Risk Assessment of Azaspiracids (AZAs) in Shellfish

August 2006

A Report of the Scientific Committee of the Food Safety Authority of Ireland (FSAI)

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Forward

Relative to other marine biotoxins, azaspiracids are a new phenomenon that were first identified in 1995 in Ireland and have only been identified in a few other European countries since then. In 1999/2000 this toxin was responsible for the closure of many Irish shellfish growing areas for much of the season, in order to protect consumers' health. This 2006 risk assessment for azaspiracids in shellfish was prepared by the Sub-committee on Food Additives, Chemical Contaminants and Residues and adopted by the Scientific Committee of the Food Safety Authority of Ireland. It is the second risk assessment on azaspiracids conducted by the Scientific Committee, the first being reported in 2001. There have been three international risk assessments on azaspiracids since the publication of the 2001 Irish risk assessment, all of which have used, as a basis, the lowest observable adverse effect level determined in that work. However, there has been significant progress in standardising analytical techniques for azaspiracids since 2001 and this work underpins an increase in the scientific data concerning this emerging but rare biotoxin. It is therefore timely to re-assess the risk to human health posed by azaspiracids based on these new data and to establish an acute reference dose allowing for the determination of a safe level for AZAs in shellfish.

Executive Summary

Certain marine biotoxins pose a serious health risk when present above maximum permitted limits in bivalve molluscs, echinoderms, tunicates or marine gastropods and therefore legislation has required the establishment of national marine biotoxin monitoring programmes. These programmes are responsible for monitoring shellfish harvesting areas for the presence of toxins produced by marine phytoplankton and have generated new and useful data for use in risk assessments.

Azaspiracids (AZAs) were first identified in 1995 in Ireland and have only been identified in a few other European countries since then. Human intoxication, termed azaspiracid poisoning (AZP), associated with the consumption of shellfish contaminated with AZAs, has been recorded. However, toxicological data on the effects of AZAs are limited. Available data clearly indicate that AZA produces profound biological perturbations at the cellular level. Observations in humans and in animal studies, as well as *in vitro* studies report the ability of AZA to affect the gastrointestinal tract. In addition, *in vivo* studies suggest a potential tumourigenic action, although no definitive decision can be based on these limited studies. However, the lack of statistically robust toxicological data for AZAs in animal models means that the determination of a safe level (Acute Reference Dose, ARfD) for AZAs in mussels must therefore still be based on the acute toxic effects of AZAs in humans and an exposure assessment that relies on information from documented incidents of AZP.

The first risk assessment of AZAs was carried out by the Food Safety Authority of Ireland (FSAI) in 2001, aimed at establishing a 'lowest observable adverse effect level' (LOAEL) for AZAs. Three risk assessments have been carried out by international bodies since 2001, and these have all been based on the intake level for AZAs associated with symptoms of AZP calculated in the FSAI risk assessment. This was between 6.7µg (5%) and 24.9µg (95%) per person.

A meeting of a European Commission Working Group on Toxicology on DSP and AZP (EC, 2001) considered new data on AZA heat stability and derived a recalculated range of AZA intake of between 23 µg (5%) and 86µg (95%) per person with a mean value of 51.7µg. An 'acute reference dose' (ARfD) of 0.127µg/kg body weight (b.w.) was subsequently calculated using a safety factor of 3. Based on an intake level of a maximum of 100g shellfish meat per eating occasion it was stated that an allowance level of 8µg AZAs/100g of shellfish meat per meal should result in no appreciable risk for human health. However, to allow for detection by the mouse bioassay, a level of 16µg/100g was proposed as a maximum regulatory limit. This was later adopted into European law (EC, 2002).

The evaluation of the Joint FAO/IOC/WHO ad hoc expert consultation on marine biotoxins in bivalve molluscs (FAO, 2004) established a provisional ARfD of 0.04µg/kg b.w., based on the lowest reported "LOAEL" of 23µg per person in humans (EC, 2001) and using a safety factor of 10. This risk assessment preferred a consumption level of 250g of shellfish meat per person. Hence, a derived guidance level for AZAs in shellfish of 0.0096mg/kg (0.96µg/100g) was advised. As there were insufficient data on the chronic effects of AZA, no tolerable daily intake (TDI) could be established.

The most recent risk assessment for AZAs was carried out by the European Commission Working Group on Toxicology in 2005 (Community Reference Laboratory on Marine Toxins

(CRLMB)), in which a guidance level of 0.032mg/kg (3.2µg/100g) shellfish meat for AZAs was considered appropriate (subject to future re-evaluation). This level was based on the existing lowest LOAEL from epidemiological studies of 0.38µg/kg b.w. (2 µg per person divided by a 60kg reference body weight), a shellfish portion size of 250g and a safety factor of 3 (Anonymous, 2005).

The current risk assessment has revisited the 2001 Irish risk assessment and re-evaluated the calculations therein, whilst utilising new data available since that time. Since 2001, published scientific evidence has become available concerning the following key parameters of the 2001 Irish risk assessment:

- Tissue distribution of AZAs in mussels,
- Ratios of different analogues of AZAs,
- Effect of cooking on AZAs.

A probabilistic exposure assessment approach has been used for the exposure assessment as a means of indicating the range of possible outcomes and their relative likelihoods. The uncertainties in this approach are due in part to the assumptions made and also in part to the quantity and accuracy of the data used in the risk assessment to describe natural variability.

In relation to **tissue distribution of AZAs in mussels**, expert opinion on the relative proportions of hepatopancreas to whole flesh was used in the 2001 FSAI risk assessment to calculate the likely concentration of AZA-1 in whole flesh, given a measurement of concentration in hepatopancreas. However, Hess *et al* (2005) recently reported a series of ratios based on measurements of AZAs in mussel hepatopancreas relative to whole flesh, and the use of these new data resulted in a reduction of a source of uncertainty in the original risk assessment and allowed for a more accurate description of the natural variability of this ratio. These new data increased the range of estimates of AZA-1 in mussel whole flesh with a higher average estimate (2µg/g) compared to the previous estimate (1.3µg/g) (FSAI, 2001).

In relation to the **ratios of different analogues of AZAs** in mussel flesh, in the 2001 FSAI risk assessment a single proportion for AZA-2 and AZA-3 relative to AZA-1 was used based on data from Ofuji *et al* (1999b). New data from the 2005 Irish biotoxin programme has generated a range of 72 different proportions for AZA-2 and AZA-3 relative to AZA-1, confirming that the relative proportions of the three analogues in mussels are highly variable and positively correlated. In the current risk assessment these new data were used to provide a much more accurate basis for the calculation of total AZAs than the single value used in the 2001 FSAI risk assessment, thus reducing the uncertainty in the original 2001 risk assessment. The data of Ofuji *et al* (1999b) were within the range of these new data but towards the upper end particularly for the ratio of AZA-3 to AZA-1.

In relation to the **effects of cooking on AZA,** a previous report in abstract form by James *et al*, 2001 indicated that AZAs were inactivated in mussels during traditional home cooking procedures. These data, used in the 2001 risk assessment, resulted in a reduction in the estimation of total AZAs in whole cooked mussel meat of 70%. However, a more recent study by Hess *et al*, 2005 showed that steaming of raw fresh mussels resulted in a two-fold higher concentration of azaspiracids in the cooked flesh compared to the uncooked flesh. This applied to both whole flesh and for digestive gland tissue and was attributed to the loss of water/juice from the matrix. The overall behaviour of azaspiracids in the cooking process was therefore, in the opinion of the authors, very similar to that of other lipophilic marine

biotoxins, e.g. okadaic acid. The heat stability of AZAs was also recognised in a previous international risk assessment (EC, 2001). This finding also allowed for a simplification in the 2001 exposure assessment model by calculating AZA intakes based on mussel consumption expressed in terms of raw weight, rather than having to account for the reduction in mussel meat weight due to cooking (~50%). The combined effect of the changes on heating alone led to a 7 fold increase in estimates of AZA than calculated in the previous exposure assessment (FSAI, 2001). However, a degree of uncertainty still exists in this part of the exposure assessment due to the lack of knowledge on mussel meat weight in the Arranmore growing site in 1997.

The use of the new data on the above key parameters and a simplified calculation approach has resulted in an increased estimate of AZA intake that led to AZP on Arranmore. The revised estimates of AZA intakes believed to have caused human intoxication are now between 50.1µg (5%) and 253.3µg (95%) per person. The comparable intakes of AZA reported in the original Irish risk assessment were between 6.7µg (5%) and 24.9µg (95%) per person.

The median ARfD for AZA, derived from the above distribution of intake estimates, is 0.63µg/kg b.w., obtained by application of a safety factor of 3 to the estimates of the lowest observable adverse effect level (LOAEL) for AZAs based on the Arranmore AZP incident. This is comparable to the maximum intake value of 0.67µg/kg b.w. for a 60kg person consuming 250g mussels contaminated with AZAs at the current regulatory limit of 0.16mg/kg. The validity of an ARfD of 0.63µg/kg b.w. is supported by the absence of reported incidents of AZP since the adoption of the 0.16mg/kg maximum regulatory limit for AZAs in shellfish and strengthening of national biotoxin monitoring programmes to enforce it.

The current risk assessment remains based on a number of unavoidable assumptions, in turn leading to uncertainties in the outcome of the assessment. In particular it is recognised that the epidemiological data on which the assessment is based are limited in number. However, it is considered that despite the deficiencies in these data the rarity of AZP means that the Arranmore incident is still the best documented and most thoroughly studied incident today. The new data available on key parameters used in the original risk assessment has meant that the inherent uncertainties have been substantially reduced in the current assessment. Further refinement of the risk assessment will only be possible when a more substantial toxicological database from animal studies and/or additional epidemiological data in humans become available.

Main Report

Background

European legislation has been established to secure a high level of consumer protection with regard to food safety. As certain marine biotoxins pose a serious health risk when present above maximum permitted limits in bivalve molluscs, echinoderms, tunicates or marine gastropods, legislation has required the establishment of national marine biotoxin monitoring programmes. These programmes are responsible for monitoring shellfish harvesting areas for the presence of toxins produced by marine phytoplankton and have generated new and useful data for use in risk assessments.

Azaspiracids (AZAs) are a class of marine biotoxins that have emerged in the past ten years and have caused food poisoning. They were first identified in Ireland in 1995 (McMahon & Silke, 1996) and have been identified intermittently since then. Toxic events are still relatively rare compared to other biotoxins, but periodically AZAs can affect shellfish growing areas for long periods of time.

Following the identification of AZAs and preliminary work on its structure, causative agent and toxicology, the Food Safety Authority of Ireland (FSAI) carried out a risk assessment to help with the establishment of initial regulatory limits (FSAI, 2001). The general lack of toxicological data and monitoring data at the time meant that the risk assessment had to be based on epidemiological information recorded during an azaspiracid poisoning (AZP) incident on Arranmore Island in 1997. Consequently, the risk assessment was underpinned by assumptions and expert opinion, necessary to facilitate the assessment, and the uncertainty inherent in the outputs of the risk assessment was high. The FSAI concluded that the risk assessment would need to be revisited when more data on the toxicology and occurrence of AZAs were available. The European Commission eventually set a regulatory limit of 0.16mg/kg in raw mussels based on a re-evaluation of the 2001 Irish risk assessment (EC, 2001 & 2002).

There are now more data on the occurrence of AZAs, a better characterisation of their chemistry and more reliable, standardised analytical methods. However, the advancements in the toxicology of AZAs have been limited by the amount of material available and therefore it is still not possible to derive a NOAEL, a LOAEL or an acute reference dose (ARfD) for AZAs using classical toxicological studies in animals. Despite this, it is timely to update the original FSAI risk assessment using the new data available on other parameters used in the risk assessment, to revise its assumptions and replace the use of expert opinion where possible to reduce the uncertainty in the assessment of risk.

Hazard Identification

Shellfish Associated with AZAs and Causative Organism

An unusual biotoxin was first observed in mussels (*Mytilus edulis*) from Ireland in 1995 after an illness similar to diarrhoeic shellfish poisoning (DSP) was observed (McMahon and Silke, 1996). With the identification of AZA as the causative agent (Satake *et al*, 1998a), the illness was named azaspiracid poisoning (AZP) (Ofuji *et al*, 1999a). Following the discovery in

mussels, several other bivalve molluscs have been identified as containing AZAs including oysters (*Crassostrea gigas, Ostrea edulis*), scallops (*Pecten maximus*), clams (*Tapes phillipinarium*), cockles (*Cardium edule*) and razor fish (*Ensis siliqua*) (Hess *et al*, 2001; Furey *et al*, 2003). The European Commission Rapid Alert System (RASFF) has also reported the occurrence of AZAs in crabs.

