

## **Marine biotoxins in shellfish – Azaspiracid group<sup>1</sup>**

### **Scientific Opinion of the Panel on Contaminants in the Food chain**

**(Question N° EFSA-Q-2006-065B)**

**Adopted on 9 June 2008**

#### **PANEL MEMBERS**

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#### **SUMMARY**

Azaspiracids (AZAs) are a group of shellfish toxins causing AZA poisoning (AZP) which is characterized by symptoms such as nausea, vomiting, diarrhoea and stomach cramps. Approximately 20 different analogues have been identified, of which AZA1, AZA2 and AZA3 are the most important ones based on occurrence and toxicity. AZAs can be found in various species of filter-feeding bivalve molluscs such as oysters, mussels, scallops, and clams. Monitoring of AZAs in shellfish in Ireland has shown that mussels are the most affected species for this group of toxins. Only recently has the dinoflagellate that produces the AZA toxins been isolated. AZAs are nitrogen-containing polyether toxins comprising a unique spiral ring assembly, a heterocyclic amine (piperidine) and an aliphatic carboxylic acid moiety. AZAs in shellfish are not decomposed at temperatures relevant for cooking.

The toxicological database for AZAs is limited and comprises mostly studies on their acute toxicity. The following toxic equivalence factors (TEF) have been applied in some countries: AZA1 = 1, AZA2 = 1.8 and AZA3 = 1.4. Because the available data (lethality of very few mice

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following intraperitoneal (*i.p.*) administration) are not sufficient to establish robust TEF values, the Panel on contaminants in the food chain (CONTAM Panel) adopted these TEF values as an interim measure in order to provide a best estimate of the toxicity of AZAs.

Only a few limited repeated-dose toxicity studies of longer duration (maximum duration 1 year) were available for AZA1. Pathological changes were observed in multiple organs; lungs, stomach, small intestine and liver. Occasionally lung tumours were observed. Because these tumours were only observed at doses causing severe toxicity, the CONTAM Panel considered this observation of limited relevance. No data on genotoxicity have been reported for AZAs.

The data on the chronic effects of AZAs in animals or humans were insufficient for a tolerable daily intake (TDI) to be established. In view of the acute toxicity of AZAs, the CONTAM Panel decided to establish an acute reference dose (ARfD) based on the available human data.

There were only data available from one incident of human poisoning involving AZAs that could be used for the derivation of an ARfD. The CONTAM Panel concluded that the most probable estimate of a lowest-observed-adverse-effect-level (LOAEL) resulting in AZA poisoning was 113 µg AZA1 equivalents per person (1.9 µg AZA1 equivalents/kg body weight (b.w.) for a 60 kg adult). Uncertainty factors were required to extrapolate from the LOAEL to a no-observed-adverse-effect level (NOAEL), and for variability within the human population. Because the effects considered were mild and reversible a factor of three was applied for the extrapolation of the LOAEL to NOAEL. The CONTAM Panel decided that the usual factor of 10 for human variability was not required because the reported incident was expected to have occurred in sensitive, rather than average, individuals. However, an additional factor of three was applied because the available data related to a small number of individuals from a single incident. Consequently the Panel established an ARfD of 0.2 µg AZA1 equivalents/kg b.w.

In order to protect against the acute effects of AZAs, it is important to use a high portion size rather than a 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 the high portion size to be used in the acute risk assessment of marine biotoxins.

It was noted that consumption of a 400 g portion of shellfish meat containing AZAs at the current EU limit of 160 µg AZA1 equivalents/kg shellfish meat would result in a dietary exposure of 64 µg AZA1 equivalents. For a 60 kg adult this is approximately 1 µg AZA1 equivalents/kg b.w. This figure is 5-fold higher than the ARfD established by the CONTAM Panel. Therefore, it cannot be excluded that this intake could exert effects in susceptible consumers. Based on the consumption and occurrence data, there is an approximately 4% chance for 60 kg adults of exceeding the ARfD of 0.2 µg AZA1 equivalents/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 not exceed the ARfD, a 400 g portion of shellfish should not contain more than 12 µg AZA1 equivalents, i.e. 30 µg AZA1 equivalents/kg shellfish meat.

The mouse and the rat bioassay are the officially prescribed reference methods in the EU for the detection of AZAs. The CONTAM Panel noted that both methods have shortcomings e.g. they are not specific and not quantitative, and that method performance characteristics for AZAs have not been established for the mammalian assays. Based on limited data on acute *i.p.* toxicity in mice, it is not clear whether the mouse bioassay (MBA) can detect levels at the current EU regulatory level of 160 µg AZA1 equivalents/kg shellfish meat.

The current EU legislation permits the replacement of the bioassays, provided that the alternative methods have been validated according to an internationally recognised protocol. At this point however, none of the methods for the determination of toxins from the AZA group have been validated by interlaboratory studies. The evidence available at this moment suggests that liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) based methods have the greatest potential to replace the mammalian assays. Moreover, they are able to detect AZAs at concentrations well below the current regulatory limit of 160 µg AZA1 equivalents/kg shellfish meat. The LC-MS/MS based methods also have the possibility for multi-toxin group detection/quantification. The CONTAM Panel noted that, while application of single laboratory validation according to recognised international guidelines to demonstrate their fitness-for-purpose can be an impetus for implementation of alternative instrumental analyses of marine biotoxins for regulatory purposes, method performance criteria should be stipulated where possible and validation by interlaboratory trials should be the long-term objective.

**KEYWORDS:** Marine biotoxins, azaspiracids, AZA1, AZA2, AZA3, AZA4, AZA5, shellfish, bivalve molluscs, mammalian bioassays, 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, 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<sup>2</sup>. Two additional groups, palytoxins (PITX) and ciguatoxins (CTX), may also be considered. STX and its derivatives cause Paralytic Shellfish Poisoning (PSP), and DA causes Amnesic Shellfish Poisoning (ASP). Diarrhoeic Shellfish Poisoning (DSP) is caused by OA-group toxins (OA and dinophysins toxins (DTX)). 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<sup>3</sup>, are established maximum levels for ASP, PSP and DSP toxins. Annex III of

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<sup>2</sup> [ftp://ftp.fao.org/es/esn/food/biotoxin\\_report\\_en.pdf](ftp://ftp.fao.org/es/esn/food/biotoxin_report_en.pdf)

<sup>3</sup> 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

Commission Regulation No 2074/2005/EC<sup>4</sup> of 5 December 2005 lays down the recognised testing methods for detecting marine biotoxins. Annex II Chapter II (14) to Regulation (EC) 854/2004<sup>5</sup>, 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/EEC<sup>6</sup> 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<sup>6</sup> 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<sup>6</sup> 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.

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<sup>4</sup> 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.

<sup>5</sup> 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.

<sup>6</sup> 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.

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.

### **Recognised testing methods for marine biotoxins and maximum levels**

Commission Regulation (EC) No. 2074/2005<sup>4</sup> 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 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 853/2004<sup>3</sup> 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<sup>7</sup> 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.

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<sup>7</sup> 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 azaspiracids, respectively.



### 3. 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)<sup>8</sup> 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 100g, 250g or 380g 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.

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<sup>8</sup> 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 <sup>8</sup>	Derived Guidance Level/ Max Level based on consumption of 100g (1), 250g (2) and 380g (3)	Limit Value currently implemented in EU legislation
<b>AZA</b>	0.4 (1)	10(H)	0.04 µg/kg 2.4 µg/adult <sup>a)</sup>	0.024 mg/kg SM (1) 0.0096 mg/kg SM (2) 0.0063 mg/kg SM (3)	0.16 mg/kg SM
<b>BTX</b>			N/A		0.8 mg/kg SM as Pb Tx-2
<b>Cyclic Imines</b>			N/A		
<b>DA</b>	1,000 (1)	10(H)	100 µg/kg 6 mg/adult <sup>a)</sup>	60 mg/kg SM(1) 24 mg/kg SM(2) 16 mg/kg SM(3)	20 mg/kg SM
<b>OA</b>	1 (1)	3(H)	0.33 µg/kg 20 µg/adult <sup>a)</sup>	0.2 mg/kg SM (1) 0.08 mg/kg SM (2) 0.05 mg/kg SM(3)	0.16 mg/kg SM
<b>PTX</b>			N/A		
<b>STX</b>	2 (1)	3(H)	0.7 µg/kg 42 µg/adult <sup>a)</sup>	0.42 mg/kg SM(1) 0.17 mg/kg SM(2) 0.11 mg/kg SM(3)	0.8 mg/kg SM
<b>YTX</b>	5,000 (2)	100(A)	50 µg/kg 3 mg/adult <sup>a)</sup>	30 mg/kg SM(1) 12 mg/kg SM(2) 8 mg/kg SM(3)	1 mg/kg SM

SM = shellfish meat

<sup>a)</sup> Person with 60 kg bodyweight (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<sup>9</sup> or III<sup>10</sup> 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.

#### **4. 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

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<sup>9</sup> 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.

<sup>10</sup> 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.

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 (100g, 250g and 380g) 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.

## ACKNOWLEDGEMENT

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## ASSESSMENT

### 1. Introduction

Azaspiracids (AZAs) are a group of shellfish toxins causing AZA poisoning (AZP) which is characterised by symptoms such as nausea, vomiting, diarrhoea and stomach cramps. Approximately 20 different analogues have been identified, of which AZA1, AZA2 and AZA3 are the most important ones based on occurrence and toxicity. These toxins accumulate in various species of filter-feeding bivalve molluscs such as oysters, mussels, scallops, and clams. AZAs were first detected in mussels (*Mytilus edulis*) in Ireland in 1995 (McMahon and Silke, 1996). A research group from Germany has succeeded in isolating a small hitherto unknown dinoflagellate as the producer of AZAs (Cembella, 2008). Since 2001, continuous monitoring of

AZAs in shellfish in Ireland has shown that mussels are the most affected species for this compound group, with some concentrations detectable in oysters (*C. gigas*) (Hess *et al.*, 2003). Currently 21 different analogues (Table 2) have been identified for AZAs (Satake *et al.*, 1998; Ofuji *et al.*, 1999; 2001; James *et al.*, 2003; Rehmann *et al.*, 2008). While AZA1 was discovered first, other analogues (AZA2 and AZA3) were later discovered to also frequently occur at significant concentrations and to also contribute significantly to the AZAs present in most shellfish samples. Therefore, regulatory surveillance in Ireland and Norway and possibly other Member States that have reported quantitative data for this opinion, would add the toxicity-weighted sum of AZA1, AZA2 and AZA3 to obtain the total AZA-toxicity equivalent. In the weighting, Ireland and Norway have used the relative toxicity factors published by Satake *et al.* (1998), i.e. 1.8 for AZA2 and 1.4 for AZA3 (based on scarce data for intraperitoneal (*i.p.*) lethality). Occasional analyses of AZA4 and AZA5 in monitoring and control programs in Ireland and Norway showed that these analogues occur at levels significantly lower than AZA1, AZA2 and AZA3 (Alfonso *et al.* 2008; Hess and Aune, 2008; Rehmann *et al.* 2008).

