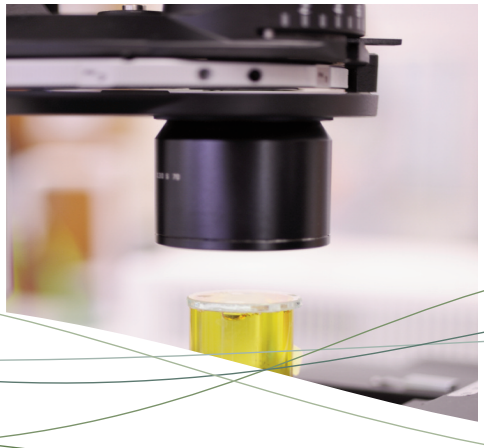
Marine Research Sub-Programme
(NDP 2007–'13) Series

SeaChange
Casadh na Taoide

AZASPIRACIDS - Toxicological Evaluation, Test Methods and Identification of the Source Organisms (ASTOX II)

Project-based Award

Lead Partner: Marine Institute



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NDP Marine Research Sub-Programme 2007-2013

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THE BIOLOGICAL SOURCE, CHEMICAL AND TOXICOLOGICAL STUDIES ON AZASPIRACIDS (ASTOX 2)

Lead Partner: Marine Institute

Project Partners: Ifremer, Alfred Wegener Institute, Norwegian School of Veterinary Science, University of Michigan, NOAA, National Research Council, Canada and Dublin Institute of Technology.

Collaborating Partners: Norwegian Veterinary Institute, Queen's University Belfast and University of Trieste.

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Project Duration: 01/07/2009 to 29/11/2013

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Disclaimer

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Abbreviations

ACN	acetonitrile
ADAM	9-anthryldiazomethane
ALT	alanine-aminotransferase
AP	alkaline phosphatase
AR	absorption rate
AS	artificial saliva
AST	aspartate-aminotransferase
AWI	Alfred Wegener Institute
AZA	azaspiracid
AZP	azaspiracid poisoning
bw	body weight
CB	chromatin bound
CE	cytoplasm
CGC	cerebellar granular cells
CID	collision induced dissociation
CL	confidence level
COSY	correlation spectroscopy
CPK	creatine phosphokinase
CR	clearance rate
CRM	certified reference material
DCM	dichloromethane
DG	digestive gland
DIC	differential interference contrast
DIDs	4,4-diisothiocyanatostilbene-2,2-disulfonic acid
DNA	deoxyribonucleic acid
DSP	diarrhetic shellfish poisoning
DTX-2	dinophysistoxin -2
EC_{50}	half maximal effective concentration
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay
EQUIV	equivalents
EtOAc	ethyl acetate
EU	European Union
EU-RL	European Union – reference laboratory
FAO	Food and Agricultural Organisation
FSAI	Food Safety Authority Ireland
FTA	feeding time activity
GGT	gamma-glutamyl transpeptidase

GI	gastrointestinal
GLDH	glutamate dehydrogenase
GPCR	G-protein coupled receptors
HABs	harmful algal blooms
hERG	human Ether-à-go-go-Related Gene
HPLC	high performance liquid chromatography
IP	Intraperitoneal
ITS	internal transcript spacer
LC-FLD	liquid chromatography – flouresence detection
LC-MS/MS	liquid chromatography – mass spectrometry
LC-PDA	liquid chromatography – photo diode array
LC-UVD	liquid chromatography – ultra violet detection
LD_{50}	median lethal dose
LOAEL	lowest observable adverse effect level
MBA	mouse bioassay
MI	Marine Institute
MeOH	methanol
ME	membrane
ML	maximum likelyhood
MP	maximum parsimony
NaOH	sodium hydroxide
NDP	National Development Plan
NE	nuclear
NJ	neighbour joining
NOAA	National Oceanic and Atmospheric Administration
NOAEL	no observable adverse effect level
NoE	nuclear overhauser effect
NOESY	nuclear overhause effect spectroscopy
NMDA	N-methyl-D-aspartic acid
NMR	nuclear magnetic resonance
NRCC	National Research Council Canada
NRL	National Reference Laboratory
NSB	Non-specific binding
NSVS	Norwegian School of Veterinary Science
NVI	Norwegian Veterinary Institute
OA	okadaic acid
PBS	phosphate buffered saline
PCD	programmed cell death
PCR	polymerase chain reaction
PE	pellet

QC	quality control
qNMR	quantitative nuclear magnetic resonance
QTof	quadrapole time of flight
QUB	Queen's University Belfast
RDA	retro Diels Alder
RF	remaining flesh
RM	reference material
ROESY	rotating frame overhause effect spectroscopy
RP	reverse phase
RT-PCR	reverse transcription - polymerase chain reaction
SAR	structure activity relationship
SD	standard deviation
SE	standard error
SEC	size exclusion chromatography
SEM	scanning electron microscopy
SGF	simulated gastric fluids
SIF	simulated intestinal fluids
SIM	selected ion monitoring
SLV	single laboratory validation
SOP	standard operating procedure
SPE	solid phase extraction
SPR	surface plasmon resonance
SRM	selected reaction monitoring
TDI	tolerable daily intake
TEER	transepithelial electrical resistance
TEF	toxic equivalent factor
TEM	transmission electron microscopy
TEQ	toxic equivalents
TFR	total filtration rate
TOCSY	total correlated spectroscopy
TSQ	triple stage quadrapole
UM	University of Michigan
UV	ultra violet
VGSC	voltage-gated sodium channel
WP	work package
YTX	yessotoxin

1. Executive Summary

1.1. Background

Since the Irish monitoring program was set up in 2001 azaspiracids (AZAs) have been detected in shellfish above the regulatory limit every year with the exception of 2004 (Figure 1). The SouthWest coast of Ireland is especially prone to the onsets of AZA events. Over this period a number of poisoning incidents associated with this toxin group have occurred, all related to Irish shellfish (Table 1). In 2003 the Marine Institute was awarded funding for a research project named ASTOX. This project was very successful in producing a range of reference materials (RMs), which are essential for accurate detection and monitoring, and which up to this point were unavailable. The project also examined the toxicity of AZAs, primarily using *in vitro* cell assays but some *in vivo* studies were also performed. Functional assays based on lymphocyte T (Jurkat) and Caco-2 cells were developed and both indicated that AZAs are cytotoxic. A study on medaka fish embryos found AZAs to be teratogenic, while mouse studies revealed multiple organ damage. Although much was learned about the toxicity of AZAs, the mode of action still remained elusive. Additional questions remained regarding the causative organism, effects of exposure to combined groups of toxins, oral toxicity and bioavailability.

1.2. Project aims

The overall aims of the ASTOX 2 project were to strengthen knowledge on the causative organism and toxicity of AZAs. The project aims were grouped into three areas: ecology, chemical support and toxicology.

The project was designed to investigate the primary causative organism(s) and address the trophic transfer of AZAs through the marine food web. The identification and monitoring of the producing organism(s) using molecular probes would enable more effective management of shellfish production zones (warning system for onset of blooms).

Chemical support outcomes were to include the provision of purified AZA analogues for the preparation of RMs (to ensure sustainable supplies for monitoring laboratories) and for toxicological assessment. Suitably contaminated feed was additionally to be prepared for the *in vivo* studies. Validated methods of analysis were to be developed for the analysis of phytoplankton and animal tissues and to explore stability, metabolism and bioavailability.

The toxicological studies were aimed at answering four major questions:

- What are the molecular targets of AZAs in mammalian cells i.e., the mode of action at molecular level?
- What is the pharmacokinetic behaviour of AZAs?
- What are the relative toxicities of AZAs?
- What are the possible consequences of the co-occurrence of AZAs with other lipophilic shellfish toxins?

The project incorporated the use of *in vitro* (Jurkat T lymphocytle cell) and *in vivo* (mouse and pig) assays to try and answer these questions. Information from these toxicity studies was necessary to underpin or revise the current risk assessments.

1.3. Producing organism(s) and culturing

1.3.1. Identification of new and related *Azadinium* species

In 2007, whilst on a survey in the North Sea, Alfred Wegener Institute scientists discovered a small (10–15 µm in length) photosynthetic thecate dinoflagellate, subsequently named *Azadinium spinosum*, that was found to produce AZA1 and -2. Over the course of the ASTOX 2 project eight additional *Azadinium* species (*A. obesum, A. poporum, A. polongum,* two varieties of *A. caudatum, A. dalianense* and three other species (yet to be named)) were identified, in addition to a related species *Amphidoma languida.* Yet another *Azadinium* species was discovered by an Italian research group off the coast of Naples subsequently named *Azadinium dexteroporum*.

Thus currently there are 11 species (three of which are not yet formally described), reflecting a rapidly increasing body of knowledge on the diversity of this genus, considering the short interval since this first identification of *Azadinium*. Some of the species are available as multiple strains. It is noteworthy that multiple strains of the type *A. spinosum* from different locations have consistently been found to produce AZA1, -2 and -33. The next two species, including *A. poporum*, have initially been described as non-toxigenic, as none of the known AZAs were found. However, with the recent detection of new AZAs in two species, including *A. poporum*, it became evident that the species diversity within this group is also reflected by a high chemical diversity, with AZA production even found in the related genus *Amphidoma*. In particular, *A. poporum* turned out to be a rich source of different AZA compounds, but with a large variability of AZA-profile among different strains.

1.3.2. Quantitative analysis of *A. spinosum*

A. spinosum cells were collected from bioreactor cultures, using centrifugation or filtration. Different extraction procedures were evaluated for formation of methyl-ester artefacts, yield, and matrix effects. Filtration of cultures using glass-fibre filters led to increased formation of methyl-esters. Hence centrifugation is recommended for recovery of cells. The type of extraction solvent (methanol, acetone, acetonitrile) did not significantly affect the yield of AZAs so long as the organic content was 80% or higher. However, the use of methanol led to increased formation of methyl-ester artefacts. AZA1 recovery over two successive extractions was 100% at 95% confidence level for acetone and methanol. In standard addition experiments, no significant matrix effects were observed in extracts of *A. spinosum* or *A. obesum* up to sample intake of 4.5×10^9 μm^3. Moreover, experiments carried out to clarify the formation and structure of methylated AZA analogues, led to the description of two new AZAs and the correction of the chemical structures of AZA29–32.

1.3.3. Nutritional and environmental parameters affecting toxin production

An in depth study on the environmental parameters affecting toxin growth and toxin production of *A. spinosum* was performed. Growth rate and maximum cell concentration were highest at 35 psu, at temperatures between 18 to 22 °C, and with aeration. Concerning AZA cell quota, the most significant effect was observed at low temperature; the AZA cell quota was more than 20 times higher at 10 °C (220 fg/cell) than at temperatures between 18 and 26 °C. High irradiance also enhanced (but to a lesser degree) cell quotas. Low K modified medium concentration (0.5 Kmod) improved AZA cell quota while higher concentration (2 Kmod) improved maximal cell concentration in the photobioreactor.

1.3.4. Bulk culturing

Continuous pilot scale culturing was carried out to evaluate the feasibility of AZA production using *A. spinosum* cultures. Algae were harvested using tangential flow filtration or continuous centrifugation. AZAs were extracted using solid phase extraction (SPE) procedures, and subsequently purified. When coupling two stirred photobioreactors in series, cell concentrations reached 190,000 and 210,000 cells/mL at steady state in bioreactor 1 and 2, respectively. The AZA cell quota decreased as the dilution rate increased from 0.15 to 0.3 per day, with optimum toxin production at 0.25 per day. After optimisation, SPE procedures allowed for the recovery of 79 ± 9% of AZAs (AZA1, -2, -33 and -34).

Harvesting of *A. poporum* cultures was also performed. Approximately 300 L of each strain was grown to high density (>100,000 cells/mL) in 5 L flasks using gentle aeration. Lipophilic toxins were

harvested by adding HP-20 to cultures where cells had been lysed by acetone addition (7% final concentration). HP-20 was subsequently collected, dried, and extracted with 100% acetone to get a crude extract containing the previously undescribed toxins AZA36 and -37.

1.4. Molecular probes

A molecular probe was developed specifically for the detection and discrimination of *A. spinosum, A. obesum* and *A. poporum.* Good correlation was observed between results from the molecular probe analysis of seawater and the results of chemical analysis of shellfish samples. This probe will serve as a very useful tool in the effective monitoring of AZA producing organisms in seawater samples.

1.5. Trophic transfer and metabolism

1.5.1. Vector species

Preliminary studies with copepods found that these plankton grazers ingested minor amounts of *A. spinosum* and almost no AZA accumulation was found. Of the protistan grazers that were investigated the large ciliate *Favella ehrenbergii* showed substantial toxin accumulation.

1.5.2. Trophic transfer – toxin accumulation and biotransformation

Two separate experiments were performed, involving the feeding of *Mytilis edulis* on *A. spinosum* both at the MI and at Ifremer. The original algal toxins AZA1 and -2, as well as mussel metabolites AZA3 to 12, -17, -19, -21 and -23 were found during these trials. After as little as 6 h, AZA contents in mussels reached the EU regulatory limit (160 µg/kg), and metabolites were observed in all conditions at ~25% of the total AZA content. This increased to 50% after 24 h, and continued to increase until the end of the studies. AZA17 and -19 were found to be the main metabolites, with AZA17 concentrations estimated to be in the same order of magnitude as that of the main algal toxin, AZA1. A previous study showed that AZA3, -4, -6 and -9 are formed when shellfish are cooked due to the chemical conversion (thermally driven) of AZA17, -21, -19 and -23 respectively.

1.5.3. Dissolved AZAs

Experiments were performed to determine whether dissolved AZAs released from decaying blooms of *A. spinosum* could be relevant for the shellfish industry; human health; or play a role in ecological interactions with other aquatic organisms (e.g., bivalve larvae, plankton, fish embryos). In the study, two levels of cellular concentrations were investigated using lysed *A. spinosum* cells and the results

compared with those using live *A. spinosum* cells. The conditions corresponded to a bloom of 1×10^5 cells/mL of *A. spinosum* and a more realistic scenario of a bloom of 1×10^4 cells/mL. The study showed that dissolved AZAs were bioavailable for mussels and that AZA accumulation may reach concentrations above the regulatory limit and thus might be considered as a food safety issue.

Additional experiments to assess the ability of mussels to produce AZA metabolites by exposure to semi-purified AZA resulted in formation of metabolites, however, the conversion efficiency and the recovery were too low to justify using this procedure for preparative isolation.

1.5.4. Effects of *A. spinosum* on feeding behaviour and physiology

A study was designed to compare the feeding behaviour of mussels fed toxic and non-toxic diets of *A. spinosum* and *Isochrysis aff. galbana* (T-Iso), respectively. Physiological factors such as feeding time activity (FTA), clearance rate (CR), filtration rate (TFR) and absorption rate (AR) were followed to confirm the negative effect of *A. spinosum* on mussels.

A. spinosum had a significant effect on mussel feeding behaviour compared to T-Iso: CR was lower by a factor of 6, FTA by a factor of 5, TFR by a factor of 3 and AR even decreased to negative values for the last day of exposure. Furthermore, direct feeding with *A. spinosum* revealed slightly increased mussel mortality and negative effects on the thickness of mussel digestive gland tubules compared to the non-toxic diet. These results show a negative effect of *A. spinosum* on blue mussel feeding activity and indicate a possible regulation of AZA uptake by decreasing filtration and increasing pseudofaeces production.