Recent evidence has suggested the dinoflagellate *Protoperidinium crassipes* as the progenitor of AZA-1, AZA-2, and AZA-3 (James *et al*, 2003). Due to the predatory nature of this organism, it cannot be excluded that AZA could accumulate through consumption of another prey species (Latz and Jeong, 1996). The accumulation of an AZA precursor from a prey species, which was subsequently metabolised into AZA-1, AZA-2, or AZA-3 cannot not be precluded. Due to the widespread distribution of *Protoperidinium* spp., confirmation of this as the causative organism of these toxins may have serious consequences in countries where Protoperidinium is present (Tomas *et al*, 1997).

Geographical Distribution of AZAs and Reported AZP Incidents

AZAs are a recent addition to the biotoxin family. Data on their geographical distribution is limited because few countries have the capability of identifying them other than through the general mouse bioassay which is not specific for these toxins. AZAs were first identified in Ireland in 1995 (Satake *et al*, 1998a) with infrequent re-occurrence since. AZAs have also now been reported in four other countries, Norway, UK, France and Spain. However, given the difficulties in identification methodology, the possibility of wider distribution cannot be ruled out. Analysis of the European Commission rapid alert system demonstrates the infrequent nature of AZA occurrence in Europe (Table 1) but also demonstrates that AZA contaminated product has been placed on the market and presumably consumed without reported AZP.

Table 1: Reported AZA contamination of shellfish on the EC rapid alert system 2001-2006

Reporting Country	Country of Origin	Species affected	Date	AZA detected	Level reported	Weight of product & reported consumption
Belgium	UK (Scotland)	Queen Scallops (*Chlamys Opercularis*)	29th March 2002	No (reported as DSP/AZA due to failure of mouse bioassay)	None	Not applicable
Belgium	Italy	Mussels (*Mytulis galloprovinciales*)	25th June 2002	No (reported as DSP/AZA due to failure of mouse bioassay)	None	Not applicable
Belgium	Fished in Ices area VII E by Belgian vessel	Scallops *(pectin maximus)*	17th October 2002	No (reported as DSP/AZA due to failure of mouse bioassay)	None	Not applicable
UK	Canada	Mussels (*Mytilus edulis)*	26th May 2005	No (test results only for DSP)	None	Not applicable
Norway	Norway	Crabs *(Cancer pagurus)*[a]	1st November 2005	Yes	399µg AZA eq/kg	1045kg "presumed product already consumed"

Reporting Country	Country of Origin	Species affected	Date	AZA detected	Level reported	Weight of product & reported consumption
Norway	Norway	Crabs *(Cancer pagurus)* [a]	9th November 2005	Yes	177µg – 269µg AZA eq/kg	6200kg "presumed product already consumed"
Norway	Norway	Crabs *(Cancer pagurus)* [a]	14th November 2005	Yes	217µg AZA eq/kg	3094kg "distribution on the market possible-withdrawn"

[a] presumed to have become toxic by consumption of bivalve molluscs in the wild

However, AZP has been reported in five countries, all in the European Union (Table 2) with all cases linked to the consumption of Irish shellfish prior to 2001 when a legislative limit for AZAs was adopted in Europe and a significantly improved biotoxin monitoring programme was implemented in Ireland and some other countries. There appears to have been no reported cases of AZP since 2001.

Table 2: Reported cases of Azaspiracid poisoning (AZP) 1995-2006

Location of AZP	Date	Implicated food source	Amount consumed	Area of production	Number of illnesses recorded
Netherlands	November 1995	Mussels (*Mytilus edulis)*	Not recorded	Killary, Harbour, Ireland	8
Ireland	September/ October 1997	Mussels (*Mytilus edulis)*	"As few as 10-12 mussels"	Arranmore Island, Ireland	8 confirmed (Estimated 20-24)
Italy	September 1998	Mussels (*Mytilus edulis)*	Not recorded	Clew Bay, Ireland	10
France	September 1998	scallops (*Pecten maximus*)	Not recorded	Bantry Bay, Ireland	Estimated 20-30
United Kingdom	August 2000	Frozen Mussels (*Mytilus edulis)*	Not recorded	Bantry Bay, Ireland	12-16

Adapted from James *et al* 2004

To place the observations on reported illness in context, it must be noted that for a significant proportion of 2001-2006, AZAs did not re-occur in significant quantities in Ireland. However, there were major AZA incidents in 2001 and again in 2005. During these incidents, a considerable quantity of shellfish had been placed on the market containing AZAs at levels at or below the maximum regulatory limit of 0.16mg/kg. However, there were no reported instances of AZP, unlike the situation prior to the adoption of this regulatory limit (table 2).

In Ireland, attempts have been made to retrospectively assess the amount of shellfish that have been placed on the market with AZA levels at or below the legal limit. For example, data provided by sea-fisheries officers in Ireland show that 135,731kg of oysters (*C. gigas*) were harvested and marketed (not sent for relaying or depuration) from areas where

oysters have been found to contain AZA at or below the regulatory limit (AZA levels measured between 0.1 and 0.16mg/kg). Assuming all were grade one (the largest size) and given that a typical grade one oyster weighs 0.1kg (source: FAO) this weight of oysters equates to approximately 1.3 million oysters. A portion of oysters approximates to six oysters per portion which suggests that over 216,000 portions have been consumed without reported AZP. Although these calculations are crude, they demonstrate that a significant quantity of shellfish containing AZA has been consumed in Europe without apparent AZP effects. This would not support the hypothesis of a very low ARfD for AZAs, a point that will be revisited in the risk characterisation section of this report.

Chemical Characteristics of Azaspiracids

AZAs are nitrogen-containing polyether toxins with a unique spiral ring assembly, a cyclic amine and a carboxylic acid and were first detected in mussels (*Mytilus edulis*) in Ireland in 1995. Currently, 11 different congeners have been identified (Satake *et al*, 1998b; Ofuji *et al*, 1999a and 2001; James *et al*, 2003). Extensive characterisation by mass spectral analysis was carried out for six analogues by Brombacher *et al*., 2002, and for 11 analogues by James *et al*, 2003. Recently the total synthesis of AZA-1 has been accomplished (Nicolaou *et al*, 2004a,b).

Some mouse intraperitoneal studies have been published for the first five analogues, AZA-1 to AZA–5 (Satake *et al*, 1998b; Ofuji *et al*, 1999b and 2001). These studies allow for a crude assessment of the relationship between the structure of AZA analogues and their relative activity. It appears that the hydroxylated analogues AZA-4 and AZA–5 are significantly less toxic (factor 2-5) compared to AZA-1. This reduced toxicity, in combination with these toxins occurring at lower concentrations, has led to the overall conclusion that only AZA-1, AZA-2 and AZA–3 are of public health significance.

Analytical Methods

There are currently two types of methodology used for the detection of azaspiracids, biological and chemical assays. The current official European Union (EU) reference method is the mouse bioassay. To date, this method has never been validated. A mouse bioassay can be used to detect AZAs, involving acetone extraction of the shellfish with liquid/liquid partition steps in ethyl acetate/water or dichloromethane/water to remove potential interferences if necessary. AZA detection at the regulatory levels, by means of this procedure, requires the use of an extract equivalent to 5g of the hepatopancreas or 25g whole body, as the test portion. The death of two out of three mice over a 24-hour period should be considered a positive response (EC, 2002). The lower limit of detection is reported to be in the range of 2 to 4µg of AZA equivalents per 20g mouse, producing death within 24 hours of observation after intraperitoneal (i.p.) administration (Ofuji *et al*, 1999b). With the regulatory limit for AZAs in shellfish in the EU set at 0.16mg/kg in the whole body or any edible part (EC, 2002; Ofuji *et al*, 1999b), the detection limit of the mouse bioassay is inadequate.

An alternative biological detection assay is the oral DSP rat bioassay, which is capable of detecting the diarrhoetic properties of AZAs (Kat, 1983; McMahon and Silke, 1996). A diarrhoetic response in any of three rats is considered a positive response. This method yielded a strongly positive result with the contaminated mussel samples from the first incident in the Netherlands in 1995. The lower limit of detection has yet to be determined.

Both types of bioassays are not capable of determining quantitatively the levels of AZAs present in a matrix. Hence, these assays cannot be used in the evaluation of amounts of AZA ingested during cases of human illness. As a result, sensitive, quantitative techniques such as liquid chromatography coupled to mass spectrometry (LC-MS) have been developed. The initial reports by Satake *et al*, 1998b, and Ofuji *et al*, 1999a and 2001, have made use of the pure AZAs isolated following the initial incidents in 1995/6 and 1997. The analysis has been improved since then through a number of quality control procedures, including ISO 17025 accreditation of the LC-MS based methodology at the National Reference Laboratory for Marine Biotoxins in Ireland, the Marine Institute, who carry out this test routinely. This accredited method has been used in the studies by Hess *et al*, 2003 and 2005. Current efforts are being undertaken by the Marine Institute to obtain internationally accepted standards and reference materials for AZAs through collaboration with the National Research Council Canada, an accepted producer of certified reference materials. Also, further efforts are being undertaken by the Marine Institute to establish an internationally accepted protocol for the LC-MS analysis through collaborative trials, both in an EU project (BIOTOX) and with the EU Community Reference Laboratory for Marine Biotoxins in Vigo, Spain.

An important consideration for *in vivo* assays is the route of administration. Extrapolation of i.p. *in vivo* data in mice to oral intake in man has to be taken into account when evaluating *in vivo* toxicity data. A study comparing different methods for diarrhoetic shellfish poisoning, including the mouse and rat bioassays highlighted several different experimental parameters that might influence the assay outcome (Gucci *et al*, 1994). Highlighted was the possibility that the presence of fatty acids could affect the systemic toxicity of samples or that the presence of other toxins could lead to synergistic effects.

Factors Influencing the Detectable Levels of AZA in Shellfish

Distribution of AZAs in Shellfish

The movement of AZA from the digestive gland to the general mussel tissue reported by James *et al*, 2002, has not been reproduced in other studies. More recently, it has been reported that AZAs typically accumulate in the digestive gland of mussels, *Mytilus edulis*, similar to other lipophilic toxins (Hess *et al*, 2005). This study investigated both a bulk shellfish sample from Norway and 28 samples obtained during the routine shellfish monitoring programme in Ireland over a 2.5 year period. It was concluded that there was on average a five-fold ratio in the digestive gland alone compared to the whole mussel tissue (including the digestive gland). As suggested in the study, these results may justify the practice to only analyse the digestive gland. Very similar results were found by Brana Magdalena *et al*., 2003, who studied the content of AZA in the scallop, *Pecten maximus*. The authors found the toxin mostly concentrated in the digestive gland (> 85%), with very small amounts detectable in only one edible part of the scallop, the gonad (=roe). The gonad in scallops has a particular feature in that an intestinal loop is integrated into this tissue, hence, some of the toxin contained in the gut of the scallop may occur also in the gonad.

Depuration

Initial reports suggested that AZA may be retained in shellfish longer than other toxins (James *et al*, 2000; James *et al*, 2002). However, as published by Hess *et al*, 2003, other

lipophilic toxins may be similarly retained if they appear late in the year, when the metabolic activity of mussels is reduced. Therefore, it can be assumed that the depuration behaviour of azaspiracids is very similar to other toxins and can be modelled for the okadaic acid group of toxins with a two-compartment model, as suggested by Blanco *et al*., 1999. Similar behaviour was again observed in the Irish monitoring programme 2005/6, where depuration of azaspiracids was slow during winter months.

Effects of Cooking on AZAs

A previous report in abstract form by James *et al*, 2001 indicated that AZAs were inactivated in mussels during traditional home cooking procedures. These data, used in the 2001 risk assessment (FSAI, 2001), resulted in a reduction in the estimation of total AZAs in whole cooked mussel meat of 70%. However, a more recent study by Hess *et al*, 2005, fresh mussels showed that steaming of raw fresh mussels resulted in a two-fold higher concentration of azaspiracids in the cooked flesh compared to the uncooked flesh. This applied to both whole flesh and for digestive gland tissue and was attributed to the loss of water/juice from the matrix. The overall behaviour of azaspiracids in the cooking process was therefore, in the opinion of the authors, very similar to that of other lipophilic marine biotoxins, e.g. okadaic acid. The heat stability of AZAs was also recognised in a previous international risk assessment (EC, 2001).

