Performance of the EU Harmonised Mouse Bioassay for Lipophilic Toxins for the Detection of Azaspiracids in Naturally Contaminated Mussel (*Mytilus edulis*) Hepatopancreas Tissue Homogenates Characterised by Liquid Chromatography coupled to Tandem Mass Spectrometry

Philipp Hess[a, b], Tim Butter[c], Andrew Petersen[c], Joe Silke[a] and Terry McMahon[a]

[a] *Marine Institute, Rinville, Oranmore, Co. Galway, Ireland*

[b] *Author's present address : Ifremer, Rue de l'Ile d'Yeu, Nantes, France*

[c] *En-Force Laboratories Ltd., Spring Lane, Ballyvolane, Cork, Ireland*

email of the corresponding author: Philipp.Hess@ifremer.fr

Abstract

Azaspiracids (AZAs) are a group of lipophilic polyether toxins that were discovered in shellfish from Ireland in 1995, following a food poisoning incident. Both the limited availability of pure AZAs and the co-occurrence in shellfish of other toxins in combination with AZAs have so far prevented an in-depth evaluation of the performance of the EU reference test, the mouse bioassay (MBA), for this toxin group at the regulatory limit.

The present study evaluated the performance of the mouse bioassay at the example of a mussel tissue homogenate, naturally contaminated with AZAs, diluted with uncontaminated tissues to appropriate concentration levels. Concentrations were determined using liquid chromatography coupled to tandem mass spectrometry (LC-MS-MS) (7 levels ranging from levels less than the limit of quantification to a maximum of ca. 2.24 mg/kg in hepatopancreas, which corresponds to a maximum whole flesh AZA1-equivalent of ca. 0.34 mg/kg). Replicate homogenates of each concentration level were analysed by MBA on 7 independent

occasions over 6 weeks. Inhomogeneity between replicate aliquot portions was evaluated using LC-MS-MS and ranged from 1.8 to 6.6% RSD for the six levels contaminated above quantification limits. This variation was similar to the variability of the LC-MS-MS method within a batch, and the difference between replicate aliquots could thus be considered negligible. Other uncertainties considered in the study included the short- and long-term variability of the LC-MS-MS method, toxic equivalence factors, relative response factors in mass spectrometric detection, additional analogues and matrix effects.

A concentration-response curve was modelled as a 4-parametric logistic fit to a sigmoidal function, with an LC_{50} of 0.70 mg AZA1-equivalent/kg hepatopancreas tissue. Furthermore, the mathematical model of the lethality data from this study suggests that occasional negative mouse assays at high concentrations, previously observed in the Irish statutory monitoring, are at least partly due to the biological variation of mice and can be understood on a statistical basis. The mathematical model of the concentration-response curve also describes the probability of a positive mouse bioassay at the current regulatory limit of 0.16 mg/kg to be ca. 95%. Therefore, it appears that the mouse bioassay performs very well in the implementation of this limit. Hence, the present study very strongly suggests that the MBA and LC-MS-MS techniques can be considered equivalent in the implementation of the current regulatory limit of 0.16 mg/kg for Azaspiracids in shellfish.

Keywords

Phycotoxins, shellfish, public safety, assay performance, qualitative analysis, lethality, matrix effects, mass spectrometry, instrument response factor, toxic equivalence factor

Introduction

Azaspiracids (AZAs) are a group of lipophilic algal toxins that may accumulate in shellfish to levels causing acute sickness in shellfish consumers (FSAI, 2006). However, since their first discovery in 1995, following a food poisoning incident from Irish mussels (McMahon and Silke, 1996; Satake et al., 1998), only five further poisoning events could be traced back to the presence of these compounds in shellfish. Due to this rare occurrence of AZA shellfish poisoning, the extent of toxicity of the compounds remains a matter of international debate. Apart from Ireland, AZAs have been found in shellfish all along the European Atlantic coast from Norway (Hess et al., 2005) to Portugal (Vale et al., 2008), and also in Morocco (Taleb et al., 2006). The ecology, chemistry and toxicology of AZAs were reviewed extensively by Twiner et al., 2008. More recently, a small dinoflagellate has been identified as a causative organism of AZAs in the North Sea and has been shown to also produce these toxins in culture (Krock et al., 2008).

A regulatory limit for AZAs in shellfish (0.1 mg/kg) was first proposed in Ireland in 2001 (Anderson et al., 2001). The European Community revised its legislation in 2002, and recommended a limit of 0.16 mg/kg for AZAs in shellfish as - at the time - this was the limit believed to be detectable by the mouse bioassay (Anon., 2002, now superceded by Anon., 2005). More recently, this regulatory limit has been given additional credibility through a revised risk assessment of the Food Safety Authority Ireland (FSAI, 2006). Both rat and mouse bioassays have been used for the detection of AZAs since 1995 (McMahon and Silke, 1996), however, the performance and in particular the limits of detection of these assays could not be established due to a lack of large enough quantities of shellfish test materials.

In 1995, following establishment of a network of national and community reference laboratories for marine biotoxins, the mouse bioassay was introduced as detection method in official monitoring of shellfish toxins in Ireland, replacing the rat bioassay which had been

used until then. Initially, an assay injecting the concentrated, resuspended acetone extract was used (Yasumoto et al., 1978); later, in 2001, the amended protocol (Yasumoto et al., 1984) was introduced. The observation time had been 24 h since 1995, as this duration of observation had been recommended for the detection of delayed symptoms from esters of the OA-group toxins (Yanagi et al., 1989). Following toxic events in 1999 and 2000, official control of shellfish production areas in Ireland has been carried out since 2001 using two tests in parallel: the mouse bioassay, as per EU-harmonised protocol (H-MBA), and an in-house validated method based on liquid chromatography coupled to tandem mass spectrometry (LC-MS-MS). While the mouse bioassay gives a simple positive or negative response, the LC-MS-MS method allows for the acquisition of quantitative results. Due to the qualitative nature of the MBA, it is not possible to describe the repeatability of the assay for a particular shellfish tissue without using further tests to characterise the shellfish tissue quantitatively.

