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SCIENTIFIC OPINION

Marine biotoxins in shellfish – Domoic acid[1]

Scientific Opinion of the Panel on Contaminants in the Food Chain

(Question No EFSA-Q-2006-065H)

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PANEL MEMBERS

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SUMMARY

Domoic acid (DA) and its isomers are marine biotoxins causing amnesic shellfish poisoning (ASP) in humans. Symptoms of ASP include gastrointestinal symptoms (vomiting, diarrhoea or abdominal cramps) and/or neurological symptoms (confusion, loss of memory, or other serious signs such as seizure or coma) occurring within 24-48 hours after consuming contaminated shellfish. DA is a water-soluble cyclic amino acid mainly produced by marine red algae of the genus *Chondria* and diatoms of the genus *Pseudo-nitschia.* The first confirmed outbreak of ASP occurred in Canada in 1987 and was related to mussels affected by a bloom of the *Pseudonitzschia f. multiseries*. DA isomers have also been detected in shellfish in the United States and in a number of European countries. Although several isomers of DA (diastereoisomer epi-domoic acid (epi-DA) and isodomoic acids (iso-DAs)) have been identified data on the occurrence only of DA and epi-DA (expressed as sum DA) have been reported.

The toxicological database for DA is limited, comprising mostly studies on the acute toxicity in rodents and Cynomolgus monkeys following administration by parenteral routes and with few studies with oral administration. Neurotoxicity is the critical toxicological effect identified in experimental animals as well as in humans. The toxic effects of DA are mediated through its high affinity binding and agonist activity on some forms of glutamate receptors particularly in certain regions (e.g. hippocampus) of the brain. The few data available indicate that Cynomolgus monkeys are more sensitive than rodents.

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Following oral administration DA shows low systemic absorption. Absorbed DA is rapidly cleared from the body by renal excretion and impaired renal function increases the susceptibility to DA.

Data on the genotoxicity of DA are inconclusive. No data on the chronic effects of DA in animals are available, therefore the Panel on Contaminants in the Food Chain (CONTAM Panel) could not establish a tolerable daily intake (TDI). In view of the acute toxicity of DA the CONTAM Panel decided to establish an acute reference dose (ARfD) based on the available human data on acute toxicity.

The few data on exposure to DA associated with adverse effects in humans (9 individuals) in ASP outbreaks indicate that severe and irreversible effects occurred at about 4 mg/kg bodyweight (b.w.) and the lowest-observed-adverse-effect-level (LOAEL) for mild signs and symptoms was 0.9 mg/kg b.w. Although the acute oral toxicity is not well characterised, the CONTAM Panel considered it appropriate to base the establishment of the ARfD on this LOAEL.

Taking into account the steep dose-response relationship, the CONTAM Panel decided to apply a factor of 3 for extrapolation from a LOAEL to a no-observed-adverse-effect level (NOAEL). The CONTAM Panel concluded that a factor of 10 should be applied to allow for human variability and also for fact that sensitive methods for the detection of neurotoxic effects had not been used in the investigation of affected individuals.

The CONTAM Panel therefore established an ARfD of 30 µg DA/kg b.w. by applying the overall uncertainty factor of 30 to the LOAEL of 0.9 mg/kg b.w. Because DA can be converted to epi-DA during storage, the ARfD applies to the sum of DA and epi-DA.

In order to ensure protection against the acute effects of DA, it is important to use a large portion size rather than the long-term average consumption in the health risk assessment of shellfish consumption. Consumption data for shellfish species across the European Union (EU) were limited, therefore the European Food Safety Authority (EFSA) requested the Member States to provide information on consumption of relevant shellfish species. Based on data provided by five Member States, the CONTAM Panel identified 400 g of shellfish meat as a large portion size to be used in the acute risk assessment of marine biotoxins.

Consumption of a 400 g portion of shellfish meat containing DA and epi-DA at the current EU limit of 20 mg DA/kg shellfish meat would result in a dietary exposure of 8 mg DA (equivalent to about 130 µg DA/kg b.w. for a 60 kg adult). This is about four times higher than the ARfD of 30 µg DA/kg b.w. (equivalent to 1.8 mg DA per portion for a 60 kg adult) and is considered to constitute a potential health risk. Based on current consumption and occurrence data there is a chance of about 1 % of exceeding the ARfD of 30 µg DA/kg b.w. when consuming shellfish currently available on the European market.

The CONTAM Panel concluded that in order for a 60 kg adult to avoid exceeding the ARfD of 30 µg DA/kg b.w. a 400 g portion of shellfish should not contain more than 1.8 mg DA corresponding to 4.5 mg DA/kg shellfish meat. Of the currently available occurrence data for samples in compliance with the EU regulatory limit of 20 mg/kg shellfish meat, 3.5 % exceed this value of 4.5 mg/kg shellfish meat.

DA is heat stable and cooking does not destroy the toxin, although normal home cooking processes, such as boiling and steaming, could reduce the amount of DA in shellfish meat due to partial leaching of the toxin into the cooking fluids. In scallops, redistribution of DA from the hepatopancreas into the other tissues could occur. For other types of shellfish it is unlikely that processing would have a major effect on the DA concentration in shellfish meat.

Several analytical methods are available for the determination of DA and its isomers. High performance liquid chromatography-ultraviolet detection (HPLC-UV) methods are widely used. One HPLC-UV method has been validated (AOAC method 991.26) and standardised (CEN method 14176). In addition, a method based on enzyme-linked immunosorbent assay (ELISA) has been validated (AOAC method 2006.02) and is officially allowed to be used in the EU for screening purposes. These methods have limits of detection that are sufficiently low to adequately detect DA at the concentration of 4.5 mg/kg shellfish meat, for which consumption of a large portion would not lead to the ARfD being exceeded. The available evidence suggests that liquid chromatography-mass spectrometry (LC-MS), particularly using electrospray ionisation in the tandem mode, can also be a valuable tool for rapid and selective determination of DA and its isomers in crude extracts. Other chemical methods have been developed but they are only rarely used and they have not been validated in interlaboratory studies.

Key words: **Marine biotoxins, domoic acid (DA), epi-domoic acid (epi-DA), isodomoic acids (iso-DAs) , shellfish, bivalve molluscs, acute reference dose, portion size, methods of analysis, human health, risk assessment.**

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

Marine biotoxins, also commonly known as shellfish toxins, are mainly produced by algae or phytoplankton.

Based on their chemical structure, the toxins have been classified into eight groups, namely, the azaspiracid (AZA), brevetoxin (BTX), cyclic imine, domoic acid (DA), okadaic acid (OA), pectenotoxin (PTX), saxitoxin (STX) and yessotoxin (YTX) groups, as agreed at the Joint FAO/IOC/WHO *ad hoc* Expert Consultation held in 2004[2]. Two additional groups, palytoxins (PlTX) and ciguatoxins (CTX), may also be considered. STX and its derivatives cause Paralytic Shellfish Poisoning (PSP), and DA causes Amnesic Shellfish Poisoning (ASP). Diarrhetic Shellfish Poisoning (DSP) is caused by OA-group toxins (OA and dinophysis toxins (DTX)), and AZA group toxins cause Azaspiracid Shellfish Poisoning (AZP). These toxins can all accumulate in the digestive gland (hepatopancreas) of filter-feeding molluscan shellfish, such as mussels, oysters, cockles, clams and scallops, and pose a health risk to humans if contaminated shellfish are consumed. Marine biotoxin-related illness can range from headaches, vomiting and diarrhoea to neurological problems, and in extreme cases can lead to death.

To protect public health, monitoring programmes for marine biotoxins have been established in many countries, which often stipulate the use of animal models (for example, the mouse bioassay (MBA) and the rat bioassay (RBA)), for detecting the presence of marine biotoxins in shellfish tissues.

In the European Union (EU), bioassays are currently prescribed as the reference methods. Various stakeholders (regulators, animal welfare organisations, scientific organisations) have expressed their concerns about the current legislation in Europe, not only with regard to the use of large numbers of animals, involving procedures which cause significant pain and suffering even though non-animal based methods are available, but also since the scientific community argues that the animal test may not be suitable for all classes of toxins and that the state-of-the-art scientific methodology for the detection and determination of marine biotoxins is not fully reflected in current practices.

1. Legal framework

In 2004, the purported *EU Hygiene Package* of regulations, bringing together and replacing the existing hygiene regulations for the food sector previously contained in numerous individual vertical Directives was published. In Annex II Section VII Chapter V (2) to Regulation 853/2004/EC[3], are established maximum levels for ASP, PSP and DSP toxins. Annex III of Commission Regulation No 2074/2005/EC[4] of 5 December 2005 lays down the recognised testing methods for detecting marine biotoxins. Annex II Chapter II (14) to

[2] ftp://ftp.fao.org/es/esn/food/biotoxin_report_en.pdf

[3] Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. OJ L 139, 30.4.2004, p. 55–205

[4] Commission Regulation (EC) No 2074/2005 of 5 December 2005 laying down implementing measures for certain products under Regulation (EC) No 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No 854/2004 of the European Parliament and of the Council and Regulation (EC) No 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004 OJ L 338, 22.12.2005, p. 27–59.

Regulation (EC) 854/2004[5], gives the monitoring authorities in the EU Member States the mandate to examine live molluscs for the presence of marine biotoxins. The *EU Hygiene Package* came into effect on 1 January 2006.

2. The Council Directive 86/609/EEC

Council Directive 86/609/EEC6 makes provision for laws, regulations and administrative provisions for the protection of animals used for experimental and other scientific purposes. This includes the use of live vertebrate animals as part of testing strategies and programmes to detect identify and quantify marine biotoxins. Indeed, the scope of Article 3 of the Directive includes the use of animals for the safety testing of food, and the avoidance of illness and disease.

Directive 86/609/EEC sets out the responsibilities that Member States must discharge. As a result of this use of prescriptive language, Member States have no discretion or flexibility, and most of the provisions of the Directive must be applied in all cases. It is clear that Member States have to ensure that: the number of animals used for experimental and other scientific purposes is reduced to the justifiable minimum; that such animals are adequately cared for; and that no unnecessary or avoidable pain, suffering, distress or lasting harm are caused in the course of such animal use.

Member States may not (Article 7, 2) permit the use of live animals in procedures that may cause pain, suffering, distress or lasting harm: "if another scientifically satisfactory method of obtaining the result sought and not entailing the use of live animals is reasonably and practicably available". When animal use can be justified, Directive 86/609/EEC specifies a range of safeguards that Member States must put in place to avoid or minimise any animal suffering that may be caused. All justifiable animal use should be designed and performed to avoid unnecessary pain, suffering, distress and lasting harm (Article 8). Member States must ensure (Article 19, 1) that user establishments undertake experiments as effectively as possible, with the objective of obtaining consistent results, whilst minimising the number of animals and any suffering caused.

This latter requirement necessitates the use of minimum severity protocols, including appropriate observation schedules, and the use of the earliest humane endpoints that prevent further suffering, once it is clear that the scientific objective has been achieved, that the scientific objective cannot be achieved, or that the suffering is more than can be justified as part of the test procedure. The EC and Member States are also required (Article 23, 1) to encourage research into, and the development and validation of, alternative methods that do not require animals, use fewer animals, or further reduce the suffering that may be caused, whilst providing the same level of scientific information.

3. Recognised testing methods for marine biotoxins and maximum levels

Commission Regulation (EC) No. 2074/20054 specifies a mouse bioassay (MBA) for the determination of paralytic shellfish poisoning toxins (PSP) and a MBA or the rat bioassay (RBA) for lipophilic marine biotoxins. Alternative test methods can be applied if they are

[5] Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. OJ L 139, 30.4.2004, p. 206–320.

[6] Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animal used for experimental and other scientific purposes. OJ L 358, 18.12.1986, p. 1–28.

validated following an internationally recognised protocol and provide an equivalent level of public health protection.

Besides paralytic shellfish poisoning toxins, okadaic acid, dinophysistoxins, pectenotoxins, azaspiracids and yessotoxins, also cyclic imines, (gymnodimine, spirolides and others which are currently not regulated in the EU), all give a positive response in MBAs.

The reference method for the domoic acid group (the causative agent of ASP) is based on high-performance liquid chromatography (HPLC).

Chapter V (2) (c) and (e) of Section VII of Annex III to Regulation (EC) No 853/20043 establishes that food business operators must ensure that live bivalve molluscs placed on the market for human consumption must not contain marine biotoxins in total quantities (measured in the whole body or any part edible separately) that exceed the following limits:

- 800 micrograms per kilogram for paralytic shellfish poison (PSP),
- 20 milligrams of domoic acid per kilogram for amnesic shellfish poison (ASP),
- 160 micrograms of okadaic acid equivalents[7] per kilogram for okadaic acid, dinophysistoxins and pectenotoxins in combination,
- 1 milligram of yessotoxin equivalents per kilogram for yessotoxins,
- 160 micrograms of azaspiracid equivalents per kilogram for azaspiracids.

4. Joint FAO/IOC/WHO *ad hoc* Expert Consultation on Biotoxins in Bivalve Molluscs (Oslo, September 26-30 2004)

Based on the available information, the Joint FAO/IOC/WHO ad hoc Expert Consultation suggested provisional acute reference doses (ARfDs)8 for the AZA, OA, STX, DA, and YTX-group toxins, respectively (summarized in the Table 1). The Expert Consultation considered that the database for the cyclic imines, brevetoxins and pectenotoxins was insufficient to establish provisional ARfDs for these three toxin groups. In addition, guidance levels were derived comparing results based on the consumption of 100 g, 250 g or 380 g shellfish meat by adults. However, the Expert Consultation noted that the standard portion of 100 g, which is occasionally used in risk assessment, is not adequate to assess an acute risk, whereas a portion of 250 g would cover 97.5 % of the consumers of most countries for which data were available.

Available methods of analysis were reviewed for the 8 toxin groups and recommendations made for choice of a reference method, management of analytical results and development of standards and reference materials.

The Joint FAO/IOC/WHO ad hoc Expert Consultation, however, did not have sufficient time to fully evaluate epidemiological data and to assess the effects of cooking or processing for deriving the provisional guidance levels/maximum levels for several toxin groups (especially the AZA and STX groups). The Consultation encouraged Member States to generate additional toxicological data in order to perform more accurate risk assessments and to facilitate validation of toxin detection methods in shellfish.

[7] Equivalents: the amount of toxins expressed as the amount of okadaic acid that gives the same toxic response followed intraperitoneal administration to mice. This applies similarly for the group of yessotoxins and azapiracids, respectively.

[8] The acute reference dose is the estimate of the amount of substance in food, normally expressed on a body-weight basis (mg/kg or µg/kg of body weight), that can be ingested in a period of 24 hours or less without appreciable health risk to the consumer on the basis of all known facts at the time of evaluation (JMPR, 2002)

Table 1. Summary data used in the derivation of the ARfD and current guidance levels

Group toxin	LOAEL(1) NOAEL(2) µg/kg body weight	Safety Factor (Human data (H) Animal data (A))	Provisional Acute RfD[8]	Derived Guidance Level/ Max Level based on consumption of 100g (1), 250g (2) and 380g (3)	Limit Value currently implemented in EU legislation
AZA	0.4 (1)	10 (H)	0.04 µg/kg 2.4 µg/adult a)	0.024 mg/kg SM (1) 0.0096 mg/kg SM (2) 0.0063 mg/kg SM (3)	0.16 mg/kg SM
BTX			N/A		
Cyclic Imines			N/A		
DA	1,000 (1)	10 (H)	100 µg/kg 6 mg/adult a)	60 mg/kg SM (1) 24 mg/kg SM (2) 16 mg/kg SM (3)	20 mg/kg SM
OA	1 (1)	3 (H)	0.33 µg/kg 20 µg/adult a)	0.2 mg/kg SM (1) 0.08 mg/kg SM (2) 0.05 mg/kg SM (3)	0.16 mg/kg SM
PTX			N/A		0.16 mg OA equivalents/kg SM
STX	2 (1)	3 (H)	0.7 µg/kg 42 µg/adult a)	0.42 mg/kg SM (1) 0.17 mg/kg SM (2) 0.11 mg/kg SM (3)	0.8 mg/kg SM
YTX	5,000 (2)	100 (A)	50 µg/kg 3 mg/adult a)	30 mg/kg SM (1) 12 mg/kg SM (2) 8 mg/kg SM (3)	1 mg/kg SM

SM=shellfish meat, LOAEL=lowest-observed-adverse-effect level, NOAEL = no-observed-adverse-effect level, N/A = not available, EU = European Union

a) Person with 60 kg body weight (b.w.)

The Joint FAO/IOC/WHO *ad hoc* Expert Consultation also indicated that there were discrepancies between different risk assessments, especially for determining methods of analysis for certain marine biotoxins and in relation to established maximum limits.

Test methods for the eight toxin groups were reviewed and recommendations for Codex purposes made. Mouse bioassays are widely used for shellfish testing but for technical and ethical reasons it is highly desirable to move to new technologies which can meet Codex requirements more adequately. Most currently available methods do not meet fully the strict criteria for Codex type II[9] or III[10] methods and have therefore not been widely used in routine shellfish monitoring. However, the recommendations made by the Expert Consultation represent the best currently available methods. Liquid chromatography-mass spectrometry (LC-MS) has much potential for multi-toxin analysis and has been recommended for consideration and recommendation by Codex. The Joint FAO/IOC/WHO *ad hoc* Expert Consultation is of the opinion that the complexity and chemical diversity of some toxin groups is such that validated quantitative methods to measure all toxins within a group will be extremely difficult. Thus the implementation of a marker compound concept and the use of functional assays should be explored.