Hazard Characterisation

Introduction

The most relevant toxicological data for hazard characterisation purposes is that generated from human exposure, i.e. epidemiological data. The benefit of epidemiological data is the removal of the need to extrapolate animal studies to humans due to differences in mechanisms of action, etc. However, inter-individual sensitivities should also be factored in. Depending on the nature of the substance, these data are not always readily available and do not account for mixtures of substances contributing to the observed effect. If human data are absent or lacking, then animal studies are used, however inter-species differences (e.g. metabolic pathways) mean that the results of such studies cannot always be extrapolated to humans. Nevertheless, animal studies can be performed to identify potential mechanisms of action. Extrapolation from a NOAEL established for a substance in an animal study often has an uncertainty factor of at least 10 applied to it as humans are considered to be more sensitive than animals. With limited information, the potential hazard of a toxin can be further assessed with *in vitro* data, physico-chemical properties and structure-activity relationships.

The hazard characterisation for AZA is primarily based on limited human epidemiological data, since there are deficiencies in the available *in vivo* data in animals, due to a lack of pure toxin to carry out such studies. In comparison, the commercial availability of the diarrhoetic shellfish toxin okadaic acid has enabled the generation of large quantities of toxicological data. This is an issue that can only be addressed with additional purified AZA from extensive toxic mussel events or synthesis of AZA analogues.

Observations in Humans

The major human route of exposure to AZA is ingestion, resulting in toxicity characterised by gastrointestinal disturbances e.g. diarrhoea, vomiting, abdominal pain and cramps. Epidemiological data on AZA incidents to date are limited, despite large quantities of shellfish being sold containing AZA equivalents at or near the regulatory level (0.16mg/kg). AZA poisoning is dependent on the dose ingested rather than the presence of the toxin alone. The most complete information available is from an incident in 1997 on Arranmore Island, Ireland, affecting eight people. Although the Arranmore Island data has a number of deficiencies, to the best of our knowledge no other incidents provide more extensive epidemiological data. However, limited epidemiological data do not necessarily correspond with limited AZA poisonings as these incidents are not necessarily acknowledged or reported by the consumer.

Studies in Animals

Limited *in vivo* studies have been carried out to date. These studies highlight several toxicological effects of AZAs. However, several factors limited the scope of these studies. The largest factor was the limited availability of purified AZAs. As a result, a reduced number of animals were used thus impairing statistical analysis. Therefore neither a LD_{50} (lethal dose that causes death in 50% of the population) nor a NOAEL could be determined.

Acute Toxicity

Oral Studies

Acute oral studies with AZA in mice have been performed. AZAs were extracted from mussels collected in Killary Harbour, Ireland, in February 1996. Male ICR mice aged 5 or 8 weeks, receiving orally by gavage a single dose of 500 (n = 7), 600 (n = 6), 700 (n = 2) or 900 (n = 2) µg purified AZA/kg b.w., showed no behavioural changes within four hours (Ito *et al*, 2000). The number of mice that survived after 24 hours were 0/2 administered 500µg/kg (8 weeks old, n=2), 3/6 administered 600µg/kg (5 weeks old, n=6) and 1/2 administered 700µg/kg b.w. (5 weeks old, n=2). The p.o. lethal dose was estimated to be 500µg/kg, i.e. 2.5 times higher than the i.p. lethal dose reported below. No diarrhoea was observed in mice treated with 500µg/kg of AZA within 24 hours (Ito *et al*, 2000).

At a sub-lethal oral dose of AZA (300µg/kg), mice exhibited macroscopic changes in the small intestine as visualised by congestion and pooled watery substances in the lumen. Degenerating cells were seen, coupled with atrophic lamina propria. After eight hours treatment with 600 or 700µg/kg b.w. villi became shorter, coupled with further degeneration of both lamina propria and epithelial cells. Recovery was seen to begin after 24 hours in the epithelia. No marked changes were observed in the stomach mucosa. Fatty changes coupled with cellular necrosis and degenerating cells were observed in the liver (Ito *et al*, 2000).

The toxicological effects of 2 administrations of AZA to a total of 25 mice at levels of 300-450µg/kg followed by an extended recovery phase included erosion and shortened villi in the stomach and small intestine, persisting for more than 3 months, oedema, bleeding, and infiltration of cells in the alveolar wall of the lung, persisting for 56 days, fatty changes in the liver, persisting for 20 days; and necrosis of lymphocytes in the thymus and spleen, persisting

for 10 days (Ito *et al*, 2002). Administration of the highest dose of 450µg/kg b.w. to four week old mice resulted in death of 11/16 treated mice.

It should be stressed that these oral studies were carried out by gavage and this method of administration itself has the potential to induce gastrointestinal effects

Intraperitoneal Studies

Satake *et al* (1998b), in a study using 2 male ddY mice, reported an i.p. lethal dose of purified AZA of 200µg/kg b.w. The intraperitoneal lethal doses for AZA-2 and -3 in mice were 110 and 140 µg/kg b.w., respectively (Ofuji *et al*, 1999b). The i.p. lethalities for AZA-4 and AZA-5 were approximately 470 and less than 1000µg/kg b.w., respectively, indicating that they were less toxic than AZA-1 (Ofuji *et al*, 2001; FAO, 2004). Due to the limited number of mice used in establishing these i.p. lethalities, the relative toxicities between the different analogues are not fully established, however the structural similarities between the different analogues does not suggest major toxicity differences between AZA-1, -2 or -3.

Table 3 summarises available data on the acute lethal dose of AZA in mice.

Table 3: Lethal dose of azaspiracid-1 in mice

Species age	Sex	Route	Lethal dose (µg/kg bw)	Reference
Mouse ICR 4-5 week	male	oral	~ 450	Ito *et al*, 2002
Mouse ICR 5 month	male	oral	~ 250	Ito *et al*, 2002
Mouse ddY	male	i.p.	200	Satake *et al*, 1998b

Repeated Dose Toxicity

Oral Studies

Oral doses of 50, 20, 5 and 1µg AZA/kg b.w. were administered twice a week, up to 40 times (145 days), to four groups of mice (4 weeks old at the start of the study) (Ito *et al*, 2002). Nineteen control mice were used. Nine out of ten mice at 50 µg/kg and three out of ten at 20µg/kg became so weak (as indicated by inactivity and weight loss) that they were sacrificed before completion of 40 injections (between 18th and 40th administration). All these mice showed interstitial pneumonia and shortened small intestinal villi. Lung tumours were observed in four mice, one out of ten (10%) at 50µg/kg and three out of ten (30%) at 20µg/kg. Tumours were not observed in 11 mice treated at lower doses and in 19 control mice. Hyperplasia of epithelial cells was also observed in the stomach of six mice out of ten administered at 20µg/kg.

No signs of weakness were observed in the 5 and 1 µg/kg b.w. groups. Mice in these groups had normal body weights and appearance after 30 treatments, but showed reduced villi heights (5 and 1µg/kg groups). Small intestinal villi had not fully recovered after three months post-withdrawal. The accumulation of fat droplets in the liver previously seen with

acute or lethal oral doses, were not observed in mice treated by repeated administration. (Ito *et al*, 2002)

A second study using more animals was performed in an attempt to confirm the previous incidences of AZA-induced tumours. A further five tumours were observed in the second study. The highest dose that was not lethal at chronic exposure (boundary dose) consistently caused tumours in both experiments, i.e. three tumours were observed in both studies for the mice exposed at 20 μg/kg. Amongst the nine tumours observed, seven were lung tumours and two were lymphomas. The ratio of tumour-bearing mice was >7% (9/126), while control mice did not show any tumours. Since ICR mice show a relatively high ratio of spontaneous tumours in the lung, liver and whole body (21.1, 17.2 and 7.5% respectively) at two years old (Giknis, 2000), AZAs may possibly be either tumourigenic or a promoter to their early appearance. Future work should consider a larger number of mice, to enable better statistical assessment of the observed effects and to establish whether the observed lymphomas will lead to malignant tumours after the exposure is ceased (Anonymous, 2005; Ito *et al*, 2004).

Genotoxicity: No data on the possible genotoxic effects of azaspiracids has been reported.

Effect of AZA on DNA fragmentation: *In vivo* administration of azaspiracid was studied for the ability to cause DNA fragmentation. Mice organs (ICR male four week) were stained with Apoptotic Peroxidase *in situ* (apoptosis kit) following oral administration of AZA. Following this, the livers showed apoptosis in all examined cases (300μg/kg: 1, 2 and 4 hours, and 600 μg/kg: 4, 18 and 24 hours), but not in the lung and kidney (Ito, unpublished observation).

Reproductive toxicity: The teratogenic potential of AZA-1 was examined in the Japanese medaka (*Oryzias latipes*) fish model. Microinjection of AZA-1 caused dose-dependent effects on heart rate, growth rate, hatching success and viability in medaka embryos. Within four days of exposure to doses of ≥ 40 pg/egg of AZA-1, substantial retardation in development was observed as reduced somatic growth and yolk absorption, and delayed onset of circulation and blood pigmentation. Embryos treated with ≥ 20 pg/egg AZA-1 had slower heart rates (bradycardia) for the nine day *in ovo* period followed by reduced hatching success. These studies demonstrate that AZA-1 is a potent teratogen to finfish (Colman *et al*, 2005). No data on the possible reproductive effects of azaspiracids in mammalians have been reported.

In vivo Toxicokinetics

Absorption, distribution and excretion/ toxicokinetics: No data reported.

Biotransformation: No information on pathways of azaspiracid metabolism in animals has been reported.

In vitro Toxicological Studies

At present there are very few data on the mechanism of action of AZAs. A functional *in vitro* model of human gastrointestinal cells, aimed at reflecting the human indications of toxicity, namely diarrhoea, has been established. The human colonic cell line Caco-2 was used to assess the impact of AZA-1 on an *in vitro* model of gastrointestinal permeability, namely confluent Caco-2 cells, grown on microporous filters to reflect the *in vivo* gastrointestinal cell layer. AZA-1 was capable of increasing epithelial paracellular barrier permeability in a dose-dependent fashion over time. Disruption of the paracellular barrier is a contributing factor to increased fluid secretion in diarrhoea. A significant decrease was observed with 5 nM AZA at 24 hr (Ryan *et al*, 2004). This disruption of the paracellular barrier was characterised by alterations in tight junction proteins. Tight junctions are responsible for regulating paracellular barrier function, and AZAs resulted in upregulation of the tight junctional protein claudin-2. This indicates that this *in vitro* functional assay may provide information on the precise mechanism of how AZAs induce diarrhoea in humans and on the acute toxicity of AZAs.

Cytotoxic and cytoskeletal effects of AZA-1 on mammalian cell lines

The cytotoxic potential of AZA-1 has been examined in a range of human and non-human cell lines. Investigations reported that AZA-1 is differentially cytotoxic to several different cell types. Calculated EC_{50} values for the Jurkat cell line (lymphocyte T cells) were 3.4, 1.1 and 0.9 nM for 24, 48 and 72 hour exposures respectively.

AZA-1 affected membrane integrity as determined by the significant release of the cytosolic enzyme, glucose-6-phosphate dehydrogenase (G6PD), from Jurkat cells, with preliminary EC_{50} values of 0.2 and 0.07 nM for 24 and 48 hours of exposures. AZA-1 was also reported to be capable of re-arranging cellular F-actin in Jurkat cells. This was apparent with the concurrent loss of pseudopodia and cytoplasmic extensions that function in mobility and chemotaxis, prior to cytotoxicity (Twiner *et al*, 2005).

Studies by Roman *et al*, (2002), have provided some information on targets at the cellular level. In excitable neuroblastoma cells, AZA-1 did not modify mitochondrial activity but decreased F-actin concentration. These results indicate that the toxin did not have an apoptotic effect but used F-actin for some of its effects, highlighting the cytoskeleton as an important cellular target for AZA-1 effects. AZA-1 did not induce any modification in membrane potential, suggesting that AZAs may not have neurotoxic effects. In human lymphocytes, AZA-1 induced Ca^{2+} increase was negatively modulated by agents which regulate protein kinase C (PKC) activation, protein phosphatases 1 and 2A (PP1 and PP2A) inhibition and cAMP increase (Roman *et al*, 2002).