1.6. Isolation and reference materials

1.6.1. Isolation from shellfish (*Mytilus edulis*)

Research into analytical methods, chemistry, metabolism and toxicology of AZAs has been severely constrained by the scarcity of high-purity AZAs. Consequently, since their discovery, considerable efforts have been made to develop methods to isolate AZAs in sufficient amounts and purities for toxicological studies, in addition to the preparation of calibrant RMs. A seven-step procedure, involving a series of partitioning and column chromatography steps, was improved (compared with previously reported procedures) for the isolation of AZAs. Recoveries increased 2-fold (to ~52%) compared to the method employed in the ASTOX project and led to the isolation of 12 AZA analogues (seven of which were fully characterized for the first time).

1.6.2. Isolation from phytoplankton

The preparative isolation procedure developed for shellfish was optimised for algal extracts (*A. spinosum* and *A. poporum*), such that only four steps were necessary to obtain purified AZA1 and -2. A purification efficiency of ~70% was achieved, and isolation from 1,200 L of culture yielded 9.3 mg of AZA1 and 2.2 mg of AZA2 (purities >95%). This work demonstrated the feasibility of sustainably producing AZA1 and -2 from *A. spinosum* cultures. In addition to AZA1 and -2, sufficent amounts of AZA33, -34, -36 and -37 were isolated for structural determination by NMR and toxicity assays.

Purity and structural determination were performed by LC-MS/MS, LC-UVD and NMR. Overall 16 AZA analogues were purified.

1.6.3. Reference materials (RMs) and proportions in shellfish

Calibrant RMs were prepared for each analogue and subsequently used for identification and accurate quantitation in cooked *Mytilus edulis* shellfish extracts. AZA1, -2, -3 and -6 were found to be the most relevant toxins in terms of proportions. Very high levels of AZA3 (2.5-fold that of AZA1) and -6 (2.5-fold that of AZA2) were found in some samples. This finding is significant considering the EU stipulates that raw shellfish be analysed, with only AZA1, -2 and -3 being regulated for, which means that levels of AZA equivalents may be underestimated in shellfish under current monitoring practices.

1.6.4. Certified reference materials (CRMs)

A number of CRMs are currently available from the NRCC that were produced as part of the ASTOX project. Stocks isolated as part of the ASTOX 2 project will ensure the supplies of AZA calibrants will be sustained.

1.7. Toxicity

1.7.1. *In vivo*

1.7.1.1. Pig feeding study

Pigs are often used in toxicology studies because they have similar anatomical and physiological characteristics to humans, including the cardiovascular, urinary, integumentary and digestive systems. Two trials were performed whereby mini-pigs were fed AZA1, -2 and -3 contaminated feed at a dosage of ~350 µg/kg (i.e., 120 g of feed with an AZA concentration of 23 µg/g (144 times the regulatory limit) fed to an 8 kg pig). During the trials, no sickness (diarrhea, vomiting, etc) was noticed, however, the following was observed:

- Pigs were slightly drowsy after 24 h compared with the control
- Analysis of blood samples revealed AZA concentrations peaked ~4 h after feeding
- Histology showed some cell damage to the duodenum villi, jejunum, ileum, colon, and liver
- Enzyme expression characteristic of liver damage was observed in haematology
- Prolonged food retention (compared to control) possibly due to reduced GI tract mobility
- Chemical analysis of mini pig samples showed wide distribution of AZAs throughout the body, thus, AZAs were absorbed from the feed across the intestine
- Phase 2 metabolism is likely to play a significant role as only 14% of administered AZAs were accounted for

1.7.1.2. Mouse intraperitoneal (*ip*) study

The current regulatory limits are based on the initial mouse studies *(ip)* performed by Satake *et al* (1998) and Ofuji *et al* (1999) which indicated higher toxicity for AZA2 and -3 relative to AZA1. The study was repeated producing similar results for AZA2 (117 µg/kg) and -3 (164 µg/kg) but a lower LD_{50} (higher toxicity) for AZA1 (74 µg/kg). In parallel, the LD_{50} was determined for AZA6 (100 µg/kg) for the first time.

Chemical analysis of the tissues revealed wide distribution with similar distribution profiles for each toxin. Higher recoveries were observed for AZA1 and -2 (~15–37%) while only 2–15% of AZA3 could be accounted for possibly due to the greater instability of this compound.

1.7.1.3. Mouse oral study

Limited data was previously available on the oral toxicity of AZAs with studies only performed on AZA1 which showed that the GI tract, liver, spleen and thymus are the main organs affected. A study was performed for AZA1, -2 and -3 and the results were very similiar to those obtained in the *ip* study.

Chemical analysis of the tissues revealed wide distribution of toxin. Higher recoveries were observed for AZA1 and -2 (~4–19%) while lower recoveries were obtained for AZA3 (2–8%) after 24 h.

1.7.1.4. Combined toxicities (mouse oral)

AZAs have been shown to co-occur with other toxin groups (OA group toxins in Ireland and YTX in Norway), therefore it was important to investigate whether exposure to combinations of these toxins could have additive, synergistic or antagonistic effects.

Oral mouse studies demonstrated no additive or synergistic effect on lethality or absorption into the body when administered in combination with OA. Similar results were obtained in a separate experiment examining combinations of AZA1 and YTX. The results indicate that combinations of these toxin groups in shellfish does not present an increased risk for consumers.

1.7.2. *In vitro*

1.7.2.1. Mode of action studies

A series of screening experiments were performed whereby the effects of AZA1 towards various ion channels were directly monitored. A distinct and significant inhibition of the hERG (a gene that codes for an ion channel protein) potassium channel was observed. Further exploration of this phenomenon demonstrated that AZA1–3 were open state blockers of hERG potassium channels. These channels are best known for their contribution to the electrical activity that co-ordinates the heart's beating, but are additionally transcriptionally expressed in a broad array of other cell/tissue types including the brain, liver, kidney, breast, pancreas and colon. Considering that the concentration of AZA necessary to inhibit hERG channels (IC_{50} range: 640–840 nM) are at least two orders of magnitude higher than those capable of causing cytotoxicity and cytoskeletal effects, it is likely that yet another target and mechanism of action exists for AZAs.

1.7.2.2. Cytotoxicity and apoptosis

Multiple human cell lines (Jurkat T lymphocytes, Caco-2 intestinal cells, and BE(2)-M17 neuroblastoma cells) were used to characterize cytotoxicity and pathways of apoptosis. Cytotoxicity experiments were consistent with the published literature showing that AZA1 was cytotoxic in both a concentration and time-dependent manner to each cell type tested, with mean EC_{50} values ranging between 1.1 and 7.4 nM. Despite the absence of morphological changes indicating apoptosis, caspase-3/7 activity was higher in all cell types treated with AZA1. Furthermore, in T lymphocytes, the most sensitive cell type, the activities of the initiator caspases-2 and -10 and concentrations of intracellular cytochrome c were elevated. DNA fragmentation was also observed for T lymphocytes exposed to AZA1, -2, and -3. Collectively, the data confirm that AZA1 is highly

cytotoxic to multiple cell types and that cells exposed to AZA1 undergo atypical apoptosis, possibly in conjunction with necrotic cytotoxicity.

1.7.2.3. Structure activity relationships (SARs)

The Jurkat T lymphocyte cell assay was used to assess SARs for the AZA analogues purified over the course of the project. Using this model relative potencies after are: AZA2 > AZA6 > AZA34 ≈ 37-*epi*-AZA1 > AZA8 ≈ AZA3 > AZA1 > AZA4 ≈ AZA9 > AZA5 ≈ AZA10 > AZA33 ≈ AZA36 > AZA37 > AZA26.

The results indicate AZA potency is gained by methylation of C8 and/or C22 (AZA2, -6) while AZA potency is reduced by C3 and/or C23 hydroxylation (AZA4, -5). AZA33 (AZA1 missing A/B/C rings) was significantly less potent than AZA1 (~5-fold), whereas AZA34 (AZA1 missing C4/C5 alkene) was 5.5-fold more potent than AZA1. Similarly, 37-*epi*-AZA1 was 5.1-fold more potent. Semi-synthetic hydrogenated AZA1 analogues, 7,8-deuterohydroAZA1 and 4,5,7,8-tetrahydroAZA1, were also tested. Both compounds were found to be approximately equipotent to AZA1 suggesting that the C4/C5 alkene and the C7/C8 olefin bonds do not contribute to the toxicological activity.

1.8. Scientific publication output

To date this project has led to the publication of 32 peer reviewed papers (with more than 7 additional manuscripts in preparation), 3 book chapters and 72 presentations at national and international conferences.

1.9. Recommendations summary

1.9.1. Benefits to the Industry

- Improved monitoring and forecasting becomes possible with the development of a molecular probe and the isolation of purified stocks of AZAs for CRMs
- Toxicity and cooking studies indicate current regulatory limit is sufficiently low and the EFSA recommendation to reduce the limit may be unwarranted
- Feeding and vector studies peformed in ASTOX 2 will contribute towards developing mitigation strategies

1.9.2. Recommendations to regulators

- Introduce a cooking step in the legislated method to more accurately reflect toxin profiles (due to heat induced toxin conversions)
- Include AZA6 in legislation (high levels were detected in some cooked *Mytilus edulis* tissues)
- Review current regulatory limit based on all the information gathered in the ASTOX 2 project, with a revision of the TEFs for AZA2 and -3 based on oral and mouse *ip* studies

2. Introduction

2.1. History of AZA research

Azaspiracid poisoning (AZP) was first reported in 1995 when several people in the Netherlands became ill after eating contaminated shellfish harvested in Ireland.[1] Contaminated mussels from this and subsequent incidents were sent to Tohoku University in Japan where the causative toxins AZA1, -2 and -3 were isolated and characterized.[2,3] In a study using two male ddY mice, an *ip* lethal dose of purified AZA1 of 200 µg/kg bw was reported[2], while the lethal doses for AZA2 and -3 were 110 and 140 µg/kg bw, respectively.[3] In 2002, AZA1–3 were introduced into EU biotoxin legislation with the limit being set at 160 µg/kg.[4]

The Irish national monitoring program was set up in 2001, and since that time, the detection of AZAs in shellfish samples has led to significant protracted shellfish farm closures with severe economic consequences for the industry[5] (Figure 1). To date, seven human AZP events have been confirmed (Table 1), but it is quite possible, due to the similarity of symptoms observed for people with DSP or other types of food poisoning (e.g., bacterial enteritis), that many more undocumented events may have occurred. Coincidently, each of the confirmed AZP events have been traced to contaminated Irish shellfish (*Mytilus edulis*). AZAs have been detected in other European countries[6], Morocco[7], North[8] and South America[9], Japan[10] and elsewhere (Figure 2).

In 2003 the Marine Institute received National Development Plan (NDP 2003–2006) funding for a three year project on AZAs named ASTOX. The primary aims of the project were to purify sufficient amounts of AZA1–3 to produce CRMs to aid in the monitoring of these toxins and to further study the toxicological effects. Through collaborations with other international research teams the project aims were achieved; successful isolations of AZA1–3 from shellfish, enabling the production of CRMs and some *in vitro* toxicity testing.[11] Over the course of the project 8.4 mg of AZA1, 0.9 mg of AZA2 and 1.3 mg of AZA3 were isolated. The majority of this material was used for the preparation of calibrant CRMs – a first for this toxin group.[12] A number of tissue CRMs were also prepared for the first time.[13–16] The availability of CRMs is essential to ensure accurate results are being produced by monitoring laboratories. Other findings from the project included information on shellfish distribution, toxin ratios and the effects of cooking.[17,18]

The toxicological studies focused mainly on cell based assays with the development of lymphocyte T (Jurkat) and Caco-2 cell assays. Caco-2 cells are often used as a model for studying intestinal drug

transport[19] and/or TEER[20], and were putatively chosen as an *in vitro* model to parallel the extensive intestinal effects that AZAs are known to have on mammals when tested *in vivo*.[21–24] Two of the ASTOX related studies suggested that AZA1 had little or no effect on Caco-2 cells[11,25], whereas one separate investigation found AZA1 to be somewhat cytotoxic.[26] Despite these different findings regarding cytotoxic endpoints, monolayers of Caco-2 cells exposed to AZA1 have shown significant reductions in TEER assays when exposed to low concentrations of AZA1 (i.e., 5 nM)[11] suggesting altered permeability and/or cellular function. The Jurkat T lymphocyte assay was shown to be particularly sensitive to AZAs demonstrating the time- and concentration-dependent effects as well as differences in AZA analogue potencies.[11,27] This assay supported the original mouse *ip* studies in terms of the relative toxicities of AZA1, -2 and -3.[11]

To date, no effects (of AZAs) other than gastro-intestinal disorder have been described in humans. However, a number of studies on animals (within the ASTOX project and by other research groups) suggest that there is a potential for sub-acute effects. An early study by Ito *et al.*[24], on chronic exposure of mice to AZA suggested that more tumors were observed in AZA-exposed mice compared to the control population. Unfortunately, the study was not considered conclusive as the number of animals used was quite low and there was no clear dose-response relationship (i.e., more lung tumors were observed at the lower dose of 20 µg/kg twice weekly (3/10 mice) than at the higher dose of 50 µg/kg twice weekly (1/10 mice)). Still, 9 out of 10 mice at the higher dose level were sacrificed during the study due to extreme weakness and the lung tumor that was observed at the highest dose level was the earliest tumor observed (the other two tumors being observed at the lower dose level during the period of recovery post exposure). Hence a tumorigenic effect of AZAs cannot be completely ruled out at this stage. The study by Colman *et al.*[28], even though only based on a single dose exposure, also showed strong sub-acute effects on embryonic development of Japanese medaka fish. This study suggests a teratogenic potential of AZAs at sub-acute levels in this model and transfer across the placental barrier in pregnant women should be considered a hazard that needs to be investigated further. This hazard appears of particular importance in light of the facile uptake observed for AZA1 across the intestinal barrier in small rodents.[29] Initial observations of AZA-exposed mice had also suggested that AZA should be considered a neurotoxin as symptoms had included jumping behaviour.[30] An additional indication of neurotoxicity was found by Kulagina *et al.*[31] who detected effects of AZA1 on neuronal cells. Hence, further confirmation of neurotoxic effects should be made once the mechanism(s) of action is (are) clarified in case sub-acute effects in humans may be caused.

In 2008, based on all the information gathered, another risk assessment was performed by EFSA who suggested a lowering of the regulatory limit.[32] Up to this point, little information on the oral toxicity, bioavailability and mode of action of AZAs was available. Furthermore, uncertainty remained regarding the causative organism and the effects of exposure to combined groups of toxins. For such studies large amounts (mg quantities) of purified toxins would be required. Apart from AZA1–3, numerous other analogues were identified in the ASTOX project[33], however, due to time limitations their significance had not been established. In 2009, funding was provided by the NDP (2007–2013) for another three year project on AZAs, named ASTOX 2, which was to address these outstanding issues.

Table 1. Confirmed cases of azaspiracid poisoning (AZP).