[9] A Type II method is the one designated Reference Method where Type I methods do not apply. It should be selected from Type III methods (as defined below). It should be recommended for use in cases of dispute and for calibration purposes.

[10] A Type III Method is one which meets the criteria required by the Codex Committee on Methods of Analysis and Sampling for methods that may be used for control, inspection or regulatory purposes.

5. Working Group Meeting to Assess the Advice from the Joint FAO/IOC/WHO ad hoc Expert Consultation on Biotoxins in Bivalve Molluscs, Ottawa, Canada, April 10-12, 2006

The working group (WG) discussed available reference methods in particular and concluded that they should be highly specific, highly reproducible, and not prone to false positives or false negatives. The methods are expected to be definitive and may well result in significant rejections of products and must therefore withstand the most robust legal and scientific scrutiny.

In considering their weaknesses and merits, the meeting noted that the various mouse bioassays should be discussed individually since the level of performance and success differs markedly between the official method for PSP by mouse bioassay, the American Public Health Association (APHA) method for brevetoxins and the multiple mouse bioassay "DSP" procedures employed for the other lipophilic toxins such as okadaic acid, azaspiracids and others.

Recognizing that the majority of the currently available methods do not meet all Codex criteria for reference methods (Type II), the WG concluded that Codex Committee for Fish and Fishery Products (CCFFP) should consider a variety of biotoxin analytical methods. Wherever possible, reference methods should not be based on animal bioassays. Functional methods, biochemical/immunological and chemical-analytical methods currently in use, and considered to be validated according to Codex standards, should be recommended by CCFFP to the Codex Committee on Methods of Analysis and Sampling (CCMAS) for review and designation as Type II or Type III methods.

Because the Expert Consultation has offered 3 different guidance limits associated with three levels of consumption (100 g, 250 g and 380 g) for most toxin groups, it is important to determine which consumption level is appropriate for the protection of consumers.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

In accordance with Art. 29 (1) (a) of Regulation (EC) No 178/2002, the Commission asks EFSA to assess the current EU limits with regard to human health and methods of analysis for various marine biotoxins as established in the EU legislation, including new emerging toxins, in particular in the light of

- the report of the Joint FAO/IOC/WHO *ad hoc* Expert Consultation on Biotoxins in Bivalve Molluscs (Oslo, September 26-30 2004), including the ARfDs and guidance levels proposed by the Expert Consultation,
- the conclusions of the CCFFP working group held in Ottawa in April 2006,
- the publication of the report and recommendations of the joint European Centre for the Validation of Alternative Methods (ECVAM)/DG SANCO Workshop, January 2005,
- the report from CRL Working group on Toxicology in Cesenatico October 2005,
- any other scientific information of relevance for the assessment of the risk of marine biotoxins in shellfish for human health.

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ASSESSMENT

1. Introduction

This scientific opinion deals with domoic acid (DA) which is a marine biotoxin causing amnesic shellfish poisoning (ASP) in humans. Symptoms of ASP include gastrointestinal effects (nausea, vomiting, diarrhoea or abdominal cramps) within 24 hours of consuming shellfish contaminated with DA and/or neurological symptoms or signs (confusion, loss of memory, or other serious signs such as seizure or coma) occurring within 48 hours.

DA is a water-soluble cyclic amino acid. It is produced mainly by marine red algae of the genus *Chondria* and diatoms of the genus *Pseudo-nitschia* although other species are known to produce DA (FAO/IOC/WHO, 2004). The first confirmed outbreak of ASP occurred in Canada in 1987 related to mussels affected by a bloom of the *Pseudonitzschia f. multiseries*. DA has also been detected in the United States and in a number of European countries.

2. Chemical characteristics

DA is a cyclic amino acid (311 Da) with three carboxylic acid groups which are responsible for its water solubility and its relatively high polarity (Quilliam *et al*., 2001) (Figure 1). DA is structurally very similar to another known neurotoxin kainic acid. For the three carboxylic acid groups the acid dissociation constants (pK_a values) are 2.10-4.97 and for the cyclic amino group 9.82 (Piñeiro *et al*., 1999), and hence DA can exist in different charged states depending on pH (Jeffery *et al*., 2004).

Several isomers of DA (epi-domoic acid (epi-DA), (domoic acid C5'-diastereomer)) and isodomoic acids A, B, C, D, E, F, G and H (iso-DA A-H)) have been reported (Figure 1) (Maeda *et al*., 1986; Wright *et al*., 1990a; Walter *et al*., 1994; Zaman *et al*., 1997; Holland *et al*., 2005). Iso-DA A, B and C have not been detected in shellfish tissue (Wright and Quilliam, 1995). Based on the data of Zhao *et al*. (1997), Jeffrey *et al*. (2004) estimated that the concentrations of iso-DA D, E and F in shellfish extracts are much lower than the concentration of DA. DA transforms into epi-DA through long-term storage (Quilliam *et al*, 1989) and degrades and transforms to epi-DA and iso-DAs through exposure to ultra violet light (Wright *et al*., 1990; Wright and Quiliam, 1995; Djaoued *et al*., 2008). Epimerisation is also accelerated by heating (Quilliam, 2003a).

Due to the conjugated double bond in the aliphatic side chain DA absorbs ultra violet (UV) light. The conjugated double bond is also the cause of radical-mediated oxidative metabolism. DA does not degrade at ambient temperature or when it is exposed to light in sterile saline solution (Johannessen, 2000). As a contaminant in shellfish tissues, DA is heat stable and cooking does not destroy the toxin (McCarron *et al*., 2007).

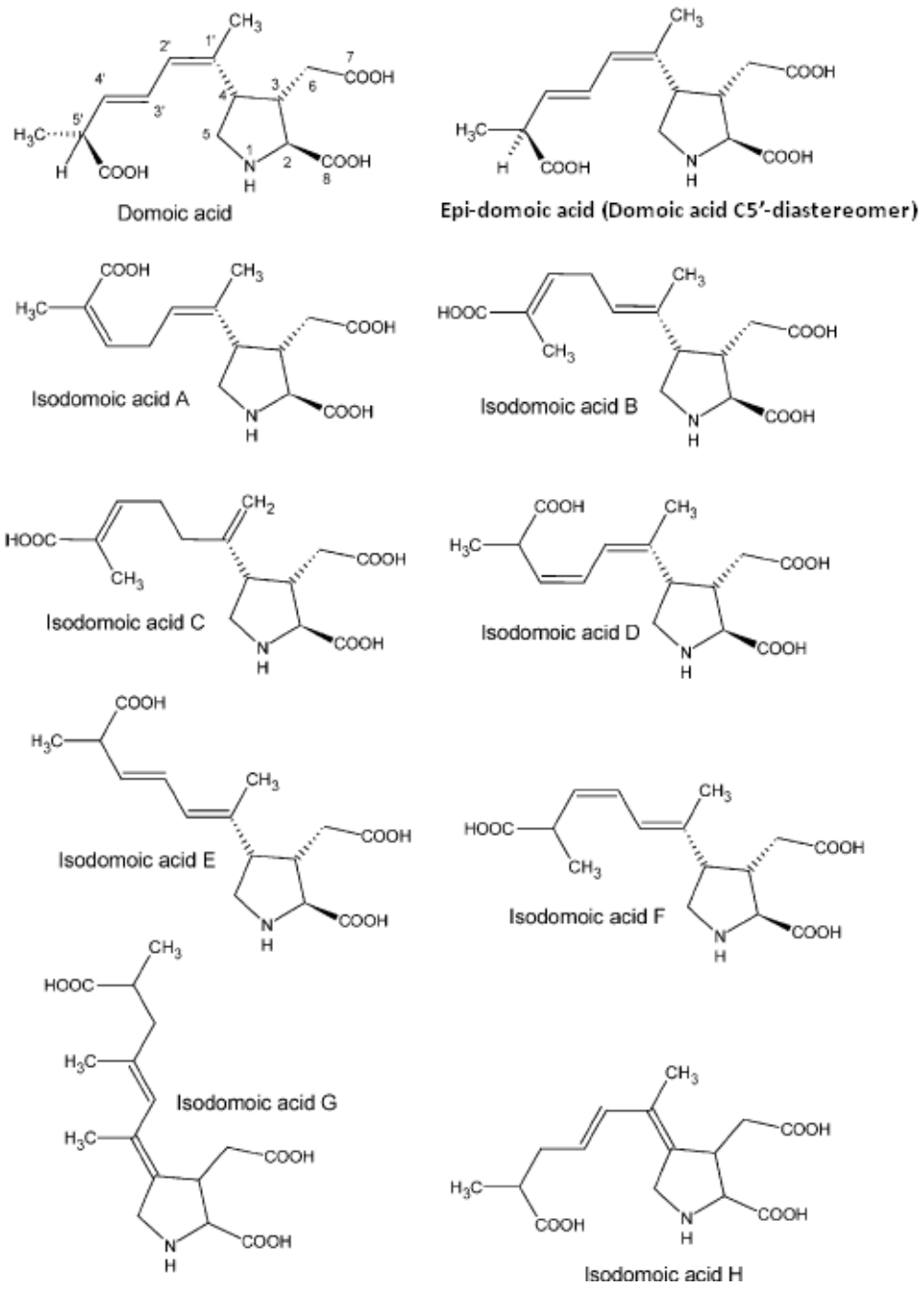

Figure 1. Chemical structures of domoic acid and its isomers

3. Regulatory status

For the control of DA in the European Union (EU), Commission Regulation (EC) No 853/2004[3], provides details in section VII: "Live bivalve molluscs", chapters II and V. Chapter II: "Hygiene requirements for the production and harvesting of live bivalve molluscs. A. Requirements for production areas" states: "*Food business operators may place live molluscs collected from class A production areas on the market for direct human consumption only, if they meet the requirements of chapter V*". Chapter V: "*HEALTH STANDARDS FOR LIVE BIVALVE MOLLUSCS states: "In addition to ensuring compliance*

with microbiological criteria adopted in accordance with Regulation (EC) No 852/2004[11]*, food business operators must ensure that live bivalve molluscs placed on the market for human consumption meet the standards laid down in this Chapter". "They must not contain marine biotoxins in total quantities (measured in the whole body or any part edible separately) that exceed the following limits: for amnesic shellfish poison (ASP): 20 milligrams of domoic acid per kilogram."*

This limit corresponds with limits established in countries outside the EU, such as Canada, the United States (guidance limit) and New Zealand (FDA, 2001; FAO/WHO, 2004).

In Commission Decision 2002/226/EC[12], the European Commission allows a restricted harvesting regime under which bivalve molluscs belonging to the species *Pecten maximus* and *Pecten jacobaeus* (scallops) with a DA concentration over 20 mg/kg but lower than 250 mg/kg in the whole body can be harvested. This is the case if two consecutive analyses of samples, taken between one and no more than seven days, show that the DA concentration in whole mollusc is lower than 250 mg/kg and that the DA concentration in the parts intended for human consumption, (the adductor muscles and/or the gonads) is lower than 4.6 mg/kg and if the hepatopancreas, soft tissues and any other contaminated parts are removed. After removal of these parts the adductor muscle and/or gonads intended for human consumption must not contain a DA level detectable by the high performance liquid chromatogrpahy (HPLC) method exceeding 20 mg/kg of DA. Harvesting of *Pecten maximus* and *Pecten jacobaeus* must not be allowed during the occurrence of an ASP outbreak in the waters of the production area.

Commission Regulation (EC) No 2074/2005[4], provides details about the "Recognized testing methods for detecting marine biotoxins". Annex III, Chapter II of this regulation deals with DA detection methods. This chapter has been amended by Commission Regulation (EC) No 1244/2007[13] to read:

"Chapter 2: Amnesic Shellfish Poison (ASP) detection method

The total content of amnesic shellfish poison (ASP) of edible parts of molluscs (the entire body or any part edible separately) must be detected using the high-performance liquid chromatography (HPLC) method or any other internationally recognised method.

However, for screening purposes, the 2006.02 ASP ELISA method as published in the AOAC Journal of June 2006 may also be used to detect the total content of ASP of edible parts of molluscs.

If the results are challenged, the reference method shall be the HPLC method."

In conclusion, the EU legislation for DA requires the use of an HPLC method but also allows alternatives, provided these have been validated according to an internationally agreed protocol. Currently, the Association of Analytical Communities (AOAC) method 991.26, which is the same as CEN method 14176, fulfils this requirement and can therefore be used. Another internationally recognised and often used HPLC method is the so-called Quilliam-method (Quilliam *et al*., 1995) which is currently pending standardisation in the European

[11] Commission Regulation (EC) No 852/2004 of 29 April 2004 on the hygiene of foodstuffs. OJ L 139, 30.4.2004, p. 1–54

[12] Commission Decision 2002/226/EC of 15 March 2002 establishing special health checks for the harvesting and processing of certain bivalve molluscs with a level of amnesic shellfish poison (ASP) exceeding the limit laid down by Council Directive 91/492/EEC.OJ L 75, 16.3.2002, p. 65–66

[13] Commission Regulation (EC) No 1244/2007 of 24 October 2007 amending Regulation (EC) No 2074/2005 as regards implementing measures for certain products of animal origin intended for human consumption and laying down specific rules on official controls for the inspection of meat. OJ L 281, 25.10.2007, p. 12–18

Committee for Standardization (CEN). The EU-legislation does not specify which HPLC-method should be used for analysis of DA in shellfish.

4. Methods of analysis

Several methods are available for the determination of DA. For screening of DA, molecular methods such as the enzyme-linked immunosorbent assay (ELISA) and the surface plasmon resonance (SPR) biosensor are used. Methods applying HPLC with ultra violet (UV) or mass spectrometric (MS) detection are widely used. Alternative approaches like thin layer chromatography and capillary electrophoresis have also been proposed. Reference materials and analytical standards have been commercially available for several years.

4.1. Supply of appropriate reference material

Currently, the National Research Council Canada (NRCC) provides for DA a certified reference calibrant solution and a mussel tissue certified reference material (CRM). The calibrant solution and the mussel tissue also contain known amounts of epi-DA, in addition to DA. The certified value for the calibrant and the mussel CRM relates to the sum of DA and epi-DA.

4.2. Mammalian bioassay

4.2.1. Mouse bioassay

The mouse bioassay (MBA) for DA is based on the AOAC mouse bioassay for saxitoxin (STX)-group toxins (AOAC Official method 959.08), involving acidic aqueous extraction with boiling and intraperitoneal injection of 1 mL of the extract into the mouse. Although it was used to detect DA during the first ASP incident in Canada, signs (scratching of shoulders by the hind leg, sedation-akinesia, rigidity, loss of posteral control, convulsions and death) are observed in mice when DA is present at levels starting at approximately 40 mg/kg, while the regulatory limit is at 20 mg/kg (Ciminello *et al*., 2005). Therefore the MBA is not capable of checking for compliance with the regulatory limit and HPLC is prescribed as the detection method in the EU legislation.

4.3. Biomolecular methods

Biomolecular methods for DA are based on two different strategies, use of receptors and use of antibodies. Only an immunological assay based on ELISA has been validated through the AOAC International Official Methods Program.

4.3.1. ASP Enzyme-Linked Immunosorbent Assay (AOAC official method 2006.02)

The use of an indirect ELISA for analysing DA was first studied by Garthwaite *et al*. (1998). Later this indirect ELISA, called ASP ELISA, was converted to direct format using horseradish peroxidase-conjugated primary antibodies, and validated for DA and its isomers in shellfish. The calibrants and reference materials used in the study contained DA, iso-DA A, D, E, F and epi-DA (Kleivdal *et al*., 2007). In the ASP ELISA the samples are extracted with 50 % methanol-water solution without cleanup or a preconcentration step. Later the method was evaluated in a collaborative study involving 16 laboratories and it was adopted as official AOAC method 2006.02 (Kleivdal *et al*., 2007). The results obtained from the parallel HPLC-

UV analyses (e.g. AOAC official method 991.26 and Quilliam method) showed that ASP ELISA is a suitable alternative to the HPLC-UV method (Kleivdal *et al*., 2007). In 2007 Commission Regulation (EC) No 2074/2005[4] was amended by Commission Regulation (EC) No 1244/2007[13] stating that ASP ELISA can be used as a screening method, alternative to the HPLC method. The ASP ELISA has a limit of detection (LOD) of 0.003 mg/kg and a limit of quantification (LOQ) of 0.01 mg/kg and it was validated for the determination of DA and its isomers at levels >0.12 mg/kg in shellfish (mussels, scallops and oysters).

The main advantages of the ASP ELISA (AOAC official method 2006.02) are:

- it is sensitive, rapid and provides a high sample throughput;
- the equipment needed is relatively cheap;
- it is easy to perform, can be automated and requires minimal training;
- it has been formally validated in an interlaboratory study.

4.3.2. Surface Plasmon Resonance Biosensor

An immuno-based screening method was developed using a surface plasmon resonance (SPR)-based optical biosensor (Traynor *et al*., 2006). The assay parameters suggested that the method allows a screening of DA at the European regulatory limit of 20 mg/kg, but the method has not been validated so far in an interlaboratory study.

The main advantage of the SPR method is:

- it allows for automatisation and high sample-throughput.

The main disadvantages of the SPR method are:

- the necessary equipment is relatively expensive;
- it has not been validated in an interlaboratory study.