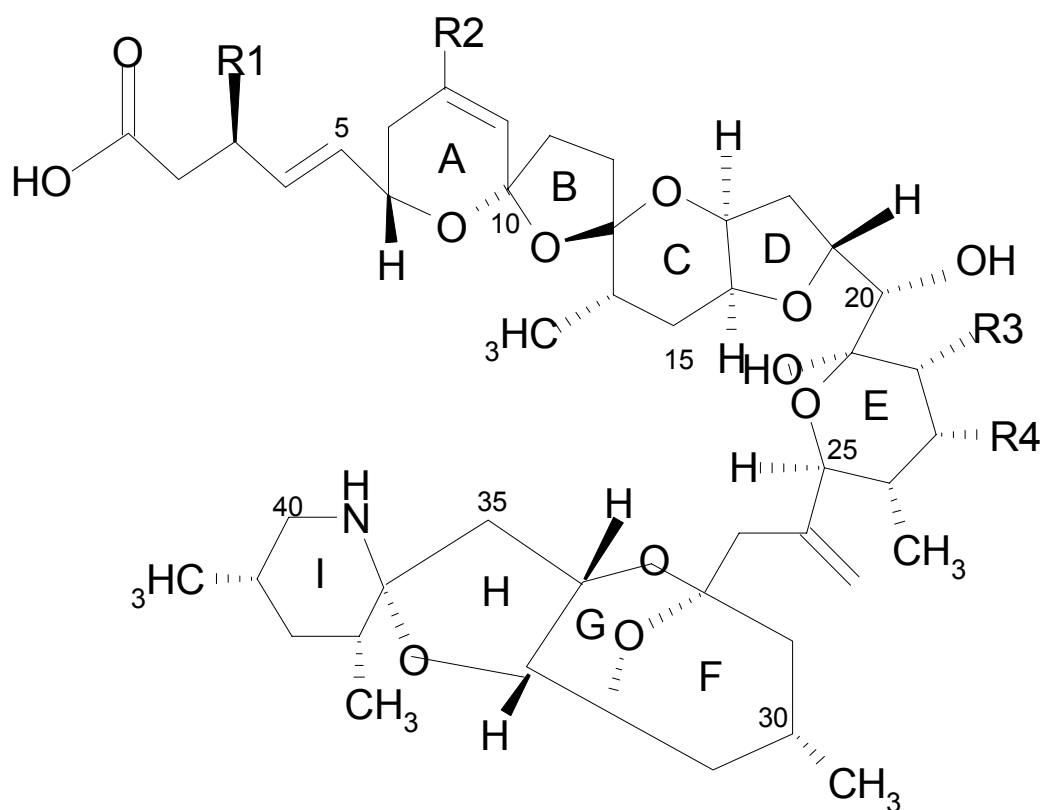
While 21 analogues have been identified (Rehmann *et al.*, 2008), only AZA1, AZA2, AZA3, AZA4, and AZA5 (see figure 1) have been isolated in quantities allowing limited toxicity studies. AZA1 and AZA2 have been shown to be produced by algae isolated from the North Sea (Krock, 2008). All other analogues are likely to be formed through metabolism in shellfish as they have been only identified at significant levels in shellfish. More recently, AZAs have been found in scallops and crab (FSAI, 2006). Since their initial discovery in 1995, AZAs were also identified in shellfish from UK and Norway at significant levels, while trace amounts were also found in shellfish from France and Spain (Brana-Magdalena *et al.*, 2003b), Morocco and Portugal (Taleb *et al.*, 2006; Vale *et al.*, 2008).

The amount of toxin-producing algae cells can vary considerably over the year. Periods of explosive growth (“algae bloom”) can occur during changes in weather conditions, but other factors such as upwellings, temperature, transparency, turbulence or salinity of the water, and the concentration of dissolved nutrients may also play a role (FAO, 2004). Consequently also the levels of marine biotoxins present in filter-feeding bivalve molluscs will vary over the year.

## 2. Chemical characteristics

Azaspiracids are nitrogen-containing polyether toxins comprising a unique spiral ring assembly containing a heterocyclic amine (piperidine) and an aliphatic carboxylic acid moiety (FAO, 2005). AZA1 is a colourless amorphous solid with no UV absorption maxima above 210 nm. Recently its synthesis has been accomplished (Nicolaou *et al.*, 2004a,b). Unpublished work by Yasumoto, Satake, Ofuji and Rehman *et al.*, showed that treatment with strong acid or base (HCl or NaOH) led to very rapid destruction of AZAs. Amongst all analogues, AZA3 appears to be the most easily degraded analogue, which may be the reason why it occurs naturally at lower

concentrations than AZA1 and AZA2 in mussels. Although studies with AZA in acidic methanolic solutions have shown that AZA1 and AZA2 are unstable above 70°C (Alfonso, 2008), in shellfish tissues a study on the heat stability of AZAs (McCarron, 2007) showed that temperatures above 100 °C are required to decompose or rearrange AZAs.



	<u>R<sub>1</sub></u>	<u>R<sub>2</sub></u>	<u>R<sub>3</sub></u>	<u>R<sub>4</sub></u>
azaspiracid (AZA)	H	H	CH <sub>3</sub>	H
azaspiracid-2 (AZA2)	H	CH <sub>3</sub>	CH <sub>3</sub>	H
azaspiracid-3 (AZA3)	H	H	H	H
azaspiracid-4 (AZA4)	OH	H	H	H
azaspiracid-5 (AZA5)	H	H	H	OH

**Figure 1:** Chemical structures of azaspiracids 1-5 (Nicolaou *et al.*, 2006a,b), the only 5 AZAs that have been preparatively isolated and characterised by nuclear magnetic resonance to date.

**Table 2:** List of all AZAs with nomenclature including AZA-methyl esters.

Abbreviation	Original analogue	Substituent	Name
AZA1			azaspiracid
AZA2			8-methylazaspiracid
AZA3			22-desmethyl-azaspiracid
AZA4	AZA3	OH	22-desmethyl-3-hydroxyazaspiracid
AZA5	AZA3	OH	22-desmethyl-23-hydroxyazaspiracid
AZA6			22-desmethyl-8-methylazaspiracid
AZA7	AZA1	OH	3-hydroxyazaspiracid
AZA8	AZA1	OH	23-hydroxyazaspiracid
AZA9	AZA6	OH	22-desmethyl-3-hydroxy-8-methylazaspiracid
AZA10	AZA6	OH	22-desmethyl-23-hydroxy-8-methylazaspiracid
AZA11	AZA2	OH	3-hydroxy-8-methylazaspiracid
AZA12	AZA2	OH	23-hydroxy-8-methylazaspiracid
AZA13	AZA3	2 OH	22-desmethyl-3,23-dihydroxyazaspiracid
AZA14	AZA1	2 OH	3,23-dihydroxyazaspiracid
AZA15	AZA6	2 OH	3,23-dihydroxy-8-methylazaspiracid
AZA16	AZA2	2 OH	22-desmethyl-3,23-dihydroxy-8-methylazaspiracid
AZA17	AZA3	COOH	carboxy-22-desmethylazaspiracid
AZA18	AZA1	COOH	carboxyazaspiracid
AZA19	AZA6	COOH	carboxy-8-methylazaspiracid
AZA20	AZA2	COOH	carboxy-22-desmethyl-8-methylazaspiracid
AZA21	AZA3	COOH + OH	carboxy-22-desmethyl-3-hydroxyazaspiracid
AZA22	AZA1	COOH + OH	carboxy-3-hydroxyazaspiracid
AZA23	AZA6	COOH + OH	carboxy-22-desmethyl-3-hydroxy-8-methylazaspiracid
AZA24	AZA2	COOH + OH	carboxy-3-hydroxy-8-methylazaspiracid
AZA25	AZA3	-H <sub>2</sub> O	21-22-dehydro-22-desmethylazaspiracid
AZA26	AZA1	-H <sub>2</sub> O	21-22-dehydroazaspiracid
AZA27	AZA6	-H <sub>2</sub> O	21-22-dehydro-8-methylazaspiracid
AZA28	AZA2	-H <sub>2</sub> O	21-22-dehydro-22-desmethyl-8-methylazaspiracid
AZA29	AZA3	COOCH <sub>3</sub>	22-desmethyl-azaspiracid-1-methyl ester
AZA30	AZA1	COOCH <sub>3</sub>	azaspiracid-1-methyl ester
AZA31	AZA6	COOCH <sub>3</sub>	22-desmethyl-8-methyl-azaspiracid-1-methyl ester
AZA32	AZA2	COOCH <sub>3</sub>	8-methyl-azaspiracid-1-methyl ester

The last 4 analogues (methyl-esters) are artefacts of storage of AZAs in methanolic solution and have not been identified as shellfish contaminants specifically. AZA18, AZA20, AZA22, AZA24, AZA26, AZA27 and AZA28 have not been observed experimentally but are postulated.

### 3. Regulatory status

For the control of the AZAs in the EU, Commission Regulation (EC) No 853/2004<sup>3</sup>, provides details in section VII: “Live bivalve molluscs”, chapters II and IV. 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

*production areas on the market for direct human consumption only, if they meet the requirements of chapter IV*". Chapter IV: "Hygiene requirements for purification and dispatch centres. A. Requirements for purification centres" states: *"Food business operators purifying live bivalve molluscs must ensure compliance with the following requirements: 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 azaspiracids 160 µg of AZA equivalent per kg"*. AZA equivalent is not specified here, but it is assumed that AZA equivalent should be expressed as AZA1 equivalents.

Commission Regulation (EC) No 2074/2005<sup>4</sup> provides details about the "Recognized testing methods for detecting marine biotoxins". Annex III, Chapter III of this regulation deals with lipophylic toxin detection methods. Biological methods are to be used for the detection of azaspiracids: both a mouse bioassay and a rat bioassay may be used. When the mouse bioassay (MBA) is applied for the detection of azaspiracids at regulatory levels, it is mandatory to use the whole body as the test portion. Newer findings about the distribution of azaspiracids (see chapter 4) within shellfish would justify the practice to analyse the digestive gland and not only the whole body, as prescribed in the regulation. Commission Regulation (EC) No 2074/2005 also states the following concerning alternative detection methods:

*"A series of methods, such as high-performance liquid chromatography with fluorescence detection, liquid chromatography, mass spectrometry, immunoassays and functional assays, such as the phosphatase inhibition assay, shall be used as alternative or supplementary to the biological testing methods, provided that either alone or combined they can detect at least the following analogues, that they are not less effective than the biological methods and that their implementation provides an equivalent level of public health protection.*

- *okadaic acid and dinophysistoxins: a hydrolysis step may be required to detect the presence of DTX3.*
- *pectenotoxins: PTX1 and PTX2.*
- *yessotoxins: YTX, 45 OH YTX, homo YTX, and 45 OH homo YTX.*
- *azaspiracids: AZA1, AZA2 and AZA3.*

*If new analogues of public health significance are discovered, they should be included in the analysis. Standards must be available before chemical analysis is possible. Total toxicity shall be calculated using conversion factors based on the toxicity data available for each toxin. The performance characteristics of these methods shall be defined after validation following an internationally agreed protocol"*.

Currently there is no detailed guidance on how a non-animal-based method can become an accepted alternative method, i.e. which performance criteria should be fulfilled. In addition, conversions factors have not been established. The Commission Regulation (EC) No 2074/2005<sup>4</sup> (Annex III, Chapter III) also states that *"Biological methods shall be replaced by alternative detection methods as soon as reference materials for detecting the toxins prescribed in Chapter*



*V of Section VI of Annex III to regulation (EC) No 853/2004 are readily available, the methods have been validated and this Chapter has been amended accordingly”.*

In conclusion the legislation stimulates the replacement of the biological methods, provided that alternatives have been validated according to an internationally agreed protocol. The application of single laboratory validation (SLV) according to international guidelines to demonstrate their fitness-for-purpose in practice can be an impetus for implementation of instrumental analysis (e.g. liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS)) in regulatory analysis.

#### **4. Methods of analysis**

The EU regulation on analytical methodology for AZAs prescribes the use of the whole body as the test portion for analysis (see chapter 3). The text of the regulation was formulated based on the initial assumption that AZAs can move from the digestive gland to the remaining mussel tissue (James *et al.*, 2002). However, more recent information has shown that AZAs typically accumulate in the digestive glands of scallops (Bran-Magdalena *et al.*, 2003a) and mussels (Hess *et al.*, 2005; McCarran, 2008). These findings indicate that the analysis of the digestive gland is the safe practice for raw shellfish, rather than the whole body as prescribed in the regulation.

Two types of methods are used for the detection of AZAs: biological and chemical methods. The biological assays include the mouse bioassay (*imp.* injection with animal death as toxicity criterion) and the rat assay (oral administration with diarrhoeic response as toxicity criterion) (see 4.1). The mammalian bioassays for the AZAs are still applied widely, despite the growing resistance against the use of these assays for reasons of animal welfare, and their inherent analytical variability. AZA contamination of shellfish can be accompanied by other lipophilic toxins (okadaic acid group toxins, yessotoxins, Pectenotoxins) (FAO, 2004) which may cause positive results in animal bioassays thereby requiring further confirmatory testing to evaluate actual risks. Chemical methods (i.e. LC-MS/MS) are applied largely for this purpose, but their significance for determinative purposes is growing rapidly (see 4.2).

No Biomolecular methods for AZAs are currently available, largely due to the fact that AZAs have been difficult to obtain in sufficient quantities and with sufficient purity to produce viable antibodies. Several European projects have been commissioned with the aim of obtaining an adequate supply of pure material, but it is unclear what the outcome of these efforts will be. The development of functional assays has yet to be undertaken because the mechanism of action of AZAs is not yet known.