Event	Location of AZP	Date	Implicated food source	Amount consumed	Area of production	Number of illnesses recorded	[AZA_{total}] (μg/g)	Comments	Reference
Confirmed AZP	The Netherlands	Nov-95	Mussels (*Mytilus edulis*)	Not recorded	Killary Harbour, Ireland	8	1.43[a,b]		8,35,36
Confirmed AZP	Arranmore, Ireland	Sep / Oct-97	Mussels (*M. edulis*)	"As few as 10–12 mussels"	Arranmore Island, Ireland	Estimated 20–24 (8 seen by a doctor)	30[c]	Equivalent to ~6 μg/g whole mussel meat; AZA4,-5 were also present.	8,34
Confirmed AZP	Ravenna, Italy	Sep-98	Mussels (*M. edulis*)	Not recorded	Clew Bay, Ireland	10	1.0[c]		8,34
Confirmed AZP	France	Sep-98	Mussels (*M. edulis*)	Not recorded	Bantry Bay, Ireland	Estimated 20–30	1.1–1.5[a]	Were tested for DSP toxins using mouse bioassay and deemed "safe".	8,34
Confirmed AZP	United Kingdom	Aug-00	Frozen mussels (*M. edulis*)	Not recorded	Bantry Bay, Ireland	12–16	0.85[a]		8,34
Confirmed AZP	France	Apr-08	Frozen mussels (*M. edulis*)	Not recorded	Ireland	"large outbreak"	>0.16[a]		34,37
Confirmed AZP	USA	Jul-08	Frozen mussels (*M. edulis*)	~113 and 340 g	Bantry Bay, Ireland	2	0.086–0.244[a]	~150 tonnes were voluntarily destroyed; AZA6 was also present.	38

[a]Whole mussel meat
[b]Sampled in April 1996 (5 months after event)
[c]Digestive glands / hepatopancreas

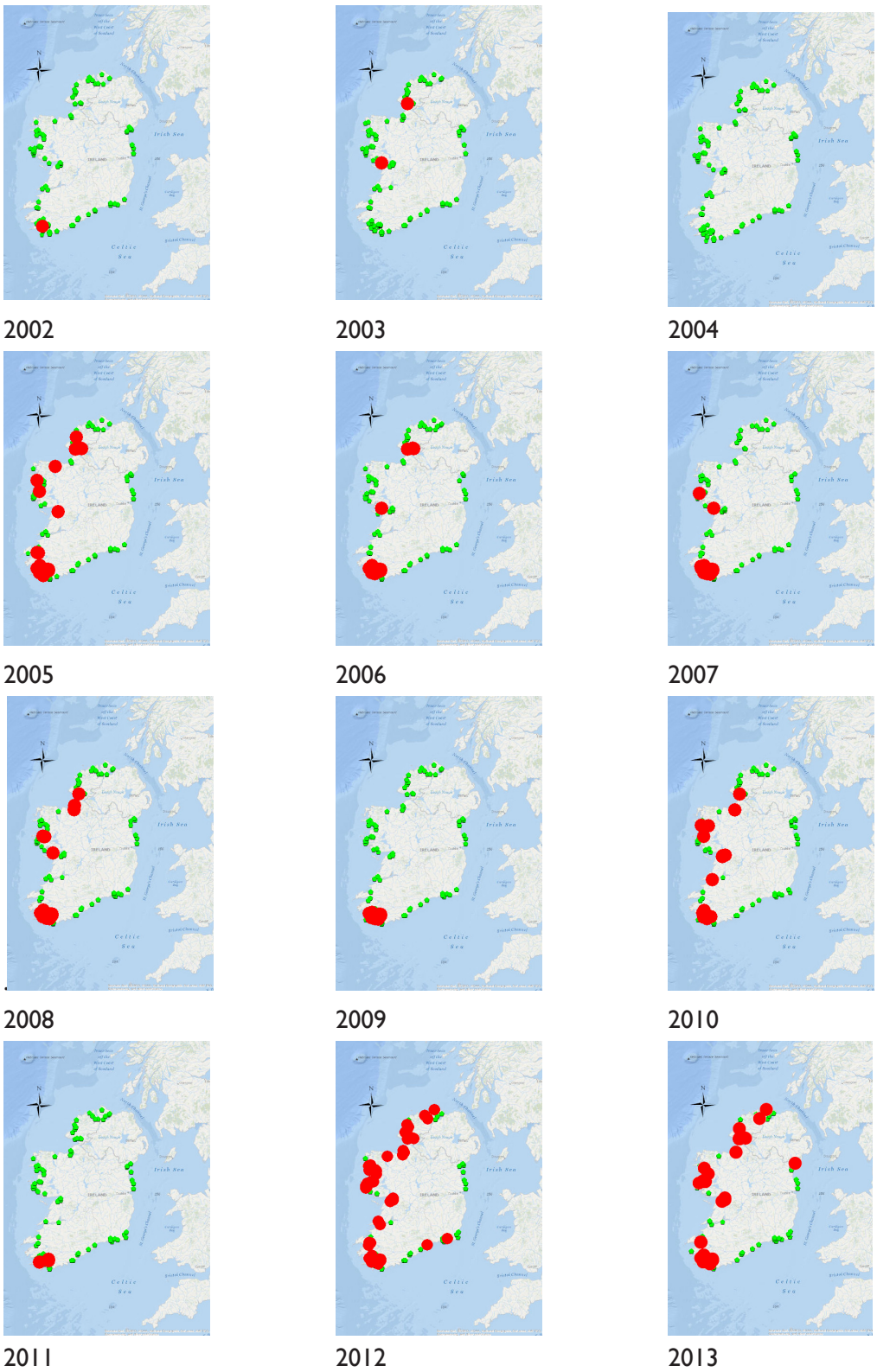


Figure 1. Closure of Irish shellfish harvesting sites due to AZAs from 2002–2013.

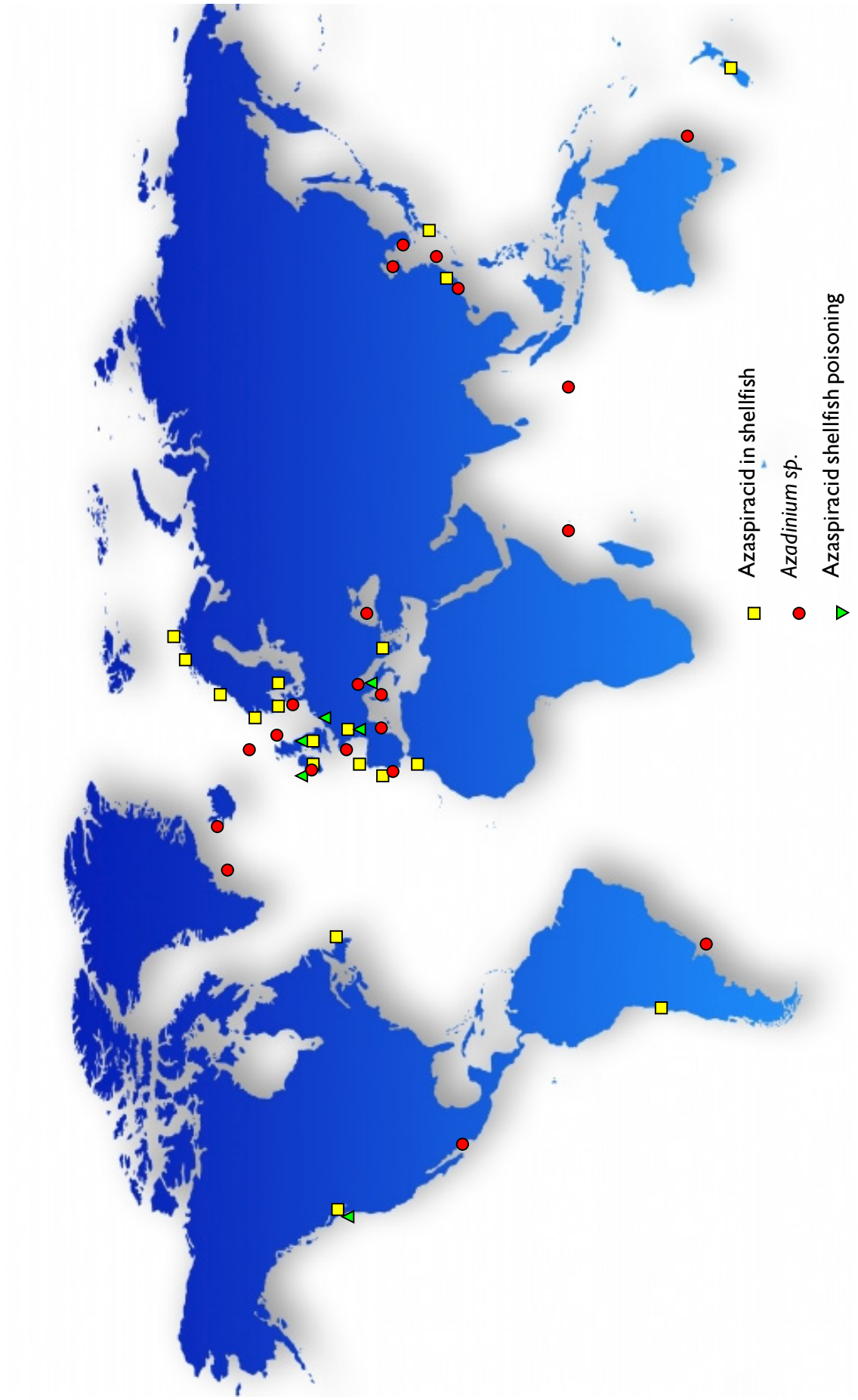


Figure 2. Global distribution of AZAs.

2.2. ASTOX 2 project design

The project was divided into 10 work packages (WPs) (Table 2, Figure 3). Two of the WPs dealt with project management and dissemination (WP1 and 10). WP 2 dealt with bulk culturing of the producing organism and involved Ifremer, the Marine Institute (MI) and the Alfred Wegener Institute (AWI). Confirmation of the causative organism and development of molecular probes was covered in WP3 involving the MI and AWI. WP 4 led by AWI and Ifremer encompassed trophic transfer of *A. spinosum*. WP5 focused on the isolation of AZAs and involved the MI, the Norwegian Veterinary Institute (NVI) and the National Research Council, Canada (NRCC). The remaining WPs (6–9) dealt with the toxicological aspects – *in vivo* and *in vitro* testing with subsequent analysis of tissues for metabolites and involved the MI, Norwegian School of Veterinary Science (NSVS), the University of Michigan (UM) and the Centre for Coastal Environmental Health and Biomolecular Research (NOAA). Additional collaborators included Queen's University Belfast (QUB) and the Univerisy of Trieste.

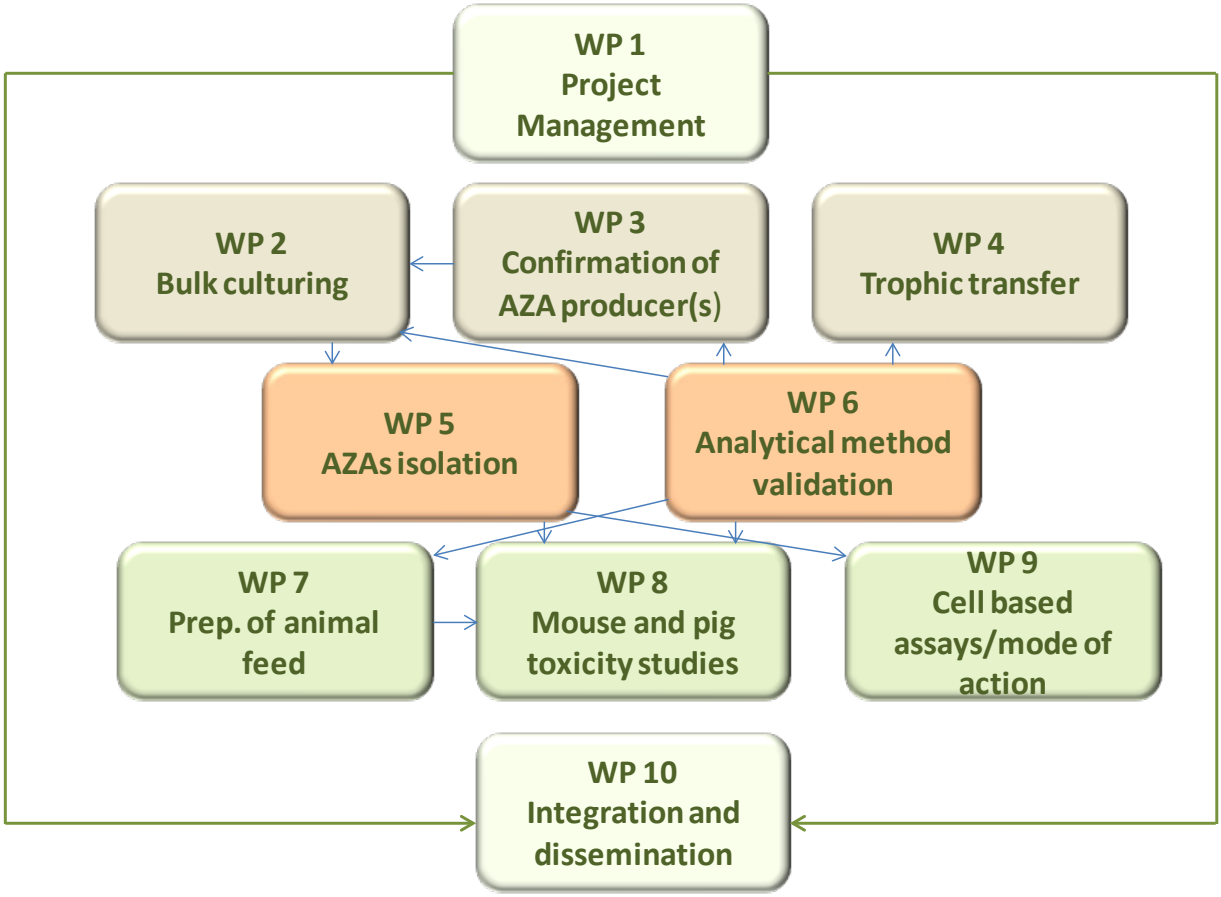


Figure 3. ASTOX 2 work package (WP) tasks.

Table 2. WP titles and deliverables.

WP	Title	Deliverables	Partner responsible	Work packages involved
1	Project management	• Effective day to day project management • Collation of annual reports • 6 monthly cost statements • Annual steering committee meetings • Final project report	MI	1–10
2	Bulk culture of AZA producer	• Sucessful establishment of a bulk culture of AZA producing organism • Optimal conditions for maximum toxin retrieval	Ifremer, AWI, MI	5
3	Confirmation of AZA producer in Irish waters	• Sampling during an AZA event • RT-PCR molecular probe developed • RT-PCR screening method set up at MI • Sucessful sourcing of AZA producer from Irish waters	MI, AWI	-
4	Trophic transfer of AZAs	• Experimental design for trophic transfer across planktonic species • Sucessful examination of trophic transfer into heterographic organism and other *Protists*	AWI, Ifremer	-
5	Isolation of AZAs	• Isolation of sufficient amounts of AZA1, -2 and 3 for toxicological studies and the preparation of CRMs • Isolate minor AZAs	MI, NRCC	2, 5, 8, 9
6	Validation of analytical techniques	• Method developed and validated for AZAs in phytoplankton • Method developed and validated for AZAs in pig feed • Method developed and validated for AZAs in mouse and pig tissues • Analysis of mouse and pig tissues following toxicology studies	MI, Ifremer	8
7	Preparation of AZA contaminated feed	• Pig feed prepared for toxicological studies	MI	8, 9
8	*In vivo* studies of AZAs in mice and pigs	• LD_{50} *ip* for AZA2 and -3 • Combined effects of AZA1 and OA • Bioavailability in pigs	NSVS	5, 7
9	*In vitro* studies investigating mode of action of AZAs	• Model of cumulative toxicity of lipophilic toxins at cellular level • Interaction of AZAs with cholesterol pathway clarified • Characterisation and mode of action of AZAs	UM, NOAA	5
10	Integration and dissemination	• Dissemination workshop • Final project report	MI	1–10

3. AZA PRODUCING PHYTOPLANKTON

3.1. Introduction

For many years following the identification of AZAs the source remained elusive. The seasonal accumulation of these toxins in shellfish, particularly mussels *(Mytilus edulis)* and the structural homologies with other known lipophilic phycotoxins, pointed towards a dinoflagellate producer. *Protoperidinium crassipes* had been reported as the producer[38], however this organism was later found not to produce these toxins in the laboratory[39] and is more likely a vector of the producing organism.

