4. Amnesic Shellfish Poisoning (ASP)

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

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

4.1 Chemical structures and properties

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

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

- Domoic acid
- C5'-Diastereomer
- Isodomoic acid A
- Isodomoic acid B
- Isodomoic acid C
- Isodomoic acid D
- Isodomoic acid E
- Isodomoic acid F
- Isodomoic acid G
- Isodomoic acid H
4.2 Methods of analysis

4.2.1 Bioassays

**in vivo assays**

**mouse bioassay**

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

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

**in vitro assays**

**receptor binding assays**

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

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

4.2.2 Biochemical assays

immunoassays

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

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

Garthwaite et al. (1998) used ovine antibodies raised against conjugates, linked through the secondary amino group of DA, together with activated-ester-derived conjugates of DA as the plate coater, to develop an indirect competitive ELISA for shellfish and seawater. The ELISA has a detection limit below 0.1 ng/ml, a limit of quantitation (LOQ) of 0.15 ng/ml and a working range of 0.15-15 ng DA/ml. The LOQ is equivalent to 38 µg DA/kg shellfish flesh, 500 times less than the regulatory limit of 20 mg/kg flesh. This ELISA was also shown to be appropriate for analysis
of DA in algal cultures and seawater collected from the field in New Zealand, and thus has the potential to provide early warning of developing algal blooms. Analogous compounds in seawater, such as kanaic acid, do not interfere with this assay.

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

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

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

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

### 4.2.3 Chemical assays

**thin layer chromatography**

DA can be determined by thin layer chromatography as a weak UV-quenching spot that stains yellow after spraying with a 1 percent solution of ninhydrin (Quilliam et al., 1998). Normal amino acids that are present in crude extracts will interfere and must be separated from DA. This can be accomplished for plankton samples by two dimensional TLC. Crude extracts of shellfish tissues cannot be analysed directly, as they are too complex. A clean-up procedure (strong anion change solid phase extraction [SAX-SPE] with minor modification) yields fractions that can be used directly or concentrated in vacuum before applying to a silica gel plate. Only one-dimensional TLC is required when this clean-up is used as almost all interfering amino acids are removed. The detection limit for DA is about 0.5 ng by this method, which permits detection in shellfish tissues.
at about 10 ng/g. It is also possible to detect DA on the TLC plate using some other spray reagents. For example, after spraying a TLC plate with vanillin, a yellow colour with domoic (or kainic) acid forms first and changes to pink on standing (Wright and Quilliam, 1995).

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

**amino acid analysis**

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

**liquid chromatography (LC)**

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

If crude extracts (either acidic or aqueous methanol) are analysed without clean-up, the practical limit for quantitation is about 1 ng/g (Lawrence et al., 1989a). This is suitable for most regulatory laboratories concerned with detecting contamination levels greater than 20 ng/g. However, interferences are commonly encountered that can give false positives with crude extracts. For example, it has been shown that tryptophan and some of its derivatives are often present in substantial concentrations in shellfish and finfish tissues and that these compounds elute close to DA. A photo-diode array detector can be used to examine UV spectra in order to confirm DA. An alternative approach is to prepare a chemical derivative and to analyse the sample with comparison to a known standard carried through the same procedure. Derivatization techniques involving reaction with phenyl- or butyl-isothiocyanate or esterification with isopropanol have been developed for this purpose (Lawrence et al., 1989b; Lawrence and Ménard, 1991). A more sophisticated technique of confirmation is to replace UV detection by electrospray mass spectrometry with selected ion monitoring (Lawrence et al., 1994). Application of this technique
offers the possibility of confirming the presence of DA down to levels of 0.1 mg/g without additional sample treatment.