## Supply of appropriate reference material

The only reference material currently available for AZAs is the certified calibrant for AZA1, supplied by NRC-IMB, Halifax, NS, and CA. Further calibrants for AZA2 and AZA3 are under development as well as mussel tissue reference materials.

A research group in Germany has succeeded in isolating the AZA producer (Cembalo, 2008, Personal communication). The AZA producer is a dinoflagellate that is growing well in culture. Consequently, this presents a promising source of toxins from the AZA group for future studies.

### 4.1 Mammalian bioassays

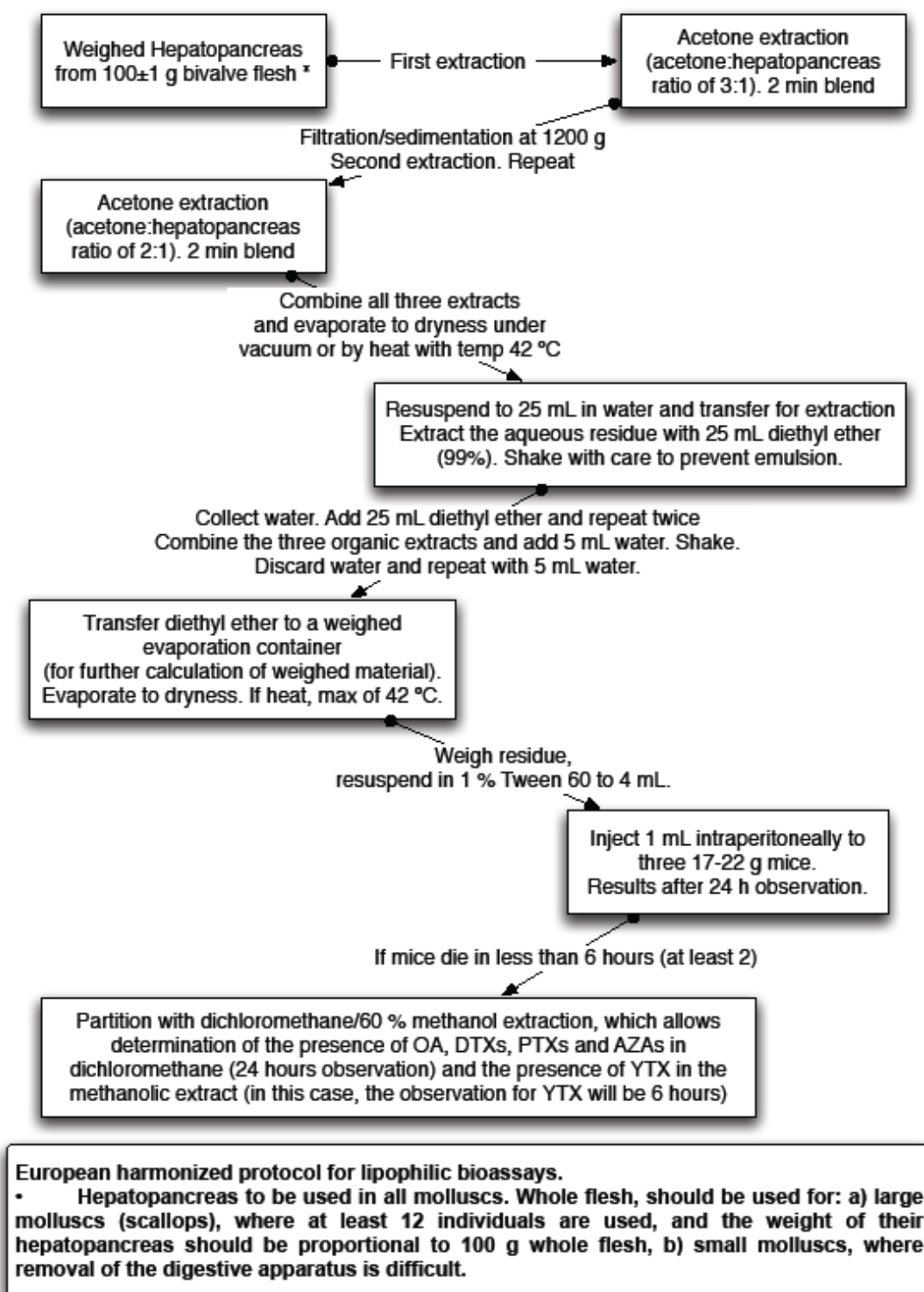
Regulation (EC) No. 2074/2005<sup>4</sup> allows for the use of two types of mammalian bioassays for the detection of the AZA-group toxins; neither of which have been formally validated. These are described below:

#### Mouse bioassay

Historically, the MBA has been used extensively in biotoxins monitoring and as such is incorporated into EU legislation (Commission Regulation (EC) No 2074/2005<sup>4</sup> Annex III, Chapter III). The MBA was developed by Vasomotor and colleagues (1978) as an investigative tool for the determination of the causative agents responsible for a food poisoning outbreak associated with the consumption of molluscs in Japan. Essentially, the assay uses acetone extraction of the whole flesh (or the hepatopancreas (HP)) of molluscs followed by evaporation and resuspension of the residue in a 1% solution of Tween 60 surfactant. Mice are then exposed to the extract via *i.p.* injection and survival monitored over a 24 hour period (see Figure 2).

In efforts to improve the specificity of the assay, several modifications to the technique (generally involving an additional partitioning step) were developed (Yasumoto *et al.*, 1984; Lee *et al.*, 1987; Marcaillou-Le Baut *et al.*, 1990; Fernández *et al.*, 2002). Commission Regulation (EC) 2074/2005<sup>4</sup> allows for the use of different solvents in the liquid/liquid (water) partition step including ethyl acetate, dichloromethane and diethyl ether. A positive result is defined as the death of 2 out of three mice within 24 hours of injection with an extract operationally equivalent to 25 g whole flesh (including HP). The detectability and selectivity depends on the choice of solvents used for extraction and partitioning.

Clearly it is not ideal for a regulatory method to allow for such procedural variation, so in an effort to harmonise the methodology used within the EU, the Community Reference Laboratory for marine biotoxins (CRL-MB) has developed a standard operating procedure based on acetone extraction with either diethyl ether or dichloromethane partitioning against water. The Standard.



**Figure 2.** Sample preparation and extraction methods of hepatopancreas for the MBA (CRL-MB, 2007).

Operating Procedure (SOP) for this method has been available at the CRL web page since 2007 (CRL-MB, 2007). Although this protocol was developed for OA-group toxins AZAs may also be detected in this test. The method performance characteristics (LOD, recovery etc.) for the AZAs have not been established for the MBA

Due to the restricted supply of pure AZA analogues, only few mice were used when studying acute *i.p.* toxicity of AZA1 to AZA5. Long-term comparison of the MBA with LC/MS testing in Ireland (2001-2008) has shown that on a small number of samples (10-20 *per annum*, i.e. <1% of total number of samples), the MBA has given negative results despite high concentrations being present (0.4-0.8 mg/kg) (McMahon, 2008).

These data also seem insufficient to derive an LOD for the MBA. Therefore, the data from the above studies shows some applicability of the MBA in principal without giving a specific cut-off level.

The advantages of the MBA include:

- the provision of a measure of total toxicity based on the biological response of the animal to the toxin(s);
- it does not require complex analytical equipment.

The major disadvantages of the MBA include:

- the outcome depends on the choice of solvents used;
- it is labour intensive and cannot be readily automated;
- it requires specialised animal facilities and expertise;
- the high variability in results between laboratories due to e.g. specific animal characteristics (strain, sex, age, weight, general state of health, diet, stress);
- the potential for false positive results due to interferences (e.g. free fatty acids);
- the potential for false negative results;
- it is not selective for solely the AZAs;
- it is not quantitative; LOD has not been established.
- the injection volume of one mL exceeds good practise guidelines (less  $\leq 0.5$  mL) intended to minimise stress to mice;
- in many countries the use of the MBA is considered unacceptable for ethical reasons.

### **Rat bioassay**

In the original procedure (Kat, 1983) shellfish hepatopaneas mixed with normal rat feed is fed to pre-starved white female rats. In the procedure currently applied in the Netherlands (Van der Hoeven, 2007) 10 g of shellfish hepatopaneas (if possible and desired) or 10 g of shellfish meat (e.g. for cockles) is collected and fed to female rats, that have starved for 24 hours. After a 16 h-period the consistency of the faeces (softening) is observed along with the quantity of food eaten.

The test results are expressed as -, +/-, +, ++ or +++, where a response of + and ++ in the rat corresponds with severe diarrhoea and nausea in man. This method yielded a strongly positive result with the contaminated mussel samples from the first AZA incident in the Netherlands in 1995. The method has official status in EU legislation and is still in use in the Netherlands. The limit of detection for AZAs has yet to be determined.

The advantages of the rat bioassay include:

- it does not involve extraction of toxin and therefore it avoids any toxin loss due to methodology;
- it does not require complex analytical equipment.

The disadvantages of the rat bioassay include:

- lack of specificity, since it will also detect other diarrhoeic agents in the sample;
- it requires specialised animal facilities and expertise;
- variation in sensitivity and symptomology amongst rats;
- it is not quantitative; LOD has not been established.

## 4.2 Chemical methods

AZAs lack a chromophore for liquid chromatography with ultra violet absorption detection (LC-UV) determination and conditions for fluorescence derivatisation have not been established. For the determination of AZAs, sensitive LC-MS/MS techniques have been developed that are routinely used in some laboratories in Ireland, Norway, Germany and New Zealand. One multi-toxin protocol that includes AZAs has been subjected to a limited interlaboratory validation study (McNabb *et al.*, 2005). The LOQ for this method was 0.05 mg/kg, but lower limits were anticipated to be readily achievable, should this be desirable for enforcement of lower limits. A problem for the analytical community is the limited availability of reliable calibrants for AZAs. Currently a certified standard is only available for AZA1. Quantification of other analogues is currently undertaken through cross-calibration assuming equal response factors. However, efforts are being undertaken by the Marine Institute in Ireland to obtain internationally acceptable standards and reference materials for AZAs through collaboration with the National Research Council Canada.

Attempts to advance and validate chemical methodology of the AZAs (LC-MS/MS) are being undertaken by the EU-CRL for Marine Biotoxins (Vigo, Spain) and under the auspices of the EU-funded project “BIOTOX” (2005-2008).

The major advantages of LC-MS/MS methods include:

- it is highly specific and sensitive;
- it can screen and quantitatively measure AZAs individually;
- it gives information on the AZAs profiles in samples;
- it can be automated.

The major disadvantages of LC-MS/MS methods include:

- it requires costly equipment and highly trained personnel;
- it requires reference material for identification and quantification.

### 4.3 Summary on methods

From the above review of methods it can be seen that although currently prescribed by EC legislation, the mammalian bioassays have not been fully validated. Very limited quantitative data on the mouse and rat bioassays are available. Additionally, Council Directive 86/609/EEC<sup>6</sup> states that Member States may not 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.

Currently none of the methods for the determination of toxins from the AZA group have been validated by interlaboratory studies. The evidence available at this moment suggests that LC/MS based methods have the greatest potential to replace the mammalian assays. Moreover, they are able to detect AZAs at concentrations well below the current regulatory limit of 160 µg AZA1 equivalents/kg shellfish meat. The LC-MS/MS based methods also have the possibility for multi-toxin group detection/quantification. However before these methods can be used, there are a number of obstacles to overcome, such as validation results that support their use. While full collaborative validation studies are complex, costly and time-consuming, the application of single laboratory validation (SLV) according to international guidelines to demonstrate their fitness-for-purpose in practice should be further explored.

## 5. Occurrence of AZA

Following a request by EFSA for data on AZA1, AZA2, and AZA3, Germany, Ireland, Norway, Spain, and UK provided data on the occurrence of AZAs in shellfish. Therefore, the reported data comprised information on these analogues. A total of 12,275 samples were submitted. The numbers of analyses presented by the countries are considerably different. Table 3 shows a summary of the number of samples provided by each country including years of harvesting, type of sampling, analytical method and limit of detection (LOD) and limit of quantification (LOQ).