Further studies by Roman and colleagues (Roman *et al,* 2004) indicated that AZA-2 and -3 could increase cytosolic cAMP levels. AZA-2 increased intracellular Ca^{2+} by release from internal stores and Ca^{2+} influx from extracellular medium when cultured initially in Ca^{2+}-free medium. AZA-2 induces Ca^{2+}-influx through store-operated Ca^{2+} channels. AZA-3 did not, however, empty intracellular Ca^{2+} stores, but did increase cytosolic Ca^{2+} levels. In a similar model, AZA-4 was reported not to modify cytosolic calcium in resting human lymphocytes. These effects were reversible and not regulated by cAMP pathway. AZA-4 appeared to be a novel inhibitor of plasma membrane Ca^{2+} channels, affecting store operated channels, showing an effect different from other AZA analogues (Alfonso *et al*, 2005). The authors suggested that the lower toxicity observed with AZA-4 and AZA-5 compared to AZA-1 and -

3, is due to the inability of AZA-4 and -5 to increase Ca^{2+} influx. The different mechanistic behaviour between the different analogues in the human lymphocyte model indicates a need to correlate structural activity with toxicity.

Extracellular recordings of action potentials (APs), or spikes from cultured networks of spinal cord neurons derived from E13 mice were made using a neuronal network-based biosensor. The mean spike rate for a network was computed as a function of time and used as an assay for the efficacy of AZA-1 to alter the behaviour of the neurons in the network. AZA-1 decreased the mean spike rate of the spinal cord neurons with an IC_{50} of ~2.5 nM. However, a small sub-population of neurons continued to fire APs even at high concentrations of AZA-1 (10 nM). This suggested that the underlying ion currents responsible for AP generation were not being affected by AZA-1. These data suggest that AZA-1 may be affecting synaptic transmission in the neuronal networks through a mechanism that does not involve voltage-gated channels (Kulagina *et al*, 2004).

Conclusion on Hazard Characterisation

The classical *in vivo* toxicological studies required to carry out and establish a NOAEL have been hampered by the lack of pure AZAs, and were not designed with this objective in mind. A proper statistical analysis of the data derived from available animal studies is not possible, and the routes of administration applied, namely gavage (for oral) and intraperitoneal also have problems in relation to extrapolation to human oral consumption.

It was therefore concluded, that for the purposes of risk characterisation, the key toxicological effects to be taken into account were the acute effects experienced by humans following ingestion of AZAs, since the normal consumption pattern of shellfish would dictate that chronic exposure to AZAs is less important in terms of characterising the health risks. Chronic effects of AZAs will be addressed in a future assessment of AZA toxicology, when better animal data become available.

The risk characterisation addresses these acute effects by deriving an ARfD, based on the epidemiological data provided by the Arranmore incident. The ARfD has been defined by the WHO-FAO Joint Committee on Pesticide Residues (JMPR) as:

''The estimate of the amount of a substance in food and/or drinking water, normally expressed on a body weight basis, 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 the evaluation'' (JMPR, 2002).

The relative rarity of AZA occurrence and hence the limited availability of purified toxin standard led to the conclusion in 2001 that there was insufficient statistically robust toxicological data for AZAs in animal models to enable the determination of an acute reference dose (ARfD) in the conventional toxicological manner. This situation has not changed, following the first hazard characterisation and risk assessment in 2001 (FSAI, 2001). The determination of a safe level (ARfD) for AZAs in mussels must therefore still be based on an exposure assessment that relies on information from documented incidents of AZP.

Exposure assessment

Ideally epidemiological studies of AZP incidents should detail the amount of mussels consumed by each person, determine the concentration of AZAs in the mussels that were eaten and record the attack rate (number of people ill as a proportion of the number of people consuming contaminated mussels) as well as any physiological details from patients. Unfortunately, no report to date has captured all of these essential data. However, the AZP incident that occurred in Arranmore in 1997 (McMahon and Silke, 1998) did provide some of these details although they were never published. Despite the age of this incident, the rarity of AZP means that it is still the most thoroughly studied incident today.

Consequently, in the absence of any further studies since the 2001 Irish risk assessment (FSAI, 2001), this revised exposure assessment was based on the facts of the Arranmore incident. The uncertainty in this approach is due in part, to the need to make assumptions and also in part to the quantity and accuracy of the data used in the risk assessment to describe natural variability. A probabilistic exposure assessment approach was used for the exposure assessment as a means of indicating the range of possible outcomes and their relative likelihoods.

Since 2001, published scientific evidence has become available concerning the following key parameters of the exposure assessment model in the 2001 Irish risk assessment:

- Tissue distribution of AZAs in mussels
- Ratios of different analogues of AZAs
- Effects of cooking on AZAs

An additional piece of information used in the exposure assessment came from unpublished experimental observations:

- The variation of raw mussel meat weight in raw mussels

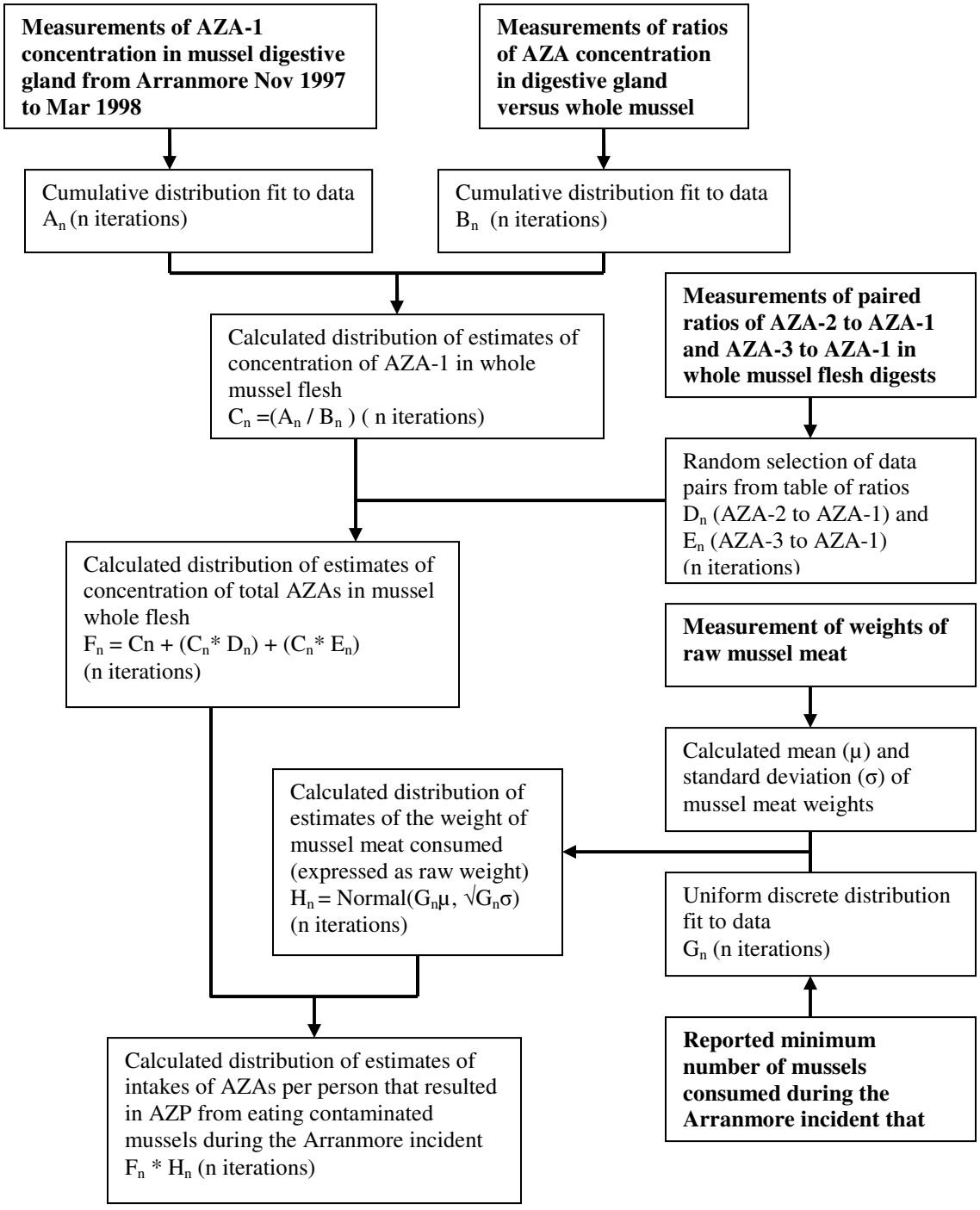
Summary of the Arranmore AZP Incident in 1997

Details of the Arranmore AZP incident were supplied by Dr. Terry McMahon (pers. comm.). 20-24 individuals were affected in the outbreak and 7-8 of these were examined by a doctor. Symptoms were vomiting, diarrhoea and nausea. There were no indications of any hepatotoxic effects and no individuals subsequently presented with illnesses that could be related to the initial intoxication. Some patients reported illness following the consumption of as few as 10 -12 mussels. All patients made a complete recovery after 2 - 5 days.

Estimate of the Level of AZA Exposure in the Arranmore Incident

The objective of the exposure assessment was to calculate the AZA intake from the consumption of mussels by individuals who experienced AZP in the Arranmore incident. Figure 1 shows the schematic pathway for the exposure assessment model. The detail of the two main steps is outlined in the remainder of this section.

Figure 1: Schematic model pathway



Estimating the distribution of possible concentrations of AZAs in whole flesh of Arranmore mussels

Monitoring of toxins in mussels in the Arranmore area showed that the toxin persisted in the shellfish from this location from October 1997 through to June 1998. Dr. Satake measured levels of AZA-1 in the hepatopancreas of raw Arranmore mussels, beginning two months after the toxic event and continuing through to May 1998 (reported in a fax to the Irish Marine Institute dated 17th August 1998) (Table 4). All mouse bioassays were positive throughout this period.

Table 4: AZA-1 concentrations in the HP of mussels from Arranmore, Ireland.

Date	AZA 1 concentration (μg/g hepato pancreas)
12th Nov 1997	8.2
2nd Dec 1997	7.2
15th Dec 1997	9.2
8th Jan 1998	10.7
13th Jan 1998	10.7
4th Mar 1998	5.7
22nd Apr 1998	<0.025
11th May 1998	<0.025
18th May 1998	0.05

Shaded data used for the distribution of possible estimates of AZA-1 concentration in Arranmore mussels during the incident (see text for explanation)

Studies were also carried out on comparative levels of AZA-1, AZA-2 and AZA-3 in Arranmore mussels by two different groups of workers in November 1997 (Ofuji *et al*, 1999b; James and Furey, 2000), around two months after the incident. Table 5 shows a comparison of these results.