In the LC-MS-MS test, concentrations of individual analogues have to be measured and converted into toxic equivalents. At least 20 analogues of AZAs have been identified in naturally contaminated shellfish (Rehmann et al., 2008). However, not all of these are believed to be toxicologically relevant and thus only 3 analogues have been included in the EU regulation due to their comparatively high toxicity, namely AZA1, -2 and -3 (Anon., 2005). The relative toxicity of individual analogues is typically expressed via a toxic equivalence factor (TEF) which is a multiplier to be applied to concentrations of analogues, transforming these concentrations into concentration equivalents of a reference compound. For AZAs, the reference compound is set to AZA1, which is the compound contributing most to the total toxic equivalent (TEQ), in most natural events. These TEF-multipliers have been derived from initial studies on mice using purified AZA1, -2 and -3, resulting in TEFs of 1, 1.8 and 1.4, respectively (Ofuji et al., 1999); these TEFs should be verified again once certified pure compounds become available.

For the period from 2001 to 2006, the LC-MS based method in Ireland had been calibrated using standards isolated in Japan in 2001. Since 2007, certified AZA1 has been available (NRCC, 2007) and has been used for calibration of LC-MS-MS analysis in Ireland. Currently, AZA2 or -3 are still not available for instrument calibration. However, the MS response of these compounds may be estimated using relative response factors and comparing the instrument response to AZA1. A relative response factor of ca. 1.43 has been established for both AZA2 and -3, compared to AZA1, by two independent studies (Ofuji et al., 1999; Rehmann, 2008).

The use of the LC-MS-MS methodology has allowed for collection of appropriately contaminated shellfish and subsequent isolation and purification of AZA reference compounds (Hess et al., 2007a). Similarly, the possibility to obtain shellfish of different contamination levels has also allowed for the preparation of shellfish tissue reference materials and testing of the stability of such materials (Hess et al., 2007b, McCarron et al., 2007a and b). However, even though these reference materials have been used for the in-house validation of LC-MS-MS methods for the detection of AZAs, they were not appropriate for the verification of the performance of the MBA because, in addition to AZAs, these materials also contained toxins from *Dinophysis spp*., i.e. okadaic acid and analogues (OA and DTXs) and pectenotoxins (PTXs). Until now, the effects of combinations of different compound groups have not been established, again due to the lack of pure reference compounds. The sensitivity of mice to a compound can be expressed as LD_{50} for individual compounds once these are purified. However, the LD_{50} is not the only performance characteristic of the MBA, as the use of pure compounds does not account for other effects, e.g. recovery losses during sample preparation and matrix effects when injecting contaminated extracts of mussel matrix into mice.

In 2007, for the first time since 2001 when quantitative LC-MS-MS analysis was introduced in the Irish national monitoring plan, shellfish were observed to be exclusively contaminated with AZAs, without the presence of other toxin groups. Bulk samples of mussels were obtained from this event, and these tissues were used in the present study for the verification of the performance of the MBA in its current format, i.e. the EU-harmonised protocol (CRL-MB, 2007). Azaspiracids effectively concentrate in the hepatopancreas of mussels (Hess et al., 2005), however, the current LC-MS analysis in Ireland is based on whole flesh as a matrix tested. This difference to the bioassay (which is carried out on hepatopancreas) potentially leads to discrepancies between bioassay and chemical testing. The present study attempted to avoid this problem through the preparation of both HP tissues to be tested by MBA and LC-MS and the parallel preparation of reconstituted whole flesh tissues, tested by LC-MS only.

Materials and Methods

Reagents

Methanol (MeOH) and acetonitrile (ACN) were obtained as Pestiscan grade solvents from Labscan (Dublin, Ireland). A reverse osmosis purification system (Barnstead Int., Dubuque, IA) supplied water for the mobile phase. Formic acid and ammonium formate were from Sigma–Aldrich (St Louis, MO). AZA1 calibrants for LC-MS analysis were dilutions of the certified AZA1, supplied by National Research Council Canada. Acetone and diethylether for extraction and partitioning of extracts in the MBA was obtained from Sigma-Aldrich (Dublin, Ireland) as *puriss.* grade.

Preparation and subsampling of Homogenate Mussel Tissues

All tissue dissections, mixtures and homogenates were prepared at the Marine Institute (Fig. 1). Preparation of these tissues closely followed the procedures for the preparation of wet shellfish tissue reference materials previously described by McCarron et al., 2007b, and Hess et al., 2007b. Blue mussels (*Mytilus edulis*) contaminated with AZAs were obtained from Bantry Bay (Southwest of Ireland) during a contamination event in October 2007. These shellfish were analysed by LC-MS-MS and found to contain only AZAs present above the limit of quantification (LOQ_{AZA1} = 0.02 mg/kg; LOQ_{OA} = 0.04 mg/kg). The mussels were cooked to remove them from the shell in a commercial processing factory in Ireland, and were stored frozen prior to further treatment. After arrival at the Marine Institute, the mussels were defrosted, and hepatopancreas (HP) tissue was dissected and homogenised using a Waring™ blender (Fig. 1, tissue A), and three 1 g aliquots were taken for LC-MS-MS analysis. The water content of the homogenate was determined gravimetrically after drying five 1g aliquots overnight in a centrifugal evaporator (Jouan, RC10.22, with RCT90 condenser and Edwards R8 pump).

In January 2008, uncontaminated mussels (Fig. 1, mussels B) were obtained from Carlingford Lough (East coast of Ireland), an area where generally no AZAs have been observed above the LOQ. Hepatopancreas tissue (1.75 kg) was dissected from the uncontaminated, raw shellfish and was homogenised using a Waring™ blender (Fig. 1, tissue C), and the water content of the homogenate was determined gravimetrically as above; absence of AZAs and OA-group toxins was verified by LC-MS-MS analysis of aliquots.

Homogenates of HP tissue (Fig. 1, tissues E, levels 1 to 7) were prepared by mixing the contaminated, cooked HP tissue (Bantry Bay) with the uncontaminated, raw HP tissue from Carlingford Lough. All dilutions of contaminated with uncontaminated tissues were carried out by weight. The initial design of the study aimed at obtaining concentrations in the final HP tissues that would have equidistant whole flesh concentrations of AZA1-equivalents

of ca. 0, 0.04, 0.08, 0.12, 0.16, 0.20 and 0.24 mg/kg. Replicates (9 aliquots of 30 g each) were dispensed using a peristaltic pump while mixing the homogenate for each of the seven concentration levels (Fig. 1, tissues F). For each concentration level, subsamples (3 x 2 g) were taken from five of the 9 aliquots (replicates 1, 3, 5, 8, and 9) for assessment of homogeneity of the aliquots after these had been dispensed. Replicates 1 to 7 of each of the seven concentration levels (49 in total) were then sent on ice-packs to En-force for mouse bioassay testing, while one replicate (8) of each level was retained as back-up in case further studies were required or an aliquot needed replacement due to damage in transit. At En-force the tissue homogenates were stored in a freezer until analysis (ca. -20ºC). One of the remaining 30 g replicates (9) was used to prepare reconstituted whole flesh mussel homogenates by dilution of the contaminated HP tissue from Bantry Bay with remainder (REM) tissue from the uncontaminated Carlingford mussels. In this dilution of the HP tissue, also checked by weight, a ratio of ca. 1:4 for HP: REM was used, i.e. ca. 20% HP in the final homogenate. Replicate 9 (3 analytical subsamples of all seven levels) was also used for the characterisation of the AZA1-equivalents in the HP tissues using triple-stage-quadrupole MS.