In 2007, during a cruise on the research vessel Poseidon in the North Sea the causative organism was discovered from the NorthEast coast of Scotland, provisionally named as 3D9, and the strain isolated had been described as a new species in a new genus.[40] This organism, a small (10–15 µm in length) armoured dinoflagellate named *A. spinosum* was shown to produce AZA1 and -2 (2–20 fg/cell AZA1) in culture and effectively identified as *de novo* producer of AZAs.[41]

3.2. *Azadinium spinosum*

Following the discovery of *A. spinosum* further isolation of *Azadinium*-like species was performed on plankton samples collected during a cruise to coastal waters of the southern North Sea along the Danish coast. A large number of crude cultures from these stations were established by use of serial dilution and screened for the presence of *Azadinium*-like dinoflagellates and AZAs. Four new isolates could be grown as clonal cultures. All cultures were carefully analysed morphologically (light and electron microscope), with respect to their DNA sequences (18S and 28S rDNA, internal transcribed spacer (ITS) and cytochrome oxidase I (COI)), phylogenetically and chemically (for the detection of AZAs). One isolate (UTHE2) was found to contain AZAs. Subsequent electron microscopic examination confirmed this strain to be *A. spinosum* showing the characteristic Kofoidean plate pattern for the genus *Azadinium* (po, x, 4′, 6′′, 6C, 5(?)S, 6′′′, 2′′′′) and exactly resembling the *A. spinosum* described by Tillmann *et al.* (2009).[40]

In August 2009, shellfish samples of blue mussel *(Mytilus edulis)* from the SouthWest of Ireland were analysed using LC-MS/MS and found to be above the regulatory limit (0.16 µg/g AZA equivalents) for AZAs. Water samples from this area were collected and the armoured dinoflagellate *A. spinosum* was isolated, cultured and shown to produce AZA toxins. This was the first strain of *A. spinosum* isolated from Irish waters.[42] Molecular phylogenetic analysis of the LSU rDNA gene shows that the

Irish *A. spinosum* was identical to the other two available *A. spinosum* strains from Scotland (3D9)[40] and Denmark (UTHE2).[43,44] The toxin concentration of the Irish strain was found to be ~15–25 fg/cell AZA1 and ~1–5 fg/cell AZA2.

In 2011 another *A. spinosum* isolate was discovered from a coastal station off the Shetland Islands (on the research vessel Heincke) that displayed similar characteristics to other *A. spinosum* isolates.[45]

3.3. Discovery of several new *Azadinium* and related species

The first isolation and description of *A. spinosum* as a source of AZA toxins stimulated further isolation and characterization of *Azadinium*-like organisms. The description of *A. spinosum* in 2009 was soon followed by the discovery of a second species of the genus, now described as *Azadinium obesum*.[46] This species was isolated from the same water sample collected from the NorthEast coast of Scotland, indicating co-occurrence with *A. spinosum*. Intensive chemical analysis by LC-MS/MS, however, failed to detect known AZAs or potential analogues in *A. obesum*.[46,47]

Further *Azadinium* like isolates were collected from Danish coastal waters that did not contain any of the known AZAs. The isolates resembled *Azadinium* sp. in terms of size, shape and swimming pattern, however, the morphological differences, DNA sequences and phylogenetic analysis indicated a new species subsequently named *Azadinium poporum*.[43] Subsequent surveys around Korea[48] and China[49] led to the isolation of additional *A. poporum* strains, some of which produced known and novel AZAs (Table 3). Considerable differences in the ITS and 28 S gene sequences were observed between the Asian and European strains. In an LSU/ITS tree, all available strains of *A. poporum* comprised 3 well supported clades. The first clade included multiple strains originating from the coast of China, as well as a Korean strain. The second clade included strains from the East China Sea and South China Sea, and the third consisted of strains from Europe. Therefore there is a considerable cryptic diversity within *A. poporum* with sympatric occurrence of two distinct ribotypes in China.

Another isolate from Irish coastal water samples turned out to represent a yet un-described new species, to be placed in the genus *Amphidoma*. Interestingly, this species, although with a distinctively different plate pattern to *Azadinium*, show some clear similarities (e.g., the configuration of the apical pore complex, the number and arrangement of sulcal plates) that indicate a close phylogenetic relationship between the genera *Azadinium* and *Amphidoma*. This morphological evaluation was

confirmed by a detailed sequence-based phylogenetic study. LC-MS/MS analysis showed that *Amphidoma languida* produced two novel AZAs; AZA38 and -39.[47]

Another species collected from the NorthEast coast of Scotland, was named *Azadinium caudatum*. Both sequence and morphometric data clearly showed that the species occurred with two distinct varieties, var. *caudatum* and var. *margalefii*, that are easily distinguished by the different shapes of the antapical projection.[50] A. *caudatum* is easily distinguished from other species of *Azadinium* by light microscopy due to its larger size; characteristic triangular shape; and clearly visible antapical projection. The basic plate pattern is the same as other *Azadinium species*, nevertheless there are a couple of minor morphological differences visible at SEM level. LC-MS/MS analysis of the two strains (var. *caudatum* and var. *margalefii*) did not show any evidence of AZA production.[51]

During the 2011 expedition on the research vessel Heincke another *Azadinium* was isolated from a coastal station off the Shetland Islands. This isolate was described as a new species, *Azadinium polongum*. By light microscopy, it was very similar to *A. spinosum*, *A. obesum* and *A. poporum*. Similar to *A. spinosum*, *A. polongum* also had an antapical spine (Figure 4) making diffrentiation by light microscopy very difficult. A distinctive morphological feature (but only visible at the SEM level) of A. *polongum* is the shape of the pore plate (elongated) which differs from other *Azadinium species* (round to slightly ellipsoid pore plate). Detailed LC-MS/MS analysis showed that *A. polongum* does not produce any known AZAs in measureable amounts.[45]

The most recently described species *A. dalianese* was isolated from Chinese coastal waters. This species is clearly different from all other species of *Azadinum* by having a reduced number of apical and epithecal intercalary plates. No known AZAs could be detected.[52]

By using high resolution light microscopy during an expedition on the research vessel Merian to Greenland and Iceland, the presence of *Azadinium* on a few stations in the Irminger Sea between Greenland and Iceland and on a few coastal stations of Western Iceland could be shown (Figure 4). Among those, a number of new species were identified, which currently are being described taxonomically. Together with the records of *Azadinium* from the Shetland Islands, these observations clearly show the presence of the genus in cold water and Sub-Arctic areas.

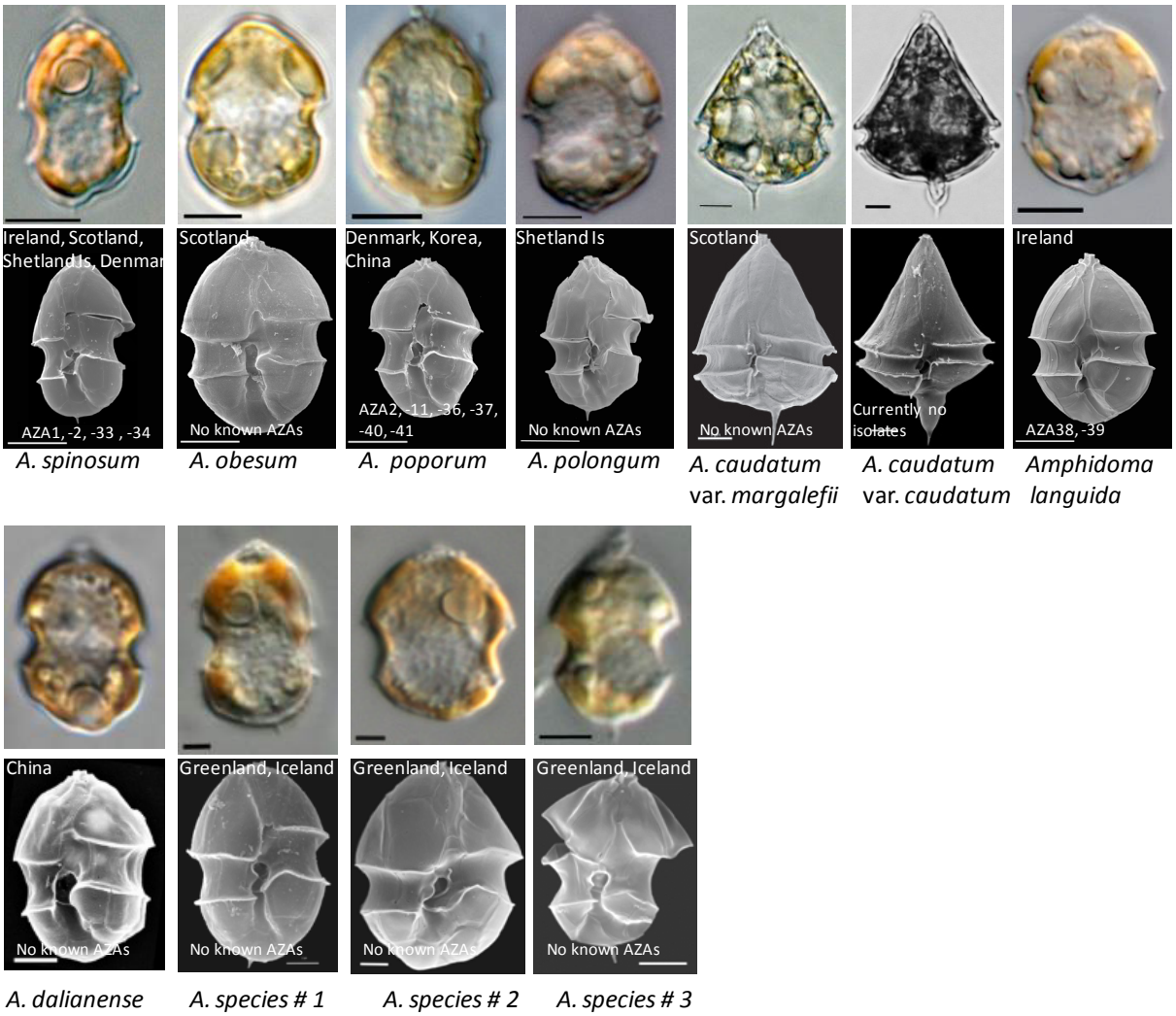


Figure 4. Light (colour) and electron (black and white) microscope pictures of the *Azadinium* and related species detailing location of strain isolation and toxins identified.

Table 3. Summary of *Azadinium* species and AZA producers identified to date.

Species	Strain	Origin	AZAs	AZA cell quota
Azadinium spinosum	3D9	Scotland 2007	AZA1, -2, -33, -34	~20 fg
	UTH E2	Denmark 2008	AZA1, -2, -33, -34	~20 fg
	SM2	Ireland 2009	AZA1, -2, -33, -34	~20 fg
	SHET F6	Shetland Is. 2011	AZA1, -2, -33, -34	~20 fg
Azadinium obesum	2E10	Scotland 2010	none	-
Azadinium poporum	UTH D4 UTH C5 UTH C8	Denmark 2008	AZA37	~10 fg
	HJ2010	Korea 2010	AZA36	~2 fg
	G25	Bohai Sea 2007	AZA36	1–2 fg
	G42	East China Sea 2011	AZA2	n.a.
	G64	East China Sea 2011	AZA2	8–23 fg
	G60	East China Sea 2011	-	-
	G66	East China Sea 2011	AZA11	2–8 fg
	G68	South China Sea 2011	AZA2	2–5 fg
	16 new strains	South China East China Sea Yellow Sea	AZA2; AZA11 AZA36; AZA40 AZA41, none	0.2–8 fg
	10 new strains	Argentinian coast	AZA2	2–5 fg
Azadinium caudatum var *margalefii*	AC1 AC2	Scotland 2011	-	-
Azadinium polongum	SHET B2	Shetland 2011	-	-
Amphidoma languida	SM1	Ireland 2009	AZA38 AZA39	~10 fg
	Merian 2A11	Greenland Iceland 2012	AZA38 AZA39	~10 fg
Azadinium dexteroporum[53]	Mediterranean	Naples	AZA? AZA?	2–4 fg
	Merian 1D12	Greenland Iceland 2012	-	-
Azadinium dalianense	AZH02	China 2012	-	-
Azadinium species #1	Merian 48A, 4B11, A2D11	Greenland, Iceland	-	-
Azadinium species #2	Merian 3D6	Greenland, Iceland	-	-
Azadinium species #3	Merian 1C6	Greenland, Iceland	-	-

Although the number of available strains of the genus *Azadinium* is still low, the results up to now support the view that toxin production is a stable characteristic of the species *A. spinosum*. However, for *A. poporum*, both AZA-producing strains as well as strains without any detectable AZAs have been reported[49,54], indicating the presence of intraspecific variability in toxin production. It is clear that different species of the genus do co-occur.[43,55] The increasing diversity of the genus and the co-existence of toxigenic and non-toxigenic species in the same water mass, complicate all attempts to identify/quantitate the source organism of AZAs by routine monitoring programmes using light microscopy. Thus molecular probes able to discriminate toxic *A. spinosum* from other similar species are required.

3.4. Morphology, taxonomy and phylogeny

Detailed studies revealed morphological details potentially important to elucidate the taxonomic position of the genus *Azadinium* within the higher order system of dinoflagellates. These include the description of the plate overlap pattern[46,56], which was shown to be particular in some aspects among dinoflagellates. The identified type of growth bands (the way thecal plates grow and increase in size) for *A. spinosum* has been found to be typical for the order gonyaulacales (Figure 5). Finally, the cell division type of *A. spinosum* was identified.[57] Cells divide by sharing the thecal plates, which is characteristic of the gonyaulacales (Figure 6).

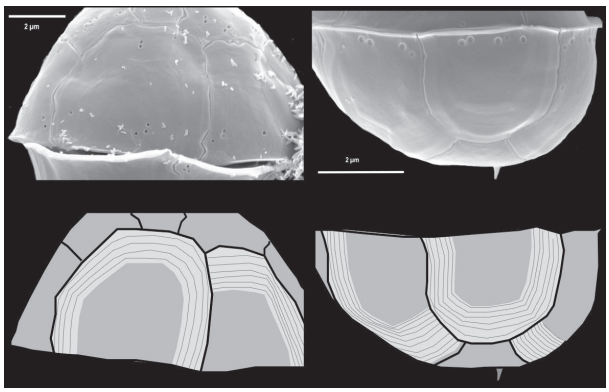


Figure 5. *A. spinosum.* "Growth bands" are restricted to overlapping plate margins (typical for Gonyaulacales) and do not occur on any plate margin (as in Peridiniales).