Table 5: AZA analogues measured in Arranmore mussels 1997

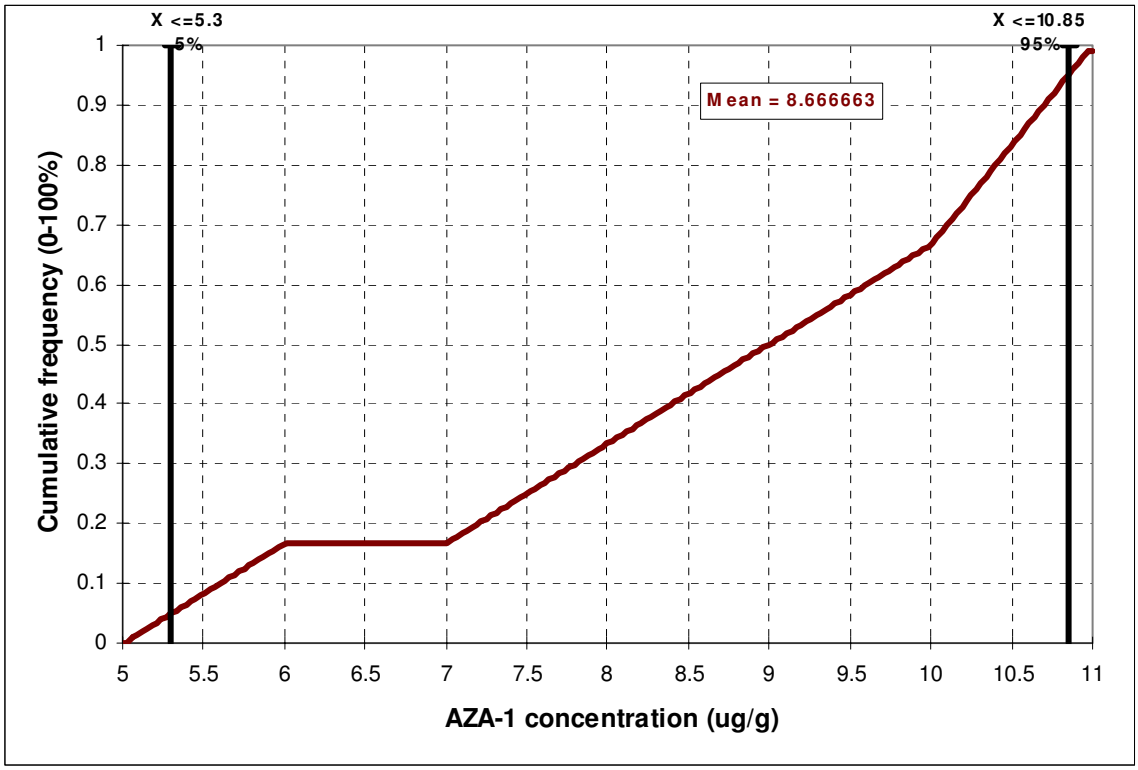
Azaspiracid compound	Concentration in raw mussel meat (μg/g)[a]	Concentration in raw mussel hepatopancreas (μg/g)[b]
AZA-1	0.87	14.7
AZA-2	0.25	13
AZA-3	0.24	8
Total AZAs	**1.36**	**35.7**

a) Ofuji *et al*, 1999b. 3rd Nov 1997; b) James and Furey, 2000. (Sometime in Nov 1997)

The measurements in Tables 4 and 5 were reasonably comparable when the different matrices were accounted for. Any remaining differences could have been due to natural variability both within mussel populations and with time, as well as the lack of a certified standard for the toxin at the time of these studies, which could have led to methodological differences.

For the purposes of the exposure model, the data shaded in Table 4 were used. The last three data points were discarded as AZA-1 levels had dropped to the detection limit or below. The remaining data were checked for outliers using the Cochran outlier test (99% confidence) and none of these data points could be identified as outliers. Consequently all data from 12th Nov 1997 to 4th March 1998 was used as the basis of a distribution of possible AZA-1 concentrations in hepatopancreas of mussels during the Arranmore incident. This distribution is shown in Figure 2.

Figure 2: Cumulative distribution of possible AZA-1 concentrations in mussel hepatopancreas from Arranmore based on data in table 4



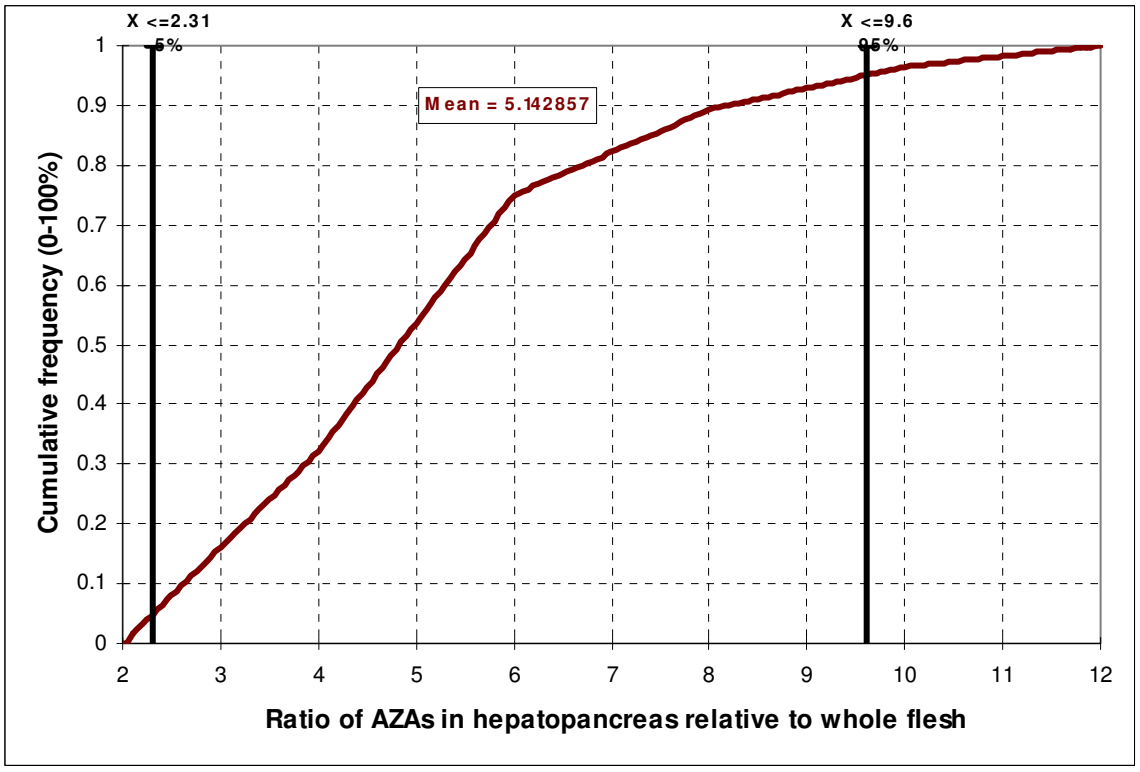
Two assumptions were applied to these data (Table 4) to estimate the level of AZA-1 in mussels during the Arranmore incident, namely:

- It was assumed that no natural depuration of mussels occurred between the date of the incident and the 4th March 1998. This assumption was not supported by surveillance of AZAs conducted in the Irish national biotoxin monitoring programme to 2006, that showed that natural depuration of mussels does occur. Hence, the level of AZAs ingested in the Arranmore incident could have been higher than those measured after the incident (table 4). However, the consequence of using this assumption in the exposure assessment was to assign a lower level of AZA ingestion causing AZP than may have actually occurred in the Arranmore incident. This was a conservative assumption and remains a source of uncertainty in the model.

- It was assumed that no significant increase in the level of AZA-1 in mussels occurred between the date of the incident and the start of measurement in November 1997. This assumption was supported by subsequent surveillance of AZAs conducted in the Irish national biotoxin monitoring programme to 2006, showing that AZA levels peak in summer and in autumn rather than winter. The consequence of this assumption if incorrect would be to assign a higher level of AZA ingestion causing AZP than may have actually occurred in the Arranmore incident. This is a source of uncertainty in the model.

To calculate the concentration of AZA-1 per g of whole mussel flesh, the ratio of AZA-1 in hepatopancreas to whole flesh was required, since the data in Table 4 are based on measurements of AZAs in hepatopancreas. In the previous Irish risk assessment (FSAI, 2001), estimates of this ratio were calculated from expert opinion on the ratio weights of mussel hepatopancreas to whole flesh. However, since this time Hess *et al.* (2005) demonstrated a range of ratios for AZAs in digestive gland and whole flesh (shown in Table 2 of their paper). These data were used to generate a cumulative distribution describing the variability in the measured ratios. This reduced the uncertainty in this exposure assessment compared to the previous assessment (FSAI, 2001). The new distribution is shown in Figure 3.

Figure 3: Cumulative distribution of AZA ratios fitted to data by Hess et al (2005)

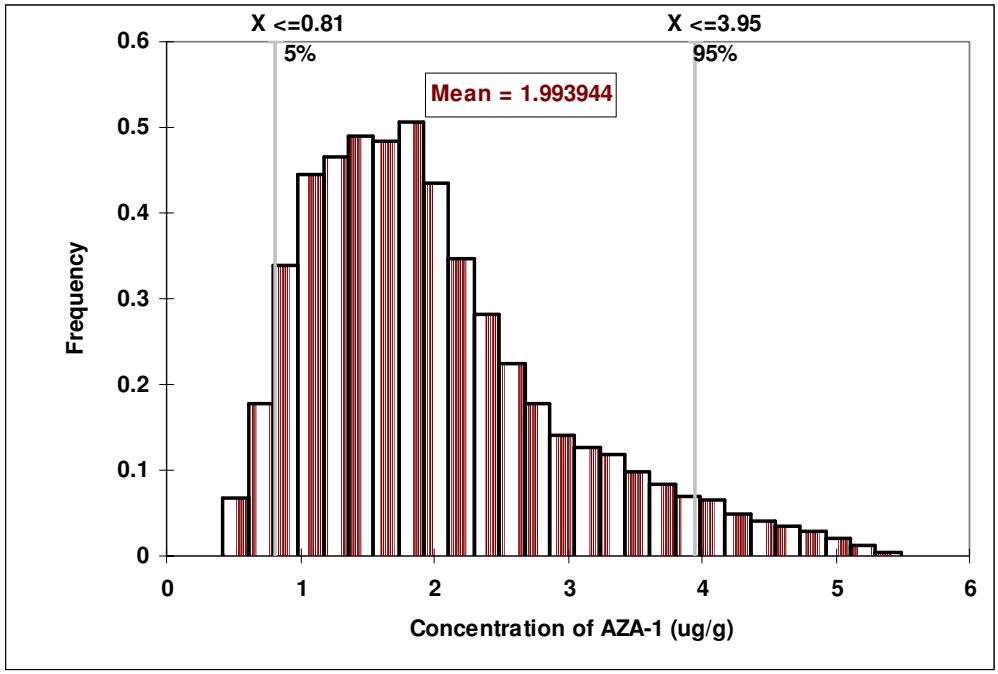


Although these ratios were not measured in mussels from Arranmore it was assumed that:

- The cumulative distribution of the ratios of AZAs in digestive gland relative to AZAs in whole flesh was also representative of the relative distributions of AZAs in Arranmore mussels. Lower ratios would ultimately result in higher estimates of AZA intake by people who suffered with AZP on Arranmore and vice versa.

The distribution of estimated concentrations of AZA-1 in the whole flesh of mussels in Arranmore was calculated by dividing random values from the distribution of AZA-1 in mussel hepatopancreas (Figure 2) by random values from the distribution of ratios of AZAs in hepatopancreas relative to whole flesh (Figure 3). Figure 4 shows the resultant distribution of estimates.

Figure 4: Distribution of estimates of AZA-1 concentration in whole flesh of Arranmore mussels



However, AZA-1 is not the only azaspiracid isomer that has toxicity and AZA-2 and AZA-3 were also present in mussels from Arranmore (Table 5). In the previous Irish risk assessment (FSAI, 2001), the ratios for AZA-2 and AZA-3, relative to AZA-1, used in the exposure model were based on the data generated by Ofuji *et al*, 1999b (Table 5). Since that time, the Irish biotoxin monitoring programme has generated a much more comprehensive data set on the relative co-existence of the different AZA isomers in Irish mussels (Marine Institute, 2005). These data were generated in separate 100g digests of mussel whole flesh (table 6).