Extraction of Homogenate Tissues for LC-MS-MS analysis

All extractions and LC-MS-MS analyses were carried out at the Marine Institute. Unless otherwise stated, the extraction procedure was the same as that accredited at the Marine Institute, however, additional dilutions were carried out for extracts of the HP tissues to investigate the influence of matrix effects.

Aliquots (1 ± 0.1 g) of the undiluted, contaminated HP tissue (Fig. 1, tissue A) were extracted with 15 mL of 100% MeOH using a vortex mixer for 1 min at full speed. The sample was then centrifuged (CR4-22 Jouan, Thermo Electron Corp., CA, USA, 5 min at 2660 g, 15 °C; and: Sorvall, Heraeus 3 S-R, Kendro Lab. Prod., Hanau, Germany, 5 min at

3600 g, 15 °C). The remaining pellet was successively re-extracted twice (2 x 15 mL of 100% MeOH) using an Ultraturrax™ high speed blender (T25, IKA, Staufen, Germany) at 11,000 rpm and centrifuged as above. The two decanted supernatants were combined in a 50 mL volumetric flask and the solvent was filled up to the mark with MeOH.

Aliquots (2 ± 0.5 g) of the contaminated HP tissues diluted with uncontaminated HP tissues (Fig. 1, tissues F) were extracted with 9 mL of 100% MeOH using a vortex mixer for 1 min at full speed. The samples were then centrifuged as above, decanted and the remaining pellet was re-extracted with a further 9 mL of 100% MeOH using an Ultraturrax™ high speed blender at 11,000 rpm and centrifugation as above. The two decanted supernatants were combined in a 25 mL volumetric flask and the solvent was filled up to the mark with MeOH. Subsequently, aliquots (2 mL) of the crude extract were diluted 5 times by weight, making up the volume of a 10 mL volumetric flask with MeOH. The crude and diluted extracts were filtered (0.2 µm; Schleicher & Schuell, Whatman, Maidstone, UK), and stored in the freezer until LC-MS-MS analysis. Thus, the sample-to-solvent ratio (SSR) of the crude HP extracts was 1 : 12.5 while the SSR of the diluted HP extracts was 1 : 62.5. Aliquots (2 ± 0.5 g) of the reconstituted whole flesh tissues (Fig. 1, tissues G) were extracted in the same way as described above for the HP tissues, however, only crude extracts were prepared at the SSR ratio of 1 : 12.5.

LC-MS-MS analysis of Homogenate Tissues

Analysis of the original contaminated HP (Fig. 1, tissue A) and the 105 subsamples for the homogeneity study (Fig. 1, triplicate subsamples of replicates 1, 3, 5, 8 and 9 of all seven levels) were carried out using the Micromass Quadrupole-Time-of-Flight Ultima (Q-ToF) instrument, with an LC-method that had previously been shown to achieve adequate within-batch repeatability of < 5% (McCarron, 2008). A Waters 2795 HPLC coupled to a Q-

ToF Ultima mass spectrometer, equipped with a z-spray electrospray ionization source, was used in positive ion mode. The Q-ToF was used in TOF-MS-MS mode with capillary voltage 3.0 kV, cone voltage 40 V, source temperature 140 °C, desolvation temperature 350 °C, and collision energy 50 eV. A binary mobile phase was used, with: A, water, and; B, 95% aqueous acetonitrile, each containing 2 mM ammonium formate and 50 mM formic acid. Isocratic separation was performed over 6 min using a ACE C18 column (30 × 2.1 mm, 3 µm; Alpha Column Technologies, Aberdeen, UK) with a guard (10 × 2 mm, 3 µm), 60% B. The following molecular ions ($[M+H]^+$) were selected as parent ions for Q-ToF analysis: AZA3 m/z 828.5, AZA1 m/z 842.5, AZA2 m/z 856.5. Fragment ions were scanned over the range from m/z 100 to 900 over 1 s with a 0.1 s interscan delay.

LC-MS-MS analysis of the 21 subsamples for verifying the accurate concentrations of the 30 g HP aliquots (Fig. 1, tissues F, triplicate subsamples of replicate 9), the five-fold diluted extracts and the 21 subsamples for verifying the accurate concentrations of the 30 g WF aliquots (Fig. 1, triplicate subsamples of tissues G) was carried out using the triple stage quadrupole (TSQ) instrument, with an LC method for which most performance data were available at the Marine Institute. A Waters 2690 HPLC coupled to a Micromass Ultima TSQ mass spectrometer, equipped with a z-spray electrospray ionization source was used in positive ion mode. The TSQ was used in MRM mode with capillary voltage 3.2 kV, cone voltage 100 V, source temperature 130°C, desolvation temperature 350°C, and collision energy 50 eV. A binary mobile phase was used, with: A, water, and; B, 95% aqueous acetonitrile, each containing 2 mM ammonium formate and 50 mM formic acid. A linear gradient was run on a BDS-hypersil C8 column (50 × 2 mm, 3 µm; Thermo Fisher Scientific, Loughborough, UK) with a guard (10 × 2 mm, 3 µm), from 30–90% B over 8 min, held for 3 min, decreased to 30% B over 0.5 min, and equilibrated for 3 min until the next run. The following transitions were selected for MRM analysis: AZA3 (m/z 828.5 → 640.4 and

828.5 → 658.4), AZA1 (m/z 842.5 → 654.4 and 842.5 → 672.4), AZA2 (m/z 856.5→ 654.4 and 856.5 → 672.4). Instrument calibration was carried out using only AZA1, hence a relative response factor (RRF) of 1.43 has been used for AZA2 and -3 to transform instrument response of these analogues into AZA1-concentrations (Ofuji et al., 1999; Rehmann 2008). AZA1-equivalents of AZA2 and -3 were calculated by weighting the RRF-corrected concentrations of AZA2 and -3 with their respective TEFs of 1.8 and 1.4. Total AZA1-equivalents were the sum of the individual AZA1-equivalents of AZA1, -2 and -3.