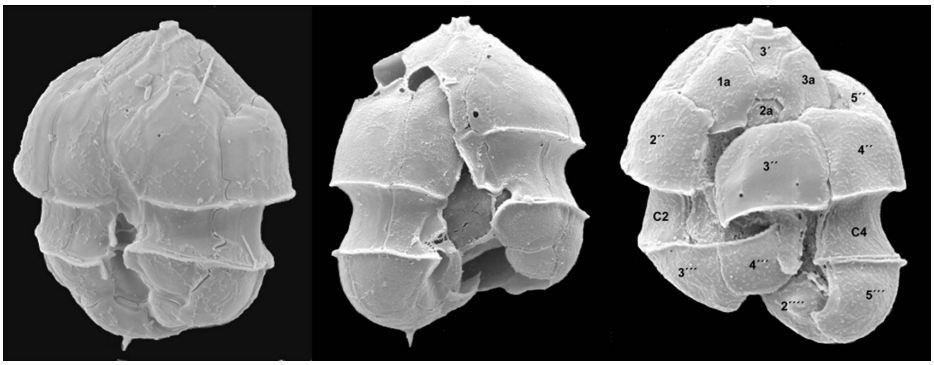


Figure 6. *A. spinosum.* Cell division type is desmoschisis. Plates are shared between sister cells. This type of cell division is common among Gonyaulacales.

Morphological criteria used to separate Peridiniales and Gonyaulacales indicate that the genus *Azadinium* has more affinities to Gonyaulacales. Phylogeny, however, showed that the genus *Azadinium* is closely related to the Prorocentrales, Gymnodiniales and Peridiniales, and is clearly separated from the Gonyaulacales. The ordinal position is therefore not fully clarified and requires more information on the phylogeny of potentially related genera.

Morphology and pigment profiles of the new species of the genus *Amphidoma* and the genus *Azadinium* revealed increasing evidence for a close relationship that led to including *Azadinium* and *Amphidoma* in the family *Amphidomataceae.*[55]

Using transmission electron microscopy the ultrastructures of three *Azadinium* sp. (*A. spinosum*, *A. obesum* and *A. poporum*) were investigated and found to be quite common for dinoflagellates (Figure 7). Ultrastructural differences included the lack of stalked pyrenoids in *A. obesum* and an "apical striated complex", that had never been observed in *A. spinosum*. Intracellular bacteria are missing and thus - for *A. spinosum* - not involved in toxin production.

Interestingly, in *A. poporum* two types of pyrenoids (stalked pyrenoids, internal pyrenoids) may be simultaneously present, together with starch grains located in the plasma (Figure 8).

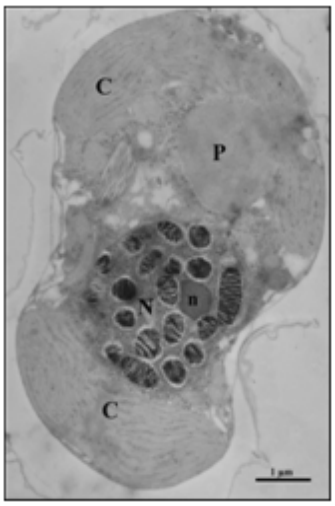


Figure 7. TEM of *A. spinosum*. C=chloroplast, N=nucleus, n=nucleolus, P=pyrenoid.

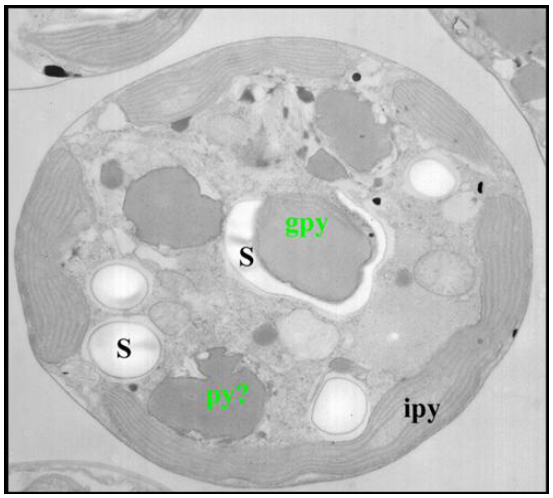


Figure 8. TEM of *A. poporum* (cross section). Ipy=internal pyrenoid, pgy=stalked pyrenoid, S=starch.

3.5. Development of a molecular probe

3.5.1. *Azadinium* 28s rRNA probes

Ribosomal RNA probes specific for representatives of the genus *Azadinium* (*A. spinosum*, *A. obesum* and *A. poporum*) were designed for fast and reliable characterisation, identification and numeration of these *Azadinium species*. The probes are ~17–18 base pair oligonucleotide sequences which are specific for D1D2 region of the LSU (28S rRNA) of the target algae. They are applied in two methods of detection – standard Fluorescence *InSitu* Hybridisation (FISH) and Catalysed Reported Deposition - Fluorescence *InSitu* Hybridisation (CARD-FISH), as well as Real Time qPCR.[58]

3.5.2. CARD FISH

The probes bind efficiently to a specific sequence of the rDNA of the target cells. They are labelled with a fluorescent dye, which emits a signal that can be detected via epifluorescent microscopy. The cells are stained with DAPI as a positive stain for intact cell identification (Figure 9). In addition to applying target specific probes, negative controls are performed to differentiate between a background and a positive signal (Figure 10). The probes hybridise with the cells of target *Azadinium species* giving a bright signal.

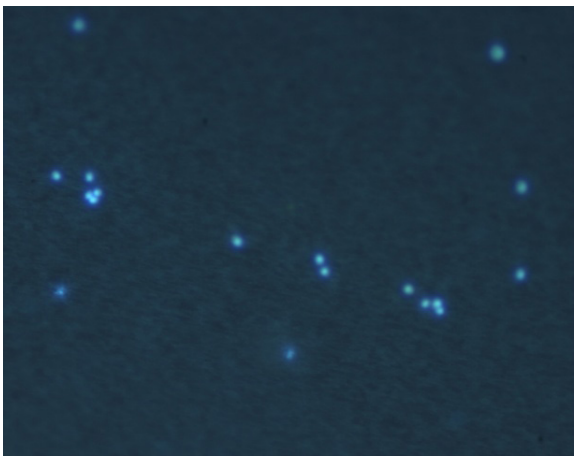


Figure 9. DAPI Citifluor stained cells of *A. spinosum* on a 1.2 µm polycarbonate filter (Isopore, Millipore, USA) at 10× magnification.

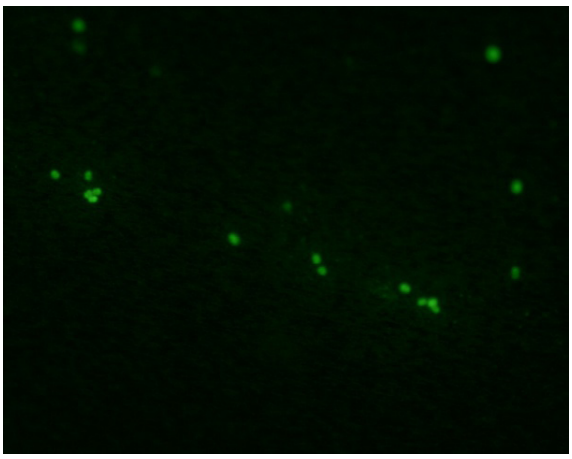


Figure 10. *A. spinosum* cells showing bright positive signal after CARD FISH at 10× magnification (same field as DAPI staining).

3.5.3. Quantitative real time PCR

TaqMan®MGB probe and primer sets (assays) were designed for the three *Azadinium species* as in the case of CARD FISH. The designed primer sets were first tested with the SYBR Green qPCR approach to determine if a specific PCR amplicon was formed and to evaluate the efficiency of the assay. After the standard curves for all sets were made, the TaqMan MGB probes were applied. The primer and probe sets gave good standard curves, with an efficiency ranging ~95–105%. The standard curves, for later quantitation approaches were made using dilution series of known concentrations of the DNA extracts corresponding to exact cell numbers of unialgal cultures (Figure 11).

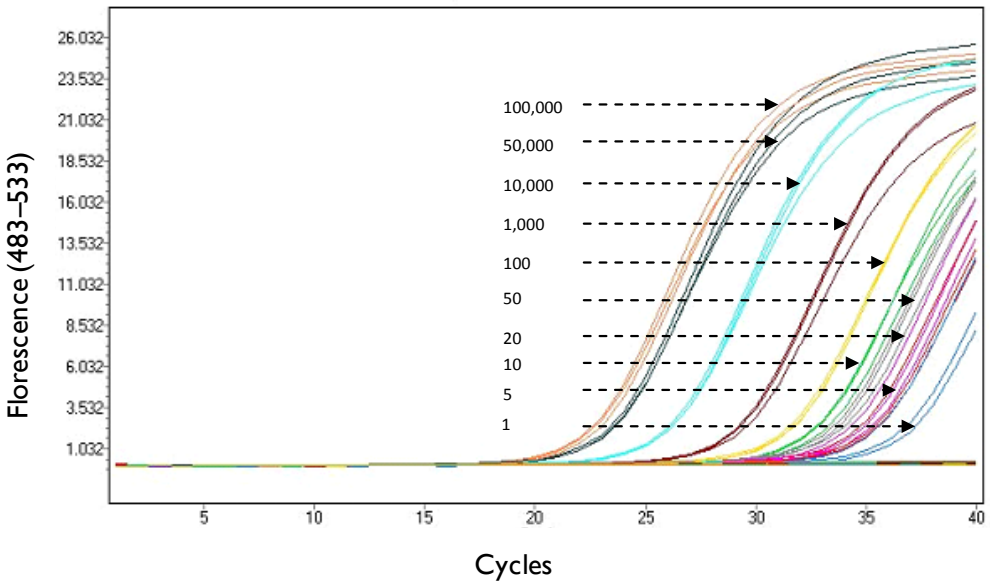


Figure 11. The real time measurements of the fluorescence plotted against the number of cycles. DNA extracts of *A. spinosum* starting with 10 ng per well diluted 1:10. The standard curve is obtained from this graph.

All three qPCR assays form products with their target sequences in efficient reactions and bind specifically to the target species (*A. spinosum*, *A. obesum* and *A. poporum*).

3.5.4. The role of qPCR in the detection of *Azadinium* species in routine monitoring programmes

The three qPCR assays developed were further optimised and their effectiveness evaluated when implemented in detecting target *Azadinium* DNA in field samples submitted to the Irish phytoplankton monitoring programme. The parameters of the assay optimised included extraction (volume, centrifuging, filtration, extraction kits, cell disruption, lysis incubation) and analysis (probe-primer concentration, annealing temperature). The introduction of controls to distinguish between true positives and true negatives from false positives and false negatives have also been implemented into the method (controls include extraction efficiency and recovery, inhibition, contamination and quantitation (cells/L)).

Identification and enumeration of *Azadinium* down to species level is difficult by light microscopy due to their small size and morphological characteristics. The use of the qPCR methodology as a tool in identifying the presence of and distinguishing between species within the genus provided valuable information on AZA events observed in Ireland during 2012 and 2013, particularly in Killary Harbour and the SouthWest. In all cases where corresponding phytoplankton samples were taken at the time of the event, the presence of *A. spinosum* DNA was detected by qPCR. Figure 12 shows the concentration levels of *A. spinosum* (cells/L) in phytoplankton samples and how they related to AZA equivalent concentrations (µg/g) detected in *Mytilus edulis* during June–August 2013. Initial observations from this event and other AZA events observed in the period 2012–2013 indicate *A. spinosum* can bloom rapidly, with subsequent rapid intoxification and accumulation of AZAs in mussels. The results also show that there is a rapid decline in cell densities following the inital bloom.

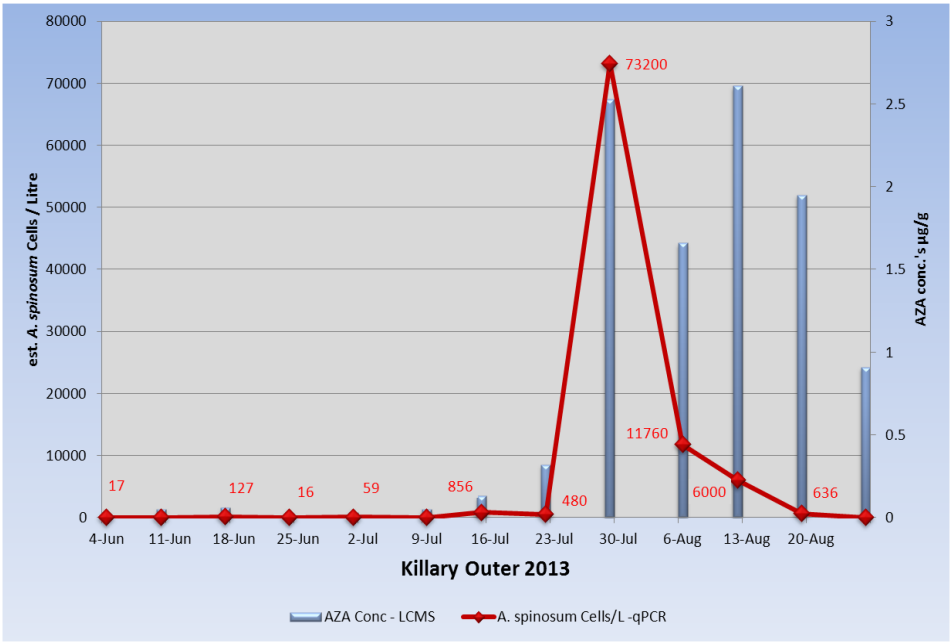


Figure 12. AZA concentrations in *M. edulis* and *A. spinosum* cells/L (qPCR) from Killary Outer July–August 2013.

A survey of Irish coastal waters for blooms of *A. spinosum* was undertaken in August 2012 aboard the Celtic Voyager (Figure 13). Chlorophyll max and depth layer samples (fractionated in the particle size range 3–20 µm and collected on 3 µm TSTP filters) were analysed for the presence of *A. spinosum* by qPCR. Surface layer samples collected from the majority of the stations contained *A. spinosum* DNA, and to a slightly lesser extent in the chlorophyll max layers. *A. spinosum* DNA was present in just under half of the stations in the depth layer.

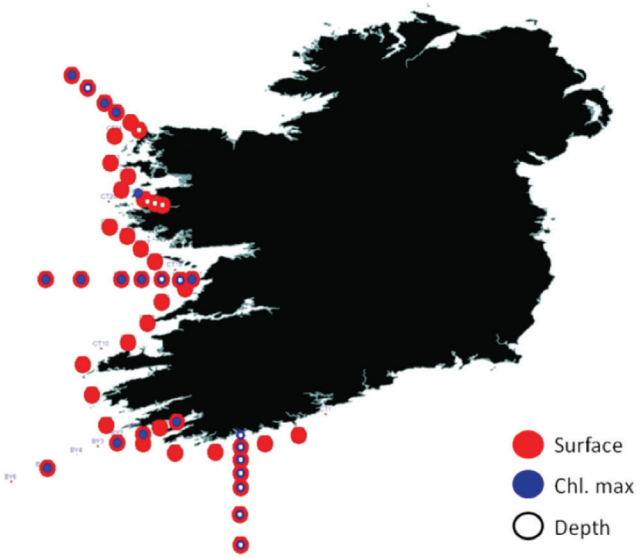


Figure 13. Survey samples collected during August 2012.