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

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

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

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

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

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

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

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

mass spectrometry

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

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

An example of extended use of LC-multiple MS was reported by Furey et al. (2001) in applying an Ion Trap mass spectrometer for qualitative and quantitative analysis of domoic acid. A handsome figure shows the fragmentation pathway for domoic acid. Good linearity was demonstrated for LC-MS \(^{1-3}\) for calibration plots over 2-3 decades using extracts from spiked scallop tissue while it was found that MS\(^4\) and MS\(^5\) resulted in poor linearity.
In the second half of 2002, an inter-laboratory study has taken place of a new LC-MS method for determination of ASP and DSP toxins in shellfish (Holland and McNabb, 2003). The eight participating laboratories generally obtained consistent sets of data for the broad group of analyte toxins down to low levels (< 5ng/ml, equivalent to 0.05 mg/kg). In general, sensitivity is adequate to achieve the LODs required. Most of the participating laboratories could detect the analyte toxins; greater differences were observed for quantitation of some toxins, especially when no analytical standards were present. The participants have used different MS detection modes: some used single MS detection (SIM/SIR), others used tandem MS detection (MRM), and some used both. Although the use of MRM mode is attractive in order to enhance specificity, it requires additional care for quantitation. To summarize, the study was very stimulating and encouraging for those who are interested in using an alternative method for the mouse bioassays which are not supported by any statistical validation data, are well known to have a relatively high rate of false positives, have inadequate detection capability for some toxins and are ethically unacceptable for routine food monitoring. Additionally, method 40.105 (the method tested) can reliably detect ASP toxins and a range of other toxins and metabolites e.g. azaspiracids and pectenotoxin seco acids which may not respond in mouse assays (Holland and McNabb, 2003).

4.2.4 Reference materials

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

4.3 Source organism(s) and habitat

4.3.1 Source organism(s)

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

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

DA is produced by Pseudo-nitzschia pungens f. multiseries in culture as well as under field conditions (Hasle and Fryxell, 1995). In the southwestern Bay of Fundy in Canada, Pseudo-nitzschia pseudodelicatissima appeared to be the organism producing DA which led to unacceptable levels of DA in shellfish destined for human consumption (Martin et al., 1993).
Peculiarly, isolates of *P. pseudodelicatissima* from Galveston Bay (Texas), Massachusetts Bay (Massachusetts), Monterey Bay (California) and also from Denmark and Australia failed to produce DA (Bates, 2000).

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

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

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

### 4.3.2 Predisposing conditions for growth

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

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

Upwelling of cold water with high nitrogen concentrations (such as found in Monterey Bay, California) might have stimulated the increase of *P. pungens* f. *multiseries* populations (Villac *et al.*, 1993b). A direct evidence of increased cell numbers with even small amounts of upwelling was reported. From 1991 to 1994 on the west coast of the United States, *P. australis* blooms were most common and persisted longer during late summer and autumn when hydrographic conditions were associated with the end of the upwelling season and were usually characterized by higher sea-surface temperatures, thermal stratification, and lower concentrations of nutrients. Nutrients might be even more important to phytoplankton growth than direct effects of seasonal temperature changes. *P. seriata*, another DA producing *Pseudo-nitzschia* species, was found from the Barents Sea near Spitsbergen in the north to Germany in the south (Kiel Bay in Baltic) and in the western
Atlantic from the west and east coasts of Greenland to 45°N in Canada. *P. seriata* was apparently restricted to cold water and reached low latitudes only in winter. It is known in the Pacific from Alaska to British Columbia/Washington and it may be expected to occur also in northern Japan.

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

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

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

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

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

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

Bates *et al.* (1991) stated that the availability of extra-cellular nitrate or other nitrogen source and the presence of light were required in order to produce DA during the stationary phase. Nutrient
stress has also been implicated as a causative factor in eliciting DA production in *Pseudo-nitzschia pungens f. multiseries*.

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

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

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

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

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

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

It was speculated that DA events might be limited to seasons when stratification and nutrient depletion occurred or limited to near shore regions where developing blooms depleted the dissolved nutrients. However field observations in Monterey Bay showed that DA was produced.
by the large-celled *P. australis* at low cell densities and at moderate nutrient concentrations (Van Apeldoorn *et al.*, 1999).

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

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

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

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

### 4.3.3 Habitat

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

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

#### *P. pseudodelicatissima*
- **Atlantic**: waters of Europe and Africa (Denmark Strait to Northwest Africa, including Norwegian and Danish coastal waters, Skagerrak, Kiel Bay), Canada and USA (Arctic to Gulf of Mexico) and South America (Argentina) (Ferrario *et al.*, 1999).

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

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

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

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

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

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

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

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

4.4 Occurrence and accumulation in seafood

4.4.1 Uptake and elimination of ASP toxins in aquatic organisms

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

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

The level of DA in mussels in the absence of Pseudo-nitzschia is <1 ng/g. A minimum concentration of 2-4 x 10^5 Pseudo-nitzschia cells per L over a period of at least three to four weeks was needed to produce 20 ng DA per g mussels in eastern Prince Edward Island, Canada (Todd, 1993). When blue mussels (Mytilus edulis) were presented DA for 24 hours in dissolved form (125 nM; at 5°C; salinity 28 °) or as food encapsulated in liposomes, <1% of dissolved DA
and up to 6 percent of food-borne DA was incorporated in mussel tissues. DA absorbed from solution was most concentrated in gills and kidneys, whereas DA ingested as food was most concentrated in digestive gland and kidneys. Gonad, muscle, foot and connective tissues retained the lowest concentrations of toxin. DA levels in mussel tissues did not decrease consistently over a depuration period of 48 hours, nor did DA appear to be transferred to any tissue for storage. Small amounts of DA were eliminated in faeces and larger amounts in dissolved form (Van Apeldoorn et al., 1999).