Mouse Bioassays of Homogenate Tissues

Male albino mice of the CD1 strain were bred at En-Force Laboratories Ltd. Breeding and test mice were maintained under the conditions prescribed in EU Commission Recommendation 2007/526/EC (Anon., 2007).

The test procedure was based on the EU-harmonised protocol (Version 4.0, CRL-MB, 2007). An aliquot (20 g ± 0.2g) of the tissue homogenate provided by the Marine Institute (Fig. 1 F) was weighed into a Waring blender goblet, to which acetone (100 ml) was added. The goblet was sealed with Parafilm® (Pechiney Plastic Packaging) and the mixture was further homogenised for 2 min at low speed using a Waring blender. The mixture was filtered through an Albet DF 411 24 cm diameter filter paper into a labelled 1 L round-bottom flask. The filtrate was rotary evaporated under vacuum (100 – 200 mbar at 50 ± 10 rpm) at 40 - 45°C to remove the acetone.

Purified water (10 mL) was added to the residue in the round-bottom flask, followed by 50 ml di-ethyl ether. This mixture was swirled to dissolve the residue and transferred to a 500 ml separatory funnel. Following separation of the water and di-ethyl ether phases, the water layer was drained back into the round-bottom flask. The water was re-extracted twice with 50 ml of di-ethyl ether and separated in the separatory flask yielding ca. 150 ml di-ethyl

ether, which was backwashed twice with purified water (20 ml each). The ether phase was collected in the round-bottom flask and rotary evaporated to dryness using a two-stage evaporation (600 mbar at 50 ± 10 rpm, and then 100 mbar at 50 ± 10 rpm) at 40 - 45°C.

The residue was then recovered using a cell scraper (Sarstedt) and resuspended in 1% aqueous solution of Tween® 60 (Merck) to a final volume of 4 ml in a glass 5ml graduated stoppered measuring cylinder (Fisher). This mixture was homogenised using an Ultraturrax™ (8 mm shaft, 8000 rpm) and 1 ml was then injected interperitoneally into each of three mice (19-21 g) via a 26-gauge needle. The mice were monitored post injection and any deaths or other responses were recorded. A positive test result was the deaths of at least two out of three mice within or at 24 h (± 1 min) (Anon., 2005).

On each of seven separate occasions over 6 weeks, one replicate of the all seven levels was analysed by bioassay (Fig. 1, tissues F1-7, replicate 1 of level 1 to 7 on day 1 and so on). The concentrations of the homogenate tissue samples were unknown to the bioassay analyst at the time of analysis, and the analysis was carried out "blind". One replicate of one level was spilled during analysis (i.e. a single sample of 49), and this sample was replaced from replicate 8 (spare set of replicates from Fig. 1, tissues F).

Regression Analysis

Statistical analysis of mouse lethality data was carried out using Sigmaplot 8.02 (SPSS Inc., 2002). Curve-fitting of the prevalence of death in mice subjected to specific tissue concentration levels was carried out as 4-parametric, logistic fit of data to a sigmoidal curve. All other calculations were carried out in Excel (Microsoft Office Corp., 2003). The mathematical model for the 4-parametric fit represents the sigmoidal function:

$$y = y_0 + \frac{a}{1 + \left(\frac{x}{x_0}\right)^b}$$

where y is the prevalence of death as percentage of dead mice and x is the total AZA1-equivalent in HP tissue; a, b, y_0 and x_0 are the model parameters calculated by the software using non-linear regression.

Results

All dissected tissues had a moisture content of ca. 80%, therefore, the moisture content was not further adjusted prior to mixtures and further homogenisations. The undiluted HP tissue (Fig. 1, tissue A) from the contaminated mussels contained an average of 6.51 ± 0.4 mg/kg AZA1-equivalents (n=3). This concentration was used to calculate the weights of HP to use in the tissue dilution scheme. The actual tissue dilution factors for the dilution for tissue A to E (Fig. 1) were 16.23, 9.08, 6.06, 4.56, 3.64 and 3.03 for levels 2 to 6, respectively (level 1 being completely uncontaminated, i.e. equivalent to HP tissue C, Fig. 1).

Homogeneity study

All tissues appeared of satisfactory homogeneity since the RSDs of the analyses of replicates were less than 7% in all cases (Table 1), which was close to the typical within-batch analytical variability of the LC-MS-MS method.

Assigning AZA1-equivalents toHomogenate Tissues

Two sets of analyses had been carried out on the seven levels of contaminated HP tissue homogenates: (a) the crude extracts at SSR of 1 : 12.5 and (b) the 5-fold diluted crude extracts at SSR of 1 : 62.5. The AZA1-equivalents from the analysis of diluted extracts were on average ca. 20% higher than those determined in the undiluted crude extracts of the HP homogenate tissues (Table 2). The whole flesh homogenates also gave a slightly lower response, i.e. 12%, compared to the dilute HP extracts. Thus, the diluted HP extracts showed

the least influence of matrix effects in the LC-MS-MS analysis and were subsequently used as the best estimate of the true concentrations in the HP homogenates.

Individual concentrations of AZA1, -2 and -3, as well as the sum of AZA1-equivalents, showed approx. equal differences between the 7 levels, another good indication that the initial contaminated HP was homogeneous prior to being spiked into uncontaminated HP (Table 3). The whole flesh AZA1-equivalents calculated from the analysis of diluted HP tissues (Table 3) showed that the HP homogenates were appropriate for the study design as there were three levels below and four levels above the regulatory limit of 0.16 mg/kg, assuming an average HP content of 15.25% in whole flesh.

Mouse Bioassays of Homogenate Tissues

None of the mice died at levels 1 and 2 (Table 4). At level 3, which was contaminated at ca. 0.12 mg/kg AZA1-equiv. in whole flesh, 14 mice died resulting in 4 positive assays out of 7. At all higher levels of contamination all assays were positive, despite up to three mice surviving per treatment group of 21 mice. At levels 4 and 5, at which 3 mice survived, these three mice survived in separate assays, thus resulting in all 7 assays being positive at each level. Even at the two highest levels, 1 mouse out of 21 survived at each level; these two mice had been exposed to doses of ca. 9.95 and 11.2 µg AZA1-equivalents, respectively for level 6 and 7, assuming 100% recovery of the MBA procedure and homogeneity of the final Tween 60 suspension injected.