Table 6: Relative proportions of AZA-2 and AZA-3 in mussel whole flesh relative to AZA-1 as measured in the 2005 Irish biotoxin monitoring programme

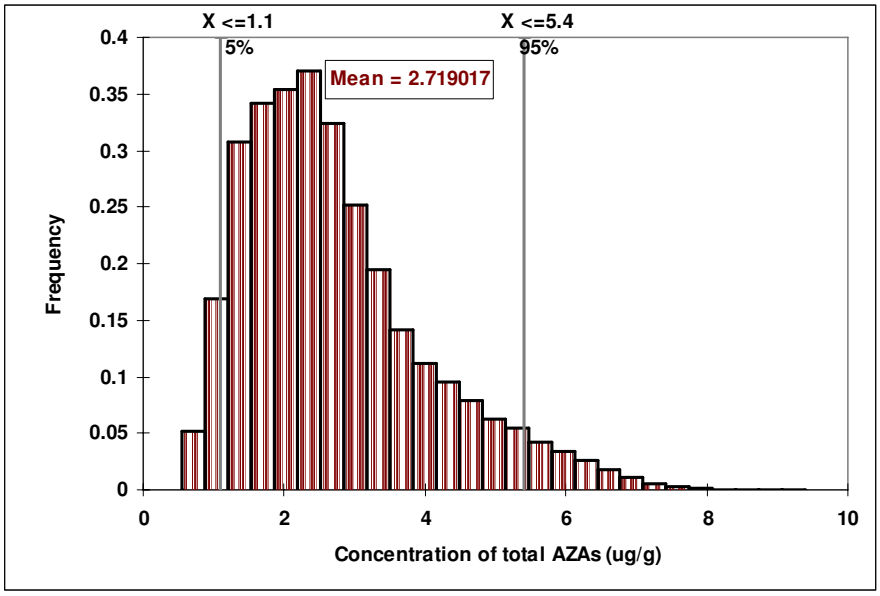
Sample number	Ratio AZA 2 to AZA 1	Ratio AZA 3 to AZA 1	Sample number	Ratio AZA 2 to AZA 1	Ratio AZA 3 to AZA 1	Sample number	Ratio AZA 2 to AZA 1	Ratio AZA 3 to AZA 1
1	0.24	0.12	*25*	0.27	0.03	*49*	0.25	0.04
2	0.23	0.07	*26*	0.28	0.06	*50*	0.29	0.04
3	0.27	0.09	*27*	0.33	0.11	*51*	0.38	0.08
4	0.26	0.04	*28*	0.30	0.04	*52*	0.38	0.05
5	0.27	0.04	*29*	0.46	0.04	*53*	0.43	0.06
6	0.24	0.04	*30*	0.26	0.05	*54*	0.42	0.11
7	0.35	0.41	*31*	0.25	0.02	*55*	0.26	0.04
8	0.23	0.03	*32*	0.25	0.02	*56*	0.32	0.04
9	0.26	0.08	*33*	0.31	0.11	*57*	0.26	0.05
10	0.28	0.08	*34*	0.25	0.04	*58*	0.51	0.09
11	0.22	0.02	*35*	0.26	0.03	*59*	0.29	0.03
12	0.21	0.07	*36*	0.27	0.05	*60*	0.29	0.03
13	0.25	0.03	*37*	0.48	0.19	*61*	0.36	0.05
14	0.30	0.03	*38*	0.31	0.06	*62*	0.30	0.02
15	0.30	0.03	*39*	0.27	0.04	*63*	0.35	0.04
16	0.23	0.06	*40*	0.30	0.05	*64*	0.37	0.08

Sample number	Ratio AZA 2 to AZA 1	Ratio AZA 3 to AZA 1	Sample number	Ratio AZA 2 to AZA 1	Ratio AZA 3 to AZA 1	Sample number	Ratio AZA 2 to AZA 1	Ratio AZA 3 to AZA 1
17	0.29	0.03	*41*	0.23	0.11	*65*	0.31	0.04
18	0.24	0.05	*42*	0.48	0.08	*66*	0.26	0.06
19	0.28	0.04	*43*	0.32	0.04	*67*	0.46	0.03
20	0.24	0.08	*44*	0.31	0.06	*68*	0.26	0.05
21	0.26	0.16	*45*	0.37	0.08	*69*	0.31	0.05
22	0.28	0.07	*46*	0.26	0.07	*70*	0.29	0.03
23	0.26	0.04	*47*	0.35	0.10	*71*	0.39	0.06
24	0.26	0.04	*48*	0.30	0.04	*72*	0.25	0.04

Analysis of the data in table 6 demonstrated a positive correlation between the relative occurrence ratios of AZA-2 to AZA-1 and the ratios of AZA-3 toAZA-1. To maintain this correlation during calculation the paired results were bootstrapped during simulation.

To calculate the distribution of estimates of total AZAs in whole mussel flesh, a random value from the distribution of AZA-1 concentration in whole flesh (Figure 4) was multiplied by a random pair of ratios from table 6 and used to calculate the levels of AZA-2 and AZA-3 relative to the concentration of AZA-1. The three values for AZA-1, AZA-2 and AZA-3 concentration were then added together to give an estimated concentration of total AZAs in whole flesh of raw Arranmore mussels. By repeating this process of random selection and calculation over several thousand iterations, a distribution of estimates of total AZAs in whole flesh of raw Arranmore mussels was generated. This is shown in Figure 5.

Figure 5: Distribution of estimates of total AZA (∑AZA-1+AZA-2+AZA-3) concentration in whole flesh of raw Arranmore mussels



The implicit assumptions in the calculation of estimated AZA concentration were:

- It was assumed that the relative proportions of AZA-2 and AZA-3 to AZA-1 measured in the Irish national biotoxin monitoring programme 2005 were applicable to the Arranmore incident in 1997. The data of Ofuji *et al*, 1999b (Table 5) shows ratios of AZA isomers in

mussel whole flesh that were within the range covered by those data in table 6, supporting this approach. In addition, the correlation between the relative concentrations of AZA-1 to AZA-2 and AZA-1 to AZA-3 seen in the data supplied by the Irish biotoxin programme was assumed to be applicable to the relative occurrence of the different AZA isomers during the Arranmore event. It is logical that the occurrence of AZA-1, AZA-2 and AZA-3 are not independent of each other and hence this is a more accurate modelling approach. By using these data the level of uncertainty was reduced in intake estimates generated in this exposure assessment compared to intake estimates generated in the previous assessment (FSAI, 2001).

- It was assumed that the relative toxicities of AZA-1, AZA-2 and AZA-3 were the same (see hazard characterisation). If AZA-2 and AZA-3 toxicities were higher than AZA-1, as suggested by preliminary results, (Satake *et al*, 1998b; Ofuji *et al* 1999a), then this would result in higher estimates of AZA intake in people who suffered AZP in Arranmore. Therefore the assumption of equal toxicity results in lower estimates of AZA intake and therefore a more conservative approach.

Estimating the intake of AZAs that caused poisoning in the Arranmore incident

To calculate the total amount of AZAs ingested by a person during the Arranmore incident it was necessary to calculate the amount of mussel meat consumed by an affected patient on Arranmore. These estimates were then combined with the estimates of AZA concentration in the mussel meat, to give estimates of the AZA intake which affected people may have been exposed to during the AZP incident in Arranmore.

Hess *et al*, 2005 demonstrated that cooking did not inactivate AZAs. Consequently, the total amount of AZA present in raw mussel meat is the same as the total amount present in cooked mussel meat, on an individual mussel basis. Therefore, it was simpler mathematically to calculate the weight of mussel meat eaten by a person during the Arranmore incident in its raw rather than cooked state. In this respect, the calculation required knowledge of the variation in the raw mussel meat weight of individual mussels. Data on raw meat weights for mussels were provided by Bantry Bay Seafoods, Ireland, based on a random sample of 30 mussels harvested in March 2006 from Mulroy Bay in Co. Donegal, Ireland (Table 7).

Table 7: Mussel meat weights of raw mussels from Mulroy Bay (Bantry Bay Mussels Ltd)

Raw mussel meat weight of individual mussels (g) n 30		
4.13	4.04	4.72
5.11	3.00	3.02
3.66	3.86	4.57
3.94	4.04	2.94
5.67	4.48	4.46
3.85	4.51	4.72
4.01	3.57	4.43
5.52	5.20	3.32
4.29	6.10	4.55
4.19	3.29	3.59

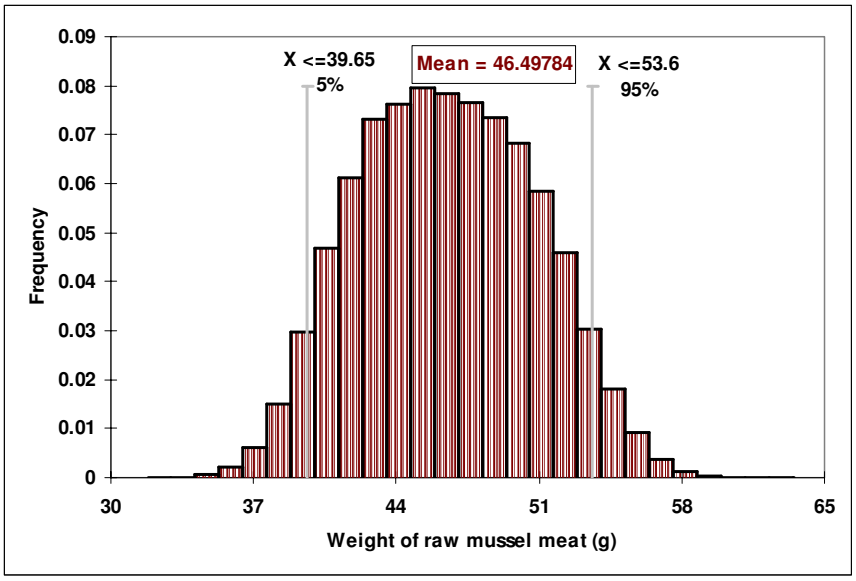
From these data a mean mussel meat weight (4.23g) and standard deviation (0.79g) was calculated.

- It was assumed that the distribution of mussel meat weights from Mulroy Bay was representative of the distribution of mussel meat weights of mussels grown in Arranmore. Geographically, the two locations are close. However, mussel meat weight varies with age, nutrient availability, temperature and water conditions. Therefore, this assumption remains a source of uncertainty that cannot be reduced because the Arranmore site is no longer in business, and no other commercial growing activity occurs on the island. These data were therefore the best estimate available and same source of uncertainty existed in the original risk assessment (FSAI, 2001).

During the Arranmore incident, it was reported that a person who went on to develop symptoms of AZP ate "as few as" 10 to 12 cooked mussels. Hence the use of these data should lead to a conservative estimate of consumption. As it is equally likely that the person ate 10, 11 or 12 mussels, this possibility was modelled using Uniform Discrete Distribution, where the three possible consumption levels have an equal probability of occurrence.

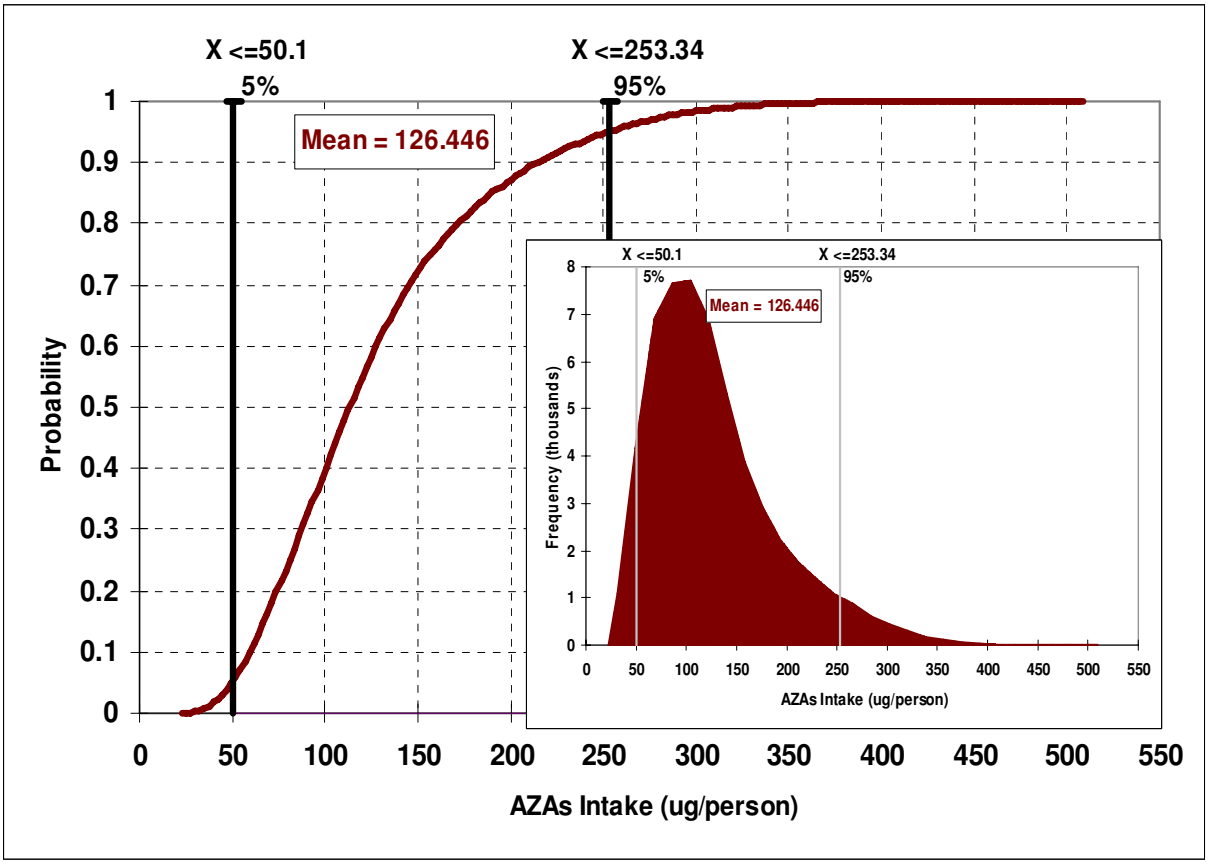
To calculate the distribution of estimates of the weight of mussel meat consumed during the Arranmore incident (expressed as raw weight), random values from the distribution describing the number of mussels eaten were combined with the mean and standard deviation of the raw mussel meat weight by applying the central limit theorem as detailed by Vose (2000). Figure 6 shows this distribution.