**Table 3:** Data on AZAs submissions obtained from Member States in the period from 2003 to 2007.

Country	Year(s) of harvesting	Number of samples	Purpose of testing <sup>a)</sup>	Method of testing	LOD (µg/kg)	LOQ (µg/kg)
Germany	2005-2006	394	Pre- and Post-MC	LC-MS/MS	<1-10 <sup>b)</sup>	2-10 <sup>b)</sup>
Ireland	2003-2006	9,847	Pre-MC	LC-MS/MS	2	20
Norway	2004-2006	1,851	Pre-MC	LC-MS/MS	6	20
Spain <sup>c)</sup>	2005-2006	5	Pre-MC	LC-MS/MS	2	10
UK	2006-2007	178	Pre-MC	LC-MS/MS	2	5
Total		12,275				

<sup>a)</sup> Pre-market control (Pre-MC) and post-market control (Post-MC) cover samples taken before products are sent to the market and product sampled at the market, respectively

<sup>b)</sup> LOD and LOQ varies between different laboratories

<sup>c)</sup> Data from Spain are statistically not relevant, therefore they were not considered in the calculations

Either whole shellfish meat or only hepatopancreas were analysed. With a few exceptions with unknown date, the submissions covered samples collected and analysed during years 2003 to 2007. Overall, 12,270 sample results were considered by the CONTAM Panel for this assessment.

As prescribed in the respective EU legislation, most of the data related to pre-market control (Pre-MC) measurements, i.e. before samples are harvested for further processing or direct marketing. Germany submitted data on both Pre-MC and post-market control (Post-MC, that is samples taken from the market). The Post-MC data from Germany revealed that samples collected at stores and supermarkets had multiple (mostly unknown) origins.

### AZA concentration in shellfish

The occurrence data for AZAs were expressed normalised to whole shellfish meat. Toxicological equivalence factors (TEF) of AZA1=1, AZA2=1.8 and AZA3=1.4 were applied by Norway and Ireland to convert the individual AZAs to AZA1 equivalents. For the further assessment the data from Germany and the UK were converted by EFSA using the same TEF values as above.

Basic statistics divided by country submitting concentration data for AZAs were calculated as shown in Table 4.



**Table 4:** Statistics of LC/MS data of AZAs in shellfish (pre- and post-market samples) sampled in 2003 to 2007 provided by Member States.

Country	Number of samples	Median	Mean	P95	Maximum	≤LOD	>160 µg AZA1 equivalents/kg shellfish meat
		µg/kg shellfish meat				%	
Lower Bound <sup>a)</sup>							
Germany	394	0	1	0	42	95.4	0
Ireland	9,847	0	21	80	1,630	85.6	2.8
Norway	1,851	0	23	141	251	73.9	4.2
UK	178	0	3	25	56	84.3	0
All	12,270	0	20	90	1,630	84.1	2.9
Upper Bound							
Germany	394	6	7	6	42	95.4	0
Ireland	9,847	6	26	80	1,630	85.6	2.8
Norway	1,851	6	27	141	251	73.9	4.2
UK	178	6	8	25	56	84.3	0
All	12,270	6	25	90	1,630	84.1	2.9

<sup>a)</sup> The percentage of samples without a value is very high (84.1 %), therefore as recommended by the FAO/WHO Workshop on Exposure assessment for Chemicals in Food of May 2005, a sensitivity analysis has been performed, comparing Upper Bound and Lower Bound scenarios. The Lower Bound is obtained by assigning a value of zero to all the samples that were reported as “not detected”. The Upper Bound is obtained by assigning an averaged value of detection to all the samples that were reported as “not detected”.

The basic statistics indicate a concentration range of AZAs in the current collection of shellfish samples from European countries ranging from “not detected” to 1,630 µg/kg shellfish meat. The reported LOD varied between 1 and 10 µg/kg. The percentage of samples ≤LOD is quite large, ranging from 73.9% for Norway to 95.4% for Germany, with an average of 84.1% of all analysed data. The sensitivity analysis (see note to Table 4) showed no difference between Upper Bound and Lower Bound scenarios at the 95<sup>th</sup> percentile level which was used in the exposure assessment.

The proportion of samples exceeding the regulatory limit of 160 µg/kg is also given. It varies among countries within the range from 0% (Germany) to 4.2% (Norway) with a total average of 2.9%. However, these results cannot be considered as representative for the respective country, taking into account differences in the objectives of the investigation, the time of sample collection (pre- or post market) and the different number of samples reported.

### ***Difference between species***

Mussels were the predominant shellfish product tested, followed by oysters, clams, cockles, crabs, scallops, gastropods in decreasing order and some individual samples of other shellfish and processed shellfish. The distribution of the sum of AZAs in the different food commodities analysed is illustrated in Table 5. Out of the 12,270 samples 12,184 were considered; 86 samples

were only described as shellfish, without any further specification, and therefore not considered in the distribution between species.

**Table 5:** Statistical descriptors for AZAs results in different shellfish products pre- and post-market samples.

Species	Number of samples	Total concentration of AZAs (µg/kg)				≤ LOD (%)	>160 AZA1 equivalents µg/kg shellfish meat (%)
		Median	Mean	95 <sup>th</sup> percentile	Max		
Mussels	8,044	6	31	130	1,630	83.5	4.1
Oysters	3,066	6	13	50	290	83.6	0.2
Clams	728	6	7	6	90	95.2	0
Cockles	158	6	8	20	80	94.3	0
Scallops	64	6	15	66	162	84.4	1.6
Gastropods	22	6	8	6	60	95.5	0
Crabs <sup>a)</sup>	102	40	70	250	250	39.2	15.7 <sup>b)</sup>

<sup>a)</sup> Currently not regulated

<sup>b)</sup> Based on brown meat and not on whole flesh

The number of samples that exceeded the regulatory limit of 160 µg/kg varied from 0 % for clams and cockles to 4.1% for mussels. As indicated in Table 5, crabs (*Cancer pagarus*) show concentrations at the median, mean and 95<sup>th</sup> percentile significantly higher than the other species. However, these data can not be directly compared with the other samples as the levels represent AZA group toxins in the brown meat (digestive organ) rather than whole flesh. This is because the brown meat (which generally makes up 30-40% of the whole flesh) is often consumed as such and separately from the remaining flesh. Crabs are currently not included in the EU regulation on marine biotoxins and thus the regulatory limit of 160 µg/kg is not applicable. Nevertheless, the high number of samples (even after conversion to whole flesh) which exceed the level of 160 µg/kg indicates the potential significance of elevated exposure to AZA group toxins for high level consumers of these species.

### *Influence of type of sampling*

The German data subset covered samples from the local monitoring programme including information on pre- and post-market control, therefore it was evaluated separately. For comparative purposes the analysis was limited to mussels and oysters (the only species present in the pre-market set). These data represent results from 2005 and 2006 (Table 6). Of the 243 mussels and oysters samples measured by means of LC-MS/MS 46 were pre-market and 197 were post-market controls.

**Table 6:** Overview of LC-MS/MS data of AZAs obtained from the German official surveillance programme of food control.

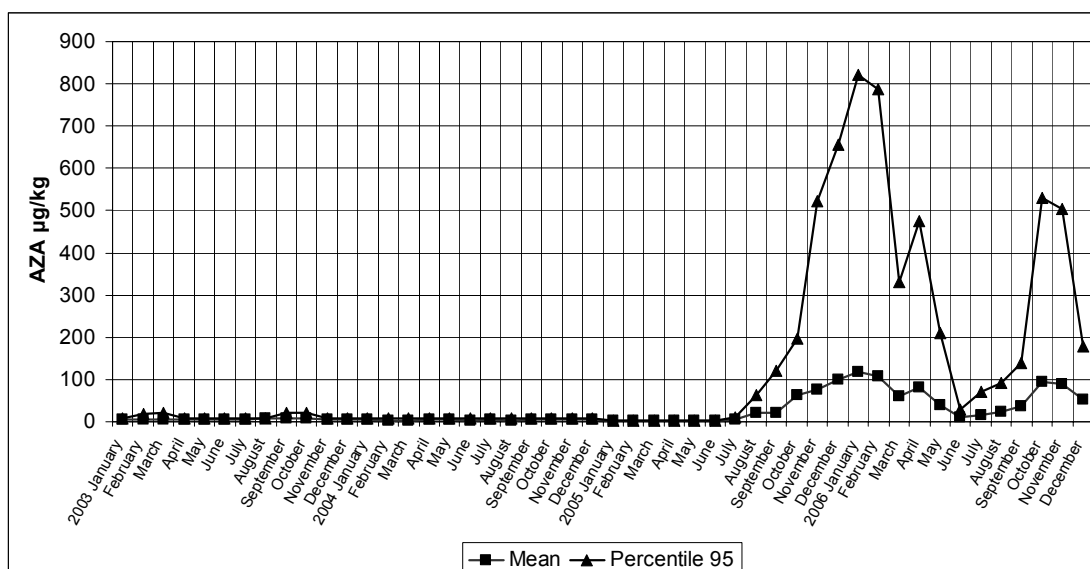
	Type of sampling	
	Monitoring (pre-MC) <sup>a)</sup>	Random (post-MC) <sup>a)</sup>
N	46	197
Median µg/kg	6	6
Mean µg/kg	8	7
95th percentile µg/kg	17	17
Max µg/kg	42	41
< LOD (%)	91.3	92.9
>160 µg AZA1 equivalents/kg shellfish meat	0	0

<sup>a)</sup> Pre-market control (Pre-MC) and post-market control (Post-MC) cover samples taken before products are sent to the market and product sampled at the market, respectively

The data from the post-marketing investigations of samples originating from other countries only revealed relatively low levels which might be an indication that the pre-marketing control to a great extent prevents lots with high concentrations from reaching the market. All German samples were cooked before analysis.

### Seasonal changes

The seasonal trend was investigated using all the Irish data from 4 years (9,847 samples dating 2003-2006). The trend is shown in Figure 3.



**Figure 3.** AZA occurrence in Ireland in the period 2003-2006.

The trend through different years is not repeatable and the occurrence seems to be in the form of separate outbreaks. The present data are not sufficient to propose a correlation between probability of outbreaks and time of the year.

## 6. Comparison of LC-MS/MS data with results of mammalian bioassays

A comparison of LC-MS/MS data with MBA results has been performed using 9,812 samples originating from Ireland, Norway and the UK that were tested with MBA. Out of these samples 966 were positive and 8,846 negative. The results are reported in Table 7.

**Table 7:** Concentration of AZAs measured by LC-MS/MS in samples comparatively tested by mammalian bioassays.

Mouse Bioassay	Number of samples	Concentration ( $\mu\text{g}$ AZAs/kg shellfish meat) determined by LC-MS/MS				$\leq \text{LOD}^{\text{b}}$ %	$>160 \mu\text{g/kg}$ N (%)
		Median	Mean	P95	Max		
Negative	8,846	6	12	40	$\geq 880^{\text{a}}$	88.8	47 (0.5)
Positive	966	6	132	800	1630	56.2	202 (20.9)

<sup>a)</sup> 880  $\mu\text{g/kg}$  was the upper limit of determination for the set of analyses that contributed to this value; for Ireland it is known that the positive MBA results were influenced by other toxins.

<sup>b)</sup> 91.5% of the MBA-negative samples has a value  $\leq \text{LOQ}$ .

As can be seen from Table 7, the majority of the MBA negative samples were well below the regulatory limit and these data are not informative with regard to the ability of the MBA to detect AZAs in the region of the regulatory limit. Of the samples tested negative in the MBA 0.5% had levels higher than 160  $\mu\text{g/kg}$  AZA1 equivalents/kg shellfish meat. The high proportion of samples with positive MBA but AZA levels below LOD (56.2 %) indicates a contribution of other lipophilic toxins, such as okadaic acid group toxins, yessotoxins, pectenotoxins or cyclic imine group toxins, or combinations thereof, to the positive response of the MBA. It can be assumed that all bivalve molluscs showing a negative response in mammalian bioassays will reach the market and will thus be consumed. From this perspective, it is not unrealistic to estimate the dietary intake of AZAs based on the LC-MS/MS data for those samples that tested negative in the mammalian bioassays.

## 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 8. The mean portion sizes for consumers only ranged between 10 g (France,

bivalve molluscs) and 136 g (The Netherlands). The data from Germany, Italy and the UK are within this range.

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 95<sup>th</sup> percentile of 400g among mussel consumers. The maximum portion size reported in this study was 1,500 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 United Kingdom 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 8:** Shellfish eating habits in France, Italy, The Netherlands, the UK, and Germany, based on national food consumption surveys.