4. Bulk culture of producing organism

4.1. Introduction

The main sources of marine algal toxins for purification purposes are the producing organisms in culture or harvested from natural blooms (e.g., okadaic acid group toxins[59], brevetoxins[60], saxitoxins[61], yessotoxins[62] and pectenotoxins[63]); from contaminated shellfish[12,64]; or from bulk extraction of environmentally contaminated HP-20 resin.[65] Isolation from bulk cultures of the producing organism is preferred; as extracts are considerably purer than shellfish extracts; and their availability is not dependent on the occurrence of natural toxic episodes. Purification of phycotoxins for the preparation of calibration standards is essential to sustain phycotoxin monitoring in food.[66] This has become particularly important as micro-algal lipophilic toxins in contaminated shellfish are now monitored in Europe using LC-MS/MS as a reference method.[67]

4.2. Validation of extraction and analysis methods

A. spinosum cells were collected from bioreactor cultures, using centrifugation or filtration. Different extraction procedures were evaluated for the formation of methyl-ester artefacts, yield, and matrix effects.[68] Filtration of cultures using glass-fibre filters led to an increase in the formation of methyl-esters. Hence centrifugation is recommended for recovery of cells. The type of extraction solvent (methanol, acetone, acetonitrile) did not significantly affect the yield of AZAs so long as the organic content was 80% or higher. However, the use of methanol as an extraction solvent led to an increase in the formation of methyl-ester artefacts. AZA1 recovery over two successive extractions was 100% at 95% confidence level for acetone and methanol. In standard addition experiments no significant matrix effects were observed in extracts of *A. spinosum* or *A. obesum* up to sample intake of 4.5×10^9 μm^3. Moreover, experiments carried out to clarify the formation and structure of methylated AZA analogues, led to the description of two new AZAs and the correction of the chemical structures of AZA29–32.

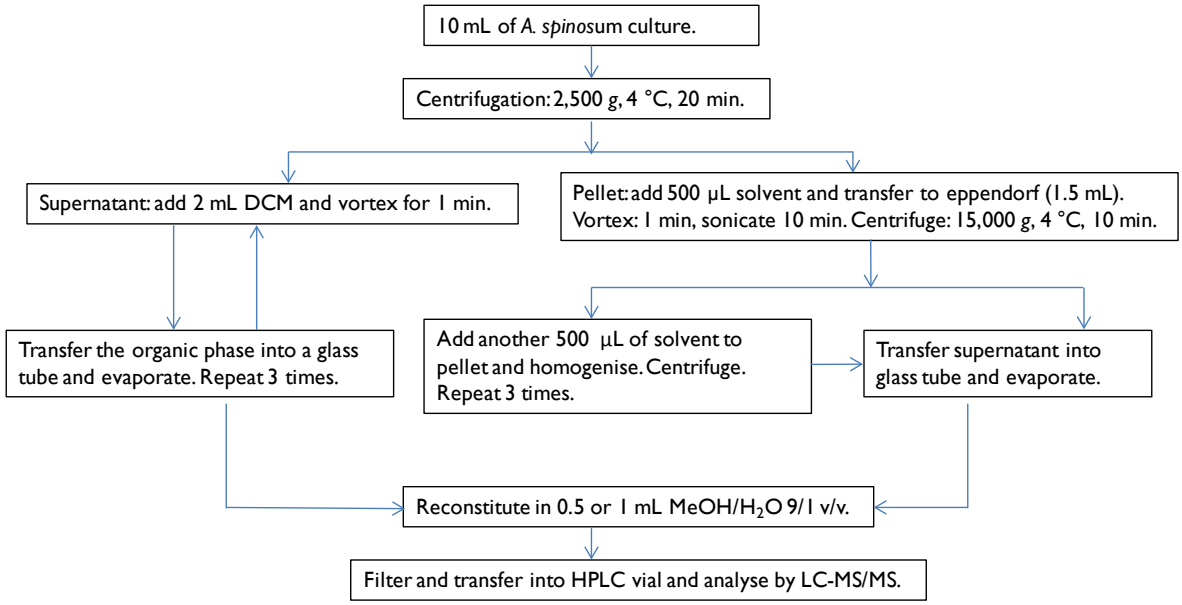


Figure 14. Sample preparation scheme for AZA extractions from *A. spinosum*.

No significant matrix effects were observed during LC-MS/MS analysis with acetone or methanol under the conditions tested.

Based on results of this study, the following procedures are recommended for the analysis of AZA1 and -2 in *A. spinosum*:

- Sample and immediately separate the cells from the culture media by centrifugation
- Extract AZAs with acetone
- Two to three successive extractions are suggested to ensure high extraction yield

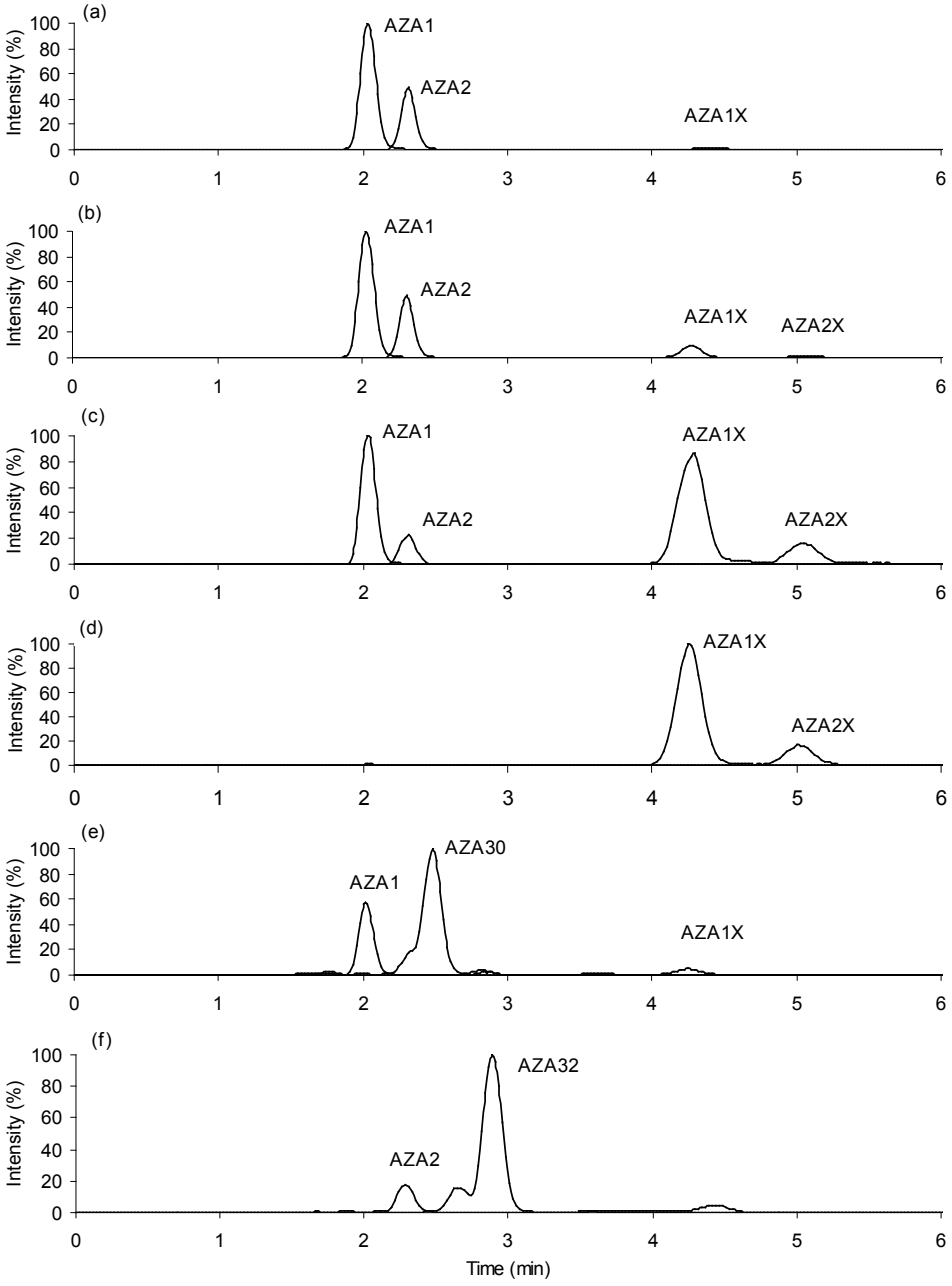


Figure 15. AZA chromatograms using an isocratic elution: A) *A. spinosum* after extraction with acetone, B) *A. spinosum* after extraction with methanol, C) partial methylated sample of AZA1 and -2, D) total methylated sample of AZA1 and -2, E) AZA1 standard after a storage experiment and F) AZA2 standard after a storage experiment. AZA1X and -2X are AZA1 and –2 methyl-esters, AZA30 and -32 are the AZA1 and -2 methyl hemiketals.

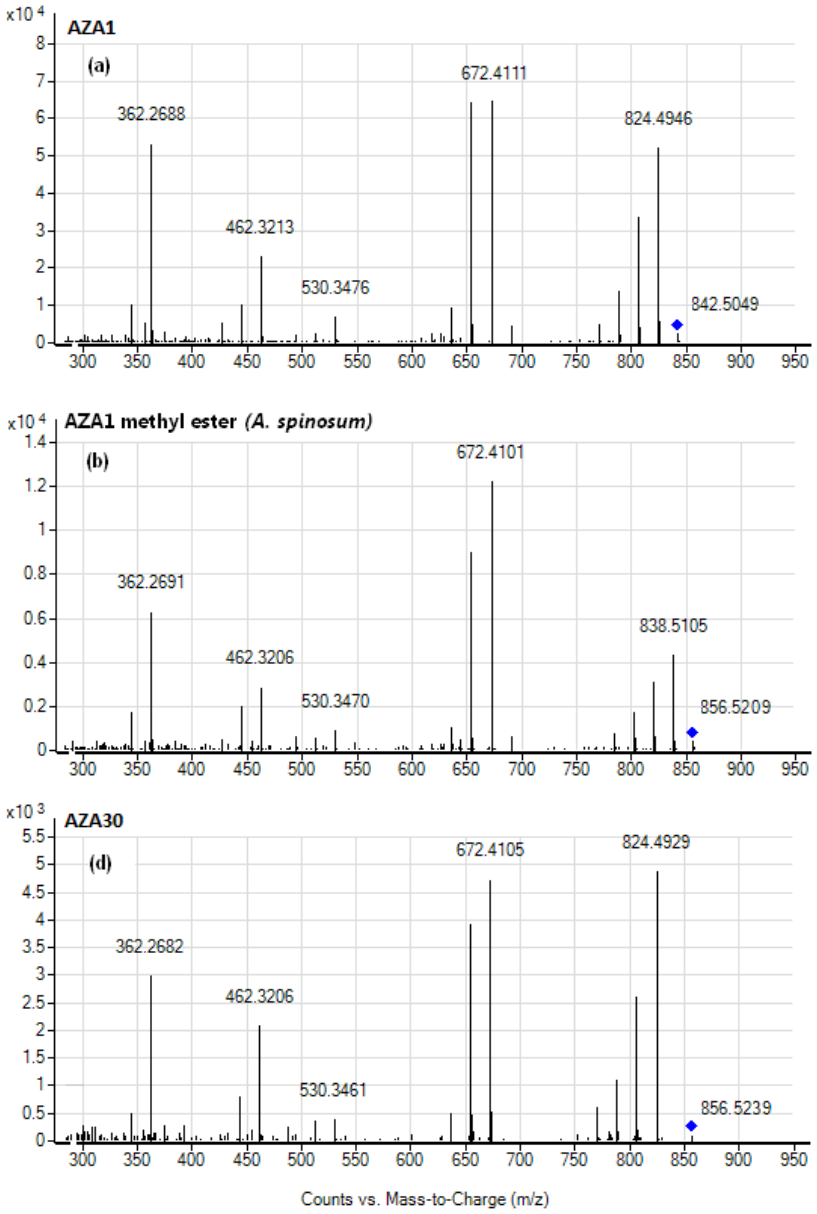


Figure 16. Mass spectra of A) AZA1, B) AZA1X (AZA1 methyl ester) and C) AZA30 (AZA1 methyl hemiketal).

4.3. Environmental parameters affecting growth and toxin production

A. spinosum was found to grow in a wide range of conditions: from 10 to 26 °C; 30 to 40 psu; 50 to 400 µmol/m^2/s and with or without aeration. The highest growth rate and/or cell concentration was obtained at 35 psu, from 18 to 22 °C and with aeration. AZA cell quota was significantly affected by low temperature; 20-fold higher at 10 °C (220 fg/cell) than at temperatures between 18 and 26 °C. Likewise, but to a lesser degree, AZA cell quota was enhanced at high irradiance in the photobioreactor and using aeration. *A. spinosum* growth rate and AZA cell quota were found to

be slightly different with all media tested. However, low K modified medium concentration (0.5 Kmod) improved AZA cell quota whilst a higher concentration (2 Kmod) improved maximal cell concentration in the photobioreactor.

These experiments on the effects of environmental and nutritional factors on *A. spinosum*[69] expanded our knowledge on its culture and facilitated growth of the organism in pilot scale photobioreactors for AZA1 and -2 production.[70] Furthermore, it gave an initial insight into factors that could impact natural blooms.

However, further studies using factorial plan designs are now required to determine the optimum parameters for AZA production using *A. spinosum* in continuous cultures. Such studies will help to determine the effect of nutrient limitations on growth, AZA toxin production and *A. spinosum* biochemical composition. Its small size, ability to grow in continuous cultures and the large differences observed under different environmental and nutritional conditions may render this organism an appropriate model for the elucidation of the genes involved in toxinogenesis.