4.3.3. Receptor assays

Van Dolah *et al*. (1997) developed a competitive microplate receptor assay using a recombinant rat GLUR6 glutamate receptor for DA determination in algal and shellfish extracts. The determination of DA was based on binding competition with [^{3}H]-kainic acid for the kainate/quisqualate glutamate receptor and included a glutamate decarboxylase pretreatment step to eliminate potential interference due to high concentrations of endogenous glutamate in shellfish. Extraction was based on the method described by Quilliam *et al*. (1995) (see section 4.4.1.2). No LOD was reported but the method could detect DA at the level of the current EU limit value (20 mg/kg shellfish meat). The results agreed well with those of the high performance liquid chromatography-fluorescence detection (HPLC-FLD) method described by Pocklington *et al*. (1990) (see section 4.4.1.3).

The main advantage of the receptor competitive binding assay for DA is:

- it is suitable for high throughput analyses.

The main disadvantage of the receptor competitive binding assay for DA is:

- it has not been validated in interlaboratory studies.

4.4. Chemical methods

4.4.1. HPLC based methods

Due to its conjugated diene structure DA exhibits an absorption peak at λmax=242 nm. This facilitates the quantitative determination of DA in shellfish tissue using HPLC followed by UV detection. The LOD for DA is dependent on the extraction procedures and the sensitivity of the UV detection. The first HPLC-UV method was developed by Quilliam in 1989 based on extraction with 50 % aqueous methanol. In 1991 Lawrence *et al*. (1991) proposed an HPLC-UV method based on extraction with 0.1 M hydrochloric acid (HCl). An improved HPLC-UV analysis procedure was developed by Quilliam *et al*. (1995). The method involves extraction with 50 % aqueous methanol combined with an additional cleanup step. Following pre-column derivatisation it is also possible to measure DA also with fluorescence detection (Pocklington *et al*., 1990; Wright and Quilliam, 1995). Finally, several HPLC methods using mass spectrometry (MS) have been developed for the specific determination of DA (Wright *et al*., 1989; Quilliam, 2003b; Furey *et al*., 2001; Holland *et al*., 2003; Pineiro *et al*., 2001; Hess *et al*. 2005; McNabb *et al*., 2005; Pardo *et al*., 2007).

4.4.1.1. Standardised HPLC-UV method (AOAC Official Method 991.26 and CEN method 14176)

The method described by Lawrence *et al*. (1991) involves a slightly modified version of the AOAC extraction procedure for the STX-group toxins. The HPLC-UV method of Lawrence *et al*. (1991) was the first standardised chemical-analytical method for DA and is still widely used for monitoring shellfish. The method has been collaboratively studied under the AOAC International Official Methods Program and has become AOAC Official Method 991.26. In addition, it has been standardised by CEN and has become CEN standard 14176. DA is extracted by boiling the homogenised shellfish tissue for 5 minutes with an equal volume of 0.1 M HCl and is measured after reverse phase HPLC-UV detection at 242 nm. The LOD is approximately 1 mg DA/kg which is well below the EU regulatory limit. Due to the absence of a cleanup step interferences can occur. In particular, tryptophan may elute close to DA or one of its isomers, making interpretation of the results ambiguous (Quilliam *et al*., 1989; Quilliam, 2003a). However, the CEN protocol considers that this problem can be solved by adjusting the mobile phase composition accordingly.

The main advantages of the HPLC-UV method (AOAC Official method 991.26 and CEN method 14176) are:

- it is sensitive;
- it gives information on the profile of DA isomers in samples;
- it can be automated;
- it has been validated in an interlaboratory study.

The main disadvantage of the HPLC-UV method (AOAC Official method 991.26 and CEN method 14176) is:

- HPLC analytical columns have a short lifetime because of the lack of sample cleanup.

4.4.1.2. HPLC-UV method based on procedures developed by Quilliam

An HPLC-UV method based on Quilliam *et al*. (1989) involves extraction of DA after boiling homogenised mussel tissue with 50 % aqueous methanol. After reverse-phase HPLC with binary gradient, DA and its isomers are detected by UV absorbance at 242 nm. The LOD is approximately 0.5 mg DA/kg which is well below the EU regulatory limit. This method was validated in a national study in Germany and became an official method of the German food law (§64 LFGB, L 12.03/04.3)[14]. It is in the process of standardisation by CEN.

Another reverse-phase HPLC-UV method was later developed by Quilliam *et al*. (1995) (so-called Quilliam-method). It consists of a single-step extraction of DA with 50 % aqueous methanol combined with a strong anion exchange solid phase extraction (SPE) cleanup, separation by HPLC with isocratic conditions and UV absorbance detection. The LOD is at 0.02-0.03 mg DA/kg, which is well below the EU regulatory limit and the LOD of the AOAC/CEN HPLC method. The Quilliam-method has also undergone a collaborative study performed by the Community Reference Laboratory for Marine Biotoxins (CRL-MB)/National Reference Laboratory (NRL) Network and its standardisation is currently ongoing under CEN including combination with the previous Quilliam-method (Quilliam *et al*., 1989).

Following the results of the CRL-MB validation procedure, CEN has eliminated the cleanup step from the method. The Quilliam-method is also recommended by Codex Alimentarius as the reference method for DA detection. These HPLC-UV methods are widely used in Europe.

The main advantages of the HPLC-UV method based on procedures developed by Quilliam are:

- it is sensitive;
- the chromatograms are free from interferences;
- the extracts are stable;
- it can be automated;
- it is validated in an interlaboratory study.

4.4.1.3. HPLC-fluorescence detection

The HPLC-fluorescence detection (FLD) method is based on pre-column derivatisation with for example 9-fluorenylmethylchloroformate to form the fluorenylmethoxycarbonyl derivative or 4-fluoro-7-nitro-2,1,3-benzoxadiazole, followed by reverse-phase HPLC analysis with FLD (Pocklington *et al*., 1990; Wright and Quilliam, 1995). It was first developed for monitoring DA in seawater and phytoplankton but has found applications with shellfish extracts (James *et al*., 2000). However this method has not led to broad applications. LOD was 15 ng DA/L for DA in seawater and aqueous extracts and 0.006 mg DA/kg in mussel tissues.

The main advantages of the HPLC-FLD method are:

- it is highly sensitive;
- it can be automated.

The main disadvantages of the HPLC-FLD method are:

[14] L12.03/04-3 „Untersuchung von Lebensmitteln - Bestimmung von Domoinsäure - ASP-Toxin – in Muscheltieren und Muscheltiererzeugnissen mittels RP-HPLC“ Amtliche Sammlung von Untersuchungsverfahren nach §64-LFBG, Beuth Verlag GmbH Berlin, December 2002.

- matrix interferences with derivatisation reactions are encountered;
- it is not validated in an interlaboratory study.

4.4.1.4. LC-MS

MS detection methods are commonly used for the determination of DA and they have been developing in recent years because they are sensitive, selective and require minimal sample cleanup. Suitable mobile phases include methanol and acetonitrile buffer systems with formic acid, acetic acid, ammonium salts of these acids or ammonium hydroxide (Quilliam, 2003b). Various LC-MS interfaces for the determination of DA have been investigated, including continuous-flow fast atom bombardment (FAB) (Wright *et al*., 1989), thermospray, atmospheric pressure chemical ionisation and electrospray interfaces. Electrospray ionisation was found to be best suited for determination of DA (Quilliam, 2003b) and work has been performed in recent years to optimise these methods, using hydrophilic interaction LC (HILIC-LC) or tandem mass spectrometry (LC-MS/MS) (Furey *et al*., 2001; Holland *et al*., 2003; Pineiro *et al*., 2001; Pardo *et al*., 2007).

One multiresidue LC-MS method (McNabb *et al.*, 2005) with a LOD of 0.015 mg/kg has undergone an intensive single-laboratory validation and a limited inter-laboratory study. However, this study included sample extracts rather than real samples.

Hess *et al*. (2001) suggested a cleanup of the extract with a strong anion exchange SPE-cartridge in order to avoid false positives. Other authors have developed more rapid methods not requiring any sample cleanup (Ciminello *et al*., 2005; Lopez Rivera *et al*., 2005) or involving cleanup inside a pressurised liquid extraction stainless-steel extraction cell packed with Florisil® material (Pardo *et al*., 2007).

An interlaboratory comparison study of HPLC-UV (Quilliam method with variations) and LC-MS methods was conducted by Hess *et al*. (2005), which showed similar results in the quantification of DA calibrant solutions and gonad homogenate. However, the limitation of this study was that shellfish extracts were used rather than shellfish samples.

The main advantages of the LC-MS methods are:

- they are rapid;
- they are highly sensitive and selective;
- they can be automated.

The main disadvantages of the LC-MS methods are:

- they require costly equipment and highly trained personnel;
- they have not been validated in an interlaboratory study.

4.4.2. Capillary electrophoresis

Since DA's imino and carboxylic groups can easily be protonated, capillary electrophoresis (CE) has been used as an alternative to HPLC for the separation and determination of DA (Nguyen, 1990; Pineiro *et al*., 1999; Zhao *et al*., 1997). After aqueous methanol extraction and strong anion exchange-SPE cleanup procedure, the method used by Zhao *et al*. (1997) allowed separation of DA and several of its isomers, that was superior to that achieved with HPLC. The LODs varied from 0.15 to 1 mg DA/kg.

The main advantages of the CE methods are:

- they enable a high resolution separation of DA and its isomers;

- they can be automated;
- they can find application with very limited sample sizes.

The main disadvantages of the CE methods are:

- they require two SPE cleanup steps to achieve reliable results;
- they have not been validated by interlaboratory studies
- they require highly trained personnel.

4.4.3. Thin-layer chromatography

Quilliam *et al*. (1998) developed a thin-layer chromatography (TLC) method for the semi-quantitative detection of DA in shellfish tissues. Samples were extracted with 50 % aqueous methanol and the extracts, purified on strong anion exchange cartridges were spotted on activated silica TLC plates and developed with a butanol-acetic acid-water system. After chromatography fluorescence quenching (absorbance of DA at 242 nm) and ninhydrin (which reacted with the secondary amine function of the DA molecule) were used for detection of DA. DA could be detected in sample extracts containing down to 10 mg DA/kg of tissue.

The main advantages of the TLC method are:

- it requires short analysis time;
- it is simple and can be used in laboratories not equipped with HPLC.

The main disadvantages of the TLC method are:

- cleanup procedure is required to avoid interference of amino acids present in crude extract;
- it is not validated in interlaboratory studies.

4.5. Proficiency tests

Proficiency testing for DA is available at EU level by the CRL-MB for the NRLs. An open proficiency test is also provided on a commercial basis for DA by QUASIMEME[15]. This proficiency testing scheme has been in operation since 2003 and has shown typically very high success rates for participating laboratories, i.e. in the order of 80 % laboratories with 100 % correct results.

4.6. Summary of methods

Regulation (EC) No 2074/2005[4] amended by Regulation 1244/2007[13] prescribes the use of HPLC methods or any other internationally recognised method for the quantification of DA and the use of the ASP ELISA method (AOAC 2006.02) for screening purposes. The method described by Lawrence *et al*. (1991) involving a slightly modified version of the AOAC extraction procedure for the STX-group toxins has become AOAC Official Method 991.26 as well as CEN standard 14176. An improved HPLC-UV analysis procedure was developed by Quilliam *et al*. (1995). This method is sensitive and selective. It has been validated and a CEN standardisation of the method without the need for a cleanup step to replace the CEN method 14176 is currently ongoing. The evidence available suggests that LC-MS, particularly using electrospray ionisation in the MS/MS mode, can also be a valuable tool for rapid and selective determination of DA in sample extracts.

[15] http://www.quasimeme.org; accessed 22/06/2009

5. Occurrence of DA and epi-DA

5.1. Data Collection

Following a request by the European Food Safety Authority (EFSA) for data on DA, 10 countries (Denmark, France, Germany, Ireland, Italy, The Netherlands, Norway, Portugal, Spain and the United Kingdom) provided data on the occurrence of DA and epi-DA in shellfish as the sum of DA and epi-DA. This means that no distinction was possible between the concentration of parent DA and epi-DA, and the CONTAM Panel decided to report the amounts of these isomers as a single DA value (sum of DA and epi-DA (sum DA)) in this opinion.

A total of 42962 analytical results were submitted. The numbers of analyses differed considerably from one country to another, but all datasets were large enough to allow for informative statistical analysis. Table 2 summarises the data submitted by each country including number of samples, purpose of testing, analytical method applied, LOD and LOQ of the method.

Table 2. Data submissions from ten European countries for the sum of DA and epi-DA in the period from 1999 to 2008

Country	Year(s) of harvesting	Number of samples	Purpose of testing [a)]	Method of testing	LOD (mg/kg)	LOQ (mg/kg)
Denmark [b)]	2005-2008	635	pre-MC	HPLC-UV	0.17	-
France	1999-2008	2792 [f)]	pre-MC	HPLC-UV	0.1-0.3	0.3-1.1
Germany	2004-2007	234	pre-MC	LC-MS/MS	0.2-0.25	0.2-0.5
	2004-2008	722	post-MC	HPLC-UV	0.1-2.0	0.5-3.0
Ireland	2002-2008	4152 [f)]	pre-MC	HPLC	1.25	2.50
Italy	2000-2008	10017	pre-MC	HPLC	0.05-0.6	0.2-2.0
The Netherlands	2006-2008	921	pre-MC	LC-MS/MS	0.18-0.35	0.35-0.75
Norway [c)]	2006-2008	1318	pre-MC	HPLC	-	0.10
Portugal	2005-2007	2567	pre-MC	HPLC-DAD	0.8	2.5
Spain	2007-2008	1508 [f)]	pre-MC	HPLC-UV	0.1-0.6	0.5-2.0
	unknown	6 [e)]	post-MC		-	1.6
United Kingdom	2000-2008	16014 [f)]	pre-MC	HPLC	0.33	1.0
	2003-2008	2076	pre-MC	SPR Biosensor	1.06-7.02 [d)]	-
Total		**42962**				

Pre/post-MC = pre-market/post-market control; LOD = limit of detection; LOQ = limit of quantification: DAD=diode array detector

a) PreMC samples are samples collected at the place of origin, before or during harvesting; PostMC are samples collected at the place of sale or along the distribution chain.

b) Denmark only reported LOD.

c) Norway only reported LOQ.

d) For the Surface Plasmon Resonance (SPR) biosensor method only one reporting threshold is given (identified by the data provider as LOD).

e) The six post-MC samples from Spain with unknown date are too few to have statistical significance and were disregarded in the further calculations.

f) Not all the samples refer to whole shellfish analysis. Samples from only dissected parts of the molluscs are 130 for France, 3147 for Ireland, 5 for Spain and 2648 for UK.

The submissions covered samples collected and tested during the years 1999-2008. Among these, 40886 samples were analysed with HPLC-based methods (including 1343 samples analysed with LC-MS/MS) and 2076 were tested with a SPR biosensor method. The reported analyses with SPR biosensor are not directly comparable with those using the

chromatographic methods, and have significantly higher LODs. Therefore, these samples will be presented only in the summary table of the occurrence data (Table 3), but will be disregarded in the following calculations.

Pre- and post-market control (pre-MC and post-MC) samples were present in the submitted datasets. Pre-MC samples, which are the samples taken before harvesting for further processing or direct marketing as prescribed in the respective EU legislation, comprised 42234 results. Post-MC samples, which are taken from the market, collected at stores and supermarkets, comprised 728 results. The dataset from Germany comprised 234 pre-MC and 722 post-MC results. Spain submitted 1508 pre-MC and 6 post-MC results (the latter results were disregarded in the further calculations because they were too few to have statistical relevance). The data submitted by Denmark (635), France (2792), Ireland (4152), Italy (10017), The Netherlands (921), Norway (1318), Portugal (2567) and UK (18090) were all from pre-MC samples.

Recognising the need to compare only homogeneous datasets it was decided to keep separate the data obtained by non-comparable analytical methods and also the pre- and post-MC data. The pre-MC data were regarded as the best suited for occurrence calculations, because the origin of the data is defined, and the occurrence is not influenced by previous screening analyses. Two groups of data were identified based on the analytical methods, HPLC-based (including LC-MS/MS) data and data obtained by the SPR biosensor method.

The SPR biosensor method measures DA without differentiating between isomers. In contrast, chromatographic methods allow the identification of DA and epi-DA but the available certified reference material only allows the quantification of the sum of them. Moreover, DA converts to epi-DA during storage. Therefore, as indicated above, the amounts of these isomers are reported as a single DA value (sum DA) in this opinion.

For the SPR biosensor method the LOD ranged approximately between 1 and 7 mg/kg DA. For the HPLC-based methods some degree of variability in LOD and LOQ was observed in different laboratories at different times in the reporting period, but in general the sensitivity was comparable between the different laboratories. Therefore, all HPLC-based samples were considered as one group.

5.2. Statistical description of DA and epi-DA in shellfish

Normally, the whole shellfish is consumed; therefore the occurrence data for DA need to be expressed for whole shellfish meat. For DA scallops are an exception. In this type of shellfish DA is found mainly in the hepatopancreas and soft tissues. Based on studies supporting this finding, the European Commission decided in the Commission Decision of 15 March 2002 (2002/226/EC)[12] "Establishing special health checks for the harvesting and processing of certain bivalve molluscs with a level of amnesic shellfish poison (ASP) exceeding the limit laid down by Council Directive 91/492/EEC" to allow a conditional harvesting of Scallops (*Pecten maximus* and *Pecten jacobaeus*) "*with a concentration of domoic acid (DA) in the whole body exceeding 20 mg/kg but lower than 250 mg/kg*", followed by removal of the contaminated parts. One of the conditions for the derogation is that "*after total removal of hepatopancreas, soft tissues and any other contaminated part the adductor muscle and/or gonads intended for human consumption must not contain an ASP level detectable by the HPLC techniques exceeding 20 mg/kg of DA*".