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

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

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

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

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

Anatomical DA distribution was studied in scallops (Pecten maximus). In one study, only hepatopancreas, muscle and gonads were analysed. In a second study, hepatopancreas, muscle and gonads combined and the remaining soft tissues were analysed. In the first study 98.8 percent of
total DA content (in hepatopancreas, gonads and muscle) was localized in the hepatopancreas, and in the second study (when total tissue is included) 79.3 percent of total DA content was localized in hepatopancreas; negligible amounts were found in the gonads and muscle and about 14.5 percent in the remaining soft tissues (Arévalo et al., 1998).

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

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

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

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

### 4.4.2 Shellfish containing ASP toxins

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

In October 1991, DA was detected in razor clams (*Siliqua patula*) from Oregon and Washington States in the United States. Levels peaked in the first week of December 1991 (maximum level in edible portion was 147 ng/g, average level was 106 ng/g for all Washington state beaches). The DA levels in the clams remained above the regulatory closure level of 20 ng/g for at least six months. DA levels declined to <10 ng/g by late spring of 1992. From the spring of 1992 until the spring of 1993, levels of DA were < 5 ng/g for most of the coastal sampling areas. DA appeared
to distribute itself throughout the various body parts of the razor clam. The highest level was found in the foot or "digger", followed by the body, viscera and siphon (or neck). The DA level in the razor clam foot reached 230 \( \mu g/g \) (Van Apeldoorn et al., 1999).

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

### 4.4.3 Other marine organisms containing ASP toxins

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

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

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

In January 1996, the death of brown pelicans (Pelecanus occidentalis) at Cabo San Lucas on the tip of the Baja California Peninsula (Mexico) was ascribed to the feeding of mackerel (Scomber japonicus) contaminated by DA-producing \( Pseudo-nitzschia \) spp. (Sierra-Beltrán et al., 1997).
Over 400 Californian sea lions (*Zalophus californianus*) died along the central California coast during May and June 1998. DA produced by *P. australis* and transmitted to the sea lions via planktivorous northern anchovies (*Engraulis mordax*) was identified as the probable causative agent. Highest DA concentrations were found in the viscera (223 µg/g), exceeding values in body tissues by a factor 7. DA levels in sea lion faeces ranged from 136.5 to 152 µg/g by LC (requires correction from interference from an unidentified compound; receptor binding assay corresponded with LC values within a factor of two) (Lefebvre *et al*., 1999).

### 4.5 Toxicity of ASP toxins

#### 4.5.1 Mechanism of action

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

DA is a glutamate analogue and binds with high affinity to glutamate receptors of the quisqualate type. Glutamate and also NMDA subclass act to open membrane channels permeable to Na⁺, leading to Na⁺ influx and membrane depolarization. Only the channel opened by NMDA receptor accessible to kainate, quisqualate and to DA, is, in addition, highly permeable to Ca²⁺ and induced lethal cellular Ca²⁺ entry. Actions at NMDA receptors can be selectively antagonized by micromolecular concentrations of magnesium ions, organic antagonists such as D-2-amino-5-phosphonovalerate (APV) and dissociative anesthetics, such as phencyclidine (Viviani, 1992). In mice, kynurenic acid, administered intraperitoneally, protected powerfully and significantly against mussel-extract induced neurotoxicity. (Pinsky *et al*., 1989). Since drugs blocking the domoate-sensitive receptor are known, their use as antidotes for domoate poisoning has been considered (Laverty, 1993). DA is a two to three times more potent neuroexcitator than the structurally related kainic acid, and is up to 100 times more potent than glutamic acid. A synergistic effect between DA and other neurotoxic amino acids normally present in mussels is possible (Ravn, 1995). DA has two primary targets in the central nervous system; the hippocampal formation and its associated regions which are involved in processing memory, and the brain stem region of the area postrema and nucleus of the solitary tract associated with visceral function (Peng *et al*., 1994).