		Number of consumers N (%)	Number of eating occasions for consumers /year	Mean portion weight (g)		Maximum portion weight (g)	Maximum frequency
France (7 days)	INCA 1999	- (11)	NA	10			NA
France (FFQ)	CALIPSO (bivalve molluscs)	962/997 (96)	NA	32	94	415	NA
France (FFQ)	CALIPSO (mussels)	862/997 (86)	NA	22	70	245	NA
Italy (7 days)	INN-CA 1994-96	212/1,981 (11)	47	83		1,000	4/week
Germany (7 days)	NVS 1985-88	150/23,239 (0.6)	171	107	400	1,500	3/week
UK (7 days)	NDNS 2000-01	212/1,631 (13)	51	114		239	4/week
The Netherlands (2 days)	DNFCS 1997-98	47/4,285 (1.1)	39	136	465	480	NA

Because AZAs have acute toxic effects, it is important to identify a high portion size rather than a long term average consumption in order to protect the health of the consumer. In the studies presented in the Table 8, the maximum reported sizes are in the range of 239 to 1,500 g. The Panel noted the highest portion sizes of 1,000 g and 1,500 g, and considered it likely that the shells were included in these weight estimates. Therefore, the CONTAM Panel considered the 95<sup>th</sup> percentile as a more realistic estimate of the portion size for high consumers. As shown in Table 8 the 95<sup>th</sup> percentile values range from 70-465 g and the CONTAM Panel chose the figure of 400 g to be used as a high portion size in acute exposure assessments. It should be noted that

this figure is at the higher end of the range of the 95<sup>th</sup> percentile reported by the Member States and is therefore likely to cover a higher percentile for the entire EU. 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 highest 97.5<sup>th</sup> percentile portion size for consumers only.

## 8. Exposure assessment

### Deterministic estimate of dietary exposure to AZAs

Consumption of a 400 g portion containing the 95<sup>th</sup> percentile of the AZA concentration from the negatively MBA tested samples, 40 µg/kg, as presented in Table 7, would result in an exposure of 16 µg AZA1 equivalents per person (equivalent to 0.25 µg/kg b.w. for 60 kg adult). For concentrations at the current regulatory limit of 160 µg AZA1 equivalents/kg shellfish meat consumption of a 400 g portion would result in an exposure of 64 µg AZA1 equivalents per person (equivalent to 1 µg/kg b.w. for 60 kg adult).

These results are conservative but not unrealistic estimates of AZAs dietary exposure in the EU.

### Probabilistic estimate of dietary exposure to AZAs

A probabilistic estimate of dietary exposure to AZAs has been performed by a Monte Carlo simulation using the distributions of both the occurrence data (summarised in Table 7) and the data on the consumption of shellfish. Compared to the deterministic estimate the probabilistic exposure estimate provides information on the chance to exceed a specific exposure level. Because a person eating shellfish will not eat the same portion size containing the same level of toxins each time, the probabilistic calculation includes all the combinations of all different occurrence and consumption data.

The results of the samples tested negative in the MBA (Table 7) also formed the basis for the probabilistic estimate<sup>11</sup>. It can be assumed that all bivalve molluscs showing a negative response

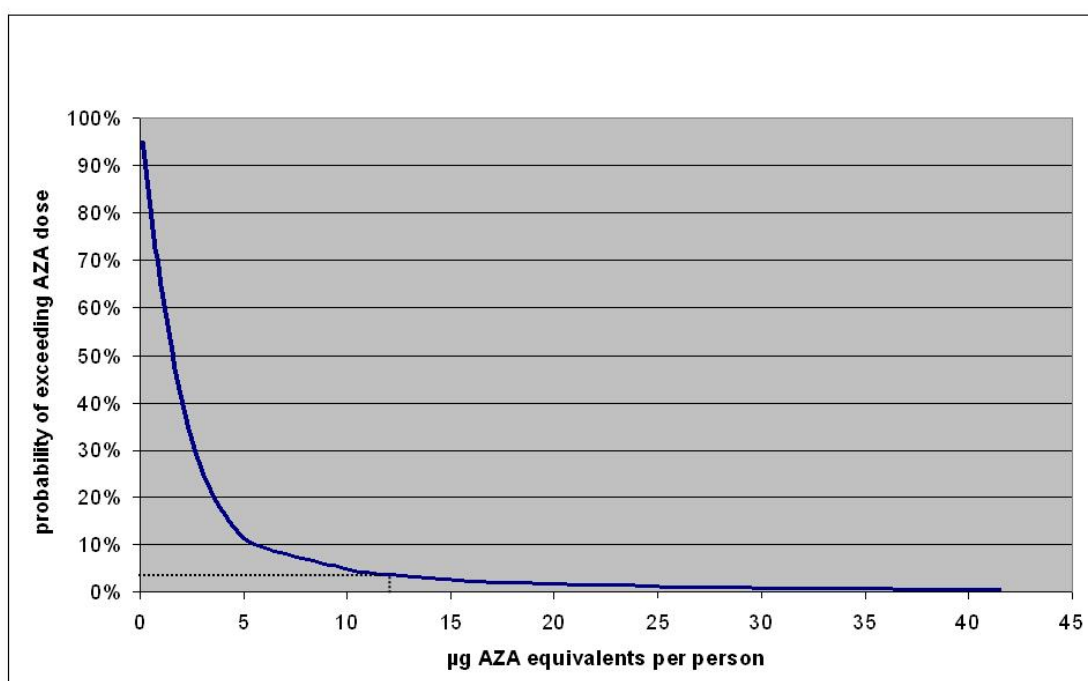
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<sup>11</sup> All samples with quantified levels (>LOQ) of AZAs (8.5 % of the total number) were described using a loglogistic distribution, which has been derived by the best fit analysis of the @RISK tool. This distribution function was truncated at 20 µg/kg [=RiskLoglogistic(21,86; 27,048; 1,6383; RiskTruncate(20;))]

The values below LOQ (20 µg/kg) were characterised as follows: a random assignment of the values was performed using a discrete distribution [RiskDiscrete({0;1};0915; 0.085] reflecting the number of samples (91.5%) at or below the LOQ and the number of samples (8.5 %) with quantified toxin concentrations. This means that the same ratio of non-quantified/quantified samples (91.5%/8.5%) was applied to characterise the values below the LOQ, leading to 91.5% values attributed as “0” (zero) and 8.5% between 0 and 20 µg/kg. These latter samples were simulated using a uniform distribution function [RiskUniform(0;20)].

in mammalian bioassays will reach the market and will thus be consumed. From this perspective, it is not unrealistic to estimate the dietary intake of AZAs based on the LC-MS/MS data for those samples that tested negative in the mammalian bioassays.

Because insufficient information is available on the distribution of portion sizes, the 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 8 the Panel chose a value of 100 g to be used as “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.



**Figure 4:** Probability of dietary exposure to AZAs resulting from consumption of a single portion of shellfish.

The resulting probabilistic dietary exposure distribution has a median value of approximately 1.6 µg/person, a mean of approximately 3 µg/person, and a 95<sup>th</sup> percentile of approximately 10 µg/person. The probabilistic exposure estimate presented in Figure 4 illustrates the chance to exceed a specific level of exposure to AZA equivalents when consuming a single portion of shellfish. The chance to exceed an intake of 12 µg corresponding with the ARfD for AZAs established in chapter 12 is about 4 %, as shown by the dotted line (Figure 4).

Based on the results of probabilistic dietary exposure distribution it is calculated that the chance to exceed the deterministic dietary exposure estimate of 64 µg AZA1 equivalents per person,



corresponding to a consumption of a 400 g portion containing AZA1 equivalents at the level of the current EU limit value, is 0.13 %. The chance to exceed an exposure of 16 µg AZA1 equivalents per person, corresponding to a consumption of a portion of 400 g containing the 95<sup>th</sup> percentile (40 µg/kg) of the AZA concentration, is about 2 %.

## 9. Toxicokinetics

AZA1 was given via gastric intubation at single doses of 100, 200 or 300 µg/kg b.w. to groups of six NMRI female mice (approximately 20g body weight) (Aasen 2008; Aune 2008). Tissue concentrations of the toxin, in µg/kg tissue (wet weight), were examined 24 hrs (three mice/dose) and seven days (three mice/dose) after intubation. AZA1 was detected in all organs examined except the brain.

The tissue concentrations increased in a dose dependent manner. After 24 hrs, the highest levels outside the gastrointestinal (GI) tract were found in spleen and kidneys (91 and 84 µg/kg), respectively. In descending order, the AZA1 concentrations at the highest dose were the following, lungs (72 µg/kg), heart (36 µg/kg) and liver (31 µg/kg). The highest levels of AZA1 after 24 hrs were found in the GI tract tissues; stomach (524 µg/kg), followed by duodenum (143 µg/kg), jejunum (37 µg/kg), ileum (34 µg/kg) and colon (14 µg/kg). The GI tract tissues were not thoroughly cleaned of food residues which may have contributed.

After seven days, AZA1 levels had decreased dramatically. In the stomach, at the highest dose, the AZA1 level was 11 µg/kg while the corresponding levels in the other GI tissues were: duodenum (22 µg/kg), jejunum (23 µg/kg), ileum (21 µg/kg) and colon (17 µg/kg).

The AZA1 levels in the other organs seven days after intubation of 300 µg/kg body weight were: kidneys (82 µg/kg), spleen (41 µg/kg), lungs (33 µg/kg), liver (8 µg/kg) and heart (4 µg/kg).

## 10. Toxicity data

### 10.1 Mechanistic considerations

The gastrointestinal tract is the main toxicological target of AZAs (Ito *et al.*, 2000). Also the lymphatic system and the liver are toxicological targets as well as other organs at higher doses. While the molecular effects of AZAS have been investigated in various cellular systems *in vitro*, the molecular mechanisms of their toxicity *in vivo* are presently unknown.

Available data obtained in the last five years, using cultured cells, show that AZAs can induce cytotoxicity and affect several cellular processes, with a notable degree of variation with regard to the effective concentrations and the time frames of responses (Table 9). Differences in properties of the cells used may partially explain these variabilities.

**Table 9:** Molecular responses elicited by azaspiracid-1 in cultured cells.

Effects	Cell lines	Effective doses (EC <sub>50</sub> , M)	Time frame	Reference
Increase of Ca <sup>2+</sup> <sub>i</sub>	Lymphocytes	10 <sup>-7</sup> -10 <sup>-6</sup>	10 min	Román <i>et al.</i> , 2002
	Primary neurons	10 <sup>-6</sup>	5 min	Vale <i>et al.</i> , 2007a
Increase of cAMP	Lymphocytes	10 <sup>-7</sup> -10 <sup>-6</sup>	10 min	Román <i>et al.</i> , 2002
Decrease in pH <sub>i</sub>	Primary neurons	10 <sup>-6</sup>	5 min	Vale <i>et al.</i> , 2007a
Cytotoxicity	Jurkat	10 <sup>-9</sup>	48 hr	Twiner <i>et al.</i> , 2005
	NCI-H460	10 <sup>-6</sup>	48 hr	Vilariño <i>et al.</i> , 2006
	BE(2)-M17	5 x 10 <sup>-8</sup>	48 hr	Vilariño <i>et al.</i> , 2006
	MCF-7	10 <sup>-9</sup>	48 hr	Ronzitti <i>et al.</i> , 2007
	Primary neurons	10 <sup>-9</sup>	24 hr	Vale <i>et al.</i> , 2007a
Altered F-actin cytoskeleton	BE(2)-M17	10 <sup>-5</sup>	1 hr	Román <i>et al.</i> , 2002
	Jurkat	10 <sup>-8</sup>	24 hr	Twiner <i>et al.</i> , 2005
	Caco-2	5 x 10 <sup>-8</sup>	48 hr	Vilariño <i>et al.</i> , 2006
Cell adhesion	Caco-2	5 x 10 <sup>-8</sup>	48 hr	Vilariño <i>et al.</i> , 2006
	MCF-7	10 <sup>-9</sup>	24 hr	Ronzitti <i>et al.</i> , 2007
	Fibroblasts	10 <sup>-9</sup>	24 hr	Ronzitti <i>et al.</i> , 2007

A prominent cytotoxic effect of AZA1 has been described in a variety of cellular systems, with effective doses ranging between 10<sup>-9</sup> and 10<sup>-6</sup> M, depending on the cell line (Twiner *et al.*, 2005; Vilariño *et al.*, 2006; Ronzitti *et al.*, 2007; Vale *et al.*, 2007a).

AZA at µM concentrations increased the intracellular Ca<sup>2+</sup> concentrations in human lymphocytes and primary cultures of cerebellar granule cells within minutes (Román *et al.*, 2002; Vale *et al.*, 2007a).