Therefore analytical data for scallops include whole shellfish analyses and separate analyses of adductor muscle, gonads, hepatopancreas and soft tissue. In some cases, data on whole

shellfish without hepatopancreas were also reported. For the remaining molluscs most of the analyses were performed on whole shellfish meat. In a few samples only hepatopancreas was analysed. In this case a factor of 5 was used to convert the value to whole shellfish meat. This factor, though not representing exactly all individual shellfish species, is considered to be a good approximation. The occurrence statistics were calculated on 37032 samples representing whole shellfish.

The "bounding" approach was applied for imputing values reported below LOD or below LOQ. It consists of attributing particular values inside the range of their possible variability. The Lower Bound (LB) is obtained by assigning a value of zero (minimum possible value) to all the samples reported as <LOD or <LOQ. The Upper Bound (UB) is obtained by assigning the value of LOD to values reported as <LOD and LOQ to values reported as <LOQ (maximum possible value).

Therefore, the CONTAM Panel decided to compare the UB and LB values (sensitivity analysis) in the tables. The two values represent the range of variability of each statistical descriptor as a function of the approach chosen to attribute a numerical value to results reported as "non-detected".

Table 3 provides an overview of the descriptive statistics of the data, grouping them by analytical method, type of sampling and country. Samples without reported results (i.e. no numerical value provided in the data submission) were assigned UB and LB values. When the statistical descriptors using the two approaches are the same the value is given, otherwise the LB and UB values are reported.

Table 3. Statistics of occurrence data for the sum of DA and epi-DA (sum DA) in shellfish sampled in the years 1999-2008, provided by European countries

Analytical method/Country	N	Median LB/UB	Mean LB/UB	P95 LB/UB	Maximum	% of samples not quantified[b)]	% of values >20 mg sum DA/kg shellfish meat
		sum DA mg/kg shellfish meat					
Pre-MC samples							
HPLC							
Denmark	635	0/0.2	0.3/0.5	0.2	30	94.8 %	0.3 %
France	2662	1	5.8/5.9	32	182	47.0 %	8.6 %
Germany	25	-	-	-	-	100 %	-
Ireland	1005	0/1.3	15.3/16.4	82	680	72.6 %	11.3 %
Italy[a)]	10017	0/0.5	0.01/1.1	0/2	5.3	99.6 %	-
Norway	1318	0/0.1	0.08/0.2	0.2	10	94.5 %	-
Portugal	2567	0/0.8	1.0/1.9	5.6	160	91.3 %	1.2 %
Spain	1503	0/1.6	2.9/4.3	10.7	173	91.7 %	3.6 %
United Kingdom	13366	0/1	30.6/31.1	250	857	75.0 %	17.1 %
LC-MS/MS							
Germany	209	-	-	-	-	100 %	-
The Netherlands	921	-	-	-	-	100 %	-
SPR biosensor							
United Kingdom	2076	-	-	-	-	100 %	-
Post-MC samples							
HPLC							
Germany[a)]	449	0/0.5	0.01/0.53	0/0.5	2	98.7 %	-
Spain	6	-	-	-	51	50 %	50 %
LC-MS/MS							
Germany	213	0/0.2	0.06/0.25	0/0.2	5	95.8 %	-

N = number of samples; LB=lower bound; UB=upper bound; P95=95th percentile; Pre/post-MC = pre-market/post-market control

For most of the data no information was available on measurement uncertainty. When two values are given they indicate the respective lower (LB) and upper bound (UB) values for samples below the limit of detection (LOD) or the limit of quantification (LOQ). The lower bound is calculated substituting 0 to all not detected samples. The upper bound is calculated substituting "<LOD" with LOD value and "<LOQ" with LOQ value; LOD and LOQ are those defined for the specific single analysis.

a) The number of not quantified samples approaches 100 %; therefore, even the P95 depends on the substitution approach (LB/UB).

b) Not quantified means no numerical value reported.

The percentage of pre-MC samples with not quantified values varies to a large extent, depending on country and year of harvesting, ranging from 47 % for France to 100 % for Germany and The Netherlands. The proportion of pre-MC samples exceeding the EU regulatory limit (20 mg/kg) also varies among countries in the range between 0 % (Germany, Italy, Norway, The Netherlands) and 17.1 % (UK).

Some areas appear to be more affected by DA than others. These areas include UK (17.1 % above 20 mg/kg, with maximum at 857 mg sum DA/kg), Ireland (11.3 % above 20 mg/kg, with a maximum of 680 mg sum DA/kg), and France (8.6 % above 20 mg/kg, with a maximum of 183 mg sum DA/kg). Lower levels are reported by Spain (3.6 % above 20 mg/kg, with a maximum of 173 mg sum DA/kg) and Portugal (1.2 % above 20 mg/kg, with a maximum of 160 mg sum DA/kg). Denmark has 0.3 % of the samples above 20 mg/kg, with a maximum of 30 mg sum DA/kg. For the remaining countries no sample exceeded the EU regulatory limit. Norway and Italy reported only a few quantified samples, with a maximum

of 10.3 mg sum DA/kg for Norway and of 5.3 mg sum DA/kg for Italy. The samples in Germany and The Netherlands were all below the LOD.

Marine biotoxins are known to show a non-homogeneous distribution in terms of time and geographical location (Ciminiello *et al*., 1999). The occurrence of DA and epi-DA is variable in time and some geographical areas are more affected than others by these toxins. This is illustrated in Figure 2, where the occurrence of DA in Ireland is shown over time. The figure shows non-detected samples as LB values.

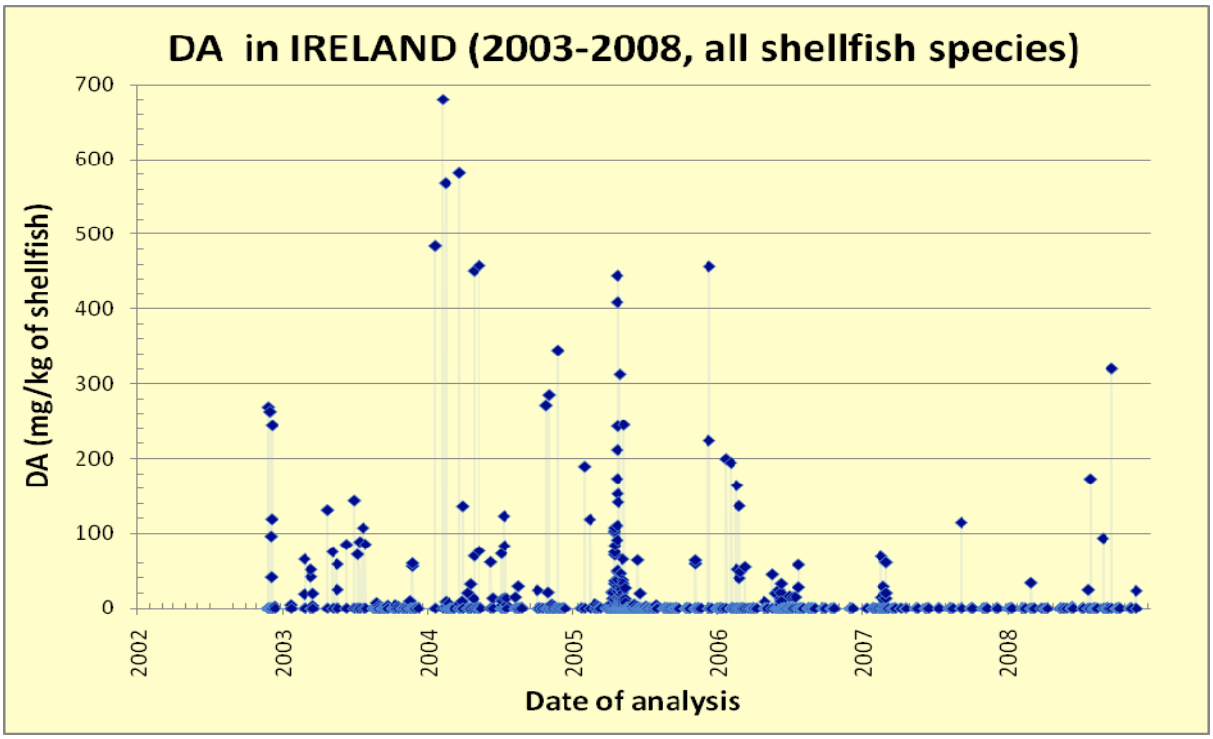

Figure 2. DA detected in Ireland over the time period of November 2003-December 2008

5.3. Difference between shellfish species

Mussels were by far the most predominant shellfish product tested, followed by clams, oysters, scallops and others. The statistical descriptors for the different species analysed with HPLC-based methods in pre-MC samples are summarised in Table 4. Samples without reported values were assigned UB and LB values. When the statistical descriptors using the two approaches are similar, the highest value is given otherwise the LB and UB values have been reported.

Table 4. Statistical descriptors for occurrence of the sum of DA and epi-DA (sum DA) in different pre-market control shellfish samples. The data reported were obtained by HPLC and LC-MS/MS analyses of whole shellfish

Shellfish	N	Total concentration sum DA mg/kg shellfish meat				% of samples not quantified[c)]	% of values >20 mg sum DA/kg shellfish meat
		Median LB/UB[a)]	Mean LB/UB[a)]	P95 LB/UB	Maximum		
Clams	3898	0/0.8	0.8/1.6	3.6	160	90.7 %	0.9 %
Cockles	1668	0/0.8	0.6/1.3	2.9	61	90.3 %	0.4 %
Gastropods[b)]	55	0/0.6	0.02/0.7	-	(1/2)	98.2 %	-
Mussels	20333	0/0.5	0.3/1.1	0/2	445	95.5 %	0.3 %
Oysters	3531	0/1	0.2/0.8	1.3	153	94.0 %	0.1 %
Scallops	4403	39	99	250	857	14.1 %	59.1 %
Others	339	0/0.2	3.8/4.1	18.1	92	74.9 %	3.8 %
All	**34227**						

N = number of samples; LB=lower bound; UB=upper bound; P95=95th percentile

a) When two values are given it indicates the respective lower (LB) or upper bound (UB) values for samples below the limit of detection (LOD) or the limit of quantification (LOQ). The lower bound is performed substituting 0 to all not detected samples. The upper bound is performed substituting "<LOD" with LOD value and "<LOQ" with LOQ value; LOD and LOQ are those defined for the specific single analysis.

b) The number of samples is not sufficient to allow the calculation of P95. The reported maximum is lower than the maximum UB value estimated for the non-detected samples.

c) Not quantified means no numerical value reported.

All shellfish species except gastropods showed maximum values well above the EU regulatory limit. However, the shellfish species most affected by DA occurrence was scallops with a 95th percentile value of 250 mg sum DA/kg and a maximum of 857 mg sum DA/kg. For the group "others", including echinoderms and unspecified species the 95th percentile value was near to the EU regulatory limit whereas all the other species showed much lower 95th percentile values. Overall it appears that all of the shellfish species considered can be contaminated with DA and epi-DA, but a high level of contamination is found only in scallops, with almost 60 % of the samples above the EU regulatory limit. This explains the derogation rules established for harvesting and processing of scallops as mentioned earlier in Chapter 5.2.

To illustrate this situation the occurrence of DA and epi-DA in the different parts of dissected scallops as reported by France, Ireland and UK is presented in Table 5. The data show that DA contamination in scallops occurs mainly in hepatopancreas and soft tissue. In some cases the gonads are also contaminated, but to a lesser extent.

Table 5. Statistical descriptors for occurrence expressed as the sum of DA and epi-DA (sum DA) in different parts of dissected scallops analysed by HPLC in France, Ireland and UK

Shellfish	N	Total concentration sum DA mg/kg shellfish meat				% of samples not quantified[c)]	% of values >20 mg sum DA/kg shellfish meat
		Median LB/UB[a)]	Mean LB/UB[a)]	P95 LB/UB	Maximum		
Gonads (roe)	4152	6.4	13.3/13.5	48.4	250	18.7 %	20.4 %
Hepatopancreas	18	522	573	(1169)[b)]	2269	0 %	88.9 %
Muscle	1557	0/2	0.6/2.1	4.3	18.7	82.9 %	-
Muscle+gonads	34	1	1.6	-	8.7	2.9 %	-
Remaining soft tissue	162	34.2	148	677	1380	25.3 %	60.5 %

N = number of samples; LB=lower bound; UB=upper bound; P95=95th percentile

a) When two values are given it indicates the respective lower (LB) or upper bound (UB) values for samples below the limit of detection (LOD) or the limit of quantification (LOQ). The lower bound is performed substituting 0 to all not detected samples. The upper bound is performed substituting "<LOD" with LOD value and "<LOQ" with LOQ value; LOD and LOQ are those defined for the specific single analysis.

b) The number of samples is not sufficient to allow calculation of reliable P95. The number reported in brackets is only an indication of the order of magnitude.

c) Not quantified means no numerical value reported.

5.4. Influence of processing

Studies have shown that normal home cooking processes, such as boiling and steaming, could reduce the amount of DA in shellfish meat due to partial leaching of the toxin into the cooking fluids and resulted in a redistribution of the DA in the tissues as a consequence of the rupture of the cellular membrane (Hatfield *et al.* 1995, Leira *et al*., 1998; McCarron and Hess, 2006). The effect on the concentration of sum DA varied depending on the species studied.

Leira *et al*. (1998) observed on average a reduction of about 20 % (from 66 to 53 mg/kg) in the concentration of DA in the hepatopancreas of scallops after steaming (5 minutes) accompanied by more than doubling (from about 1.6 to 3.5 mg/kg) in the DA concentration in the remaining tissue (whole flesh excluding the hepatopancreas). In a study investigating the effect of processing on the DA levels in Dungeness crabs, Hatfield *et al.* (1995) observed a decrease in DA concentration in the hepatopancreas of about 70 % after 20 minutes boiling, due to transfer of the toxins into the cooking fluids.

McCarron and Hess (2006) found that steaming (10 minutes over boiling water) caused a decrease of about 11 % in the DA concentration (sum of DA and epi-DA) in the hepatopancreas of blue mussels (*Mytilus edulis)* and an approximate increase in DA in whole flesh of about 20 %. They concluded that it is unlikely that processing will have a major effect on DA concentration in fresh mussels. The same authors also investigated the effects of heating on the DA concentration in the absence of water loss. For that purpose aliquots of homogenised mussel tissue and of in-house reference materials were put into tubes that were hermetically sealed and heated (water bath, 90°C; 15 minutes) or autoclaved (121°C; 15 minutes). A reduction of only 3 or 7 % in the concentration of DA was observed after steaming or autoclaving, respectively.

The effects of industrial processes such as canning and freezing have also been studied. Leira *et al*. (1998) observed an average decrease in DA levels of about 84 % in the hepatopancreas of scallops accompanied by a 3-4 fold increase of DA in whole flesh excluding the

hepatopancreas, after storage at -20°C for 60 and 180 days. This could be due to diffusion of DA from the hepatopancreas to other parts of the scallops. Overall a decrease in the DA concentration of 43 % was observed in the whole product. After canning (pickled or brined) between 30 and 65 % of the total DA content in scallops was transferred to the packing medium.

6. Considerations on samples reaching the market

In contrast to other marine biotoxins, where residual exposure was calculated on samples negative in MBA, this approach is not possible for DA and epi-DA because they are only monitored using quantitative chemical methods. Therefore products that are non compliant with the EU regulatory limit of 20 mg sum DA/kg are not expected to reach the market.

The statistical descriptors of occurrence of DA and epi-DA in products reaching the market, taking into account the results obtained by HPLC or LC-MS/MS (samples with values ≤20 mg sum DA/kg for whole shellfish meat, separated scallop muscle and scallop muscle with gonads) in ten European countries are reported in Table 6.

Table 6. Statistical descriptors for pre-market control samples compliant with the EU regulatory limit of 20 mg DA/kg shellfish meat. The samples include whole shellfish meat and also separated scallop muscle with and without gonads

Data groups	N	Median LB/UB[a)]	Mean LB/UB[a)]	P95 LB/UB[a)]	Maximum	% of samples not quantified[b)]
		sum DA mg/kg shellfish meat				
Compliant samples	33098	0/0.8	0.5/1.3	2.5	20	91.0 %

N = number of samples; LB=lower bound; UB=upper bound; P95=95th percentile; sum DA=sum of DA and epi-DA

a) When two values are given they indicate the respective lower and upper bound values for samples below the limit of detection (LOD) or the limit of quantification (LOQ). The lower bound is performed substituting 0 to all not detected samples. The upper bound is performed substituting "<LOD" with LOD value and "<LOQ" with LOQ value. LOD and LOQ are those defined for the specific single analysis.

b) Not quantified means no numerical value reported.

7. Human consumption of shellfish

Limited consumption data were available for individual shellfish species across the EU. The EFSA concise database does not yet provide sufficient information since there is no differentiation between meal sizes for fish and other seafood. Therefore, EFSA requested the Member States to provide information on shellfish consumption. Data have been submitted by France, Germany, Italy, The Netherlands and the UK. A compilation of the data received is presented in Table 7. The mean portion sizes for "consumers only" ranged between 10 g (France, bivalve molluscs) and 136 g (The Netherlands).