Figure 6: Distribution of estimated weights of mussel meat consumed (raw equivalent) by a person who subsequently developed AZP during Arranmore incident



An estimation of the total amount of AZAs (the intake) ingested by a person who went on to develop symptoms of AZP, was calculated by multiplying random values from the distribution of estimated concentrations of AZAs in the mussel meat (Figure 5), by random values from the distribution of weights of mussel meat consumed (Figure 6). Figure 7 shows the outcome of this calculation, which allows for the calculation of a LOAEL for AZA poisoning for affected individuals in the Arranmore incident.

Figure 7: Cumulative distribution of intakes of AZAs that may have caused AZA poisoning on Arranmore (insert: same data plotted as a frequency distribution)



These data shown in Figure 7 are summarised in tabular form (Table 8). This shows the distribution of estimates of the exposure of a person on Arranmore to AZAs (∑AZA-1, AZA-2 and AZA-3) from eating contaminated mussels.

Table 8: Statistics for the distribution of intake estimates for AZAs on Arranmore (µg per person)

	22.75		100.36
	509.25		106.77
	126.45		113.41
	62.22		120.34
	3871.02		127.71
	1.11		135.86
	4.21		145.58
	90.64		156.97
	50.10		171.37
	59.60		190.09
	67.19		215.13
	74.19		253.34
	80.96		
	87.46		
	93.89		

Table 8 shows that the intakes of AZA believed to have caused human intoxication were between 50.1µg (5%) and 253.3µg (95%) per person. The comparable intakes of AZA reported in the original Irish risk assessment, were between 6.7µg (5%) and 24.86µg (95%) per person (FSAI, 2001), or some 8 times lower than these new estimates.

Sensitivity analysis of the exposure model demonstrates that the outcome is dominated by one input, the measurements of AZA-1 concentration 2 months after the Arranmore incident (table 4). Consequently any failure in the assumptions surrounding the use of these data (as outlined above) would have significant consequences on the validity of the final estimate of AZA intake. All other input variables were demonstrated to have a relatively minor impact on the outcome of the model in comparison to the AZA-1 concentration data.

Risk Characterisation

To date, toxicological data on the effects of AZAs are limited. Available data clearly indicate that AZA produces profound biological perturbations at the cellular level. Observations in humans and in animal studies, as well as *in vitro* studies report the ability of AZA to affect the gastrointestinal tract. In addition, the *in vivo* studies by Ito and colleagues suggest a potential tumourigenic action, although at this stage no definitive decision can be based on these limited studies. Further, more extensive carcinogenicity studies would be required. *In vitro* studies on the mechanism of action of AZAs are ongoing and an *in vitro* model for human gastrointestinal effects has been established. Until these data become available, it is only possible to address the acute toxic effects of AZAs in this risk characterisation. This approach is in accordance with the conclusions of the Joint FAO/IOC/WHO ad hoc expert consultation on biotoxins in bivalve molluscs. (FAO, 2004)

As part of this risk characterisation, it is necessary to consider the outcome in the light of other risk assessments conducted on AZAs. Three risk assessments have been carried out by international bodies since the initial Irish risk assessment (FSAI, 2001), and these have all been based on the derivation of AZA intake levels calculated in the initial Irish risk assessment. As already indicated, the intakes of AZA, believed to have caused human intoxication reported in the original Irish risk assessment, were between 6.7µg (5%) and 24.86µg (95%) per person (FSAI, 2001).

The report of the meeting of the European Commission Working Group on Toxicology of DSP and AZP (EC, 2001) re-examined the initial Irish risk assessment. The availability of new data on heat stability resulted in a recalculated range of the LOAEL (intake per person producing toxicity). This resulted in a recalculated LOAEL of between 23 and 86µg per person with a mean value of 51.7µg. A safety factor of three was applied, to convert the LOAEL to a NOAEL, to account for individual variation, producing a range of 7.7µg to 28.7µg, with a mean of 17.2µg. Based on the lowest NOAEL of 7.7µg and a standard 60kg adult, this yielded an ARfD of 0.127µg/kg b.w.. Based on an intake level of a maximum of 100g shellfish meat/meal, it was stated that an allowance level of 8µg AZAs/100 g of shellfish meat should result in no appreciable risk for human health. However, to allow for detection by the mouse bioassay, a level in shellfish meat of 0.16mg/kg was proposed as a maximum regulatory limit.

The evaluation of the Joint FAO/IOC/WHO ad-hoc expert consultation (FAO, 2004), established a provisional ARfD of 0.04µg/kg b.w., based on the lowest reported LOAEL of

23µg per person in humans (EC working group, 2001) and a body weight of 60kg, using a ten fold safety factor, to take into consideration the small number of people involved. This risk assessment preferred a consumption level of 250g of shellfish meat per person. Hence, a derived guidance level for AZAs in shellfish meat of 0.0096mg/kg was advised. As there was insufficient data on the chronic effects of AZA, no tolerable daily intake (TDI) could be established.

The most recent conclusions and recommendations were those of the European Commission Working Group on Toxicology in 2005 (Community Reference Laboratory on Marine Toxins (CRLMB), where a guidance level of 0.032mg/kg shellfish meat for AZAs was considered appropriate (subject to future re-evaluation). This level was based on the existing lowest LOAEL from epidemiological studies of 0.38µg/kg b.w. (23µg per person divided by a 60kg reference body weight), a portion size of 250g and a safety factor of three (Anonymous, 2005).

It is worth reiterating that the quantitative conclusions and recommendations from international risk assessments have been based on the data generated by the initial Irish risk assessment (FSAI, 2001). The first European Commission risk assessment (EC, 2001) addressed the effect on estimated LOAELs of AZA heat stability and adjusted the Irish risk assessment estimates accordingly. This recalculation was subsequently adopted by the other international groups in their later risk assessments. However, none of the international risk assessments have ever examined the impact of new data on AZA intake calculations in the Irish risk assessment (FSAI, 2001) The revised risk assessment reported here revisited the initial data and addressed the initial assumptions based on the availability of new data. This resulted in three main changes to the exposure assessment model as follows:

Tissue distribution of AZAs in mussels: Expert opinion on the relative proportions of hepatopancreas to whole flesh was used in the 2001 FSAI risk assessment to calculate the likely concentration of AZA-1 in whole flesh given a measurement of concentration in hepatopancreas. However, Hess *et al* (2005) recently reported a series of ratios based on measurements of AZAs in mussel hepatopancreas relative to whole flesh, and the use of these new data resulted in a reduction of a source of uncertainty in the original risk assessment and allowed for a more accurate description of the natural variability of this ratio. These new data increased the range of estimates of AZA-1 in mussel whole flesh with a higher average estimate (2µg/g) compared to the previous estimate (1.3µg/g) (FSAI,2001).

Ratios of different analogues of AZAs: In the 2001 FSAI risk assessment a single proportion for AZA-2 and AZA-3 relative to AZA-1 was used based on data from Ofuji *et al* (1999b). New data from the 2005 Irish biotoxin programme has generated a range of 72 different proportions for AZA-2 and AZA-3 relative to AZA-1, confirming that the relative proportions of the three analogues in mussels are highly variable and positively correlated. In the current risk assessment these new data were used to provide a much more accurate basis for the calculation of total AZAs than the single value used in the 2001 FSAI risk assessment, thus reducing the uncertainty in the original 2001 risk assessment. The data of Ofuji *et al* (1999b) were within the range of these new data but towards the upper end particularly for the ratio of AZA-3 to AZA-1.

Effects of cooking on AZAs: AZAs in mussels are not affected by cooking (see hazard identification). Hence there was no reduction of AZA due to heating (a 70% reduction was

allowed for in the 2001 risk assessment). This finding also allowed for a simplification in the 2001 exposure assessment model, by calculating AZA intakes based on mussel consumption expressed in terms of raw weight rather than having to account for the reduction in mussel meat weight due to cooking (~50%). The combined effect of these changes led to a 7 fold increase in estimates of AZA compared to those calculated in the previous exposure assessment (FSAI, 2001). However, a degree of uncertainty still exists in this part of the exposure assessment due to the lack of knowledge on mussel meat weight in the Arranmore growing site in 1997.

The consequences of the adoption of new data and calculation approaches in the revised risk assessment reported here resulted in an increased estimate of AZA intake that led to AZP on Arranmore. The outputs of the two Irish risk assessments (2001 and that reported here in 2006) are compared in Table 9.

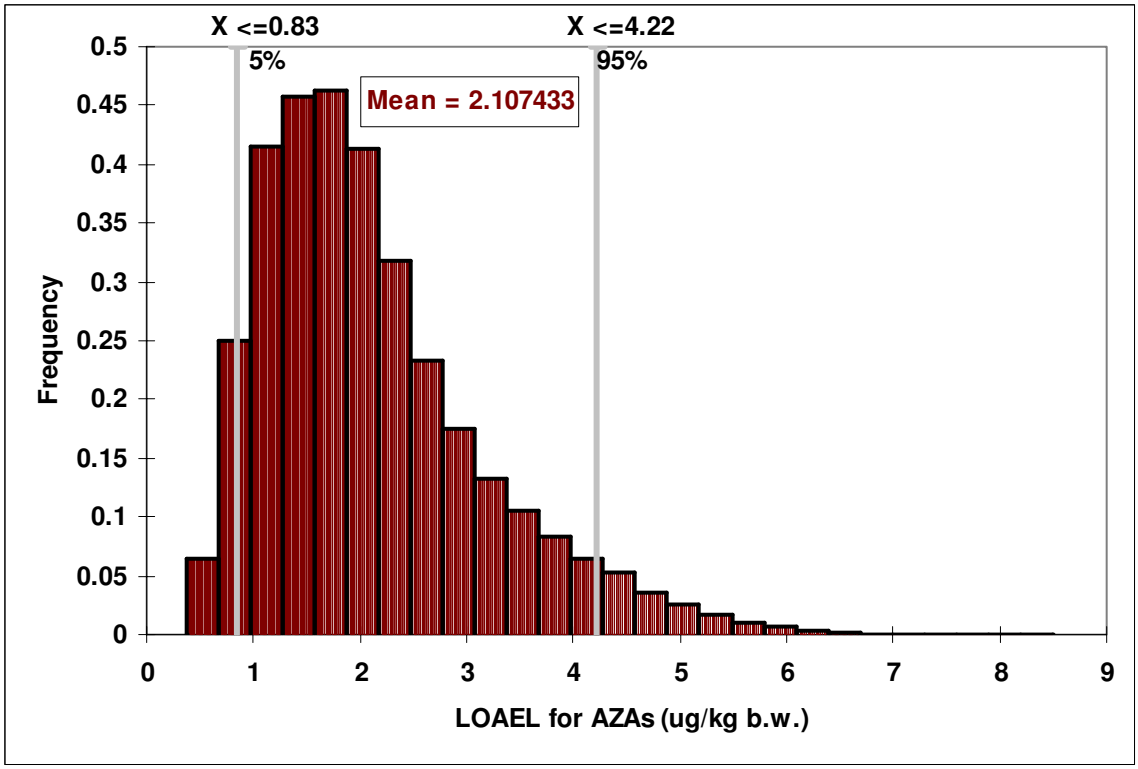
Table 9: Comparison of the statistics of the distributions of estimates of AZA intake on Arranmore reported in the 2001 FSAI Irish risk assessment and the revised 2006 FSAI risk assessment (reported here)

	AZAs intake (µg/person)	
	2006 FSAI risk assessment	2001 FSAI risk assessment
Minimum	22.75	1.96
Maximum	509.25	45.37
Mean	126.45	15.01
Std deviation	62.22	5.69
Variance	3871.02	32.41
Skewness	1.11	0.51
Kurtosis	4.21	3.14
Mode	90.64	12.25
5th Percentile	50.10	6.60
10th Percentile	59.60	7.97
15th Percentile	67.19	9.00
20th Percentile	74.19	9.88
25th Percentile	80.96	10.73
30th Percentile	87.46	11.58
35th Percentile	93.89	12.30
40th Percentile	100.36	13.07
45th Percentile	106.77	13.78
50th Percentile	113.41	14.52
55th Percentile	120.34	15.30
60th Percentile	127.71	16.07
65th Percentile	135.86	16.83
70th Percentile	145.58	17.72
75th Percentile	156.97	18.65
80th Percentile	171.37	19.75
85th Percentile	190.09	20.97
90th Percentile	215.13	22.57
95th Percentile	253.34	25.16

This new exposure assessment provides a better estimate of the intake of AZAs consumed on Arranmore than the previous exposure assessment (FSAI, 2001), because of the factors outlined above and the reduction in the uncertainty of the exposure model. However, a moderate degree of uncertainty in the assessment still exists.