#### 4.5.2 Pharmacokinetics

*studies in laboratory animals*

The absorption of DA after oral administration to rats is poor as was demonstrated by almost complete recovery in the faeces, suggesting that absorption of DA may be reduced in rodents compared to humans. After intravenous dosing to rats, thereby removing the impact of absorption, all DA was recovered in urine within 160 minutes and excretion was not affected by co-administration of probenecid. The results of this study indicated that DA was cleared from plasma by the kidneys and, more specifically, by the process of glomerular filtration. The plasma half-life in rats was 21.6 minutes compared to 114.5 minutes in monkeys. Clearly, the six-fold more rapidly elimination of DA in rats is at least partly responsible for the lack of sensitivity of the rat compared to the monkey (see also Chapter 4.5.3) (Iverson and Truelove, 1994). There was no evidence of any biotransformation of DA by the rodent or primate as it has always been recovered unchanged (Todd, 1993).
In female ICR mice, the clearance of DA from serum after single i.p. injections ranging from 0.25 to 4.0 mg/kg bw was more than 95 percent within two hours and complete clearance was seen after four hours (Peng and Ramsdell, 1996).

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

### 4.5.3 Toxicity to laboratory animals

#### acute toxicity

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

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

**mice**

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

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

Studies in mice showed that toxic actions of DA are generally insensitive to NMDA (N-methyl-d-aspartate) receptor antagonists, although the onset of some responses could be delayed by CPP (3-(2-carboxypiperazine-4-yl) propyl-1-phosphonic acid). Domoate and kainate toxicity were reduced by drugs (6,7-dinitroquinoxaline-2,3-dione=DNQX and 2,3-dihydroxy-6-nitro-7-
sulfamoyl-benzo[F]quinoxaline=NBQX) that interact preferentially with non-NMDA receptors but do not distinguish between different kainate binding sites. The newly developed isatinoxime NS-102 selectively antagonized both the behavioural and pathological toxicity of DA relative to kanaic acid (Tasker et al., 1996).

**rats**

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

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

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

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

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

Xi et al. (1997) reported that, after intraperitoneal injection, neonatal rats were approximately 80-fold more sensitive to DA induced scratching and approximately 40-fold more sensitive to DA induced seizures and lethal effects than adult rats. The i.p. LD₅₀ for postnatal day two and day ten rats was 0.25 and 0.7 mg/kg bw, respectively. Mayer (2000) stated that during neurodevelopment in neonates the blood-brain barrier is incomplete and this fact might explain the higher sensitivity to DA.

Also Doucette et al. (2000) reported that DA was a very potent neurotoxin in newborn rats and the potency decreased progressively with increasing age (interpolated ED₅₀ = 0.12, 0.15, 0.30 and 1.06 mg/kg bw at post-natal day 0, 5, 14 and 22, respectively).

In studies in rodents, the effects induced by extracts of contaminated mussels were compared with the effects induced by pure DA and the effects induced by extracts of non-contaminated mussels. The extract of contaminated mussels appeared to be the most potent formulation. This was
ascribed to potentiation of the excitatory effect of DA by glutamate and aspartate; both are excitatory amino acids found in mussel extracts (Mariën, 1996).

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

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

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

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

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

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

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

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

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

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

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

mutagenicity

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

in vitro studies

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

DA induced the death of cultured neurons of chick embryonic retina and also inositol triphosphate (ip3) accumulation 4 to 7x above basal levels, both in a concentration and Ca\(^{2+}\)-dependent manner (Duran et al., 1995).

4.5.4 Toxicity to humans

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

However, DA is toxic to both the central and peripheral nervous systems of humans. DA is an emetic causing gagging and vomiting, likely through its effect on the vomit centre in the area
postrema of the brain. It produces a syndrome of axonal sensorimotor neuropathy, amnesia, seizures, coma and death. Because of its impact on memory, among other ill effects, DA intoxication was named amnesic shellfish poisoning (ASP) (Todd, 1993 and Watters, 1995).

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

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

4.5.5 Toxicity to aquatic organisms

In a laboratory study, DA appeared to be toxic to the marine copepod (Tigriopus californicus) at low concentrations. LC50 (24 hours) was found to be 8.62 μM (Van Apeldoorn et al., 1999). In autumn 1993, an unexplained mortality among sea scallops (Placopecten magellanicus) occurred in the Bay of Fundy, Canada. The digestive gland of the scallops appeared to contain 93.4 μg DA/g. Although some bivalve molluscs have been reported to contain high levels of DA without showing any symptoms, the spiny scallop (Chlamys hastata) died rapidly (within 12 hours) after exposure to cultures of toxic P. multiseries (Douglas et al., 1997).