Regression Analysis

For statistical analysis, the 21 mice exposed to the same concentration level of AZAs were considered a single population, even though they had been exposed in groups of three in independently conducted assays. This grouping allowed for establishment of a concentration-

response curve (Fig. 2). The concentrations were expressed as AZA1-equivalents in HP tissues, as these values were measured, and are independent of the HP content of the shellfish. Non-linear regression of a 4-parametric, logistic fit to a sigmoidal function gave a correlation coefficient (r^2) of 0.9927, indicating a highly significant correlation. The values of the four parameters of the model function were a (92.5721), b (-6.6713), x_0 (-0.6733) and y_0 (-1.5149). Analysis of variance showed that with 6 degrees of freedom an F-value of 135.5 and P of 0.0011 was obtained.

Discussion

Accuracy of HP concentrations and uncertainty considerations

The differences observed between crude HP extracts and diluted HP extracts (Table 2) illustrate the importance of matrix effects in the LC-MS analysis of shellfish toxins. The matrix effects observed in this study correlated very well with those previously reported for LC-MS-MS analysis of AZAs reported by Fux et al., 2007 (23%) and Fux et al., 2008 (9.3 to 18.4%). Also, the increased response in more dilute extracts is consistent with the overall trend for the determination of AZAs by LC-MS to suffer from ion suppression. The matrix addition studies by Fux et al., 2007 and 2008, also suggested that large dilutions were necessary to circumvent the ion suppression for AZAs, hence in the present study a five-fold dilution was applied. Such large dilutions are typically not feasible when whole flesh tissues are used in the analysis since the limits of quantification required for the regulatory limit can then usually not be met. In this study, dilutions were appropriate as the concentrations of the HP tissues were designed to equate whole flesh concentrations around the regulatory limit, hence the HP concentrations were five times higher than the concentrations of interest in actual whole flesh samples. Also, the comparison of the reconsituted whole flesh samples

which were analysed undiluted, and which showed lower results by ca. 12 % on average, confirmed that the routine analysis at the Marine Institute needs to account for these matrix effects. Correction of such matrix effects will be greatly facilitated by the availability of a certified mussel tissue reference material. A candidate certified material has been prepared and is currently undergoing certification at the National Research Council Canada (McCarron et al., 2007c). Additionally, it appears attractive to use HP tissues for the analysis of shellfish toxins by LC-MS, as five-fold dilution of the matrix allows for effective removal of the matrix effects. In addition, it has been previously shown that AZAs effectively concentrate in the hepatopancreas of mussels, in particular in the raw matrix (Hess et al., 2005).

While the above section clarifies that matrix effects have been taken into account and were corrected for through analysis of the dilute HP extracts, there are further factors of uncertainty that need to be considered when interpreting the results of this study:

1. Calibration using AZA1 as sole reference standard (RRFs for AZA2 and -3)
2. TEFs for the conversion of AZA2 and -3 concentrations into AZA1-equivalents
3. Presence of other AZA analogues
4. Reproducibility of LC-MS-MS analysis on different instruments and over time

The same relative response factor (1.43) has been found by two independent studies (Ofuji et al., 1999, and Rehmann 2008). Firstly, it should be noted that the studies were carried out in different laboratories and 9 years apart. Secondly, the studies were conducted on different lots of purified standards, one set characterised by NMR, HPLC-PDA and gravimetrically, the other characterised by MS and quantitative NMR. Finally, the second study (Rehmann 2008) used a variety of MS instruments and acquisition modes, and found very little variation of the RRFs. Therefore, the extent of the uncertainty from point one is considered reasonably minimal.

The TEFs derived by mouse intraperitoneal injection (Ofuji et al., 1999) seem most relevant to this study. Unfortunately, the exact number of mice in that study is not known, and the toxicity was expressed as lethality. Thus, it is difficult to estimate the extent of uncertainty associated with these TEFs.

Even though this study did not test the presence of AZA analogues other than AZA1, -2 and -3, their presence is very likely as they are believed to be shellfish metabolites of the original AZAs. Previous studies have shown that the other AZAs tend to occur at lower concentrations, i.e. less than 5 % for the sum of the other analogues (Rehmann et al., 2008), and that at least some of these analogues are significantly less toxic (Ofuji et al., 2001). Considering the typically low concentrations in combination with a good likelihood of the analogues being less toxic, it appears reasonable to assume that the contribution of the other AZA analogues to the overall toxic equivalent is minimal.

The variability of the LC-MS-MS method over time and between instruments also needs to be considered as a potential source of error or uncertainty. There is an intrinsic check in the study which can be used to verify the reproducibility of analyses over time and between instruments: the analysis of the tissues F (as diluted HP extracts, SSR 1 : 62.5, TSQ) can be used to back-calculate the AZA1-equivalent concentration of the original contaminated HP (Fig. 1, tissue A, SSR 1 : 50, Q-TOF). The diluted tissues F result in a back-calculated mean for tissue A of 7.09 ± 0.3 mg/kg AZA1-equivalents (n=6), compared to 6.51 ± 0.4 mg/kg AZA1-equivalents (n=3) for the direct analysis of the contaminated HP. Therefore, the overall error associated with the exercise, including both the variability of LC-MS-MS analysis over 2-3 months (December 2007 to February 2008) and the procedural errors in the dilution of tissues was 8.23%. This overall error appears slightly lower than the long-term reproducibility between batches of the accredited method at MI (RSD of AZA1-equivalents for an in-house Laboratory Reference Material 19.2% for the TSQ and 9.6% for the Q-ToF).

In summary, it appears unlikely that the uncertainties considered above contributed to a very significant bias of the study.

Assigning whole flesh equivalents to HP tissues

The HP content (15.25 %) had been estimated from a previous study and this study, and was derived from 2 dissection exercises of bulk tissues (12.5 % this study and 18% in McCarron and Hess, 2006). The 2 studies appear to give a wide range of HP content, one differing by ca. 30% from the other. This natural variability of the HP content in mussels adds variability to the outcome of the mouse bioassay due to the use of HP instead of the whole flesh. If the HP tissues used in this study had only consituted 12.5% of the whole flesh, then intermediate level four would represent exactly the current regulatory limit of 0.16 mg/kg. In the previous study HP tissue constituted 18% of the whole flesh. In that case, level 3 would be closer to the regulatory limit and the MBA would appear less sensitive. The HP content is varying seasonally with feed availability and sexual status of the mussel. Therefore, it would be desirable to have access to a larger data set in order to assess the full extent of this variability.