AZA1 has also been shown to cause an increase in cellular levels of cAMP in human lymphocytes (Román *et al.*, 2002) and an increase in nuclear levels of phosphorylated (active) c-Jun-N-terminal Kinase (JNK/SAPK) in primary cultured neurons. Treatment of these cells with the JNK/SAPK inhibitor SP 600125 prevented the cytotoxic effect of AZA1 (Vale *et al.*, 2007b).

Increased transcription of genes coding for enzymes involved in cholesterol and fatty acid synthesis have been observed in human lymphocytes by microarray analysis (Twiner *et al.*, 2008).

Alterations of F-actin based cytoskeletal structures with some degree of cell-specificity have been found in model systems upon AZA1 treatment (Román *et al.*, 2002; Twiner *et al.*, 2005; Vilariño *et al.*, 2006; Ronzitti *et al.*, 2007). Furthermore, AZA1 at nM concentrations has been

shown to alter cell adhesion (Vilariño *et al.*, 2006; Ronzitti *et al.*, 2007), and to induce the accumulation of a fragmented form of E-cadherin, which is responsible for cell-cell adhesion of epithelial cells (Ronzitti *et al.*, 2007; Nollet *et al.*, 2000).

It is unclear how the various cellular effects are linked to the observed toxic effects *in vivo*. However, the gastro-intestinal effects including the destruction of intestinal epithelia (Ito *et al.*, 2000), might be explained by the alterations induced in cytoskeletal structures, focal adherens structures, and the E-cadherin system, with disruption of cell-cell- and cell-matrix interactions, and a perturbation of the intestinal barrier function (Vilariño *et al.*, 2006; Ronzitti *et al.*, 2007; Román *et al.*, 2002; Twiner *et al.*, 2005).

## 10.2 Effects in laboratory animals

The information on toxicological effects of the AZAs in laboratory animals is limited due to the low availability of pure toxins. Studies on acute toxicity following *i.p.* administration have been performed with AZA1 to AZA5. However, concerning oral toxicity, only studies on AZA1 are available. The main toxicological target appears to be the intestine. In addition, effects in the liver and lymphatic system have been observed. At high doses multiple organs may be affected.

### Short term studies:

#### *Intraperitoneal studies:*

The lethal *i.p.* dose of purified AZA1 in male ddY mice (n=2) was 200 µg/kg b.w. (Satake *et al.* 1998). Lethal doses found for AZA2 and AZA3 were 110 and 140 µg/kg, respectively (Ofuji *et al.* 1999). AZA4 and AZA5 (the 3- and 23-hydroxylated analogues of AZA3), appear to be less toxic than AZA3, with lethal *i.p.* doses of approximately 470 and 1000 µg/kg, respectively (Ofuji *et al.*, 2001).

The information on acute *i.p.* lethal doses has to be considered as indicative only, since the number of animals used was low due to the scarcity of pure toxins.

#### *Oral studies:*

AZA1 extracted from blue mussels (*M. edulis*) collected in Killary Harbour in 1996 and purified as described by Satake *et al.* (1998) was used by Ito *et al.* (2000). Male ICR mice (3-8 weeks old) were dosed by gavage with toxin diluted in 0.2 mL saline. Mice were treated with 500 µg/kg b.w. (n=7), 600 µg/kg b.w. (n=6) or 700 µg/kg b.w. (n=2) (Ito *et al.*, 2000). Eight hours after treatment with 600 or 700 µg/kg b.w., villi were shortened through loss of upper parts, and injury included both lamina propria and epithelial cells in small intestine. After 24 hours, epithelial cells showed signs of recovery, while lamina propria was slower to recover. By 24 hours, the liver weight had increased by 38% in mice (n=3) treated with 500 µg/kg b.w., and fine fat

droplets were distributed in the liver. Dose-dependent necrotic lymphocytes in the thymus, spleen and Peyer's patches of the small intestine were seen at 500 to 700 µg/kg b.w. Because of unclear information in the paper by Ito *et al.* (2000) on the number of mice that was used for histopathological studies during the first 24 hrs, it is difficult to interpret the information on lethal oral doses. However, it appears as if all the mice treated with 500 µg/kg b.w. survived 24 hrs, while half the number of mice at 600 µg/kg b.w. and 700 µg/kg b.w. died.

In the same study five mice were treated with 300 µg AZA1/kg b.w. in order to study progressive changes in the intestine at sublethal doses. After 1 hour no microscopic changes were observed in the intestine, whereas congestion of the small intestine appeared macroscopically after 3 hours. Four hours after administration, watery substances were seen in the lumen. At the same time small changes of surface epithelial cells in the small intestine could be observed by scanning electron microscopy. Histopathological examinations by light microscopy showed atrophic lamina propria spatially separated from epithelial cells and prominent vacuolization of epithelial cells. Accumulation of fat droplets in the liver was seen 1 hour after treatment (Ito *et al.*, 2000).

In another study by the same group (Ito *et al.*, 2002) 25 mice of different ages were exposed orally to single doses of AZA1 at 250, 300, 350 or 450 µg/kg b.w. by gastric intubation. Five of sixteen 4 week old mice at the highest dose survived, while all of the 4 week old mice at 300 µg/kg b.w. and the 6 week old mice at 350 µg/kg b.w. survived. However, both the 6 week old mice at 300 µg/kg b.w. and both the 5 month old mice at 250 µg/kg b.w. died. The 10 surviving mice from the initial AZA1 exposure were administered AZA1 a second time at doses ranging from 250-450 µg/kg and observed for recovery. Only one mouse died (dosed 350 µg/kg), while the others were sacrificed between 7 and 90 days. The recovery was very slow: erosion and shortened villi persisted in the stomach and small intestine for more than three months: in the lung, oedema, bleeding and infiltration of cells in the alveolar wall persisted for 56 days: in the liver, fatty changes were observed for 20 days: in the thymus and spleen, necrosis of lymphocytes was seen for 10 days.

In a recent study using synthetic AZA1 (Ito *et al.*, 2006), a lethal oral dose in 4 week-old mice (n=4) was estimated to be >700 µg/kg.

In a recent study (Aune, 2008; Aasen, 2008), six NMRI female mice (approximately 20g) were given a single oral dose of AZA1 (isolated from fresh shellfish) at 100, 200 or 300 µg/kg b.w.. Half the number of mice was sacrificed after 24 hrs, the other half after seven days. None of the mice displayed any overt signs of toxicity or diarrhoea.

The outcome of the reported studies on oral toxicity of AZA1 towards mice indicate that single oral doses causing lethality vary from 250 to 600 µg/kg. The extent to which the age of the animals is of importance is unclear.

### 10.3 Longer-term toxicity/Carcinogenicity

In the study by Ito and coworkers (Ito *et al.*, 2002) the long term effects of repeated exposure to AZA1 by oral gavage were examined. Groups of 10, 10, 5 and 6 four week old ICR male mice were exposed to oral doses of 50, 20, 5 and 1 µg AZA1/kg b.w., respectively, twice a week, up to 40 times, within 145 days. The mice that survived 40 treatments were kept for up to three months after withdrawal. Nineteen untreated mice were used as controls.

Mice in the two highest dose groups that died or were killed during the exposure period showed decreased body weight, ballooning and gastrointestinal organs containing a lot of gas. Pathological changes were observed in multiple organs; lung (interstitial inflammation and congestion), stomach (erosion), small intestine (shortened villi, oedema and atrophic lamina propria) and liver (some cases - single or focal necrosis, small inflammation, mitosis or congestion). A few lung tumours were observed among mice in the two highest dose groups, but due to the widespread toxic effects of the two highest doses, only results from the two lowest doses are considered.

No signs of weakness or illness were observed in mice from the 5 and 1 µg/kg b.w. groups (Ito *et al.*, 2002). All the mice in the two lower dose groups survived 40 treatments and an observation period of 3 months. No lung tumours were seen in the two lower AZA-dose groups or in any of the nineteen control mice. In the group exposed to 5 µg/kg b.w. one mouse showed constipation and tissue damage in the large intestine and all five mice had small intestinal erosions, which the authors attributed to unhealed injuries. In the group exposed to 1 µg/kg b.w., one of six mice had a hyperplastic nodule in the liver, with many mitotic cells present outside the nodule (Ito *et al.*, 2002).

To further examine potential carcinogenicity of AZA1, another experiment was conducted on 95 ICR mice using repeated dosing (Ito 2004). Group (1) 20 µg/kg b.w. x 2 per week (n=20, 40 doses), (2) 20 µg/kg b.w. x 2/w (n=10, 33 doses), (3) 5 µg/kg b.w. x 2/w (n=22, 40 doses), (4) 10 µg/kg b.w. x 1/w (n=23, 20 doses), (5) 5 µg/kg b.w. x 1/w (n=20, 20 doses), and control group (n=52). The dose levels in the Group (1) and (2) were changed depending on the health condition of the mice as follows, group 1 (20 µg/kg b.w. x 17 doses + 10 µg/kg b.w. x 23 doses) and group 2 (20 µg/kg b.w. x 13 doses + 15 µg/kg b.w. x 5 doses + 10 µg/kg b.w. x 5 doses), then each mouse that survived up to 20-weeks was dosed (1) 19.2, (2) 19.57, (3) 8.24, (4) 7.66 and (5) 4.34 µg/kg b.w., respectively.

Sixty-six mice were sacrificed at 8 months, but no tumours were observed among them. Among the residual 20 mice, comprising 10 mice of group (2), 6 mice of group (3) and 4 mice of group (4), five tumours appeared within 1 year. These 5 tumours comprise 2 malignant lymphomas and 3 lung tumours (one adenocarcinoma and two epithelial type tumours), in comparison with one lung tumour in the control mice (n=52). Multiple lymphatic nodules in the lung were observed in 10 out of 27 mice at 8 months from group (1) and (3). The tumour incidences of the

treated groups were not statistically different from that of the controls. The Panel noted that the tumours were observed at levels also causing severe toxicity and therefore of limited relevance.

#### 10.4 Genotoxicity

No data on genotoxicity have been reported for azaspiracids.

#### 10.5 Relative potency of analogues

According to the very few data available on acute lethal doses of AZA1 to AZA5, following *i.p.* administration to mice (see chapter 10.2), the following toxic equivalence factors (TEF) relative to AZA1 have been applied in some countries where LC-MS/MS is used to supplement the MBA: AZA1 = 1.0, AZA2 = 1.8, AZA3 = 1.4, AZA4 = 0.4, AZA5 = 0.2.

The CONTAM Panel concluded that the available data are not sufficient to establish robust TEF values but adopted these TEF values assuming that the various AZA analogues have the same mechanism of action, in order to provide a best estimate of the toxicity of AZAs. These TEF values should be revised when studies on acute toxicity for all five AZA analogues are available.

### 11. Observations in humans

The symptoms of AZA poisoning (AZP) are nausea, vomiting, diarrhoea and stomach cramps. Five incidents of AZP have been reported, from the Netherlands, Ireland, Italy, France and the UK.

In November 1995, 8 people in the Netherlands became ill after eating mussels harvested on the west coast of Ireland. Tests for known shellfish toxins failed to identify the cause at that time (McMahon and Silke, 1996). Subsequent investigations identified a toxin initially named as spiramino acid, which was associated with a similar outbreak in November 1997 in consumers of mussels from the Arranmore Island region of northwest Ireland (McMahon and Silke, 1998). In this incident, it was estimated that 20-24 consumers were affected, with 8 of these confirmed following consultation with a physician. All made a complete recovery after 2-5 days. FSAI (2006) noted that symptoms of AZP were associated with consumption of “as few as” 10-12 mussels, but it is unclear whether this was in a single individual or more.

Because the toxin was unidentified at the time of the outbreak it was not possible to estimate the exposure to the toxin, either from left-over mussels or from mussels harvested in the same region immediately after the incident.