The German national food consumption survey performed by a weighing protocol in the late 1980s indicates a minimum meal size of mussels of 2 g (mainly as an ingredient in dishes), a median of 63 g, a mean of 107 g and a 95th percentile of 400 g among mussel consumers. The maximum portion size reported in this study was 1500 g. The French Calipso study differentiated mussels and bivalve molluscs. The maximum portions for mussels (245 g) and all bivalve molluscs (415 g) varied, whereas the mean portions were similar. A survey reported by the UK indicates a mean shellfish meal size of 114 g and a maximum of 239 g. A

Dutch study reported a mean portion size of 136 g of shellfish and a maximum of 480 g. These data are for consumers only. The surveys show a large variation in the percentage of the populations consuming shellfish and it is unclear whether the data are related to cooked or uncooked shellfish.

Table 7. Shellfish eating habits in France, Italy, The Netherlands, the UK and Germany, based on national food consumption surveys

Country	Study	Number of consumers N (%)	Number of eating occasions for consumers/year	Mean portion weight (g)	P95 (g)	Maximum portion weight (g)	Maximum frequency
France (7 days)	INCA 1999	218/1985 (11 %)	N/A	10			N/A
France (FFQ)	CALIPSO 2004 (bivalve molluscs)	962/997 (96 %)	N/A	32	94	415	N/A
France (FFQ)	CALIPSO 2004 (mussels)	862/997 (86 %)	N/A	22	70	245	N/A
Italy (7 days)	INN-CA 1994-96	212/1981 (11 %)	47	83		1000	4/week
Germany (7 days)	NVS 1985-88	150/23239 (0.6 %)	171	107	400	1500	3/week
UK (7 days)	NDNS 2000-01	212/1631 (13 %)	51	114		239	4/week
The Netherlands (2 days)	DNFCS 1997-98	47/4285 (1.1 %)	39	136	465	480	N/A

FFQ = food frequency questionnaire, 7 days = 7 day dietary record, 2 days = 2 day dietary record, N/A = not available; N=number of consumers; P95=95th percentile
INCA = Enquête Individuelle et Nationale sur les Consommations Alimentaires (Volatier, 2000).
CALIPSO = Fish and seafood consumption study and biomarker of exposure to trace elements, pollutants and omega 3 (Leblanc *et al.*, 2006)
INN-CA = Nationwide Nutritional Survey of Food Behaviour (Turrini *et al*., 2001)
NVS = Nationale Verzehrsstudie (Adolf *et al*., 1995)
NDNS = National Diet and Nutrition Survey (Henderson *et al*., 2002)
DNFCS = Dutch National Food Consumption Survey (Kistemaker *et al*., 1998)

Because DA has acute toxic effects, it is important to use a large portion size rather than long term average consumption in dietary exposure calculations, to ensure protection of the health of the consumer. In the studies presented in the table above, the maximum reported sizes are in the range of 239 to 1500 g. The CONTAM Panel noted the largest portion sizes of 1000 g and 1500 g, and considered it likely that the shells were included in these weight estimates. Therefore, the CONTAM Panel considered the 95th percentile as a more realistic estimate of the portion size for high consumers. As shown in Table 7 the 95th percentile values range from 70 g to 465 g and the CONTAM Panel chose the figure of 400 g as a large portion size to be used in the acute exposure assessment of marine biotoxins. This is in good agreement with the report of the Joint FAO/IOC/WHO ad hoc expert consultation on marine biotoxins (FAO/IOC/WHO, 2004), where 380 g was reported as the 97.5th percentile largest portion size for consumers only.

8. Exposure assessment

8.1. Deterministic estimate of dietary exposure to DA and epi-DA

Based on the assumption that only products compliant with the regulatory limit reach the market (see Table 6), the dietary exposure can be estimated as shown in Table 8.

Table 8. Deterministic dietary exposure estimate of the sum of DA and epi-DA (sum DA) based on premarket samples tested to be compliant with the EU regulatory limit

P95 of the sum DA concentration in samples on the European market	2.5 mg sum DA/kg whole shellfish meat
Exposure by eating a 400 g portion at 2.5 mg sum DA/kg	1 mg sum DA/person (0.017 mg sum DA/kg b.w.)
Exposure by eating a 400 g portion at 20 mg sum DA/kg whole shellfish meat	8 mg sum DA/person (0.13 mg sum DA/kg b.w.)

P95=95th percentile, b.w.=bodyweight

The exposure for a European consumer of a 400 g portion of shellfish meat contaminated with DA and epi-DA at the 95th percentile of occurrence in premarket samples tested compliant with the current EU regulatory limit is 0.017 mg sum DA/kg b.w. This represents approximately 12 % of the exposure (0.13 mg sum DA/kg b.w.) of a person eating a 400 g portion at the level of 20 mg sum DA/kg whole shellfish meat (current EU limit) and is approximately half of the ARfD of 0.03 mg sum DA/kg b.w. established in Chapter 12.

These results are conservative but not unrealistic estimates of DA and epi-DA dietary exposure in ten European countries.

8.2. Probabilistic estimate of dietary exposure to DA and epi-DA

A probabilistic estimate of dietary exposure to DA and epi-DA has been performed by Monte Carlo simulation using the distributions of both the occurrence data and the data on the consumption of shellfish. The probabilistic exposure estimate provides information on the chance of exceeding a specific exposure level. Because a person eating shellfish will not eat the same portion size containing the same level of toxin each time, the probabilistic calculation includes all combinations of the different occurrence and consumption data. The estimation also took into consideration the fact that the laboratories reported 13 different LODs and 15 LOQs for DA concentrations (see footnote 16).

For the probabilistic estimate all data that were available from HPLC with UV, DAD or MS detection were used[16] for exposure analysis. In accordance with the assumption that only those

[16]All samples with quantifiable levels below 20 mg/kg (n=2,966) of sum DA were characterized using a lognormal distribution, which was derived by best fit analysis with the @RISK tool. Thirteen different uniform distributions were defined, respectively, to describe the concentration for each of the 13 different LODs reported, using the distribution function *riskuniform(0;LOD*). The same procedure was used to describe the values between LOD and LOQ (*riskuniform(LOD;LOQ*). The total number of values considered for calculation was 33098, as given in Table 6).

For further exposure calculations, the fitted distribution function describing the quantified samples was truncated left hand at the lowest measured concentration of 0.02 mg/kg and right-hand at the limit value for marketing of 20 mg/kg. = RiskLognorm(5,8589; 9,6201; RiskShift(-0,1158); RiskTruncate(0,02;20)), and uniform distributions characterising values between zero and LOD, and LOD and LOQ have been taken according to the percentage of the respective values. This means that 13 different uniform distributions characterising the values below LOD and 15 different uniform distributions have been used to characterize the values between LOD and LOQ.

samples with DA and epi-DA concentrations below the EU regulatory limit of 20 mg DA/kg would reach the market, the distribution has been cut off at this level.

Because insufficient information was available on the distribution of portion sizes, the CONTAM Panel decided to use a triangular distribution as a simple and pragmatic approach. A triangular distribution is characterised by three values, the minimum, the most probable and the maximum. In the case of shellfish consumption a value of 0 was used as a minimum. From the range of 10 to 136 g reported as mean consumption figures in Table 7 the CONTAM Panel chose a value of 100 g to be used as the "most probable" value, although there is no evidence that it is the most frequently consumed portion. The better-documented large portion size of 400 g (see Chapter 7) was used to represent the maximum.

The resulting probabilistic dietary exposure distribution has a median value of approximately 0.055 mg sum DA/person, a mean of approximately 0.13 mg sum DA/person, and a 95th percentile of approximately 0.45 mg sum DA/person.

The probabilistic exposure estimate is presented in Figure 3 illustrating the chance of exceeding a specific level of exposure to DA and epi-DA when consuming a single portion of shellfish.

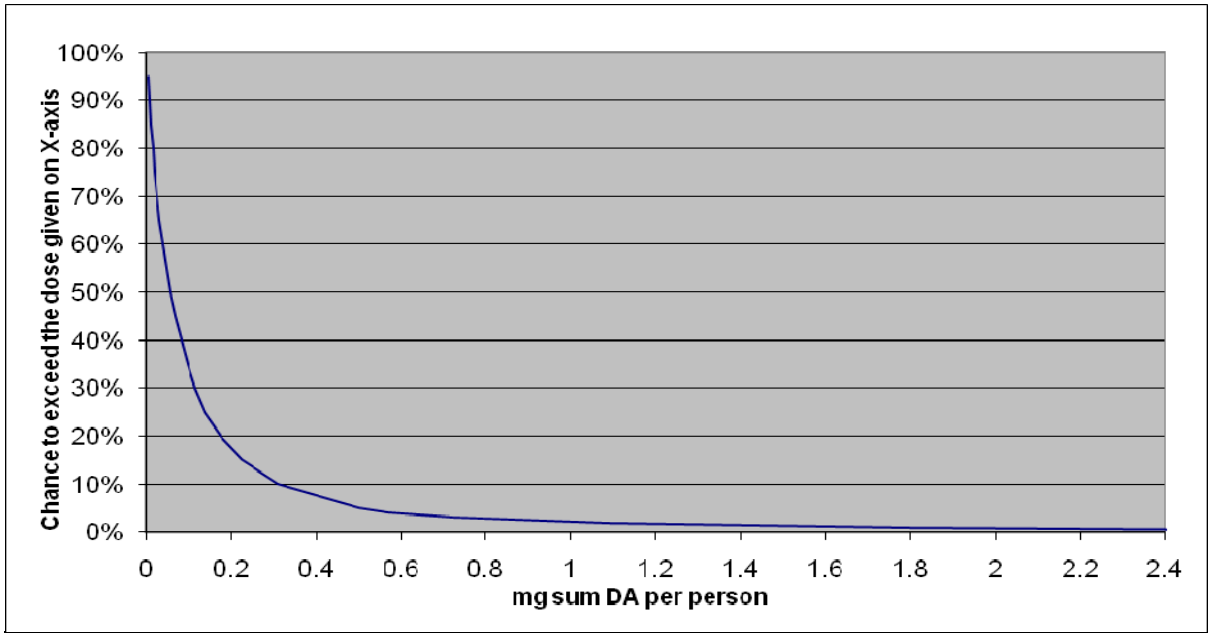

Figure 3. Probability of dietary exposure to DA and epi-DA (sum of DA and epi-DA) resulting from consumption of a single portion of shellfish

The curve in Figure 3 illustrates the chance of exceeding a dietary exposure of DA and epi-DA at the level indicated on the X-axis. For a 60 kg adult, the chance of exceeding a dietary exposure of 1.8 mg sum DA, corresponding to the ARfD of 30 µg sum DA/kg b.w. established in Chapter 12, is about 1 %, when consuming shellfish currently on the European market. The chance of exceeding the deterministic dietary exposure estimate of 8 mg sum DA (see also Table 8), corresponding to consumption of a portion of 400 g containing DA at the level of the current EU limit value of 20 mg sum DA/kg, is less than 0.1 %.

9. Toxicokinetics

Limited information exists on the toxicokinetics of DA in mammals. Initial data on the toxicokinetics of DA were obtained in 1987, from patients hospitalised as a consequence of an outbreak of DA poisoning in Canada (Perl *et al*., 1990). No DA was detected in blood, serum and cerebrospinal fluid specimens from 17 patients, whose estimated exposure of DA, as a consequence of eating contaminated mussels, was between 60 and 290 mg/person (Perl *et al*., 1990). The study authors pointed out that the analyses involved specimens that had been obtained from patients at least two days after consumption of contaminated shellfish, and the time may well have been long enough for the toxin to have been cleared from the body fluids examined (Perl *et al*., 1990). These observations suggest that DA is poorly absorbed and that systemically available DA is rapidly cleared from the body.

9.1. Absorption

Oral administration of DA in a saline solution to mice and rats has shown that the no-observed-effect-level (NOEL) is about thirty-fold higher than that found upon intraperitoneal (*i.p.*) injection of DA solutions (Iverson *et al*., 1989). This provides some evidence that DA in poorly absorbed from the gastro-intestinal (GI) tract. In the same study, following the oral administration of a mussel extract containing DA to mice and rats essentially all of the administered DA was detected in the faeces of the experimental animals, suggesting a low, if any, absorption of DA in the GI tract (Iverson *et al*., 1989). Based on the urinary excretion rate of DA, the absorption of DA in the GI tract has been estimated to be less than 2 % of a dose of 0.1 or 5.0 mg DA/kg b.w. administered to rats by gavage for 64 days (Truelove *et al*., 1996). In a subsequent study, the poor GI absorption of DA was confirmed in Cynomolgus monkeys, where after repeated oral administration of 0.5-0.75 mg.DA/kg b.w. for 30 days showed only 4-7 % of the administered DA was absorbed (Truelove *et al*., 1997).

9.2. Distribution

The intra-venous (*i.v.*) administration of tritiated DA indicated that the apparent volume of distribution of DA in the rat was about 260 mL/kg b.w. (Suzuki and Hierlihy, 1993). The volume of distribution of DA in the Cynomolgus monkey is smaller, being less than 200 mL/kg (Truelove and Iverson, 1994).

A low permeability of DA through the blood-brain barrier was hypothesised by Iverson *et al*. (1989), and was later demonstrated by Preston and Hynie (1991). Following *i.v.* injection of tritiated DA into rats (about 10 µg DA/kg b.w.), permeation through the blood-brain barrier was observed, and the measured transfer constant did not vary appreciably between different brain regions (Preston and Hynie, 1991). Concentrations between 52 and 83 pg DA equivalents/g tissue were found in different regions of the brain. Furthermore, the measured transfer constant for the permeation of DA in different brain regions did not change when the amount of DA administered was increased forty-fold. Therefore it was concluded that DA enters the brain without the involvement of a carrier mechanism (Preston and Hynie, 1991), and that conditions that might compromise the integrity of the blood-brain barrier might have an impact on the effects of DA on the brain (Perl *et al*., 1990; Preston and Hynie, 1991).

The data of Preston and Hynie (1991) have been used to develop a model for DA uptake into the brain (Kim *et al*., 1998). Some regions of the brain may not be protected from DA by the blood-brain barrier, as is the case for the area postrema. Unrestricted access of DA to the area postrema is considered to be the cause of the effect of vomiting that is found in Cynomolgus

monkeys that received DA by *i.v.* injection (Truelove and Iverson, 1994). Vomiting is also one of the effects of DA poisoning in humans (see Chapter 11).

Direct proof that DA readily crosses the placental barrier and is transferred from the mother to the fetus has been obtained by Maucher and Ramsdell (2007), who showed that a single *i.v.* injection of 0.6 or 1.6 mg DA/kg b.w. into pregnant female rats on day 13 or 20 of gestation resulted detectable levels of DA in the brain of fetuses, analyzed 1 hour post exposure. The DA concentrations in the brain of neonates were between 5.3 and 16.9 ng DA/g tissue (mean values), depending on the dose of DA injected into the dams and the day of gestation. DA was also detected in the amniotic fluid (Maucher and Ramsdell, 2007). The transfer of DA from a naturally intoxicated mother to the fetus has also been observed in sea lions (Brodie *et al*., 2006).

The transfer of DA from mother to offspring can occur through the milk. In rats it was shown that *i.p*. injection into dams of 1 mg DA/kg b.w. led to a limited transfer of DA to milk, since the concentration in milk of about 50 ng DA/mL was approximately 16 times lower than that in plasma, one hour after DA administration (Maucher and Ramsdell, 2005). In contrast to the rapid clearance of DA from blood (>99 % in the first hour after injection), the DA concentrations in milk decreased by only about 60 % in 8 hours after toxin injection. Taking into consideration that 1 hour after injection the DA levels in milk were 16 times lower than those in plasma low transfer of DA from blood to milk is implied (Maucher and Ramsdell, 2005). No DA (LOQ, 1 ng/ml) was detectable in the plasma of pups that received the milk collected from dams 4 hours after they had been injected with DA, whereas measurable levels of DA (about 27 pg/mL) were found in the plasma of pups that received a dose of 1 mg DA/kg from milk spiked with DA (Maucher and Ramsdell, 2005).

9.3. Biotransformation

The administration of tritiated DA to rats, by a single *i.v.* injection, has shown that 70-75 % of the radioactivity detected in the urine was associated with parent DA, indicating that most of the injected DA remains un-metabolised in the rat (Suzuki and Hierlihy, 1993). A separate study has provided evidence that no DA conjugates were present in the urine of rats that had received the toxin by *i.v.* injection (Truelove and Iverson, 1994).

9.4. Elimination and bioaccumulation

The relevance of renal excretion in the elimination of DA absorbed from the GI tract was initially suggested by the observation that among patients of less than 65 years of age in the Canadian incident of 1987 the most seriously ill included individuals affected by chronic renal disease (Perl *et al*., 1990). In line with this observation, Preston and Hynie (1991), found that *i.v.* injection of DA into nephrectomised rats resulted in serum toxin concentrations that were about seven-fold higher than those found in sham-operated animals, implying an important role of the kidney in DA clearance from the blood. Increased serum levels of DA in nephrectomised rats resulted in an approximately two-fold increase in the DA concentrations in different regions of the brain, confirming that conditions that impair kidney function might play an important role in the susceptibility of individuals to DA poisoning (Perl *et al*., 1990; Preston and Hynie, 1991).

Following the *i.v.* administration of tritiated DA to rats the entire dose was rapidly eliminated in the urine (Suzuki and Hierlihy, 1993). In this study, several toxicokinetic parameters of DA were determined after injection of three different DA doses (0.5 ng, 0.5 mg and 2.0 mg DA/kg

b.w.), and it was found that the elimination rate constant (0.03/minute) was not affected by the DA dose. Both the total and renal clearance were approximately 10 mL/minute/kg b.w. (Suzuki and Hierlihy, 1993). Since total and renal clearance of DA were essentially the same, it can be concluded that elimination of DA from the serum, once the toxin has been absorbed from the GI tract, occurs entirely via kidney (Suzuki and Hierlihy, 1993). Renal elimination of DA was confirmed in Cynomolgus monkeys (Truelove and Iverson, 1994), although the clearance rate in monkeys was about 10-fold less than in rats.