In summary, the effect of the variability of HP content in mussels on the applicability of the MBA to implement a legal limit expressed in whole flesh units remains poorly understood.

Performance of the mouse bioassay

The main finding of this study consisted in the description of the performance of the mouse bioassay. Firstly, it was noted that the extreme levels of high or low AZA-contamination consistently resulted in positive or negative assays, respectively; the two lowest levels gave very consistently negatives, while all replicates of the four highest levels yielded positive results. Inconsistencies only occurred around the threshold toxicity level as

would be expected from any qualitative assay. The consistent performance of the assay over seven independent batches suggested that the time course was a negligible factor in the study; this finding retrospectively validates the assumption that all 21 mice exposed to each of seven levels can be considered a single population. Furthermore, it was noted that this threshold level was very close to the regulatory limit of 0.16 mg/kg, i.e. 0.10 to 0.14 mg/kg in WF equivalent, depending on the HP content assumed. This threshold was lower than expected by the authors in view of previous observations, although more recently occasional positive bioassays had been observed during periods of lower levels of contamination when only AZAs had been present.

The mathematical model of the sigmoidal function for the concentration response-curve was useful in establishing an LC_{50} for the MBA (0.70 mg/kg AZA1-equiv. in HP). It should be noted that this LC_{50} includes not only one compound but also all the assumed TEFs and recoveries for individual analogues in the assay procedure. If recoveries were assumed 100%, the TEFs could be shown to be accurate and other analogues were completely negligeable, then this LC_{50} would be equivalent to an LD_{50} for AZA1 of 175 µg/kg bodyweight, which is very close to the previously reported lethality of 200 µg/kg bodyweight (Satake et al., 1998). The concentration-response curve was also used to estimate the probability of finding a positive assay at the current regulatory limit of 0.16 mg/kg. The probability of obtaining a positive result at the limit was derived from using the generic probability for a single mouse to live or die and the fact that in each assay three mice are used. The three mice each individually have the possibility to live or die and the probability to die was derived from the concentration-response curve to be 86.5% at the legal limit (Fig. 2). The sum of probabilities of all four scenarios in which 2 or three mice die resulted in a total probability of 95% for detecting a positive (Table 5), which is re-assuring for the implementation of the current legislation using this assay for the detection of AZAs. If on the

other hand, the legal limit were dropped to half the value, i.e. 0.08 mg/kg, as could be the case if larger portions of shellfish were used for risk assessment and management, then the probability of detecting a positive using the MBA would only be 5% at the legal limit (calculation not shown). Therefore, the MBA could not be confidently used to implement any level lower than 0.16 mg/kg. As LC-MS-MS has been used in this study to characterise the materials and obtain a very good correlation between the LC-MS-MS method and the current EU-harmonised protocol of the mouse bioassay, the two tests can be considered equivalent at the current regulatory limit.

Conclusions and further work

The present study has clearly shown that the current protocol of the EU-harmonised mouse bioassay can be implemented to ensure the assay detects positive results at the current regulatory limit of 0.16 mg/kg for AZAs at a reasonable level of confidence, i.e. with 95% probability. However, difficulties were encountered in ensuring this limit is met due to the natural variation of the content of hepatopancreas tissues in whole flesh.

Additionally, the study also outlined a possible way to implement more rugged LC-MS-MS methods for the detection of AZAs in shellfish if hepatopancreas is used for the test, as is currently the case in the mouse bioassay. The higher concentration in hepatopancreas allows for dilution of crude extracts, thus eliminating matrix effects in the MS detection effectively.

This study also allowed for the direct comparison of the mouse bioassay and the LC-MS-MS test in two laboratories, and results suggest that the two tests can be considered equivalent in their effectiveness in implementing the current regulatory limit.

Future work in the authors' laboratories will be aimed at closing in on the gaps of uncertainty, mainly through preparative isolation of AZA2 and -3 to allow for certification of

these materials. These purified, well-characterised standards should facilitate both the definitive verification of the relative response factors in the MS detection as well as a more statistically robust evaluation of the relative toxicities of AZA2 and -3. Certification of AZA2 and -3 is also required to proceed with certification of a mussel tissue reference material which is required to complete the validation of LC-MS-MS methods according to international standards.

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Tables

Table 1. Homogeneity study. Relative standard deviations (RSDs) in [%], n=5.

Level	1	2	3	4	5	6	7
RSD	n/a*	3.5	1.8	5.2	6.6	4.0	4.6

*n/a = RSD is not applicable where concentrations were not quantifyable

Table 2. AZA1-equivalents determined in mussel hepatopancreas (HP) and whole flesh (WF) homogenates. All concentrations expressed in whole flesh AZA1-equivalents [mg/kg]*, n=3. Sample-to-solvent ratios were 1 : 62.5 (diluted HP extracts), 1 : 12.5 (crude HP extracts) and 1 : 12.5 (WF extracts).

Level	1	2	3	4	5	6	7
AZA1-Equiv. (dilute HP extracts)	**<LOQ**	**0.06**	**0.12**	**0.19**	**0.24**	**0.30**	**0.34**
AZA1-Equiv. (crude HP extracts)	<LOQ	0.05	0.09	0.13	0.17	0.20	0.23
AZA1-Equiv. (reconst. WF extracts)	<LOQ	0.06	0.11	0.15	0.19	0.26	0.32

*An average HP content of 15.25% was used for transformation of the AZA1-equivalents determined in HP into whole flesh equivalents. As the reconstituted whole flesh samples had an actual HP content of 20%, the concentrations were also back-calculated to reflect the average HP content of 15.25%.

Table 3. Average concentrations measured by LC-MS-MS for individual Azaspiracid analogues in hepatopancreas tissue and toxicity weighted AZA1-equivalents in hepatopancreas (HP) and calculated whole flesh (WF) equivalents. All concentrations in [mg/kg], n=3. The TEFs for AZA2 and -3 were 1.8 and 1.4, respectively (from Ofuji et al., 1999).

Level	1	2	3	4	5	6	7
AZA1 (in HP)	<LOQ	0.16	0.31	0.50	0.65	0.80	0.90
AZA2 (in HP)	<LOQ	0.07	0.13	0.20	0.26	0.33	0.36
AZA3 (in HP)	<LOQ	0.09	0.17	0.27	0.34	0.43	0.50
AZA1-Equivalence in Hepatopancreas	**<LOQ**	**0.41**	**0.78**	**1.24**	**1.59**	**1.99**	**2.24**
AZA1-Equivalence in Whole flesh*	**<LOQ**	**0.06**	**0.12**	**0.19**	**0.24**	**0.30**	**0.34**
Estimated WF_{min}*	n/a	0.05	0.10	0.16	0.20	0.25	0.28
Estimated WF_{max}*	n/a	0.07	0.14	0.22	0.29	0.36	0.40

* The transformation of AZA1-equivalents from HP into WF equivalents is based on the estimate of the HP being 15.25 % of WF weight on average in fresh mussels. The minimum WF equivalent (WFmin) is based on the minimum HP content observed in raw mussels in the authors' laboratory (12.5%), while WFmax is based on a maximum of 18% HP in WF.