The Food Safety Authority of Ireland (FSAI) first performed a risk assessment of AZAs in shellfish in 2001 (FSAI, 2001). This was based on LC-MS data on levels of AZAs present in the



hepatopancreas of mussels collected from Arranmore in the months following the incident. Mussels were first collected 1-2 months after the incident and collections continued at regular intervals over the following 6 months. AZA1 levels were in the range of 5.7-10.7 µg/g hepatopancreas. In order to estimate the likely AZA1 intake of the individual(s) who became ill, FSAI (2001) applied probabilistic modelling to the distributions of the data relating to AZA1 concentrations in the hepatopancreas of the mussels and possible consumption, i.e. 10, 11 or 12 mussels, together with a number of assumptions related to the total amounts of AZAs likely to have been consumed. An assumption was made in relation to the likely proportion of AZA1 in whole flesh relative to hepatopancreas, based on expert opinion. To estimate the total concentration of AZAs likely to have been ingested, the assessment assumed a single value for the proportion of AZA2 (28.9%) and AZA3 (27.7%) relative to AZA1 likely to be present within contaminated mussels, based on concentrations measured in an Arranmore mussel sample collected 2 months after the incident. Evidence at that time suggested that AZA was deactivated by as much as 71% during cooking, and therefore the estimated AZA concentrations were reduced by this amount. Results of the modelling suggested that the AZA intake was likely to have been between 6.7 µg (5<sup>th</sup> percentile) and 24.9 µg (95<sup>th</sup> percentile) per person, with a median of 14.5 µg per person. These calculations were based on an assumption that the relative toxicities of AZA1, AZA2 and AZA3 were similar. FSAI (2006) noted that if AZA2 and AZA3 toxicities were higher than AZA, this assumption would result in lower estimates of total AZA intake and therefore a more conservative approach.

More recently the Scientific Committee of the Food Safety Authority of Ireland (FSAI, 2006) has re-estimated the AZA intakes associated with illness in the Arranmore incident. This assessment took into account relevant data published since the 2001 assessment which resulted in fewer assumptions than used in the earlier assessment. These data related to three key areas: the relative distribution of AZAs between hepatopancreas and total flesh of mussels, the ratios of different AZAs in mussel tissue, and the influence of cooking on AZA concentrations in mussels.

While expert opinion on the likely proportions of AZAs in mussel hepatopancreas and whole flesh had been used in the 2001 assessment, a recent publication had reported a series of measurements of hepatopancreas:whole flesh ratios in 28 mussel samples collected in Ireland between 2001 and 2003 (Hess *et al.*, 2005). These data suggested that AZA1 levels in the mussels consumed in the Arranmore incident may have been higher than originally estimated.

Information from the 2005 Irish biotoxin monitoring programme had generated a range of 75 different proportions for AZA2 and AZA3 relative to AZA1. These new data were incorporated into the exposure assessment rather than the single proportion used in 2001. Recent data indicate that steaming of raw fresh mussels results in a 2-fold higher concentration of AZAs in the cooked flesh (whole flesh and hepatopancreas) compared with the uncooked flesh (Hess *et al.*,



2005). This was attributed to the loss of water/juice from the mussels. On this basis, it was considered appropriate to calculate mussel consumption by individuals during the Arranmore incident in terms of raw weight rather than having to account for the reduction in mussel meat weight during cooking (approximately 50%). However, it was acknowledged that a degree of uncertainty remained in this part of the exposure assessment due to a lack of knowledge on mussel meat weight in the Arranmore growing site in 1997.

The use of these new data, together with consumption of 10, 11 or 12 mussels, in the probabilistic approach used by FSAI (2006) resulted in a substantially higher estimate of the AZA intake associated with AZP in the Arranmore incident compared with previous assessments. The revised estimate of AZA intake associated with human illness was calculated to be between 50.1 µg (5th percentile) and 253.3 µg (95th percentile), with a median of 113.4 µg per person. The CONTAM panel noted that, as in its 2001 assessment, the FSAI (2006) estimates assumed that the relative toxicities of AZA1, AZA2 and AZA3 were similar. The panel considered that this would have a minor impact on the estimates. For example if AZA2 was present at 25% of the total AZA, it would contribute an additional 20% AZA1 equivalents (25 multiplied by additional TEF of 0.8). AZA3 tends to be present at much lower concentrations and would contribute even less.

Other incidents were reported in September 1998 in Italy (approximately 10 cases), in September 1998 in France (20-30 cases) and in August 2000 in the UK (12-16 cases), all related to consumption of mussels or scallops imported from Ireland (James *et al.* 2004). There are no estimates of amounts of AZAs consumed in these incidents. A legislative limit for AZAs was implemented in Ireland and some other countries in 2001 and no outbreaks had been reported until March 2008. In April 2008, an outbreak occurred in France where 219 people suffered from AZP following the consumption of Irish mussels. Confirmatory analyses by LC/MS of positive MBA samples showed that the total AZA levels in 3 samples taken from consumed batches were about 5 times higher than the regulatory limit. Only the 3 regulated AZAs were searched for and the results were expressed as AZA1 equivalent using TEFs (AZA1 = 1, AZA2 = 1.8, AZA3 = 1.4). The results were reported in an EU RASFF alert (ref 2008.0426-add11). At the time this opinion was written more details about this incident were not available to the Panel.

## 12. Hazard characterisation

In a few limited repeated-dose toxicity studies of longer duration (maximum duration 1 year) occasionally lung tumours were observed. These tumours were only observed at doses causing severe toxicity, and therefore the Panel considered this observation of limited relevance. No data on genotoxicity have been reported for AZA.

The Panel considered that the available toxicity data relating to repeat-dose administration of AZAs were inadequate to establish a Tolerable Daily Intake (TDI). Because the toxicity of AZAs has been observed in humans following consumption of a single portion of affected mussels, it was considered appropriate to establish an acute reference dose (ARfD), based on the human data.

The EC Working Group on Toxicology of DSP and AZP considered the initial exposure estimates of FSAI (2001) in the light of new data suggesting that AZA levels in shellfish are not reduced during cooking (EU/SANCO, 2001). The range for the lowest-observed-adverse effect level (LOAEL) was recalculated as being between 23 µg (5th percentile) and 86 µg (95th percentile). The EC Working Group on Toxicology of DSP and AZP applied an uncertainty factor<sup>12</sup> of three, to convert the LOAEL to a no-observed-adverse effect level (NOAEL) and accounting for individual variation, to these values to derive an ARfD within the range of 7.7 µg and 28.7 µg per person, or between 0.128 and 0.478 µg of AZA1equivalents/kg b.w. for a 60 kg b.w. adult.

The CRL-MB Working Group on Toxicology derived an ARfD of 0.127 µg/kg b.w. for AZAs, based on the lower end of the LOAEL range of 23 µg/person, first converted to 0.38 µg/kg b.w. for a 60 kg b.w. adult, and an uncertainty factor<sup>12</sup> of three (CRL-MB, 2005).

FAO/IOC/WHO (2004) established a provisional ARfD for AZAs of 0.04 µg/kg b.w., based on the lower end of the LOAEL range of 23 µg per person and a 60 kg b.w., and applying a 10-fold safety factor to take into consideration the small number of people for whom data were available.

The FSAI Scientific Committee (FSAI, 2006) applied a safety factor of three to the recalculated median AZA intake estimate associated with AZP (113.4 µg per person) to derive an ARfD of 0.63 µg/kg b.w., assuming a 60 kg b.w. This uncertainty factor<sup>12</sup> was applied to account for possible intra-species variation in the toxicodynamic effects of AZAs. It was considered that a further uncertainty factor<sup>12</sup> was not required for intra-species variation in toxicokinetics, due to an absence of clear evidence for metabolism resulting in a more toxic compound. It was also suggested that metabolic activation is unlikely as the toxicity of AZA is targeted to the gastrointestinal tract.

The FSAI Scientific Committee (FSAI, 2006) noted that the derived ARfD of 0.63 µg/kg b.w. is comparable to the maximum intake value of 0.67 µg/kg b.w. for a 60 kg individual consuming 250 g of mussels at the current regulatory limit of 160 µg/kg shellfish flesh. It was considered

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<sup>12</sup> The CONTAM Panel prefers the term uncertainty factor but noted that other committees had used the term safety factor

that the validity of the proposed ARfD was supported by the absence of reported incidents of AZP since the introduction of the 160 µg/kg shellfish flesh regulatory limit for AZAs, despite evidence that approximately 216,000 portions of oysters have been legally placed on the market with AZA levels between 100 and 160 µg/kg shellfish flesh. It was also suggested that this information could be viewed as crude evidence of a much wider epidemiological data set than that provided by the Arranmore incident alone, indicating that a larger uncertainty factor<sup>12</sup> was not required to account for the small number of people involved in the Arranmore incident for whom epidemiological data are available.

The CONTAM Panel decided in its evaluation that it was appropriate to establish an ARfD based on the most probable estimate of a LOAEL resulting in AZP, i.e. 113 µg AZA1 equivalents per person (1.9 µg AZA1 equivalents/kg b.w. for a 60 kg adult), and that uncertainty factors were required to extrapolate from a LOAEL to a NOAEL, and also for variability within the human population. A factor of three was selected for the LOAEL to NOAEL extrapolation, because the effects at the LOAEL were reversible, and therefore likely to be close to the NOAEL. The usual factor of 10 for human variability was not required because the reported incidents were expected to have occurred in sensitive, rather than average, individuals. However, an additional factor of three was applied because the available quantitative data related to a small number of individuals from a single incident only.

Overall the Panel concluded that a combined uncertainty factor of nine should be applied to allow for LOAEL to NOAEL extrapolation and for human variability.

Rounding to one significant figure in view of the imprecision in the data, the CONTAM Panel established an ARfD of 0.2 µg AZA1 equivalents/kg b.w. In order to allow for toxicity of different AZA analogues the TEFs described in chapter 10.5 should be applied in calculating AZA1 equivalents for comparison with the ARfD.

### 13. Risk characterisation

Because AZAs have acute toxic effects, the Panel concluded that the identification of a high portion size rather than a long term average consumption is of importance to assess the health risk for consumers. It considered the 95<sup>th</sup> percentile as a realistic estimate of the portion size for high consumers, and chose the figure of 400 g of shellfish meat to be used in acute exposure assessments.

A 400 g portion of shellfish meat containing AZAs at the current EU limit of 160 µg AZA1 equivalents/kg shellfish meat would result in an intake of 64 µg AZAs (1 µg AZA1 equivalents/kg b.w. for a 60 kg adult). This intake exceeds the ARfD of 0.2 µg/ AZA1 equivalents/kg b.w. as established by the CONTAM Panel by about 5-fold and is only a factor

two below the most probable estimate of a LOAEL for gastrointestinal symptoms associated with AZA poisoning. Therefore, it cannot be excluded that this intake would exert effects in susceptible consumers.

As indicated in chapter 6 the Panel assumed that all shellfish samples showing a negative response in mammalian bioassays will reach the market and will thus be consumed. Therefore, the concentration data derived by LC-MS/MS for these samples (Table 7) could be used to estimate the dietary intake of AZA-group toxins.

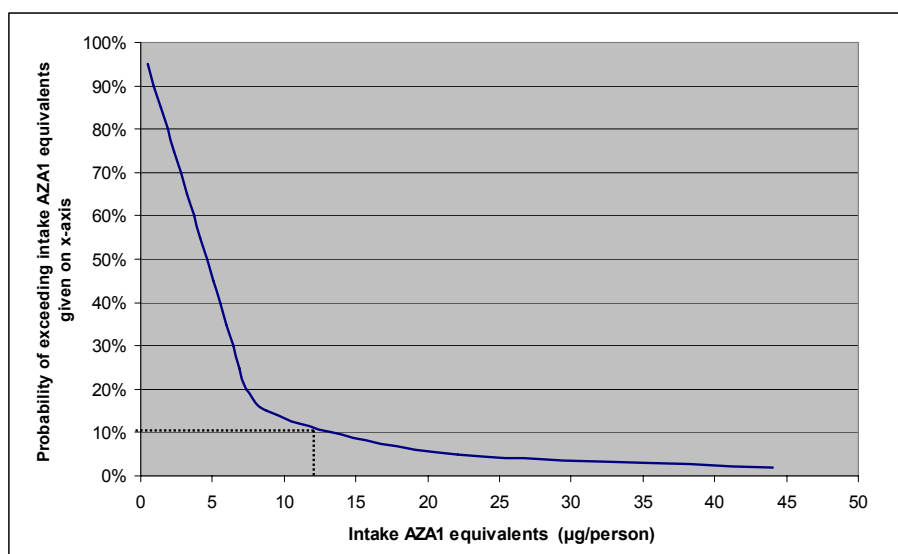
Consumption of a 400 g portion of shellfish meat containing AZAs at 40 µg AZA1 equivalents/kg shellfish meat (corresponding to the 95<sup>th</sup> percentile of the concentration, see Table 7) would result in an intake of 16 µg AZAs (0.25 µg AZA1 equivalents/kg b.w. for a 60 kg adult). This intake slightly exceeds the ARfD of 0.2 µg AZA1 equivalents/kg b.w.