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

In a laboratory study, sea scallops (Placopecten magellanicus) were fed *P. multiseries* with a high content of DA (4-6.6 pg/cell) for 22 days, followed by 14 days of feeding with nontoxic
microalgae. No signs of illness or mortality were observed during this study despite the high DA loading. However, the destructive sampling of the scallops did not allow assessment of long-term effects (Douglas et al., 1997).

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

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

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

### 4.5.6 Toxicity to water fowl

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

### 4.6 Prevention of ASP intoxication

#### 4.6.1 Depuration

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

Whole scallops (*Pecten maximus*) flesh contaminated with DA, showed a 43 percent decrease (mostly in hepatopancreas) in DA content after 180 days of frozen storage (-20 °C). During frozen storage, there was a transfer of DA from the hepatopancreas to the rest of the body, with a net average decrease in the whole product. Subsequently pickling of the scallops flesh or packing with brine and canning after frozen storage did not cause a further decrease of the DA content. During canning there was a notable transfer of toxin from the scallops to brine or the pickling
medium (more than 30 percent of total DA content in canned product) (Leira et al., 1998). Sea scallops (Placopecten magellanicus) and red mussels (Modiolus modiolus) were reported to retain DA for lengthy periods (Stewart et al., 1998). The DA level in the digestive gland of sea scallops was found to be only slightly lower at the end of a 19-month depuration study than it was at the beginning (Stewart et al., 1998). Dungeness crabs (Cancer magister) accumulated the toxin mostly in the viscera, although it can enter meat during cooking if the crabs were not eviscerated previously (Villac et al., 1993a). When contaminated whole crabs (22 mg DA/g confined to hepatopancreas) were cooked in fresh or salted water, visceral DA was reduced by 67 to 71 percent. After cooking, DA was detected not only in hepatopancreas (6.4 mg/g) but also in the body (1.9 mg/g) and leg (1.1 mg/g) meats. However, the majority of the DA was extracted out and diluted into the cook water. When cooked crabs were held for one or six days at 1 °C, DA was detected in hepatopancreas (6.1 to 8.2 mg/g) and body meats, but not in leg meats. Body meats proximal to the viscera contained higher DA levels (1.5 to 2.1 mg/g) than those distal (0.57 to 0.92). When cooked crabs were held for 90 days at -23 °C, DA was detected in the viscera (7.6 mg/g, body (0.67 to 0.79 mg/g) and leg (0.38 mg/g) meats. The storage conditions of cooked crabs had some effect on DA distribution, but no effect on the total DA content in each crab (Hatfield et al., 1995).

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

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

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

4.6.2 Preventive measures

Commercial harvest areas and aquaculture facilities are adversely and often unpredictably affected by toxic blooms. One problem is that certain algal species, which have never occurred in a certain area, may suddenly appear and then rapidly cause problems. Therefore preventive measures can hardly be taken. Extensive monitoring of the marine environment and the possibly contaminated fishery products together with regulations (see Chapter 4.8) will be required to prevent (shell)fish poisoning incidents. Data on the occurrence of toxic algal species may indicate which toxins may be expected during periods of algal blooms and which fishery products should be considered for analytical monitoring. In the case of domestic produce, several countries stopped the harvest of fishery products if levels of the toxin exceeded the limits and a waiting period was established until the concentrations of the toxin are below the acceptable limit (Shumway et al., 1995). Within the genus Pseudo-nitzschia, DA production can vary greatly with the species and it is vital to be able to distinguish species (Van Apeldoorn et al., 1999). Toxin concentrations in the fishery products can also vary with the species of fishery product involved and with the area of harvest. Harvested fishery products containing too much toxin were usually destroyed. Toxic doses are often estimated from left-over toxic seafood but these may not be always representative of the ingested food (Shumway et al., 1995).
Direct analysis of phytoplankton samples for DA will probably remain the fastest and most reliable method to confirm the presence of DA (Van Apeldoorn et al., 1999). Although the need for routine phytoplankton monitoring often has been stressed, phytoplankton monitoring has been implemented only in a few areas (Horner et al., 1997).