In addition, in an oral sub-chronic study in rats (Truelove *et al.*, 1996) the levels of DA measured in 24-hour urine samples indicated that absorbed DA is excreted entirely in urine.

The low GI absorption of DA and its rapid renal clearance would imply a marginal, if any, bioaccumulation of DA in mammals in the absence of impaired renal excretory function.

10. Toxicity data

10.1. Mechanistic considerations

DA is a recognized agonist of non-N-methyl-D-aspartate (non-NMDA) glutamate receptors, including both α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and kainate receptors (Ozawa *et al*., 1998; Lerma *et al*., 2001). Glutamate is a major excitatory neurotransmitter in the brain, and the action of DA on non-NMDA receptors perturbs neurotransmission. Non-NMDA and kainate subfamilies of glutamate receptors comprise tetrameric proteins formed by either identical or, less frequently, different subunits (Ozawa *et al*., 1998; Lerma *et al*., 2001). Nine different subunits have been identified, four of which are components of AMPA receptors (subunits GluR1-4), whereas the remaining five subunits represent the monomers of kainate receptors (subunits GluR5-7 and KA1 and KA2). Alternative splicing of mRNA precursors of different subunits can result in protein variants expressed in the cells, increasing the multiplicity of receptor forms (Ozawa *et al*., 1998; Lerma *et al*., 2001). The functional properties of AMPA and kainate receptors depend on their subunit composition, so that a marked heterogeneity is to be expected in different systems (Ozawa *et al*., 1998; Lerma *et al*., 2001; Bowie and Lange, 2002).

The glutamate receptors with high affinity for DA are expressed primarily in several regions of the central nervous system, where they are located in neuronal terminals both pre- and post-synaptically (Ozawa *et al*., 1998; Lerma *et al*., 2001), and the effects of DA ensue from a coordinated and synergistic action of receptors functioning at the two sides of the synapses (Ramsdell, 2007; Pulido, 2008; Doucette and Tasker, 2008). Direct binding of toxin to glutamate receptors expressed in peripheral tissues could also contribute to some of the peripheral effects of DA (Pulido, 2008; Doucette and Tasker, 2008).

The non-NMDA glutamate receptors represent ligand-dependent ion channels (Ozawa *et al*., 1998; Lerma *et al*., 2001), and the binding of DA determines the opening of the channel. When DA binds to AMPA channels, influx of Na^+ into the cell occurs, whereas influx of extracellular Ca^{2+} ions into the cell is induced by DA binding to kainate receptors (Ozawa *et al*., 1998; Lerma *et al*., 2001). Other ion channels and glutamate receptors, however, are recognised to participate in effects of DA in neuronal systems, although their individual contributions to the effects induced have not been fully defined (Ramsdell, 2007; Pulido, 2008). In particular, influx of extracellular Ca^{2+} ions would also occur through voltage-gated calcium channels (Xi and Ramsdell, 1996; Berman *et al*., 2002), and by reverse action of Na^+/Ca^{2+} exchangers (Berman *et al*., 2002). In both cases, the effect would involve Na^+ influx

through AMPA receptors and membrane depolarization (Berman *et al*., 2002). Furthermore, increased intracellular Ca^{2+} concentrations would also result from the action of NMDA receptors (Novelli *et al*., 1992; Berman and Murray, 1996), that would be activated by glutamate released from the synapses as a consequence of DA binding to pre-synaptic AMPA/kainate receptors (Brown and Nijjar, 1995; Malva *et al*., 1996; Berman and Murray, 1997). The influx of extracellular Ca^{2+} through NMDA receptors, in turn, would occur following the reduction of the voltage-dependent Mg^{2+}-block of calcium permeability of NMDA receptors, induced by DA acting on AMPA receptors (Novelli *et al*., 1992; Berman and Murray, 1996).

There are indications that the action of DA might be affected by other compounds present in shellfish materials (Novelli *et al*., 1992; Doucette and Tasker, 2008; Ruiz, 2009).

The net consequence of these effects of DA is an overall increase in intracellular Ca^{2+} concentrations. Intracellular calcium homeostasis, however, is lost in neurons exposed to DA, because DA binding to kainate receptors impairs their desensitisation, leading to unrestrained intracellular Ca^{2+} influx into the neurons (Lerma *et al*., 2001; Ramsdell, 2007). The prolonged calcium load then results in failure of those cellular regulatory mechanisms involving intracellular calcium homeostasis, leading to cell damage and overt neurotoxicity, that represent the major effects of DA *in vitro* and *in vivo* (Teitelbaum *et al*., 1990; Novelli *et al*., 1992; Cendes *et al*., 1995; Tasker *et al*., 1996; Jakobsen *et al*., 2002; Ramsdell, 2007; Giordano *et al*., 2007; Pulido, 2008; Doucette and Tasker, 2008).

The altered neurological and behavioural activities, as well as some GI effects (vomiting), that are apparent in humans and experimental animals poisoned by DA are explained by the neuronal damage the toxin induces in some brain regions by prolonged calcium load due to sustained activation of glutamate receptors.

10.2. Effects in laboratory animals

The literature on the toxicity of DA in laboratory animals was comprehensively reviewed by the Joint FAO/IOC/WHO *ad hoc* Expert Consultation on Biotoxins in Bivalve Molluscs (Oslo, September 26-30 2004).

10.2.1. Acute toxicity

Acute toxicity studies have been performed in mice, rats and non human primates (Cynomolgus monkeys), which were exposed to DA *via* different routes of administration, i.e. *i.p.*, *i.v.* or orally. Some of the studies have tested contaminated mussel extracts, e.g. whole mussel extracts and hepatopancreas extracts, rather than purified DA toxins. This limited their usefulness, as the DA content was not accurately quantified and the co-presence of other toxins and/or other biologically active compounds could not be ruled out.

10.2.1.1. Toxicity following oral administration

Mice

Early investigations using neutralised mussel extracts (Iverson *et al*., 1989) indicated that oral DA doses of 35 mg/kg b.w. were required to elicit the scratching response (no effects at doses of 20 and 28 mg/kg b.w.) and that death occurred at 47 and 104 mg/kg b.w. However, several doses between the latter two levels were devoid of any effect, suggesting that other factors were modifying the toxicity. With an acidified extract (pH 4.0) clinical signs were obtained at

a dose of 71 mg DA/kg b.w. and lethality at doses ranging from 71 to 83 mg DA/kg b.w. Edema and neuronal degeneration of the arcuate nucleus were observed in mice treated orally with 35 mg/kg purified DA/kg b.w.

Rats

Rats (n=1-4) given 60-80 mg/kg b.w. DA *per os* as toxic mussel extracts (in water) developed some clinical signs (flaccidity, head on floor and inactivity) but no excitation at 60 mg/kg, mastication and seizures at 70 mg/kg, mild to moderate CNS damage (hippocampus and primary olfactory cortex) and death (in just one case) at 80 mg/kg (Tryphonas *et al*., 1990d). Purified DA administered in saline also produced toxic signs (Iverson *et al*., 1989). While doses of 20 or 28 mg/kg b.w. were without effect, oral ingestion of 35 and 70 mg DA/kg b.w. elicited scratching and seizures, respectively.

Monkeys

Cynomolgus monkeys (*Macaca fascicularis*, n=11) received physiological saline (n=2), or single oral doses of neutral mussel extracts (DA equivalents dose range: 5.63-6.47 mg/kg; n=4), crude DA dissolved in Polybuffer (n=1) or purified DA dissolved in physiological saline (95 % purity, doses of 0.5, 5.0, 5.2 and 10.0 mg/kg b.w. n=4) (Tryphonas *et al*., 1990d). No clinical signs were seen when 0.5 mg/kg b.w. of purified DA was given to one monkey. Mild to moderate histopathological lesions in the central nervous system consistent with neuroexcitation were seen at doses of 5 to 10 mg/kg b.w. The hippocampus and the cortex were the most affected areas. Monkeys given crude or purified DA (5 to 10 mg/kg b.w.) developed anorexia, salivation, retching, vomiting, licking and smacking of lips and empty mastications. Similar clinical signs together with diarrhoea and prostration were also evident in animals receiving mussel extracts (~6 mg DA/kg b.w.). Signs of toxicity could last up to 70 hours.

10.2.1.2. Toxicity following intraperitoneal (*i.p.*) administration

In mice and rats the *i.p.* injection of DA has been associated with specific signs, such as a unique scratching of the shoulders by the hind leg, followed by convulsions and often death. More subtle effects included hypoactivity, sedation, akinesia, rigidity, stereotypy, loss of postural control and tremors (Wright and Quilliam, 1995; FAO/WHO/IOC, 2004).

Mice

Preliminary *i.p.* investigations in male and female CF1 mice (Iverson *et al*., 1989) using DA extracted from mussels identified a no-observed adverse effect level (NOAEL) and a lowest-observed adverse effect level (LOAEL) for scratching (taken as the earliest clinical sign of intoxication) of 0.6 and 1.2 mg/kg b.w., respectively. The non-lethal dose was 2.4 mg/kg b.w. in both males and females, whereas the median lethal dose (LD_{50}) was 5.8 mg/kg b.w. in female mice. No data are available on the LD_{50} in males. In subsequent *i.p.* studies, LD_{50} values were estimated to be 2.9-3.6 mg DA/kg b.w. (Tasker *et al*., 1991; Todd, 1993) for contaminated mussel extracts and 3.6-4.0 mg DA/kg b.w. (Tasker *et al*., 1991; Peng and Ramsdell, 1996)for the pure toxin. Peng and Ramsdell (1996) identified an *i.p.* LOAEL of 0.5 mg DA/kg b.w. for early behavioural changes in adult mice. DA at 1.0 mg/kg b.w. elicited stereotypic scratching behaviour, whereas doses ≥2.0 mg/kg b.w. were proconvulsivants. In CD female mice, the pH of the injected solution of DA (in 5 mM Tris-HCl buffer) was found to modulate the toxicity (onset times for seizure activity and death) at doses of 8.5, 11.5 mg DA/kg b.w., with toxic effects being lowest at pH 3.7 and highest at pH 7.4. Notably, the pH

effect disappeared when the dose of DA increased to 14.5 mg/kg b.w. (Nijjar and Madhyastha, 1997).

Rats

Groups of female Sprague-Dawley rats were dosed once *i.p.* with 0, 1, 2, 4, or 7.5 mg DA/kg b.w. and observed for 24 hours (Iverson *et al*., 1989; Tryphonas *et al*., 1990b, 1990c). In these studies, the dose without clinical signs was 1 mg/kg b.w. At 2 mg/kg b.w. the rats displayed equivocal transient behavioural signs (3/4 rats: withdrawal followed by hyperexcitation and scapular scratching). All rats given ≥4 mg/kg b.w. presented histopathological lesions in the hippocampus (CA3>CA1>CA4), hypothalamus, amygdala, cortex, olfactory system and retina and showed wet dog shakes, rearing with forelimb extension - "praying", loss of balance and seizures (Iverson *et al*., 1989; Tryphonas *et al*., 1990b, 1990c). In another study (Alfonso *et al*., 2000) in adult Sprague-Dawley rats, a single *i.p.* dose of 1 mg DA/kg b.w. significantly increased serum T3 and T4 levels (30 minutes after injection) and TSH levels (5 minutes after injection). These levels remained elevated for the entire study duration (60 minutes after injection). Sobotka *et al*. (1996) found that a slightly lower dose, i.e. 0.93 mg DA/kg b.w., caused hypomotility and decreased the rat body weight, and identified the next lower test dose, i.e. 0.65 mg DA/kg b.w., as the NOAEL.

Conversely, dosing rats with 1.32 mg DA/kg b.w. was sufficient to produce clinical signs (decreased body weight and exaggerated auditory startle response) suggestive of a hyperreactive syndrome, together with neuronal degeneration in the hippocampal CA1/CA3 areas and gliosis. These authors underscored the steepness of the DA dose-response curve in adult rats, whereby *i.p.* doses <1 mg/kg caused behavioural changes without apparent signs of neurological dysfunction or neuropathology, and slightly higher doses (≥1.32 mg/kg) produced in addition to behavioural effects, clinical signs of neurotoxicity, occasional morbidity, and hippocampal damage. In the study of Appel *et al*. (1997) stereotyped behaviour within 60 minutes and subsequent convulsions were detected in 60 % of male Fisher rats given 2.25 mg DA/kg b.w. Seven days after the exposure neuronal injury, astrocytosis, activation of microglia and alterations in fatty acid metabolism were observed, suggesting long lasting effects of DA on brain function.

Monkeys

One Cynomolgus monkey given a single dose of 4 mg/kg b.w. DA *i.p.* (extracted from cultured mussels) developed clinical signs of neurotoxicity after a short pre-symptomatic period. The effects included persistent chewing with frothing, gagging, emesis, loss of balance and tremors and excitotoxic central nervous system damage consisting of dendrotoxic and gliotoxic edema and nerve cell degeneration in structures of the limbic system and the retina (Tryphonas *et al*., 1990a).

10.2.1.3. Toxicity following intravenous (*i.v.*) administration

Rats

Long Evans rats that were injected intravenously (i.v.) with 0.5-1.0 mg/kg b.w. DA (Nakajima and Potvin, 1992) displayed seizure discharges in the hippocampus, tonic-clonic convulsions, and death within a few days. Convulsions and ensuing death were prevented by diazepam.

Monkeys

Cynomolgus monkeys (one animal per dose) were given a single *i.v.* dose of DA (from contaminated cultured mussels) within the range 0.025-0.5 mg/kg b.w. (Tryphonas *et al*., 1990a). Between 0.025-0.2 mg/kg b.w. DA was neuroexcitatory and strongly emetic. At 0.5 mg/kg b.w. DA was markedly excitotoxic. Clinical signs of neurotoxicity were preceded by a short pre-symptomatic period (1-3 minutes) and an even shorter prodromal period (0.5-1 minutes). The symptomatic period was characterized by persistent chewing with frothing, varying degrees of gagging, and vomiting. Additional signs at the higher dose included abnormal head and body positions, rigidity of movements, loss of balance and tremors.

Juvenile (<4 years of age, n=9) and adult (n=15) Cynomolgus monkeys were given single *i.v.* doses of 0, 0.25, 0,5, 1, 2 or 4 mg DA/kg b.w. (Scallet *et al*., 1993). All DA-treated animals showed signs of nausea (LOAEL: 0.25 mg/kg b.w.). One week later, degenerating axons and cell bodies in the hippocampus were seen by histochemical staining using a silver method. Dose ranges of 0.5-2.0 mg/kg in juvenile animals and 0.5-1.0 mg/kg in adult animals produced a small area of silver staining restricted to axons of the hippocampal CA2 stratum lucidum. One juvenile animal that received 4.0 mg DA/kg also showed a second type of lesion, characterized by widespread damage to pyramidal neurons and axon terminals of CA4, CA3, CA2, CA1, and subiculum subfields of the hippocampus. Doses higher than 1.0 mg DA/kg in the adult monkeys were either lethal or resulted in the second type of lesion.

Schmued *et al*. (1995) applied a degeneration-specific histochemical technique (de Olmos' cupric silver method) to reveal degeneration within the brains of DA doses up to 4 mg/kg b.w., given *i.v*. to 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.

10.2.2. Subchronic oral toxicity

Little is known about the effects of repeated exposure to DA at doses below the single doses inducing overt clinical symptoms (for a recent review see Pulido, 2008). Only one study in monkeys (Truelove *et al*., 1997) and one in rats (Truelove *et al*., 1996) have examined toxic effects of repeated oral dosing of DA.

DA was fed daily to 3 Cynomolgus monkeys at 0.5 mg/kg for 15 days and then at 0.75 mg/kg for another 15 days (Truelove *et al*., 1997). With both doses no toxic effects were observed in any of the parameters evaluated, e.g. body weight, food and water consumption, clinical observations, hematology, serum chemistry, light microscopy of all major organs including brain and retina, and glial fibrillary acid protein immunohistochemistry.

In adult rats of either sex, subchronic (64 days) oral administration (gavage) of 0.1 or 5 mg DA/kg b.w. per day did not cause overt clinical abnormalities, nor did it alter any haematological endpoint or serum or urine chemistry parameter (Truelove *et al*., 1996; Pulido, 2008). Irrespective of the given dose, the morphological features of brain, eyes, lung, liver, kidney and other organs were normal on observation with light microscopy. Electron microscopy analyses revealed hippocampal ultrastructural changes (especially in the CA3 region), e.g. cytoplasmic vacuolization of neurons and astrocytes, and mitochondrial damage to the pyramidal cells, in the high-dose group (Pulido, 2008). No changes were elicited by a daily dose of 0.1 mg/kg b.w., which was identified as the NOAEL. In the low-dose group, DA

concentrations in both serum and 24-hour urine samples were below the LOD of the method, 150 ng/mL. In the high-dose group, the daily urinary excretion of DA accounted for 1.8 % of the ingested dose and remained constant throughout the study (3 time-points), suggesting the lack of any changes in metabolism or accumulation of DA due to prolonged exposure (Truelove *et al*., 1996).