**Table 12:** Deterministic intake estimate of AZA group toxins as derived in chapter 8.

Concentration of toxin (µg/kg shellfish)	Portion size (kg)	Intake (µg AZA1 equivalents per portion)
160 (EU limit value)	0.4	64
40 (95th percentile concentration)	0.4	16
30 (based on ARfD)	0.4	12

From the probabilistic exposure estimate as presented in Figure 4 (chapter 8) based on the distributions of both the concentration and the consumption data, it can be estimated that there is a chance of approximately 4% to exceed the ARfD of 0.2 µg AZA1 equivalents/kg b.w., (12 µg AZA1 equivalents/person for a 60 kg adult), when consuming shellfish containing levels of AZAs that could be present in shellfish currently available on the European market.

As shown in Figure 5, using only the distribution of the concentration data, the CONTAM Panel estimated that a 60 kg person consuming a portion of 400 g of shellfish meat has a chance of approximately 10% to exceed the ARfD of 0.2 µg AZA1 equivalents/kg b.w., corresponding to 12 µg AZA1 equivalents/person for a 60 kg adult.



**Figure 5:** Probability of exceeding a certain intake of AZA1 equivalents when consuming a single portion of 400 g of shellfish meat. The dotted line indicates the chance to exceed an intake of 12 µg AZA1 equivalents/person, corresponding to the ARfD of 0.2 µg AZA1 equivalents/kg b.w.

In order for a 60 kg adult to avoid exceeding the ARfD, a 400 g portion of shellfish should not contain more than 12 µg AZA group toxins, i.e. 30 µg AZA1 equivalents/kg shellfish meat.

## 14. Uncertainty

The evaluation of the inherent uncertainties in the assessment of exposure to AZAs 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 draft report on “Characterizing and Communicating Uncertainty in Exposure Assessment” which is in preparation to be published as WHO/IPCS monograph, has been considered (WHO/IPCS, 2008).

According to the guidance provided by the EFSA opinion (2006) the following sources of uncertainties have been considered: Assessment objectives, exposure scenario, exposure model, and model input (parameters).

### *Assessment objectives*

The objectives of the assessment were clearly specified in the terms of reference and the 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.

### ***Exposure scenario***

The estimate of exposure is based on measurements made in a small number of European Countries (only Ireland and Norway) where increased concentrations of AZAs have been identified as important contaminants of shellfish. In other European regions AZAs are not a matter of concern. The exposure scenario may therefore lead to overestimation of exposure when extrapolating these data to the whole European population.

Limited information on the occurrence of AZAs in different shellfish species may introduce uncertainty as consumption figures include a large variety of non-specified shellfish species.

### ***Exposure model***

The high numbers of samples having levels below LOD may introduce uncertainties in the overall estimate. Due to the fact that the opinion concentrates on the acute risk of high levels of the AZA group toxins, the uncertainties relating to values below the LOD are considered to be negligible, as they do not have a major influence on the risk characterisation.

Uncertainties may be introduced because estimations of exposure were based on the occurrence data from mammalian bioassay negative samples in pre-market controls.

### ***Model input (parameters)***

Although the analytical methodology is assumed to deliver comparable results, appropriate calibration standards for AZA were not always available. The data were produced with non certified calibration standards which may not be appropriate for quantification. An AZA1 standard has been available for a few months. Uncertainties regarding the analytical methodology for the other AZA analogues have not been considered in this evaluation.

TEFs have been used to convert the concentrations of the AZA analogues into AZA1 equivalents. However, as pointed out in chapter 10.5, these TEFs are based on a limited database and assuming a similar mode of action for the different analogues thus producing uncertainties regarding the AZA1 equivalents.

### ***Other uncertainties:***

Regarding the human case studies used for the derivation of the ARfD the uncertainty with respect to the ingested amount of AZA-toxins is addressed in the probabilistic approach by FSAI. The major uncertainty relates to the very small number of individuals in a single incident providing the basis of the data. To take this into account uncertainty factors have been applied in the derivation of the acute reference dose.

In Table 13 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 13.** Summary of qualitative evaluation of the impact of uncertainties on the risk assessment of the dietary exposure of AZA-group toxins.

	Direction and Magnitude
Uncertainty in analytical results	+/- <sup>a)</sup>
Extrapolation of occurrence data from few European Countries to whole Europe	++
Incomplete database for shellfish consumption in Europe; data only from limited number of Member States and limited data on shellfish species other than mussels	+
Influence of non-detects on deterministic and probabilistic estimate	+/-
Consideration of shellfish sampled for premarket control for systematic dietary estimation of exposure	+/-
Use of TEFs for estimating AZA1 equivalents	+/-
Limitation in the database for establishing the ARfD	+

<sup>a)</sup> +, ++, +++ = uncertainty with potential to cause small, medium or large over-estimation of exposure/risk

-, --, --- = uncertainty with potential to cause small, medium or large under-estimation of exposure/risk (EFSA, 2006).

The CONTAM Panel considered the impact of the uncertainties on the risk assessment of exposure to AZAs 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

### *Hazard identification and characterisation*

- Azaspiracids (AZAs) are produced by dinoflagellates. They are polyether toxins which contain a heterocyclic amine and an aliphatic carboxylic acid moiety. Currently about 20 analogues have been identified of which AZA1, AZA2 and AZA3 are the most important ones based on occurrence and toxicity.
- The gastrointestinal tract is the main target organ. In humans the main symptoms are nausea, vomiting, diarrhoea and stomach cramps.
- The limited toxicological information does not allow the setting of robust toxic equivalence factors (TEFs) for AZA analogues. Assuming a common mode of action, the toxicity of the AZAs is expressed as the sum of AZA1 equivalents when determined by liquid



chromatography-mass spectrometry/mass spectrometry LC-MS/MS. Until better information is available the following factors are adopted: AZA1 = 1, AZA2 = 1.8, AZA3 = 1.4, AZA4 = 0.4, AZA5 = 0.2.

- Due to their lower occurrence and toxicity relative to AZA1, AZA2 and AZA3, AZA4 and AZA5 do not appear to pose a significant risk for public health.
- The data on the chronic effects of AZAs in animals or humans were insufficient for a tolerable daily intake (TDI) to be established.
- In view of the acute toxicity the CONTAM Panel decided to establish an acute reference dose (ARfD). The CONTAM Panel concluded that the most probable lowest-observed-adverse-effect level (LOAEL) for gastrointestinal symptoms in humans associated with AZA poisoning in a small number of individuals was 1.9 µg AZA1 equivalents/kg b.w. Overall the CONTAM Panel applied a combined uncertainty factor of 9 to allow for LOAEL to NOAEL extrapolation and for human variability.
- Rounding to one significant figure the CONTAM Panel established an acute reference dose (ARfD) of 0.2 µg AZA1 equivalents/kg b.w.

### ***Occurrence and exposure***

- There is a lack of representative occurrence data for AZAs in different species of shellfish in most Member States.
- Levels determined by liquid chromatography-mass spectrometry LC-MS/MS in samples that tested negative in the mouse bioassay (MBA) have been used for exposure assessment.
- Consumption data for shellfish are only available for a few Member States. These data do not always distinguish between shellfish species or the type of processing. In addition, different study designs were used in the collection of the consumption data. From these data, the CONTAM Panel identified the figure of 400 g as the high portion size to be used for acute exposure assessments.

### ***Risk characterisation***

- Consumption of a 400 g portion of shellfish containing AZAs at the current EU limit of 160 µg AZA1 equivalents/kg shellfish meat would result in a dietary exposure of 64 µg AZA1 equivalents per person (1 µg AZA1 equivalents/kg body weight (b.w.)). This is approximately five times higher than the acute reference dose (ARfD) of 0.2 µg AZA1 equivalents/kg b.w., established by the CONTAM Panel.

- Based on current consumption and occurrence data there is a chance of approximately 4% to exceed the ARfD of 0.2 µg AZA1 equivalents/kg b.w. when consuming shellfish currently available on the European market.
- In order for a 60 kg adult to avoid exceeding the ARfD, a 400 g portion of shellfish should not contain more than 12 µg AZA1 equivalents, i.e. 30 µg AZA1 equivalents/kg shellfish meat.

### ***Method of analysis***

- The MBA and the rat bioassay are the officially prescribed reference methods in the EU for the determination of AZAs. Both methods have shortcomings e.g. they are not specific and not quantitative. Method performance characteristics for AZAs have not been established for the mammalian assays. Based on limited data on acute *i.p.* toxicity in mice, it is not clear whether the MBA can detect levels at the current EU regulatory level of 160 µg AZA1 equivalents/kg shellfish meat.
- The LC-MS/MS based methods have the greatest potential to replace the mammalian assays, and to detect individual toxins of the AZA group below the current regulatory level.
- Neither the mammalian assays, nor the chemical alternatives to determine AZAs, have been formally validated in interlaboratory studies, following internationally agreed protocols.

## **RECOMMENDATIONS (INCL. KNOWLEDGE/DATA GAPS)**

### ***Hazard identification and characterisation***

- Reporting systems for outbreaks of azaspiracid poisoning (AZP) in Member States should be improved to better reflect the true incidence and to allow efficient follow up of causative shellfish species.
- Detailed reports on shellfish consumption and reliable data on toxin content in the event of outbreaks of AZP should be provided in order to reduce uncertainty in the ARfD for AZAs.
- There is a need for clarification of the mode of action of AZA1 and its analogues.
- Further toxicological data are needed for the establishment of robust TEFs for the most frequently occurring analogues of AZA (AZA1, AZA2 and AZA3) ideally for the oral route of administration. The assumption of dose additivity should be assessed following exposure to combinations of AZA analogues. Milligram amounts of purified AZAs should be produced for this purpose.

- Information is needed on the oral toxicity of AZAs when combined with other lipophilic toxins that often co-occur in contaminated shellfish, such as okadaic acid, yessotoxins and pectenotoxins.
- There is a need for genotoxicity data of AZAs and information about possible long term effects.

### *Occurrence and exposure*

- The database on shellfish consumption should be extended including data on portion size, frequency and individual shellfish species.

### *Methods*

- Certified standards for relevant individual AZAs and certified tissue reference materials with relevant compositions and levels of AZAs are required.
- Rapid and cost effective screening methods should be developed and validated to reliably detect AZAs at the level of interest.
- It should be investigated if reference methods can be based on performance criteria, thereby allowing the use of several methods rather than a single specific method. The feasibility of the single laboratory validation concepts should be further explored, but validation by interlaboratory trials should be the long-term objective.

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## LIST OF ABBREVIATIONS

ARfD	Acute reference dose
ASP	Amnesic Shellfish Poisoning
AZA	Azaspiracid
AZP	Azaspiracid poisoning
BTX	Brevetoxins
CCFFP	Codex Committee for Fish and Fishery Products
CONTAM	Panel on Contaminants in the Food chain
CRL-MB	Community reference laboratory for marine biotoxins
CTX	Ciguatoxins
DA	Domoic acid
DSP	Diarrhoeic Shellfish Poisoning
DTX	Dinophysis toxins
DTX3	Dinophysis toxin 3
ECVAM	European Centre for the Validation of Alternative Methods
EFSA	European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FSAI	Food Safety Authority of Ireland
Gastrointestinal	GI
HPLC	High-performance liquid chromatography
INK/SAPK	c-Jun-N-terminal Kinase
<i>i.p.</i>	Intraperitoneal administration
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-mass spectrometry/mass spectrometry
LC-UV	Liquid chromatography-ultra violet absorption detection
LOAEL	Lowest-observed-adverse-effect level
LOD	Limit of detection
LOQ	Limit of quantification
MBA	Mouse bioassay
NOAEL	No-observed-adverse-effect level
NRC-IMB	National Research Council Canada-Institute for Marine Biosciences
OA	Okadaic acid
PITX	Palytoxins
Post-MC	Post-marketing control
Pre-MC	Pre-marketing control
PSP	Paralytic shellfish poisoning
PTX	Pectenotoxin
RASFF	Rapid Alert System for Food and Feed
RBA	Rat bioassay
SLV	Single laboratory validation
SM	Shellfish meat
SOP	Standard operating procedure
STX	Saxitoxin
TDI	Tolerable daily intake

TEFs	Toxic equivalence factors
WHO	World Health Organization
YTX	Yessotoxin