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

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

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

### 4.7 Cases and outbreaks of ASP
#### 4.7.1 Europe

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

**Belgium**

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

**Denmark**

Investigations in Danish waters have shown that *P. seriata*, a widely distributed species in colder areas of the Northern hemisphere, produced DA in concentrations similar to those found in *Pseudo-nitzschia pungens f. multiseries* in Canada (1-20 pg/cell). During the survey five species and one subspecies of *Pseudo-nitzschia* were found in Danish waters: *P. delicatissima, P. fraudulenta, P. pseudodelicatissima, P. pungens, P. pungens f. multiseries* and *P. seriata*. Isolates of *P. seriata* appeared to contain DA. In isolates of three other *Pseudo-nitzschia* species detected
during this survey, *P. pseudodelicatissima, P. delicatissima* and *P. pungens*, no DA was present. *P. seriata* is one of the most common species of *Pseudo-nitzschia* in the North Atlantic. These findings support the idea that toxic and non-toxic strains occur within the same species of the diatom (Van Apeldoorn *et al.*, 1999).

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

**France**

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

**Ireland**

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

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

**Italy**

In the Gulf of Naples, high concentrations (up to 4 x 10^6 cells/litre) of *Pseudo-nitzschia* species (*delicatissima* and *pseudodelicatissima*) occurred sometimes (Montresor *et al.*, 2000). In July 2002, DA above the regulatory limit was detected in *Pecten maximus* from Scotland (EU-NRL, 2002).

**The Netherlands**

In the Dutch Wadden Sea, *Pseudo-nitzschia*-like pennate chain-forming diatom species with cell numbers ranging from 10^2 to 10^5 per litre were detected between November 1993 and July 1994. Electron microscopy of cultured isolates and field samples revealed the majority to be *Pseudo-nitzschia pungens*. At the beginning of June 1994, *P. fraudulenta* was also present and occasionally *P. delicatissima* was detected. One isolate showed the characteristic morphology of *P. multiseries*. The isolate of *P. multiseries* produced DA; after 55 days of growth about 19 pg/cell was measured. The *Pseudo-nitzschia* species found in Dutch coastal waters have not yet been implicated in shellfish poisoning (Van Apeldoorn *et al.*, 1999). In 2001, DA producing algae were detected but ASP toxins were not detected in shellfish (EU-NRL, 2001). In spring 2002, a single sample of *Spisula* contained levels up to 5 mg DA/kg (LC-UV method). Resampling three days later resulted in the absence of DA (EU-NRL, 2002).
Figure 4.2 Occurrence of ASP toxins in coastal waters of European ICES countries from 1991 to 2000

Source: http://www.ifremer.fr/envlit/documentation/dossiers/ciem/aindex.htm
Norway
Despite high abundances of *Pseudo-nitzschia* species no outbreaks of ASP have been reported (EU-NRL, 1998). In 2000 and 2001, DA was detected in mussels and scallops, however never above the regulatory limit even during a bloom of *Pseudo-nitzschia* (>4 000 000 cells/litre) (EU-NRL, 2001).

Portugal
In 1996, DA was detected in very small amounts (<20 µg/g) in almost every bivalve species all around the Portuguese coast for short periods scattered in time and coincident with the occurrence of *Pseudo-nitzschia* spp., mainly *P. australis* in concentrations below 10⁵ cells/litre. The first detected occurrence of DA in bivalves over 20 µg/g was in smooth callista (*Callista chione*) in 1995 (EU-NRL, 1998). In 1999, a total of 960 samples were tested for the presence of ASP toxins. DA was detected in 102 samples but the regulatory limit was exceeded only in two samples (EU-NRL, 2000). In 2000 and 2001, DA was detected in scallops from a wild fishery (EU-NRL, 2001). DA in Portuguese shellfish is a recurrent event that affects several shellfish resources several times a year, mainly in spring and autumn. Levels as high as twice the regulatory limit are not unusual. When several shellfish species are exploited in the same restricted area, common cockle (*Cerastoderma edule*) and carpet shell (*Venerupis pullastra*) are usually among the most toxic, followed by pepper furrow shell, clam, mussel, oyster and razor clam. In whole sardines, DA was detected in levels exceeding sometimes the regulatory limit. Fortunately, toxicity is restricted to the gut content, and does not accumulate in muscle tissue (Vale and De Sampayo, 2001)

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

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

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

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

In 1997, traces of DA were detected for the first time in Scotland (Shetland). No details were available (EU-NRL, 1998). In July 1999, a scallop fishing area of 8000 square miles on the west coast of Scotland was closed following the discovery of ASP toxins (Wyatt, 1999). *P. australis* was found to be the source of DA in king scallops (*Pecten maximus*) and queen scallops (*Chlamys opercularis*) in 1999 and 2000 (Bates, 2000). The main toxicity event during 1999 was the closure of 8000 km² of scallop fishing grounds after the detection of DA. Low levels of DA were detected in mussels but concentrations of up to 250 µg/g were detected in scallop gonads and up to 500 µg/g in total tissue (EU-NRL, 2000). During 2000 and 2001, ASP toxins above the regulatory limit were found in whole scallops and in scallop gonad tissue from all major scallop fishing areas around Scotland. Restrictions were placed on fishing activities in all affected areas (EU-NRL, 2001).