The new exposure assessment results (Table 8) can be used to characterise the risk of AZAs, and therefore to derive a figure for the LOAEL, by expressing the AZA intake relative to body weight using the standard 60kg adult commonly adopted in toxicological risk assessments. The results of this are shown in Figure 8.

Figure 8: Distribution of estimates of the lowest observable adverse effect level (LOAEL) for AZAs based on the Arranmore AZP incident



The calculation of an ARfD from the estimates of the LOAEL requires the application of a safety factor. The magnitude of the safety factor is dependent on the toxicological assessment outlined in the hazard characterisation.

There has been considerable variation in safety factors applied in international AZA risk assessments. Safety factors of 3 (Working Group on Toxicology (Community Reference Laboratory on Marine Toxins (CRLMB) (2005) and 10 (FAO/IOC/WHO ad hoc expert consultation, (FAO, 2004) have variously been used, depending on the degree of uncertainty perceived in the human epidemiological data set. Whereas international risk assessments agree that a safety factor of three is appropriate for okadaic acid, because the LOAEL is based on human epidemiological data involving over 40 people, in the case of AZAs it appears that different risk assessors have formed different views on the quality and quantity of the supporting human epidemiological data used to derive the LOAEL.

Common convention in toxicology proposes a safety factor of 10 for interspecies differences, and 10 for intra-species differences, based on uncertainty estimates derived from available data. The risk assessment reported here is based on human epidemiological data and therefore inter-species difference does not apply. Consequently, the starting point for derivation of an appropriate safety factor is 10 for intra-species differences. The safety factor of 10 is further subdivided into toxicokinetic and toxicodynamic components: 3.2 x 3.2 for intra-species differences (Renwick, 1993). Using Renwick's approach, there is a scientific basis for a

safety factor of 3. The toxicokinetic variation allows for differences in metabolism of the toxic compound between individuals. This would be particularly relevant if metabolism resulted in a more toxic compound. As indicated in the hazard characterisation section there is no clear evidence for metabolism resulting in a more toxic compound. Furthermore, as the toxicity of AZA is targeted to the gastrointestinal tract the need for metabolism is less likely. The evidence from the in vitro studies using cultured human gastrointestinal cells, where toxicity can be detected on application of AZA, also indicates that metabolism is not necessary for AZA to produce toxicity. These findings clearly indicate that a safety factor of **3** would be appropriate for AZAs. Such a safety factor is also used for many other marine toxins.

It is true that the Arranmore incident supplies limited human epidemiological data (eight people affected) and hence, if used in isolation, these data may not be considered to support a safety factor of three. However, as discussed in this report (hazard identification section), there is evidence that approximately 216,000 portions of oysters have been legally placed on the market with AZA levels between 0.1 and 0.16mg/kg, without reported ill effects. Similarly, rapid alerts suggest that a considerable quantity of Norwegian crab was placed on the market containing AZAs. Even with under-reporting of food-borne illness, it could be reasonably expected that if the ARfD for AZA was as low as suggested in recent risk assessments, then at least one report of illness would result from the consumption of such a large amount of shellfish containing AZA . This could be viewed as crude evidence of a much wider epidemiological data set than that provided by the Arranmore incident alone.

Therefore, in the current risk assessment, the risk assessors considered that a safety factor of **3** was justified, based on the toxicodynamic effects of AZAs, and human epidemiological evidence in its broadest context, that includes the absence of symptoms of AZP, following consumption of shellfish containing up to the current regulatory limit of 0.16mg/kg in raw shellfish. Application of a safety factor of **3** to the estimates of the LOAEL (figure 8) results in the distribution of estimates of ARfD shown in Figure 9 and summarised in Table 10.

Figure 9: Distribution of estimates of the Acute Reference Dose (ARfD) from the Arranmore AZP incident using a safety factor of 3

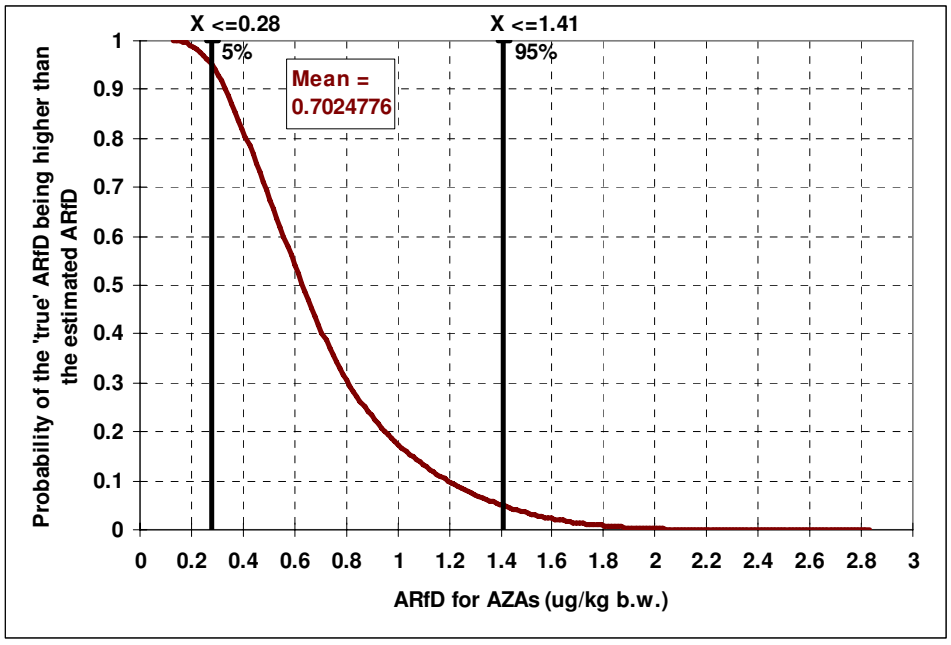


Table 10: Statistics of the distribution of ARfD estimates for AZAs on Arranmore (µg/kg b.w.) safety factor = 3

Statistics of the distribution of estimates	ARfD µg/kg b.w. (SF3)	Statistics of the distribution of estimates	ARfD µg/kg b.w. (SF3)
Minimum	0.13	**35th Percentile**	0.52
Maximum	2.83	**40th Percentile**	0.56
Mean	0.70	**45th Percentile**	0.59
Std deviation	0.35	**50th Percentile**	0.63
Variance	0.12	**55th Percentile**	0.67
Skewness	1.11	**60th Percentile**	0.71
Kurtosis	4.21	**65th Percentile**	0.75
Mode	0.50	**70th Percentile**	0.81
5th Percentile	0.28	**75th Percentile**	0.87
10th Percentile	0.33	**80th Percentile**	0.95
15th Percentile	0.37	**85th Percentile**	1.06
20th Percentile	0.41	**90th Percentile**	1.19
25th Percentile	0.45	**95th Percentile**	1.41
30th Percentile	0.49		

It should be noted that the median of the distribution, is 0.63µg/kg b.w. (Table 8). This is comparable to the maximum intake value of 0.67µg/kg b.w. for a 60kg person consuming 250g mussels contaminated with AZAs at the current regulatory limit of 0.16mg/kg. This ARfD value may be supported by the absence of reported incidents of AZP since the adoption of the 0.16mg/kg maximum regulatory limit for AZAs in shellfish and strengthening of national biotoxin monitoring programmes to enforce it.

Conclusion

The revised risk assessment reported here has revisited the initial data reported in the 2001 FSAI risk assessment and has addressed the initial assumptions based on the availability of new data on tissue distribution of AZAs in mussels, ratios of different analogues of AZAs and effects of cooking on AZAs. The use of the new data and a simplified calculation approach, coupled with a probabilistic exposure assessment approach, has resulted in an increased estimate of AZA intake that led to AZP on Arranmore, providing estimates for the intakes of AZA believed to have caused human intoxication of between 50.1µg (5%) and 253.3µg (95%) per person. The median estimate of an ARfD is 0.63µg/kg b.w. using a safety factor of 3.

The availability of new data has allowed for the recalculation of an estimate for an ARfD for AZAs. However, there remains some uncertainty associated with this estimate although pragmatic examination of the absence of reported AZP following considerable sales of shellfish with levels of AZA below the current European limit supports an estimate of this magnitude. There is a clear need for further research into the toxicology of AZAs and thorough detailed epidemiological investigation of any suspected incidents of AZP in the

future. Mechanisms of action need to be elucidated and preliminary work on long term low dose toxicological effects needs to be developed and supported with statistically significant animal studies.

Acknowledgements

We would like to acknowledge the considerable contribution of Dr. Gavin Ryan, UCD, to the development of this risk assessment and the assistance of Bantry Bay Seafoods Ireland for the provision of mussel meat data for the exposure assessment. The authors are grateful to David Vose (Vose Consulting) for reviewing the exposure model.

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Appendix 1: List of scientists associated with this report

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Dr. Wayne Anderson – Food Safety Authority Ireland
Dr. Phillip Hess – Marine Institute
Dr. Iona Pratt - Food Safety Authority Ireland
Dr. Thomasina Barron – Pesticide Control Service
Dr. Edel Healy – Health and Safety Authority of Ireland

Members of the Scientific Committee

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Prof. Michael Ryan – University College Dublin
Dr. Phillip Hess – Marine Institute
Dr Catherine Adley – University of Limerick
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Dr Sinead McCarthy – Trinity College Dublin
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Appendix 2: Risk Management Options

Risk managers using this risk assessment may wish to set a maximum regulatory level for AZAs in mussels based on an ARfD value from within the distribution of estimates and a consumption level for raw mussels that represents common consumption practice in a high proportion of the population.

In selecting an ARfD value or values, risk managers should be familiar with the assumptions in the risk assessment that affect the certainty of the estimates of ARfD. The median value or 50th percentile is the value where 50% of the estimates of ARfD are higher and 50% of the estimates of ARfD are lower (0.63 µg/kg b.w). ARfD estimates from the lower part of the distribution of estimates, reflect a more precautionary approach, than ARfD values selected from the upper part of the distribution

Equation 1 can be used to generate a maximum regulatory limit for AZAs in shellfish

ML= (pARfD*ARfD*0.6)/MC (**equation 1**)

ML *= Maximum regulatory limit for AZAs in shellfish (µg/kg)*
MC *= maximum consumption per person per meal level (kg)*
ARfD *= chosen estimate of acute reference dose for AZAs (µg/kg b.w.)*
pARfD *= percentage of the ARfD that is acceptable for a person to consume in one meal (value between 1% and 100%)*

Worked Example (for demonstration purposes only):

MC = 0.25 kg mussel meat per person per meal (250g)
ARfD = 0.63 µg/kg b.w (median value table 10)
pARfD = 100 (i.e. it is acceptable for a person to ingest AZAs up to 100% of the ARfD per day in a single meal)

ML = (100*0.63*0.6)/0.25

ML = 37.8/0.25

Maximum Regulatory Limit = 151.4 µg/kg *(~ 0.15 mg/kg raw shellfish flesh)*