Table 4. Mouse bioassay results for seven assays (3 mice each) carried out on independent days (over a 6-week period) for each of the seven concentration levels. Note, although there were three survivors at level 4 and 5, these mice survived in three separate assays, and hence did not lead to any negative assay outcome in any of the 7 independent assays at these two levels.

Level	AZA1-equiv. in WF(µg/g)*	AZA1-equiv. in HP*	Assay Neg / Pos	No. mice alive	No. mice dead	% survival	% death
1	< LOQ	**<LOQ**	7/0	21	0	100	**0.0**
2	0.06	**0.41**	7/0	21	0	100	**0.0**
3	0.12	**0.78**	3/4	7	14	33.3	**66.7**
4	0.19	**1.24**	0/7	3	18	14.3	**85.7**
5	0.24	**1.59**	0/7	3	18	14.3	**85.7**
6	0.3	**1.99**	0/7	1	20	4.8	**95.2**
7	0.34	**2.24**	0/7	1	20	4.8	**95.2**

* AZA1-equivalents in WF are calculated and indicative, those in HP are measured and adjusted for RRFs and TEFs.

Table 5. Calculation of the probability (Pr) of a positive (pos) or negative (neg) mouse bioassay for shellfish at the current regulatory limit (0.16 mg/kg). A mouse bioassay is positive when 2 or 3 mice are dead after 24 h observation.

mouse	mouse	mouse	Pr of individual			Pr (scenario)		
1	2	3	mouse to live or die					
dead	**dead**	**dead**	**0.865**	**0.865**	**0.865**	**0.647**		
dead	**dead**	**alive**	**0.865**	**0.865**	**0.135**	**0.101**		
dead	**alive**	**dead**	**0.865**	**0.135**	**0.865**	**0.101**		
alive	**dead**	**dead**	**0.135**	**0.865**	**0.865**	**0.101**	**Pr(pos)**	**0.95**
dead	alive	alive	0.865	0.135	0.135	0.016		
alive	dead	alive	0.135	0.865	0.135	0.016		
alive	alive	dead	0.135	0.135	0.865	0.016		
alive	alive	alive	0.135	0.135	0.135	0.002	Pr(neg)	0.05
					Total	1.00		

Figure Legends

Figure 1. Dissection of tissues and preparation of 7 levels of contaminated hepatopancreas (HP) tissues and one whole flesh (WF) tissue. A contaminated sample from Bantry Bay is dissected to obtain contaminated HP tissue (A). An uncontaminated sample from Carlingford Lough (B) is dissected to obtain uncontaminated HP (C) and uncontaminated remainder (REM) flesh (D). (A) and (C) are blended in different proportions to obtain seven homogenates of increasing concentration levels of AZAs (E). These seven homogenates (E) are aliquoted into nine 30 g portions each (F), replicates 1 to 9. Replicates 1, 3, 5, 8 and 9 were used for the homogeneity study (dark grey), replicates 1 to 7 were used for MBA analysis and replicate 9 was used for dilution of HP tissue with uncontaminated remainder (D) to create reconstituted WF tissues (G) for levels 1 to 7. Levels of AZA1-equivalents were determined for HP tissues (F), replicate 9 of all 7 levels, and for WF tissues (G).

Figure 2. Prevalence of death in mice as a function of AZA1-equivalents in hepatopancreas (HP) tissues. Each experimental data point was determined using 3 mice in each of 7 independent assays (21 mice per level, i.e. total of 147 mice).