In the period from 1 April 2002 to 31 March 2003, shellfish from 76 primary inshore production areas, 36 secondary areas and offshore fishing areas in Scotland were examined. A total of 5409 mollusc samples were analysed. Out of this total, 2788 samples were analysed for ASP toxins and 115 samples were determined positive (Anonymous, 2003c).

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

4.7.2 North America

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

Canada

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

Since 1988, phytoplankton samples have been collected at four stations in the western Bay of Fundy. Blooms of *P. pseudodelicatissima* in the Bay of Fundy tend to occur during late summer with highest concentrations observed during 1988 and 1995 (> 1.0x10⁶ cells/litre), during late August and early September, leading to closure of shellfish harvesting areas (Martin et al., 2001).
During August to October 1988, DA levels greater than the acceptable levels for human consumption (20 \text{mg/g}) were detected in soft-shell clams (\textit{Mya arenaria}) and blue mussels (\textit{Mytilus edulis}) from the southwestern Bay of Fundy, New Brunswick, resulting in the closure of some shellfish harvesting areas. \textit{P. pseudodelicatissima} was found to be the source of DA and was detected in all plankton tows (collected since 1987) where DA was found. \textit{P. pseudodelicatissima} was detected throughout the year with higher concentrations in June/July followed by the highest concentrations in September when water temperatures were elevated. The highest concentration (1.2 x 10^6 \text{ cells/litre}) of \textit{P. pseudo-delicatissima} was measured during 1988 and persisted throughout the water column for a longer period than during 1987, 1989 and 1990. Analysis of nutrients (chlorophyll a, salinity, nitrate, phosphate, silicate at surface and 10 and 1 m above bottom; measured, however, during 1989 and 1990 and not in 1988) did not reveal an obvious correlation between \textit{P. pseudodelicatissima} and nutrient concentrations (Martin \textit{et al.}, 1993).

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

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

\textit{Source: http://www.ifremer.fr/envlit/documentation/dossiers/ciem/aindex.htm}
The United States of America

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

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

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

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

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

A bloom of *Pseudo-nitzschia* spp. was observed in Penn Cove, Washington in July and August 1997. Levels of DA in mussels up to 3 µg/g were measured. In seawater DA levels ranged from 0.1-0.8 µg/L. Four species of *Pseudo-nitzschia* were detected, namely *P. pungens*, *P. multiseries*, *P. australis* and *P. pseudodelicatissima*. Highest *Pseudo-nitzschia* concentration was 13 x 10⁶ cells/litre on 28 July 1997 (Trainer et al., 1998a).

In the autumn of 1998, elevated DA levels prompted a coast-wide closure of the Washington razor clam fishery. In April 2001, a spike in marine toxin levels suspended razor clam digging in the Twin Harbours area but subsided enough for resumption of digging within a month. In October 2002, all three ocean beaches north of Grays Harbour, Washington, which were tentatively scheduled to open in the beginning of October, remained closed until further notice after the detection of DA in razor clams exceeding the Federal standard of 20 µg/g (Ayres, 2002). At the end of December 2002, toxin levels had declined but were still above 20 µg/g. This means that razor clams digging on Washington beaches was not allowed during the season that runs through the spring of 2003 (Ayres, 2003).
In early September 1991 (18-27 September 1991), more than 100 brown pelicans (*Pelecanus occidentalis*) and cormorants (*Phalacrocorax penicillatus*) in Monterey Bay, Central California died or suffered from unusual neurological symptoms which were attributed to the neurotoxin DA. The source was identified as a bloom of the pennate diatom *P. australis* (Horner et al., 1997).