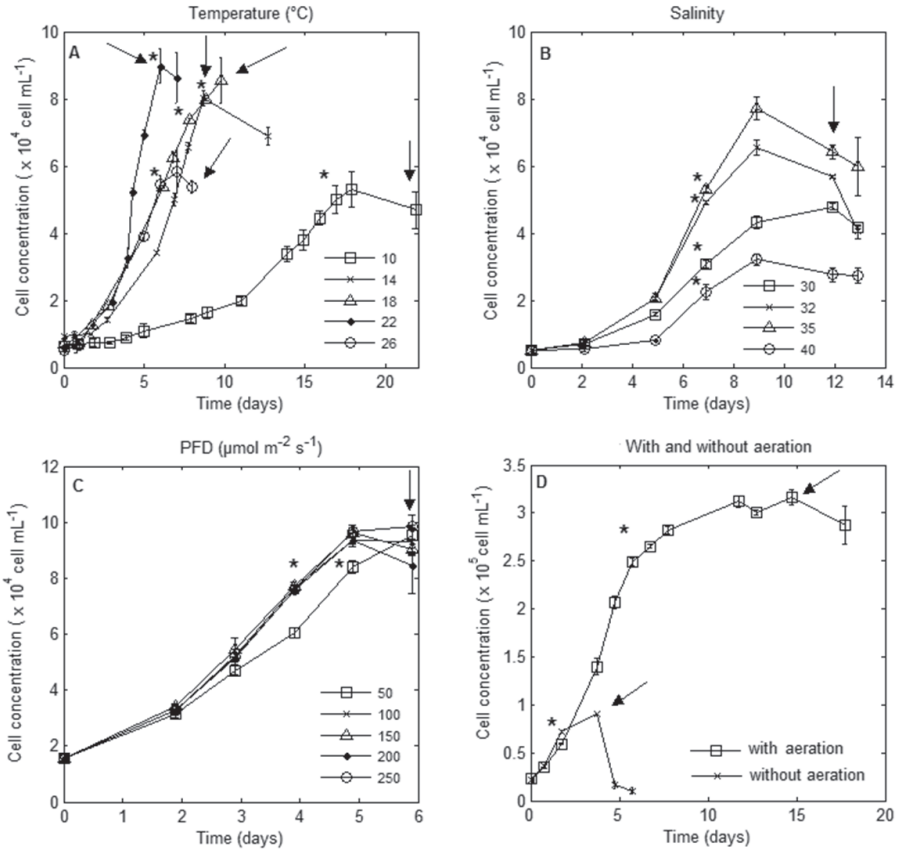


Figure 17. Growth kinetics of A) temperature (10 to 26 °C), B) salinity (30 to 40 psu), C) photon flux density (50 to 250 µmol/m^2/s) and D) with and without aeration. The arrows indicate at which day samples were taken for AZA analysis and * the day used to calculate growth rates (end of the linear growth phase).[69]

4.4. Bulk culture of phytoplankton

4.4.1. *A. spinosum*

Continuous pilot scale culturing was performed to evaluate the feasibility of AZA production using *A. spinosum* cultures. Algae were harvested using tangential flow filtration or continuous centrifugation.[70] AZAs were extracted using SPE procedures, and subsequently purified. When coupling two stirred photobioreactors in series, cell concentrations reached 190,000 and 210,000 cells/mL at steady state in bioreactors 1 and 2, respectively. The AZA cell quota decreased as the dilution rate increased from 0.15 to 0.3 per day, with optimum toxin production at 0.25 per day. After optimization, SPE procedures allowed for the recovery of 79 ± 9% of AZAs. The preparative isolation procedure previously developed for shellfish was optimized for algal extracts, such that only four steps were necessary to obtain purified AZA1 and -2. A purification efficiency of more than 70% was achieved, and isolation from 1,200 L of culture yielded 9.3 mg of AZA1 and 2.2 mg of AZA2 of >95% purity (see also section 6.3). This work demonstrated the feasibility of sustainably producing AZA1 and -2 from *A. spinosum* cultures.

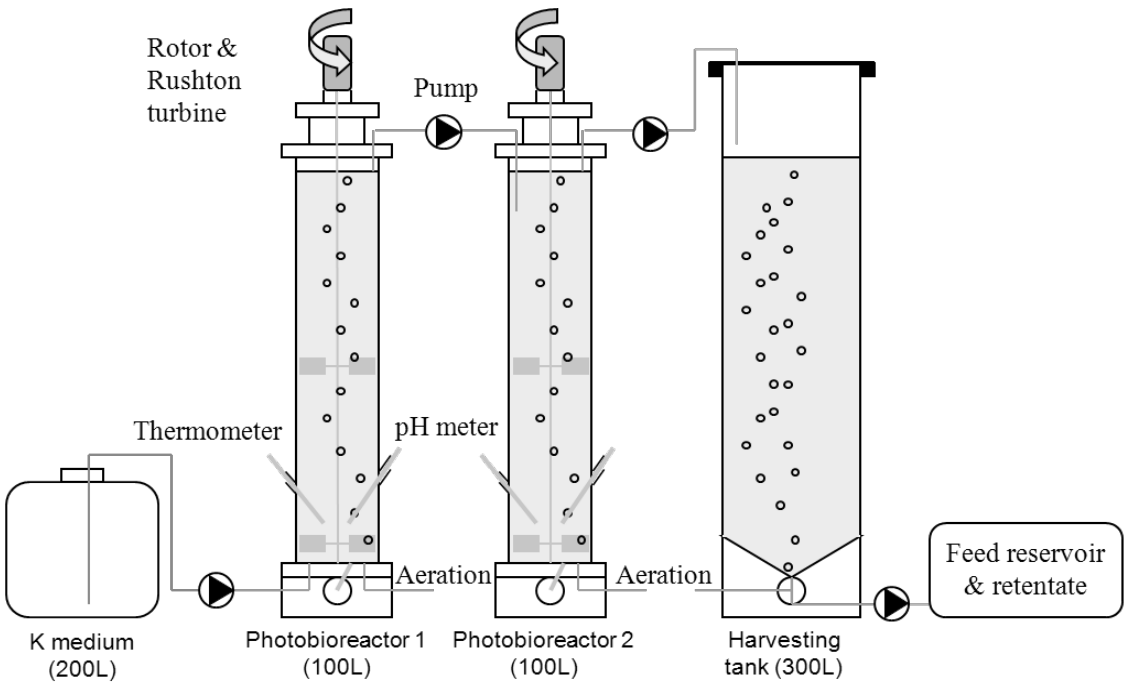


Figure 18. Schematic representation of *A. spinosum* production using photobioreactors in series.

This study[70] was the first performed on *A. spinosum* production in pilot scale photobioreactors (Figure 18). It demonstrated the ability of this small and fragile dinoflagellate to grow in this type of reactor. The effect of dilution rate on AZA cell quota and toxin production outline the secondary metabolite character of AZAs. A dilution rate of 0.25 per day was found to yield the highest

volume-specific toxin production per day. Two bioreactors coupled in series at higher dilution rates allowed for an AZA production rate of 475 µg/day (19 µg/day/L). Under these conditions ~3 mg of AZAs were obtained in crude extracts over 12 days (8 days of culture, 1 day of filtration and 3 days of extractions).

The production of large volumes of *A. spinosum* required the development of rapid procedures to concentrate and harvest algal cells for optimal toxin recovery. Both continuous centrifugation and tangential flow filtration were successfully applied to concentrate *A. spinosum* cultures. A simple and efficient procedure of extraction allowed for the retrieval of toxins from both the retentate and the permeate; providing a crude *A. spinosum* extract rich in AZAs and low in interfering matrix components.

This work showed that despite slow growth rates and low maximum cell concentrations, culturing in photobioreactors is a viable biotechnological approach to the production of toxins, with applications in research and operational food safety surveillance programs. Furthermore, metabolite production in chemostat bioreactors is stable and predictable, and there is the possibility to influence secondary metabolite production by using different culture conditions.

4.4.2. *A. poporum*

Culturing and harvesting of *A. poporum* cultures were performed on a smaller scale. Approximately 300 L of each strain were grown to a high density (>100,000 cells/mL) in 5 L flasks using gentle aeration (Figure 19). Toxins were harvested by adding HP-20 resin to the cultures following cell lysis (by the addition of acetone). The HP-20 resin was subsequently collected, dried, and extracted with 100% acetone providing a crude toxin extract ready for purification (see section 6.3.2).

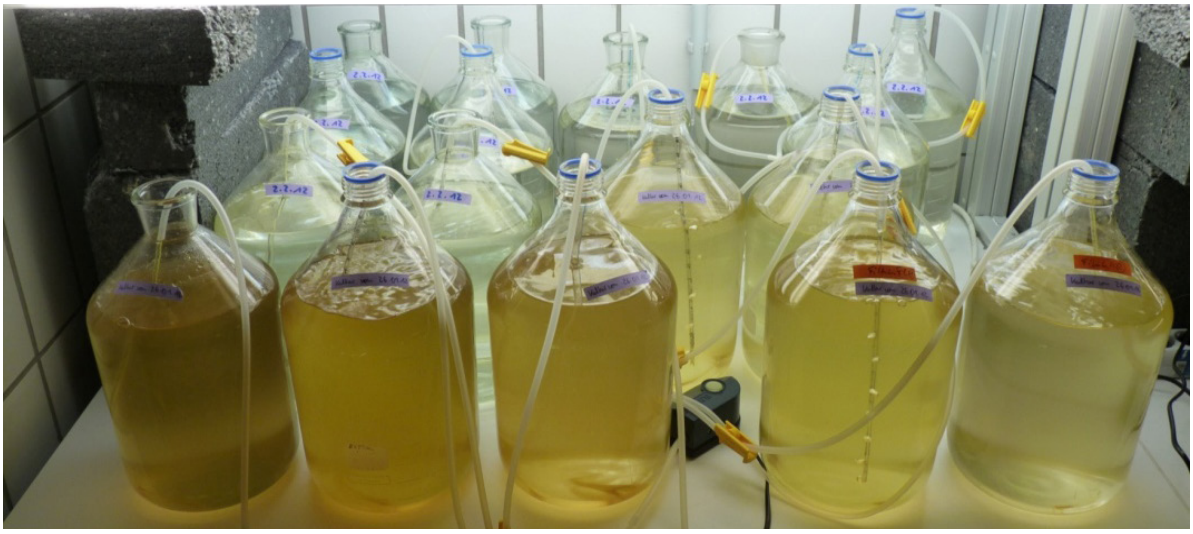


Figure 19. Mass culturing of *A. poporum* to collect toxins for structural elucidation and toxicity tests.

5. Trophic transfer of AZAs

5.1. Introduction

Due to observational deficiencies in toxic plankton (especially in detecting *Azadinium* species) and toxin monitoring programmes it had been difficult to draw a clear correlation between the detection of AZAs in bivalve shellfish and the occurrence of *Azadinium* blooms. In addition, there is also the possibility of alternative AZA sources (i.e., cryptic AZA-producing species) or toxin vectors, e.g., transfer via the pelagic food web. For a number of toxic algae, grazing within the plankton community is generally viewed as the initial pathway through which algal toxins become vectored into pelagic food webs. Subsequent accumulation and trophic transfer can intoxicate higher-trophic-level consumers such as fish, sea birds, and marine mammals.[71] For algal toxins accumulating in mussels, three transfer routes must be taken into account: (i) AZAs could accumulate in bivalve shellfish following feeding upon AZA bound to suspended particulates; (ii) via plankton vectors (e.g., copepods, tintinnids or other microplankton grazers) that have fed upon toxigenic *Azadinium* cells and (iii) direct uptake of the causative organism.

5.2. Planktonic food webs

AZAs can be present in plankton larger than the known producing species. In addition to AZA detection in the heterotrophic dinoflagellate *Protoperidinium crassipes*[38] (with an AZA cell quota of ~1.5 pg per cell), a second grazer species, *Favella ehrenbergii*, collected from field samples was also found to contain AZAs (~0.7 pg/cell).[41] In addition to the detection of AZAs in grazer species data was collected on AZAs in various plankton size fractions. During a research cruise on the research vessel Poseidon in 2007, AZA1 was detected over the entire North Sea in plankton samples collected with a plankton net (20 µm), which would not retain the small species of *Azadinium*.[41] The AZAs were mostly evenly distributed among three size fractions (20–55 µm, 50–200 µm and >200 µm) prepared from the net tows. At two stations (which had the highest amount of AZAs in net-samples) the majority were found in the 50–200 µm fractions (corresponding to a high abundance of *F. ehrenbergii*). However subsequent size fractionation of these samples revealed that >90% of the AZAs were found in the 3–8 µm and 8–10 µm fractions, which clearly corresponded to the size class of the identified AZA producing species. It is important to note that in quantitative terms, AZA found in larger size fractions were orders of magnitude lower. Although it is difficult to directly compare Niskin bottle samples and net tows, absolute AZA amounts in the small fractions (assumed to be due to *A. spinosum*) were ~2- to 3-fold higher than the low pg/L range estimated in net tow samples (assumed to be the result of trophic transfer).

Similar results were obtained on the subsequent cruise in Danish coastal areas of the North Sea.[44] AZA1 was present in a number of net tow (20 µm) samples (in the range of 50 pg per net tow), albeit in low amounts, with only traces found in the >200 µm fraction. AZA1 concentrations measured in the small size fractions (<20 µm), however, were much higher with maximum concentrations of ~2 ng/L AZA1. This is roughly 5-fold higher than amounts found in the corresponding net tow samples. These results are indicative of negligible trophic transfer, at least under this particular field situation.

5.3. Vector species

5.3.1. Feeding experiments with copepods

Preliminary and yet unpublished experiments with copepods indicated a minor grazing impact and negligible AZA accumulation in this important group of plankton grazers. During a cruise on the research vessel Heincke in 2011, cultured *A. spinosum* was added to both field plankton samples and copepods (various species). After 24 h no substantial grazing in copepods nor significant AZA accumulation in larger size fractions was detected.

5.3.2. Potential protistan grazers

A number of potential protistan predators (*Amphidinium crassum, Polykrikos kofoidii, Gyrodinium dominans, Oxyrrhis marina, Peridiniella danica* and *Favella ehrenbergii*) were established in culture and tested for their potential to ingest and grow when *A. spinosum* was provided as a food source.

Certain grazers such as *Polykrikos kofoidii* and *Amphidinum crassum* failed to ingest *A. spinosum*, most probably because of prey size limitation (for *P. kofoidii*) or due to their peduncle feeding mode (for *A. crassum*). In contrast, laboratory cultures of *Favella ehrenbergii* clearly ingested *A. spinosum* (Figure 20) and started to grow (Figure 21). Toxin accumulation was substantial (Figure 22), however, growth was limited in the presence of just *A. spinosum*.

Peridiniella dania, a small heterotrophic thecate dinoflagellate, has been identified as a promising potential grazer (Figure 23) but quantitative grazing experiments have not yet been performed. For other small heterotrophic dinoflagellates such as *Oxyrrhis marina* and *Gyrodinium dominans*, ingestion of *A. spinosum* was observed, but only rarely, with no positive growth (Figure 24).

The swimming behaviour of *Azadinium* may play a role in grazer interactions. Direct observations under the microscope show that *Azadinium* can escape by sudden jumps when attacked by these small

dinoflagellates. However, ingestion of *Azadinium* by these predators rapidly decreased from initial higher values even when immobilised prey was offered as food. Hence, it is likely that factors other than motility are involved. Clearly much more detailed studies are required to test the hypothesis that AZAs and/or other chemical compounds are involved in grazing interactions of *Azadinium*.

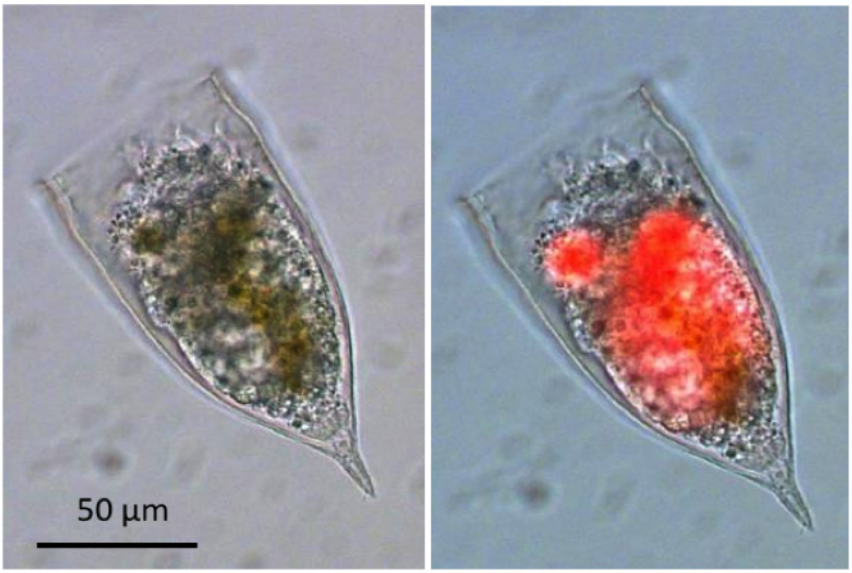


Figure 20. *Favella ehrenbergii* that has been feeding on *A. spinosum* for 16 h. Left: brightfield; right: epifluorescence showing red auto-florescence of ingested *Azadinium*.