10.2.3. Developmental toxicity

Dakshinamurti *et al*. (1993) demonstrated that *i.v.* exposure of pregnant mice to 0.6 mg DA/kg b.w. on gestational day (GD) 13 resulted in impairment of hippocampal function and morphology in the offspring, with delayed cell necrosis being detectable 30 days post partum. In a more recent study (Levin *et al*., 2005) a single subcutaneous (*s.c.*) injection of DA (0.3, 0.6 and 1.2 mg/kg) to pregnant rats on GD13 was reported to cause neurobehavioural sequelae (e.g. changes in locomotor activity and cognitive function) in the offspring, which persisted throughout adolescence and adulthood. Neither dose caused overt toxicity in terms of pups' survival and weight gain. In a teratology study (Khera *et al*., 1994), DA (0, 0.25, 0.5, 1.0, 1.25, 1.75 or 2.0 mg/kg b.w.) was repeatedly administered *i.p.* on gestational days 7 to 16 to groups of 9-15 female Sprague-Dawley rats. All dams in the 2.0 mg/kg group died within 3 days and 50 % of the rats in the 1.75 mg/kg group aborted pre-term. There was an increased incidence of retarded ossification of the sternebrae in the 1.25 mg/kg group (6 %), but the increase was comparable to that in historical controls (4 %). The NOAEL for maternal and fetal toxicity was 1.0 mg/kg/day.

DA is a potent toxicant to newborn animals, which display both immediate and permanent toxicity when exposed to doses below those considered toxic in adult animals. A time-dependent neuroexcitotoxicity involving hyperactivity, stereotypic scratching, convulsions, and death has been reported in newborn rats given DA *i.p.* (Xi *et al*., 1997). These neonates were found to be about 80-fold more sensitive to DA-induced scratching (LOAEL: 0.05 mg/kg b.w.) and about 40-fold more sensitive to DA-induced seizures and death as compared to their adult counterparts. Indeed, in neonatal rats DA at 0.1 mg/kg b.w. induced c-fos in the central nervous system and caused reproducible behavioural effects and seizures at doses as low as 0.05 and 0.2 mg/kg b.w., respectively. The *i.p.* LD_{50} values for postnatal day two (PND2) and PND10 rats were 0.25 and 0.7 mg/kg b.w., respectively (Xi *et al*., 1997). For comparison with adult animals, studies in mature rats (Tryphonas *et al*., 1990b) reported signs of moderate toxicity at *i.p*. doses of approximately 2.0 mg/kg, whereas in adult mice s the half maximal cumulative seizure score (ED_{50}) was 3.9 mg/kg following *i.p.* injection (Tasker *et al*., 1991).

Wang *et al*. (2000) reported motor seizures characterized by scratching, tail flicking, and swimming-like movement at all doses in PND7 rats which had been administered DA subcutaneously at 0.10, 0.17, 0.25, 0.33, 0.42, and 0.50 mg/kg. Doses ≥0.33 mg/kg induced paralysis (65 %), and death (47 %) in less than 2 hours, in the absence of brain damage. Spinal cord lesions (focal haemorrhage, neuronal swelling and vacuolization) were present in 73 % of the animals with paralysis/tremor in their extremities, within 2 hours after DA injection. The authors suggested that the observed behavioural changes were caused by spinal cord damage rather than seizures or brain lesion.

The neurotoxic potency of DA has been found to progressively decrease with increasing neonatal age (interpolated *s.c.*) ED_{50} = 0.12, 0.15, 0.30 and 1.06 mg/kg b.w. at PND 0, 5, 14 and 22, respectively) (Doucette *et al*., 2000). The increased vulnerability of neonates to the neuroexcitatory and lethal effects of DA has been hypothesized to be due to reduced serum

clearance of toxin, leading to increased blood levels. Indeed, the maturation of renal function correlates with the decrease in susceptibility to DA as a function of neonatal age (Doucette *et al*., 2000; Xi *et al*., 1997). Additionally, the incomplete blood-brain barrier typical of immature animals might also contribute to their higher sensitivity to DA (Mayer, 2000). Neonatal rat exposure to DA, as transferred from the blood to the milk of lactating dams, occurs, but at levels that appear to be well below symptomatic doses (Maucher and Ramsdell, 2005).

Another study by Doucette *et al*. (2003) reported physiologically relevant changes in brain development in the absence of convulsions when neonatal rats were injected daily (*s.c*.) with very low doses of DA (5 and 20 µg/kg), or pharmacologically equivalent doses of kainic acid (25 and 100 µg/kg) from PND 8-14. This study showed that while neither compound had identifiable effects on typical measures of toxicity such as weight gain, acoustic startle response, ultrasonic vocalizations, or maternal retrieval, DA administration did result in significant differences in eye opening, conditioned place preference, and spontaneous activity. These authors inferred that the second perinatal week in the rat is a critical window for the developmental neurotoxicity of DA. This period corresponds to a dynamic period for kainate receptor expression, and corresponds roughly to the latter part of the third trimester in humans. Doucette *et al*. (2004) also described a permanent and highly reproducible "seizure-like" syndrome in adult rats that had been treated daily with 5 or 20 µg/kg of DA during this critical time frame. Furthermore, using the same paradigm of early postnatal exposure with 20 µg DA/kg b.w. Adams *et al*. (2009) and Perry *et al*. (2009) also reported long lasting changes in behaviour and learning and memory tasks in rats tested as adults.

10.3. Chronic toxicity and carcinogenicity

There are no published reports on the chronic toxicity or carcinogenicity of DA.

10.4. Genotoxicity

The structure of DA contains a butadiene moiety, which raises the possibility for the formation of DNA-reactive epoxides *in vivo* (Jeffery *et al*., 2004). Reports on the genotoxicity of DA are very limited and the available data are contradictory. Negative results on mutation frequency, sister-chromatid exchange and micronucleus frequency have been reported in V79 Chinese hamster lung fibroblasts cells for DA concentrations of 27.2 and 54.4 µg/mL (i.e. 87 and 174 µM, respectively), either in the presence or absence of metabolic activation (Rogers and Boyes, 1989). In Caco-2 cells *in vitro* an increase in the frequency of micronuclei (DNA fragmentation) has been observed (Carvalho *et al.*, 2006). From another study of the same authors (Carvalho *et al.*, 2008) the Panel noted that the dose levels inducing these effects were also toxic to the Caco-2 cells.

There are no studies available on the genotoxic potential of DA in rodents *in vivo*. Genotoxicity testing has been performed in fish (*Oreochromis niloticus*, n=5 per experimental group) injected intracoelomically injected with 1, 5 or 10 µg DA/g b.w. (Çavaş and Könen, 2008). Significant increases in the frequencies of micronuclei, nuclear abnormalities as well as DNA strand breaks were observed in peripheral erythrocytes, consistent with clastogenic and DNA damaging actions of DA. However, the CONTAM Panel considered the relevance of these *in vivo* findings in a non-standard genotoxicity test to be uncertain.

10.5. Relative potency of DA isomers

There is little knowledge about the *in vivo* toxicity of DA isomers. In adult rats, intrahippocampal injection of DA, iso-DA A, iso-DA B or iso-DA C produced significant dose-dependent increases in seizure activity (Sawant *et al*., 2008). Doses producing half maximal cumulative seizure scores (ED_{50}) were 137 pmol, 171 pmol, 13000 pmol, and 3150 pmol, respectively, thus supporting an equipotent action of DA and iso-DA A when present at the local target, and a lower potency of iso-DA B and iso-DA C. Radioligand binding studies demonstrated a significant correlation between seizurogenic potency and kainate receptor affinity with Ki-values of 2.4 nM, 4.4 nM, 4990 nM and 170 nM for DA, iso-DA A, iso-DA B and iso-DA C, respectively.

Experiments with membrane fractions prepared from rat brain showed that the relative binding affinity of iso-DA F with rat forebrain membrane is about 20 times lower than that of DA itself (Wright *et al*., 1990). In a different study using rat brain membranes, it was shown that the affinity of kainate and AMPA receptors for epi-DA and iso-DA D and E is lower (1-4 orders of magnitude, depending on ligand and receptor type) than that for DA (Hampson *et al*., 1992).

A study in female Swiss Albino mice (Munday *et al*., 2008) has recently compared the acute *i.p*. toxicity of iso-DA A, iso-DA B and iso-DA C with that of DA, as estimated by the up-and-down procedure (OECD, 2006). While the LD_{50} of DA was 6.0 mg/kg b.w. (95 % confidence interval between 4.2 and 7.9 mg/kg), iso-DA C at doses of 20 mg/kg b.w. caused only minor behavioural changes (hypoactivity) and no deaths. Because of the low amounts of DA isomers available, the maximal doses tested were 5 mg/kg b.w. for both iso-DA A and iso-DA B, and 20 mg/kg b.w. for iso-DA C, with this precluding the estimation of LD_{50} values. With respect to the impact on behaviour, at a dose of 5 mg/kg b.w. iso-DA C was completely devoid of any effect, iso-DA A and iso-DA B caused transient hypoactivity, while DA caused significant alterations, e.g. forelimb tremors, without convulsions. There is no information available on potencies of DA isomers by the oral route.

Information on the relative potency of epi-DA *in vivo* is lacking. However, because the occurrence data did not make a distinction between the concentration of parent DA and epi-DA (see Chapter 5), and results were reported as sum of DA and epi-DA (sum DA), the CONTAM Panel had to assume in its assessment that DA and epi-DA are equally toxic.

Taking into account that the iso-DAs occur at much lower concentrations and are considered to be less toxic than DA, the CONTAM Panel concluded that setting of toxicity equivalency factors (TEFs) was not required for iso-DAs.

11. Observations in humans

A very early study examined the anthelminthic effect of a DA-like compound extracted from *Chondria armata* seaweed when administered to 3 children (Daigo, 1959). Oral doses of 0.4, 0.64 and 0.8 mg/kg b.w. resulted in worms being expelled without apparent toxicity to the patient. Although a pure compound was administered its structure was not determined.

The first confirmed outbreak of ASP occurred in Canada in 1987 and was related to mussels affected by a bloom of the *Pseudonitzschia f. multiseries*. (Perl *et al.,*1990). Symptoms of ASP toxicity become apparent 15 minutes to 38 hours after consumption of mussels. Intoxication was defined as the occurrence of one of more gastrointestinal symptoms (vomiting, diarrhoea or abdominal cramps) within 24 hours of consuming the mussels, or at least one of a number of neurological symptoms or signs (confusion, loss of memory, or other

serious signs such as seizure or coma) occurring within 48 hours. Of about 250 reported cases of poisoning, 107 (47 men and 60 women) fulfilled this definition. Younger patients were more likely to have diarrhoea, whereas older patients and men were more likely to have memory loss and to require hospitalisation. Nineteen were hospitalised for 4 to 101 days. Hospital charts were available for 16 of the patients and indicated that all four who were under 65 years of age had pre-existing illnesses: type 1 diabetes mellitus (2), renal disease (3) and hypertension with a history of transient ischemic attacks (1). Three died (aged 71, 82 and 84 years) in hospital 12 to 18 days after eating the mussels, two from septic shock and one from pneumonia. A fourth patient (aged 84 years) died of an acute myocardial infarction after three months and was reported to have had impaired short-term memory until time of death (Todd, 1993). A further case (aged 84 years) with prolonged impaired short-term memory developed temporal lobe epilepsy (TLE) after one year and died from pneumonia 3 years after the poisoning incident. Autopsy revealed severe bilateral hippocampal sclerosis and death was attributed to the DA poisoning (Cendes *et al.,* 1995). All deaths involved patients over the age of 68 years.

DA concentrations in samples of mussels left over from meals eaten by 9 patients with clinical symptoms and one unaffected person ranged from 310-1280 mg/kg mussel tissue. Exposure was estimated based on the number of mussels the individual recalled eating, or an average portion size, and assuming average body weights of 50 and 70 kg for elderly females and males (Todd, 1993). For those with clinical symptoms the estimated exposure ranged from 60-290 mg, equivalent to 0.9-4.2 mg/kg bodyweight. In the region of 0.9-2 mg/kg b.w., symptoms were mainly gastrointestinal, whereas two individuals estimated to have dietary exposure of 4.1 and 4.2 mg/kg b.w. DA required intensive care hospitalisation and suffered permanent neurological symptoms. The corresponding estimate for the one unaffected person was 15-20 mg (equivalent to 0.2-0.3 mg/kg b.w.). DA was not detected in samples of blood, serum or cerebrospinal fluid of 17 patients tested (samples were taken after several days) (Perl *et al*., 1990).

Teitelbaum *et al.* (1990) investigated the neurological sequelae of 14 of the more severely affected patients four to six months after the incident. Memory impairment, ranging from minor anterograde memory loss to severe retrograde amnesia, was noted in all but one patient. Motor control was also affected by DA ingestion, with patients experiencing a range of symptoms including symmetric transient hyper-reflexia, Babinski signs, hemiparesis and opthalmoplegia. The rate of glucose metabolism in the cerebral cortex was reduced in 2 severely affected patients, which correlated with their amnesia.

In the 4 patients who died, slices of brain from the hippocampus, basal forebrain, amygdala, thalamus, basal ganglia, hypothalamus, brain stem and cerebellum were examined (Teitelbaum *et al.*, 1990). Neuronal necrosis and astrocytosis were observed, with the hippocampus and the amygdaloid nucleus most severely affected. In addition, lesions that could not be attributed to age-related atrophy were also observed in the anterior claustrum, nucleus accumbens and the thalamus of these patients (Underman *et al.,* 1993; Todd, 1993).

During 1991, 11-24 cases of ASP were reported after consumption of razor clams in Washington State (USA). A retrospective epidemiological study carried out by the Washington Department of Health recorded 21 incidences of gastrointestinal symptoms associated with consumption of contaminated razor clams between September and December 1991. Mild neurological symptoms were also recorded in 13 people. There were no deaths and only 7 people sought medical attention (Horner *et al.,* 1997). The highest concentration of DA detected in the razor clams was 140 μg/g tissue. It was estimated that people with mild

gastrointestinal symptoms had ingested 0.05-0.39 mg/kg b.w. DA whilst persons consuming contaminated razor clams with no adverse effects had consumed 0-0.28 mg/kg b.w. (Todd, 1993). It is unclear how this estimate was calculated. The reporting of this incident is limited and there are conflicting reports with respect to whether causality could (Todd, 1993) or could not (Horner *et al.,* 1997) be attributed to DA.

There are no reported cases of human illness associated with DA in European countries or regions other than North America. However, in the absence of formal reporting systems, it cannot be assumed that mild cases have not occurred.

In summary, data relating to cases of human poisoning by DA are limited. They mainly relate to a single outbreak of ASP in Canada in 1987. Estimates of exposure for 9 affected individuals indicate that severe and irreversible effects occurred in the region of 4 mg/kg b.w. and the LOAEL for mild signs and symptoms was in the region of 0.9-2.0 mg/kg b.w. No exposure estimates are available for the individuals who died. The data for a single unaffected individual do not provide a basis for identifying a NOAEL.

12. Hazard characterisation

DA induced neurotoxicity is the critical toxicological effect identified in experimental animals including rodents and non-human primates, and in humans. The toxic effects of DA are mediated through its high affinity binding and agonist action on some types of glutamate receptors leading to cell death in certain regions (e.g. hippocampus) of the brain. Data on genotoxicity are inconclusive, showing DNA damage and clastogenicity *in vivo* in fish, DNA fragmentation in Caco-2 cells *in vitro*, but no effects *in vitro* in V79 cells.

There are only a limited number of studies addressing the toxicity and kinetics of DA following oral administration. Rate limiting steps in the toxicity following oral exposure to DA are gastro-intestinal absorption, transport across the blood brain barrier and renal elimination of the toxin. Rodent neonates (from dams exposed parenterally during pregnancy) appear to be more susceptible to DA than adults. When comparing the rat and Cynomolgus monkey with respect to DA kinetics, the rate of absorption from the GI-tract seems to be at least twice as high in the monkey (4-7 % versus 2 % - percentage values imply extent of absorption, not rate) and the clearance rate is about 10 times lower in the monkey. Kinetic data in humans are not available. In agreement with the kinetic differences between rodents and Cynomolgus monkeys, the latter species is more susceptible to DA toxicity following oral administration.

There are only acute and sub-acute short term studies in experimental animals and human observations include only acute toxicity following consumption of DA-containing mussels. No long term studies have been reported. The data are inadequate for establishment of a tolerable daily intake (TDI) for DA. However, the CONTAM Panel was able to establish an acute reference dose (ARfD) based on data on acute toxicity in humans and experimental animals.

In deriving an ARfD the CONTAM Panel used the outbreak of DA poisoning in humans in Canada in 1987 comprising 107 cases. In 99 patients answering a standardised questionnaire all but seven suffered from vomiting, diarrhoea, or abdominal cramps. The vomiting is possibly caused by a central nervous effect of DA. Twenty five percent suffered from loss of short term memory. Unfortunately, in only 9 individuals with symptoms was it possible to estimate the dose of DA. Of these, 6 patients had mild symptoms, including GI symptoms such as vomiting or nausea in all, memory loss in one and dizziness and/or confusion in 4.

Three patients had severe symptoms requiring hospitalisation. DA dietary exposures in these two groups were estimated to be 0.9 to 2.0 and 1.9 to 4.2 mg/kg b.w., respectively (Perl *et al*., 1990; Todd, 1993). The two individuals with the highest exposure (4.1 and 4.2 mg/kg b.w.) experienced permanent neurological symptoms (Todd, 1993). The corresponding dietary exposure estimate for the one unaffected person was 0.2-0.3 mg/kg b.w. A LOAEL for clinical symptoms of DA poisoning of 0.9 mg/kg b.w. could be inferred. However, it is not known whether these 9 patients are representative for the whole group of cases identified. In addition, the methods for detection of neurological symptoms were insensitive and subtle neurotoxic effects, which could also be permanent with loss of neurons in regions important for memory, cannot be excluded.

