clam	*Soletellina diphos*	(Hwang-Deng-Fwu, 1995)
	Alaska butter clam	*Saxidomus giganteus*	Gessner & Middaugh, 1995
	shortnecked clam	*Tapes (Amygdala) japonica*	(Asakawa *et al.*, 1993)
	little neck clam	*Protothaca staminea*	(Gessner & Middaugh, 1995)
	razor clam	*Siliqua patula*	Gessner & Middaugh, 1995
	softshell clam	*Mya arenaria*	(Martin *et al.*, 1990.)
	thick trough shell	*Spisula solidai*	CRL, 1995
	surf clam	*Spisula solidissima*	Shumway *et al.*, 1995
	pullet carpet shell	*Venerupis rhomboides*	Berenguer *et al.*, 1993
	pod razor-shell	*Ensis siliqua*	CRL, 1995
	wedge-shell clam	*Donax trunculus*	CRL, 1995
	peppery furrow shell	*Scrobicularia plana*	CRL, 1995
	striped venus clam	*Chamalea striatula*	CRL, 1995
		Venerupis pullastra (synonymus of V. rhomboides)	CRL, 1995
		Amphichaena kindermani	Rodrigue *et al.*, 1990
mussels	blue mussel	*Mytilus edulis*	Gessner & Middaugh, 1995
	California mussels	*Mytilus californianus*	Geneah & Shimizu, 1981
oysters	cultured oyster	*Crassostrea gigas*	(Asakawa *et al.*, 1993)
	common European oyster	*Ostrea edulis*	CRL, 1995
cockles	common edible cockle	*Cerastoderma edule*	CRL, 1995
	Mediterranean cockle	*Acanthocardia tuberculatum*	(Berenguer *et al.*, 1993)
		Clinocardium nutalli	(Gessner & Middaugh, 1995)
gastropoda	ormer	*Haliotis tuberculata*	Nagashima *et al.*, 1995
		Niotha Clathrata, Zeuxis scalaris	(Deng-Fwu *et al.*, 1995)

scallops	giant sea scallop	*Placopecten megallanicus*	(Laycock *et al.*, 1994)
	Japanese scallop	*Patinopecten yessoensis*	Nagashima *et al.*, 1991
	bay scallop	*Argopecten irradians*	Shumway *et al.*, 1995
	bivalve warty Venus	*Venus verrucosa*	Berenguer *et al.*, 1993
		Callista chione	Berenguer *et al.*, 1993; CRL, 1995