Fig. 1

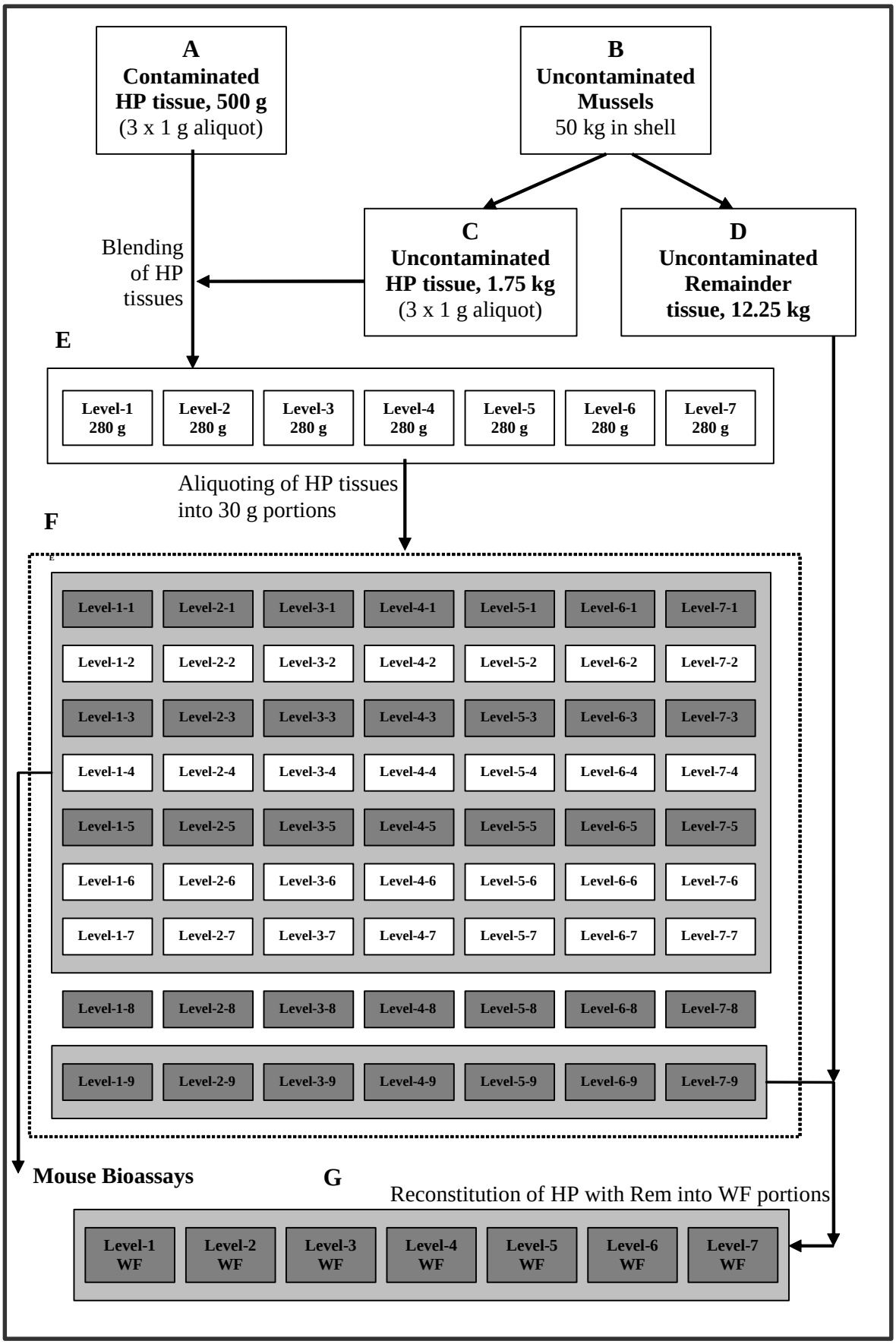
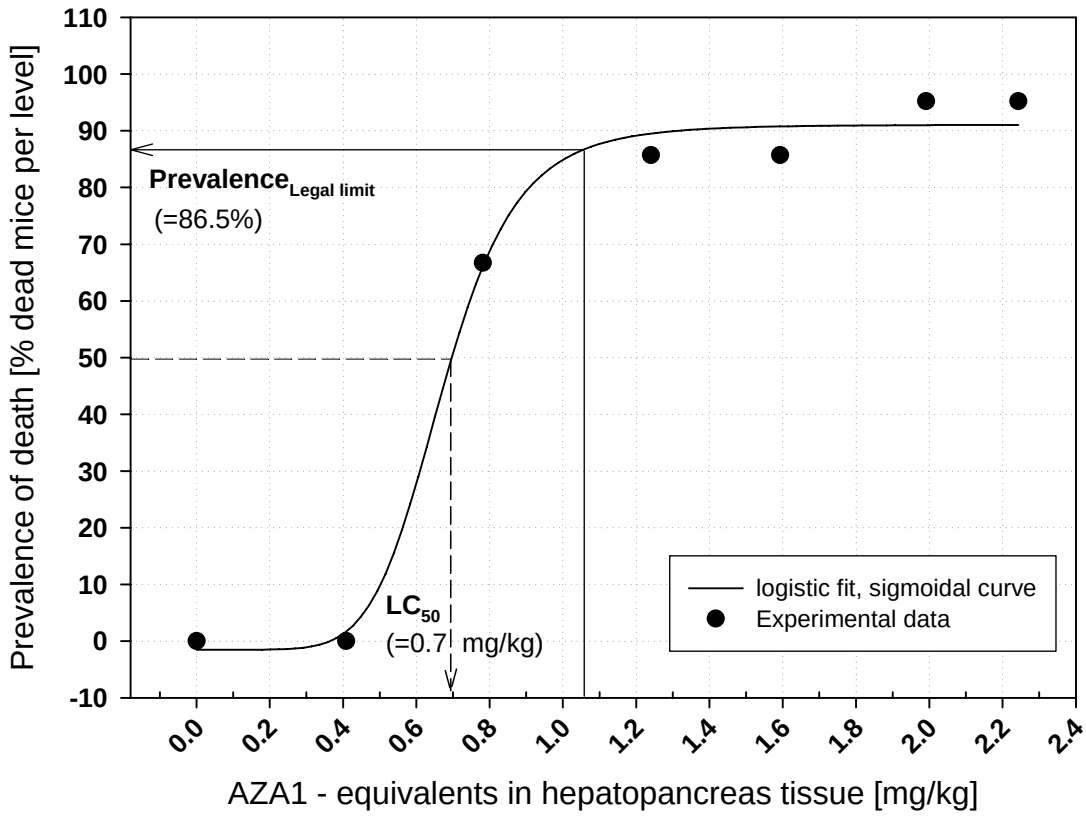

A
Contaminated
HP tissue, 500 g
(3 x 1 g aliquot)
B
Uncontaminated
Mussels
50 kg in shell
C
Uncontaminated
HP tissue, 1.75 kg
(3 x 1 g aliquot)
D
Uncontaminated
Remainder
tissue, 12.25 kg
Blending
of HP
tissues
E
Level-1 280 g
Level-2 280 g
Level-3 280 g
Level-4 280 g
Level-5 280 g
Level-6 280 g
Level-7 280 g
Aliquoting of HP tissues
into 30 g portions
F
Level-1-1
Level-2-1
Level-3-1
Level-4-1
Level-5-1
Level-6-1
Level-7-1
Level-1-2
Level-2-2
Level-3-2
Level-4-2
Level-5-2
Level-6-2
Level-7-2
Level-1-3
Level-2-3
Level-3-3
Level-4-3
Level-5-3
Level-6-3
Level-7-3
Level-1-4
Level-2-4
Level-3-4
Level-4-4
Level-5-4
Level-6-4
Level-7-4
Level-1-5
Level-2-5
Level-3-5
Level-4-5
Level-5-5
Level-6-5
Level-7-5
Level-1-6
Level-2-6
Level-3-6
Level-4-6
Level-5-6
Level-6-6
Level-7-6
Level-1-7
Level-2-7
Level-3-7
Level-4-7
Level-5-7
Level-6-7
Level-7-7
Level-1-8
Level-2-8
Level-3-8
Level-4-8
Level-5-8
Level-6-8
Level-7-8
Level-1-9
Level-2-9
Level-3-9
Level-4-9
Level-5-9
Level-6-9
Level-7-9
Mouse Bioassays
G
Reconstitution of HP with Rem into WF portions
Level-1 WF
Level-2 WF
Level-3 WF
Level-4 WF
Level-5 WF
Level-6 WF
Level-7 WF

Fig. 2


Prevalence of death [% dead mice per level]
AZA1 - equivalents in hepatopancreas tissue [mg/kg]
Prevalence Legal limit
(=86.5%)
LC50
(=0.7 mg/kg)
logistic fit, sigmoidal curve
Experimental data
110
100
90
80
70
60
50
40
30
20
10
0
-10
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4