In establishing an ARfD the CONTAM Panel used the LOAEL of 0.9 mg/kg b.w. for clinical symptoms as a starting point and applied an uncertainty factor of 3 because a LOAEL was used. Taking into account the steep dose response curve this is considered to give a reasonable estimate of a NOAEL. A factor of 10 was also used to take into account human variability and also the fact that sensitive methods for detection of neurotoxic effects had not been used. The CONTAM Panel therefore established an ARfD of 30 µg/kg b.w. Because DA can be converted to epi-DA during storage, the ARfD applies to the sum of DA and epi-DA.

The CONTAM Panel noted that in Cynomolgus monkeys given DA in single oral doses, no clinical signs or histopathological central nervous system (CNS) lesions were observed in one monkey given pure DA at the lowest dose of 0.5 mg/kg b.w., whilst vomiting and mild to moderate CNS lesions were observed in the animals given 5 mg/kg b.w. or up to 10 mg/kg b.w. of pure DA or DA in mussel extract (Tryphonas *et al*., 1990d). Analogous to the approach used for human data, the CONTAM Panel used the LOAEL of 5 mg/kg b.w. in monkeys as a starting point and a factor of 3 to extrapolate from LOAEL to NOAEL, a factor of 10 for intra-species variability and a factor of 4 for interspecies variability in toxicokinetics, to derive a value of 40 µg/kg b.w. This value is close to, and supports, the ARfD of 30 µg/kg b.w. established on the basis of the human data.

The CONTAM Panel noted that individuals with impaired renal function will be more susceptible to DA.

13. Risk characterisation

Because DA has acute toxic effects, the CONTAM Panel concluded that the use of a large portion size is more appropriate than a long term average consumption in assessing the health risk of the consumers. It considered the 95th percentiles reported by various countries as an appropriate upper bound for the high portion sizes, and identified the figure of 400 g to be used in its acute exposure assessment.

Because DA can be converted to epi-DA during storage, the ARfD of 30 µg/kg b.w. established by the CONTAM Panel applies to the sum of DA and epi-DA. Consumption of a 400 g portion of shellfish meat containing the sum of DA and epi-DA at the current EU limit of 20 mg/kg shellfish meat would result in a dietary exposure of 8 mg toxin equivalent to about 130 µg/kg b.w. in a 60 kg adult. This is about 4-fold higher than the ARfD of 30 µg/kg b.w. and is a potential concern for health.

In order for a 60 kg adult to avoid exceeding the ARfD of 30 µg/kg b.w., a 400 g portion of shellfish should not contain more than 1.8 mg of the sum of DA and epi-DA, corresponding to 4.5 mg/kg shellfish meat. Of the currently available occurrence data for samples in

compliance with the EU regulatory limit (Table 6), 3.5 % exceed this value of 4.5 mg/kg shellfish meat.

As explained in Chapter 6 the CONTAM Panel assumed that all shellfish that are compliant with the current legislation (maximum level for DA and epi-DA at 20 mg/kg shellfish meat) in pre-market controls would be representative of shellfish reaching the market and thus consumed. Therefore, the concentration data for these samples (Table 6) were used in the exposure assessments. Consumption of a 400 g portion of shellfish containing the sum of DA and epi-DA at 2.5 mg/kg shellfish meat, corresponding to the 95th percentile of the concentration (see Table 6), would result in a dietary exposure of 1000 µg (equivalent to approximately 17 µg/kg b.w. in a 60 kg adult). This dietary exposure is below the ARfD of 30 µg/kg b.w. and hence not a concern for health.

From the probabilistic exposure estimate as presented in Figure 3 (Chapter 8) based on the distributions of both the concentration and the consumption data, it can be estimated that a 60 kg person has a chance of about 1 % of exceeding the ARfD of 30 µg/kg b.w. when consuming shellfish containing levels of the sum of DA and epi-DA that could be present in shellfish currently available on the European market.

14. Uncertainty

The evaluation of the inherent uncertainties in the assessment of exposure to DA has been performed following the guidance of the Opinion of the Scientific Committee related to Uncertainties in Dietary Exposure Assessment (EFSA, 2006). In addition, the report on "Characterizing and Communicating Uncertainty in Exposure Assessment" has been considered (WHO/IPCS, 2008). According to the guidance provided by the EFSA opinion (2006) the following sources of uncertainty have been considered: assessment objectives, exposure scenario, exposure model, and model input (parameters).

14.1. Assessment objectives

The objectives of the assessment were clearly specified in the terms of reference and the CONTAM Panel prepared a risk assessment including the derivation of an ARfD, description of the different detection methods, and an exposure assessment for the current situation. The uncertainty of the assessment objectives is considered to be negligible.

14.2. Exposure scenario

The estimate of exposure is based on measurements from 10 European countries which reported occurrence data for DA as the sum of DA and epi-DA. Any uncertainty possibly introduced by non-consideration of iso-DAs is considered to be negligible as these isomers are reported to occur in shellfish at lower concentrations and are considered to be less toxic than DA. Also non-consideration of the effects of cooking in the quantitative exposure assessment is perceived to have minor impact on the final conclusions, because these toxins are heat stable and cooking does not destroy the toxin. It was shown that household processing (cooking, steaming) could lead to a slight reduction of DA in shellfish meat due to leaching-out of these compounds to the cooking fluid ("soup"), but it is unlikely that processing has a major effect on DA concentrations in shellfish meat. As no information on consumption of this "soup" was available this adds to the uncertainty of any exposure estimate. On the other hand, since it is unclear whether the consumption data related to cooked or uncooked shellfish, taking portion size as uncooked may lead to overestimation of

the exposure. Another uncertainty may be introduced into the exposure assessment due to the variability in the occurrence of DA and epi-DA with time and geographical areas.

14.3. Exposure model

The uncertainties regarding values below the LOD are considered to be negligible, as they do not have a major influence on the risk characterisation.

Uncertainty is caused by the fact that exposure was based on occurrence data from pre-market control samples. These samples may not reflect the "real" range of occurrence of DA and epi-DA in the shellfish on the market.

14.4. Model input (parameters)

A certified calibration standard is currently available only for DA (certified value relates to the sum of DA and epi-DA), but not for the other isomers which may introduce a slight uncertainty. The occurrence data which were used in the exposure assessment were all produced with HPLC based methods using different detectors but comparable and adequate LODs, which are considered to be appropriate. A further uncertainty is introduced due to the incomplete database for shellfish consumption in Europe with data only from a limited number of Member States and limited data on shellfish species other than mussels.

The oral toxicity of DA and its isomers is not well defined and the data available for establishing the ARfD are limited. However, in view of the fact that the ARfD derived from observations in humans was supported by the observations in Cynomolgus monkeys, the ARfD is considered to be sufficiently protective.

14.5. Summary of uncertainties

In Table 9 a summary of the uncertainty evaluation is presented, highlighting the main sources of uncertainty and indicating an estimate of whether the respective source of uncertainty might have led to an over- or underestimation of the exposure or the resulting risk.

Table 9. Summary of qualitative evaluation of the impact of uncertainties on the risk assessment of the dietary exposure of DA and epi-DA

Sources of uncertainty	Direction
Uncertainty in analytical results	+/- [a)]
Extrapolation of occurrence data from 10 European countries to Europe as a whole	+
Influence of non-detects on exposure estimate	+
Variability in DA and epi-DA occurrence depending on time and geographical region	+/-
Incomplete database for shellfish consumption in Europe; data only from limited number of Member States and limited data on shellfish species other than mussels	+
Consideration of shellfish sampled for pre-market control for systematic dietary estimation of exposure	+
Effect of processing, such as cooking or steaming	+/-
Establishment of ARfD	+/-

a) + = uncertainty with potential to cause over-estimation of exposure/risk
- = uncertainty with potential to cause under-estimation of exposure/risk

The CONTAM Panel considered the impact of the uncertainties on the risk assessment of exposure to DA and epi-DA from shellfish consumption and concluded that its assessment of the acute risk is likely to be conservative i.e. more likely to over- than to underestimate the risk.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

Hazard identification

- Domoic acid (DA) is a water soluble cyclic amino acid, isolated from various species of shellfish and from marine red algae of the genus *Chondria* and diatoms of the genus *Pseudonitschia*. The diastereoisomer epi-domoic acid (epi-DA) and 8 iso-domoic acids A-H (iso-DA A-H) have been reported.
- DA is neurotoxic in experimental animals, including rodents and non-human primates, and in humans. The toxic effects of DA are mediated through its high affinity binding and agonist action on some type of glutamate receptors affecting neuronal function and possibly leading to cell death in certain regions (e.g. hippocampus) of the brain. In humans DA causes amnesic shellfish poisoning (ASP).
- The available data indicate that Cynomolgus monkeys are more sensitive than rodents. There are few data on oral toxicity in experimental animals and insufficient information on genotoxicity and developmental toxicity. No long term studies have been reported, and data on genotoxicity are inconclusive.
- Taking into account that the iso-DAs occur at much lower concentrations and are considered to be less toxic than DA, the Panel on Contaminants in the Food Chain (CONTAM Panel) concluded that setting of toxicity equivalency factors (TEFs) was not required for iso-DAs.

- As there are no data on chronic effects of DA in animals no tolerable daily intake (TDI) can be established.
- The few data on exposure to DA associated with adverse effects in humans (9 individuals) indicate that severe and irreversible effects occurred in the region of 4 mg/kg b.w. The lowest-observed-adverse-effect-level (LOAEL) for mild signs and symptoms was 0.9 mg/kg b.w.
- Although the oral toxicity is not well characterised the CONTAM Panel considered it appropriate to establish an acute reference dose (ARfD) on the basis of the LOAEL of 0.9 mg/kg b.w. for neurotoxicity in humans.
- Taking into account the steep dose-response relationship, the CONTAM Panel decided to apply a factor of 3 for the extrapolation from a LOAEL to a no-observed-adverse-effect level (NOAEL). The CONTAM Panel concluded that a factor of 10 should be applied to allow for human variability and also for the fact that sensitive methods for detection of neurotoxic effects had not been used in the investigation of affected individuals.
- The CONTAM Panel therefore established an ARfD of 30 µg DA/kg b.w. by applying the overall uncertainty factor of 30 to the LOAEL of 0.9 mg/kg b.w. Because DA can be converted to epi-DA during storage, the ARfD applies to the sum of DA and epi-DA. This ARfD is supported by the observations in Cynomolgus monkeys. The CONTAM Panel noted that individuals with impaired renal function are more susceptible to DA and therefore the ARfD established by the CONTAM Panel may not be sufficiently protective for such subjects.

Occurrence/Exposure

- Consumption data for shellfish are available only for a few Member States. These data seldom distinguish between shellfish species or the type of processing. The occurrence data from Ireland indicate that scallops are more often contaminated with DA than other shellfish species. In addition, different study designs were used in the collection of consumption data.
- From the available data, the CONTAM Panel identified the figure of 400 g as an appropriate large portion size to be used in acute exposure assessments.
- DA is heat stable at temperatures relevant for cooking and steaming. The effect of cooking on DA concentrations in shellfish varies between species. In scallops redistribution of the toxin during cooking and leaching out of the toxin into the cooking fluid may lead to a reduction of the concentration of DA in the hepatopancreas and to an increase in the remaining tissue. For other types of shellfish it is unlikely that processing has a major effect on the DA concentration in shellfish meat.

Risk characterisation

- Consumption of a 400 g portion of shellfish meat containing the sum of DA and epi-DA at the current EU limit of 20 mg/kg shellfish meat would result in a dietary exposure of 8 mg toxin (equivalent to about 130 µg/kg b.w. in a 60 kg adult). This dietary exposure is about 4-fold higher than the ARfD of 30 µg/kg b.w. and is a potential concern for health.
- Consumption of a 400 g portion of shellfish containing the sum of DA and epi-DA at 2.5 mg/kg shellfish meat, corresponding to the 95th percentile of the concentration, would result in a dietary exposure of 1000 µg (equivalent to approximately 17 µg/kg b.w. for a

60 kg adult). This dietary exposure is below the ARfD of 30 µg/kg b.w. and hence not a concern for health.

- In order for a 60 kg adult to avoid exceeding the ARfD of 30 µg/kg b.w., a 400 g portion of shellfish should not contain the sum of DA and epi-DA at more than 1.8 mg (corresponding to 4.5 mg/kg) shellfish meat.
- Amongst the currently available occurrence data for samples in compliance with the EU regulatory limit, 3.5 % exceed this value of 4.5 mg/kg shellfish meat.
- Based on current consumption and occurrence data for DA and epi-DA (probabilistic dietary exposure estimation) there is about a 1 % chance of exceeding the ARfD of 30 µg/kg b.w. when consuming shellfish currently available on the European market.

Methods of analysis

- High-performance liquid chromatography (HPLC) is the officially prescribed reference method in the EU legislation for the determination of DA and epi-DA. There is one validated and standardised HPLC-ultraviolet detection (UV) method, which has a limit of detection of 1 mg/kg which is adequate to detect DA at the concentration of 4.5 mg/kg shellfish meat, at which consumption of a large portion would not lead to the ARfD being exceeded. Two other HPLC-UV methods are widely used and their standardisation is ongoing.
- Enzyme-linked immunosorbent assay (ELISA) is another approach that can be applied to determine DA and its isomers. An ELISA method for DA has been formally validated in an interlaboratory study, and it is officially permitted for use in the EU for screening purposes.
- Another technique that has the potential to determine DA and epi-DA is liquid chromatography-mass spectrometry (LC-MS) in the tandem mode. It has not yet been formally validated in interlaboratory studies, following internationally recognized protocols.

RECOMMENDATIONS (INCL. KNOWLEDGE/DATA GAPS)

Hazard identification and characterisation

- Further information on the toxicokinetics (including biotransformation), genotoxicity, oral toxicity, including developmental- and long term toxicity of DA is needed.

Occurrence/Exposure

- The low occurrence of iso-domoic acids (iso-DAs) needs to be confirmed.
- Data are needed on potential co-occurrence of DA with marine biotoxins especially those affecting the gastrointestinal tract such as the okadaic acid and azaspiracid group toxins.

Methods of analysis

- For LC-MS based methods subsequent (interlaboratory) validation studies are needed.

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ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
AOAC	Association of Official Analytical Chemists
APHA	American Public Health Association
ARfD	Acute reference dose
ASP	Amnesic Shellfish Poisoning
AZA	Azaspiracid
AZP	Azaspiracid Shellfish Poisoning
BTX	Brevetoxin
b.w.	Bodyweight
CCFFP	Codex Committee for Fish and Fishery Products
CCMAS	Codex Committee on Methods of Analysis and Sampling
CE	Capillary electrophoresis
CEN	European Committee for Standardization
CNS	Central nervous system
CONTAM Panel	Panel on Contaminants in the Food chain
CRL-MB	Community Reference Laboratory for Marine Biotoxins
CRM	Certified reference material
CTX	Ciguatoxins
DA	Domoic acid
DAD	Diode array detector
DG SANCO	Health and Consumer Protection Directorate General
DSP	Diarrhoeic Shellfish Poisoning
DTX	Dinophysis toxins
EC	European Commission
ECVAM	European Centre for the Validation of Alternative Methods
ED_{50}	Half maximal cumulative seizure scores
EEC	European Economic Community
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
FAB	Fast atom bombardment
FAO	Food and Agriculture Organization of the United Nations

FAO/IOC/WHO	Food and Agriculture Organization of the United Nations/ Intergovernmental Oceanographic Commission of UNESCO/World Health Organization
FFQ	Food frequency questionnaire
FMOC	Fluorenylmethoxycarbonyl
GD	Gestational day
GI	Gastro-intestinal
HCl	Hydrochloric acid
HILIC-LC	Hydrophilic interaction liquid chromatography
HPLC	High-performance liquid chromatography
HPLC-FLD	High-performance liquid chromatography-fluorescence detection
HLPC-UV	High-performance liquid chromatography-ultraviolet
i.p.	Intraperitoneal
i.v.	Intra-venous
LB	Lower bound
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-mass spectrometry/mass spectrometry
LD_{50}	Lethal dose – the dose required to kill half the members of a tested animal population
LOAEL	Lowest-observed-adverse-effect level
LOD	Limit of detection
LOQ	Limit of quantification
MBA	Mouse bioassay
MS	Mass spectrometry
NOAEL	No-observed-adverse-effect level
Non-NMDA	non-N-methyl-D-aspartate
NRCC	National Research Council Canada
NRL	National Reference Laboratory
OA	Okadaic acid
OJ	Official Journal of the European Union
P95	95^{th} percentile
PlTX	Palytoxins
PND	Postnatal day
Post-MC	Post-market control
Pre-MC	Pre-market control
PSP	Paralytic shellfish poisoning

PTX	Pectenotoxin
RBA	Rat bioassay
s.c.	Subcutaneous
SM	Shellfish meat
SPE	Solid Phase Extraction
SPR	Surface plasmon resonance
STX	Saxitoxin
TDI	Tolerable daily intake
TEF	Toxic equivalence factor
TLC	Thin-layer chromatography
TLE	Temporal lobe epilepsy
TSH	Thyroid-stimulating hormone
UB	Upper bound
UK	United Kingdom
UV	Ultraviolet
WG	Working group
WHO/IPCS	World Health Organization/ International Programme on Chemical Safety
YTX	Yessotoxin