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

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

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

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

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

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

**East Coast**

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

*Pseudo-nitzschia* species are often present in great abundance in Louisiana coastal waters, including areas where there are oyster beds. A multi-year study in the shelf and estuarine waters from Louisiana showed the presence of *Pseudonitzschia* spp. in 73 percent of the shelf samples.
and in 20 percent of the estuarine samples. At least six *Pseudonitzschia* species were present of which *P. multiseries* had the greatest potential of causing an outbreak of DA poisoning (Parsons *et al.*, 1998). There have been no known outbreaks of ASP in Louisiana, possibly because isolated cases have not been recognized, or oysters did not become toxic (Dortch, 2002).

**Gulf of Mexico**

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

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

*Pseudo-nitzschia* spp. were extremely abundant (up to 10⁸ cells/L; present in 67 percent of 2,159 samples) from 1990 to 1994 on the Louisiana and Texas continental shelves and moderately abundant (up to 10⁵ cell/L; present in 18 percent of 192 samples) over oyster beds in Terrebonne Bay estuary in Louisiana in 1993 and 1994. On the shelf there was a strong seasonal cycle with maxima every spring for 5 years and sometimes in the autumn, which were probably related to river flow, water column stability and nutrient availability. In contrast, in the estuary no apparent seasonal cycle in abundance was observed. The *Pseudo-nitzschia* spp. was not routinely identified during this study. However, toxin producing *P. multiseries* has been identified previously from Galveston Bay, Texas (see paragraph above), and cells from a bloom on the shelf in June 1993 were identified by scanning electron microscopy as *P. pseudodelicatissima*, which is sometimes toxic. There have been no known outbreaks of ASP in this area (Van Apeldoorn *et al.*, 1999).

**4.7.3 Central and South America**

**Argentina**

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

**Chile**

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

The percentage of shellfish samples with DA levels exceeding the regulatory limit of 20 mg/g has increased steadily since 1997. Up until 2001, no cases of ASP intoxications in humans were recorded but the situation is a potential threat to public health (Suárez-Isla, 2001).
Mexico
In January 1996, 150 dead brown pelicans (*Pelecanus occidentalis*) were found within a period of five days at Cabo San Lucas on the tip of the Baja California Peninsula. The death of these birds was the result of feeding on mackerel (*Scomber japonicus*) contaminated by DA-producing *Pseudo-nitzschia* spp. (Sierra-Beltrán *et al*., 1997).

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

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

4.7.4 Asia

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

4.7.5 Oceania

Australia, Tasmania and New Zealand

An Australian-wide taxonomic survey for species of the potentially toxic diatom genus *Pseudo-nitzschia* was carried out. The dominant bloom-forming *Pseudo-nitzschia* species in Australian coastal waters were *P. fraudulenta* (New South Wales), *P. pungens f. pungens* and *P. pseudodelicatissima* (Tasmanian and Victorian waters). *P. pungens f. multiseries* was detected on only one occasion and only as a minor component (5 percent of total biomass) of a dense *P. pungens f. pungens* bloom in a New South Wales estuary. *P. australis* was never detected in Australian waters. Cultured diatom populations of *P. pseudodelicatissima* from Tasmanian and Victorian coastal waters were consistently non-toxic. Cultures of *P. pungens f. pungens* from Australia (Hallegraeff, 1994) and Tasmania (Hallegraeff, 1994) were also non-toxic. *P. fraudulenta* has proved also non-toxic (Hallegraeff, 1994). Traces of DA have been detected in some scallop viscera by both LC and mass spectrometry, but the concentrations in edible shellfish products were all well below 20 mg/g of shellfish meat (Hallegraeff, 1994). In New Zealand, DA was not identified in 150 greenshell mussel (*Perna canaliculus*) samples and in plankton samples taken during *Pseudo-nitzschia* bloom periods (Van Apeldoorn *et al*., 1999). During the summers of 1992 and 1993, DA was detected in the marine biotoxin programme of New Zealand at low
levels in phytoplankton samples from Otago to Northland. _P. pungens_ has been found in low numbers (up to 3,000 cells per litre) at the Bay of Islands, the Hauraki Gulf and Bay of Plenty (Smith et al., 1993). Both _P. pungens_ and _P. pseudoseriata_ have been detected in New Zealand waters but ASP has never been clearly associated with shellfish from the Pacific Ocean. Chemical analysis of shellfish samples has identified low levels of DA. The highest level (16.5 mg/g) came from Manukau Harbour. Other detectable levels were well below 20 mg/g (Bates et al., 1993).

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

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

### 4.8 Regulations and monitoring

#### 4.8.1 Europe

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

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

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

_Denmark_

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

_Ireland_

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

4.8.2 North America

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

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

4.8.3 Central and South America

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

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

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

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

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

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

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

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