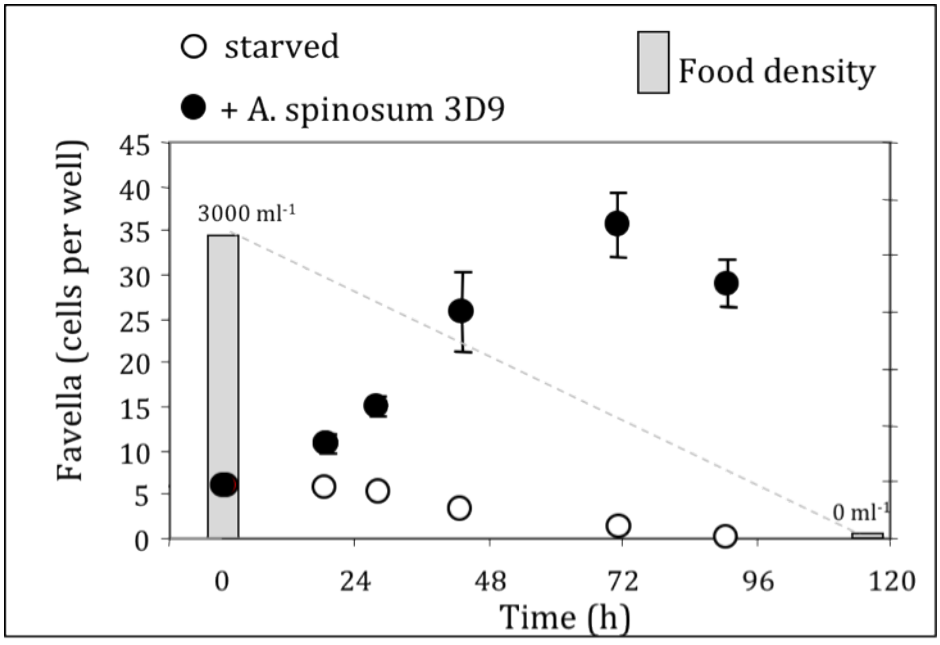


Figure 21. Change of *Favella ehrenbergii* density when starved (open circles) or fed with *A. spinosum* (black circles). Grey bar indicates the decline of *A. spinosum* from initial 3,000 cells/mL to complete food depletion.

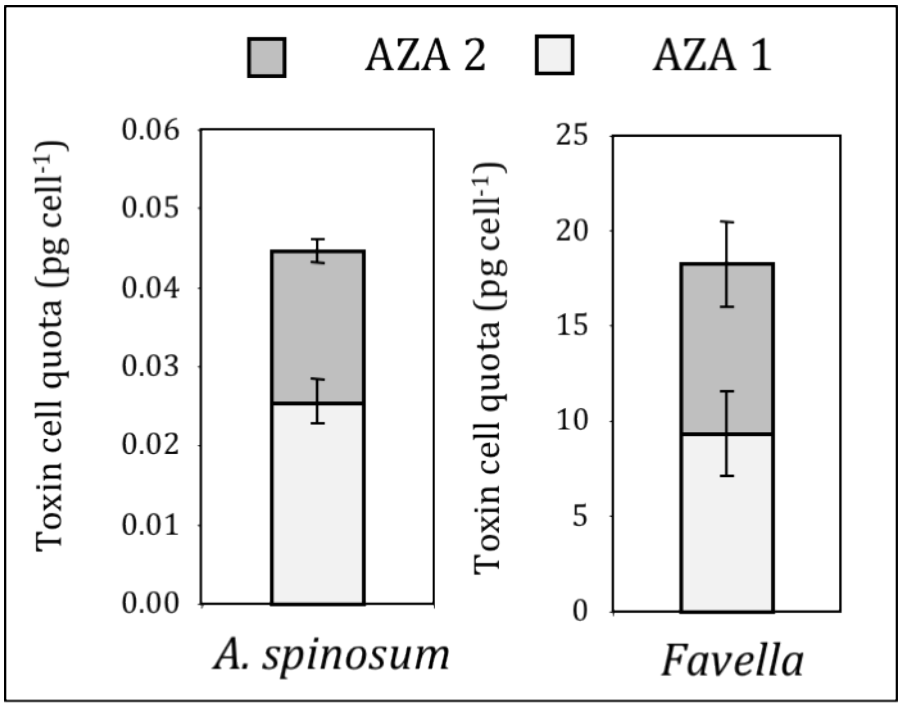


Figure 22. Feeding experiment of *Favella ehrenbergii* using *A. spinosum* as food. Initial AZA cell quota (AZA1 and -2) in *A. spinosum* (left) and final AZA cell quota in *Favella* (right). Bar height represents triplicate mean ± 1 SD. Toxin content of *Favella* would correspond to toxins accumulated from the uptake of 485 *A. spinosum* cells. The presence/absence of AZA degradation or decomposition analogues known from mussels could not be estimated due to the low number of *Favella* available in this experiment.

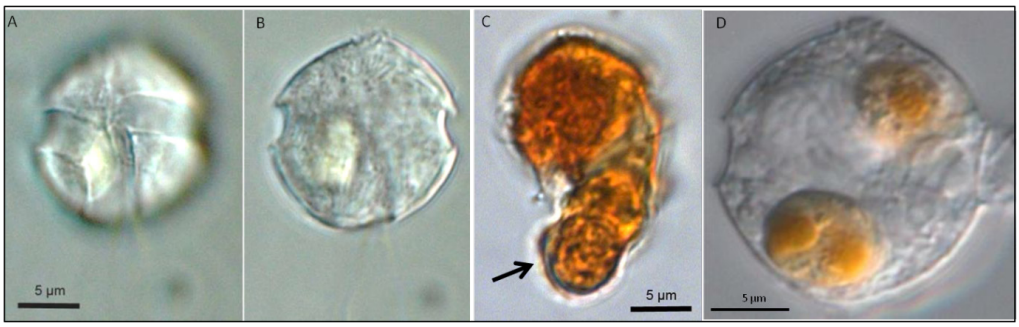


Figure 23. The heterotrophic thecate dinoflagellate *Peridiniella danica* in A) ventral view and B) dorsal view. C) A lugol-fixed cell ingesting a cell of *A. spinosum* (arrow). D) *P. danica* with remains of two ingested cells of *A. spinosum* still visible in food vacuoles.

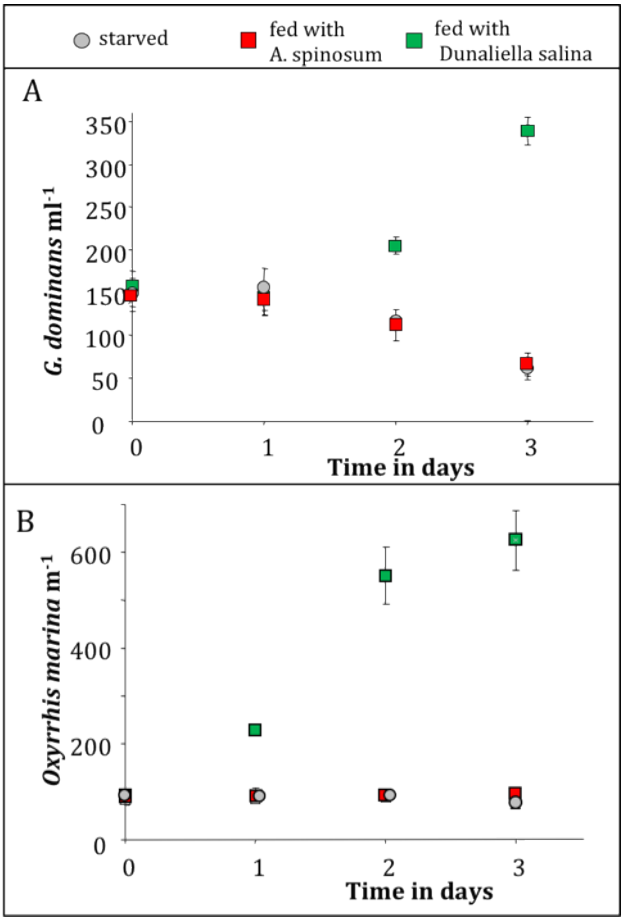


Figure 24. Change in cell density of the heterotrophc dinoflagellate grazers *Gyrodinium dominans* (A) and *Oxyrrhis marina* (B) when starved (grey circles), or fed with *A. spinosum* (red squares), or fed with Dunaliella salina (green squares). Symbols represent triplicate mean ± 1 SD.

Whereas these preliminary experiments do not contradict the possibility of vectoral transfer of AZAs to bivalves, they do not provide strong support for this mechanism. Generally, predator-prey interactions are known to be species-specific and this may be even more important for toxic species where specific chemical compounds might play a role.[71,72] Furthermore certain plankton predators have been shown to be extremely selective in their prey preferences.[73] The scant data that are available thus far do not support a view of a universal and rapid spread of AZAs among planktonic grazers; but may be indicative that certain (specialized) grazers such as *Favella ehrenbergii* may play an important role in food web transfer of *Azadinium* and AZAs. More detailed investigations and experiments are needed to clarify this issue.

5.4. Trophic transfer – accumulation, detoxification and biotransformation

A number of feeding studies were performed over the course of the project with mussels being fed *A. spinosum*. In the preliminary study[42] a 24 h feeding trial of blue mussels *(Mytilus edulis)* using an algal suspension of the Irish *A. spinosum* culture at different cell densities demonstrated that *A. spinosum* is filtered, consumed and digested directly by mussels. LC-MS/MS analysis had shown that AZAs were accumulating in the shellfish hepatopancreas. The toxins AZA1 and -2 were detected in the shellfish together with the AZA3, -6, -17 and -19 analogues, suggesting that AZA1 and -2 are metabolised in the shellfish within the first 24 h after ingestion of the algae. The levels of AZA17 detected in the shellfish hepatopancreas were equivalent to the levels of AZA1, but in the remainder tissues, the levels of AZA17 were 4 to 5-fold higher than that of AZA1. Only small quantities of AZA3 and -19 were present, with negligible amounts of AZA6 detected after the 24 h period. These results are proof of a direct toxin transfer of AZA toxins from *A. spinosum* by feeding *Mytilus edulis* without the need for a vector species. Mussels will actively filter, ingest, accumulate and bioconvert AZA toxins readily into other AZA analogues as shown in Figure 25.

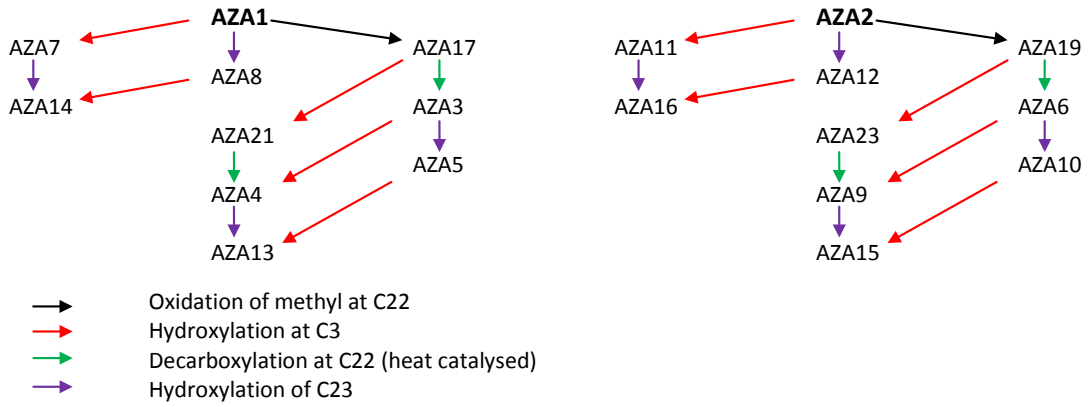


Figure 25. Mussels which feed on *A. spinosum* metabolise AZA1 and -2 to produce other AZA analogues.

In a follow up study adult mussels were continuously exposed to *A. spinosum* over 1 week in 160 L cylindrical conical tanks.[74] Three different diets were tested for contamination: 5,000, 10,000 cells/mL of *A. spinosum* and a mixture of 5,000 cells/mL of *A. spinosum* with 5,000 cells/mL of *Isochrysis aff. galbana* (T-Iso, CCAP 927/14). During the subsequent period of detoxification (2 weeks), contaminated mussels were continuously fed with 5,000 cells/mL of T-Iso.

Kinetics of accumulation, detoxification and biotransformation were evaluated, as well as the toxin

distribution and the effect of *A. spinosum* on mussel digestive gland tubules. *Mytilus edulis* fed on *A. spinosum* in the three tested conditions. The original algal toxins AZA1 and -2, as well as mussel metabolites AZA3 to 12, -17, -19, -21 and -23 were found during these trials. After 6 h, AZA contents in mussels reached the EU regulatory limit, and metabolites were observed in all conditions at ~25% of the total AZA content. This fraction exceeded 50% after 24 h, and continued to increase until the end of the study. AZA17 and -19 were found to be the main metabolites, with AZA17 concentrations estimated in the same order of magnitude as that of the main algal toxin, AZA1.

Table 4. Temporal variation in the proportion of AZA toxins (%), and AZA toxin concentrations (µg/kg and µg/kg AZA1 TEQ) in whole mussels over time for diet 1 (5,000 cells/mL of *A. spinosum*). Highlighted in grey are the toxin proportions related to AZA1 and yellow to AZA2. TEF_{AZA17} = 1.4 (as AZA17 transforms into -3 after cooking); TEF_{AZA19}=TEF_{AZA6}=1 (as AZA19 transforms into -6 after cooking, and TEF_{AZA6} is estimated the same as TEF_{AZA1}).

Algal AZAs (%)	AZA1	57.9	39.5	35.3	41.5	35.1	31.7	25.8	22.9	29.3
	AZA2	17.4	15.0	15.6	16.4	14.5	14.0	14.8	12.0	11.7
Algal AZAs (µg/kg)		*283.1*	*169.5*	*169.1*	*248.3*	*308.9*	*218.7*	*120.6*	*94.5*	*105.1*
AZAs metabolites (%)	AZA3	0.3	0.6	0.6	0.5	0.7	0.8	0.7	0.7	0.7
	AZA4	0.0	0.0	0.2	0.5	2.0	2.5	2.3	5.2	7.3
	AZA5	0.0	0.7	1.1	1.1	1.5	1.9	1.8	3.9	5.3
	AZA6	0.8	2.1	1.9	1.8	2.3	2.5	2.4	3.6	2.8
	AZA7, -8	2.9	2.6	1.7	2.0	2.9	2.7	1.7	1.6	1.9
	AZA9	0.0	0.0	0.0	0.0	0.2	0.3	0.4	0.8	1.2
	AZA10	0.0	0.0	0.3	0.4	0.5	0.6	0.6	1.1	1.5
	AZA11,-12	0.4	0.3	0.2	0.3	0.3	0.3	0.3	0.3	0.3
	AZA17	16.7	32.3	35.4	28.2	29.8	31.0	36.8	33.8	25.5
	AZA19	3.6	6.9	7.4	6.4	6.9	7.8	8.5	9.0	7.5
	AZA21	0.0	0.0	0.3	0.8	2.8	3.2	3.4	4.3	4.4
	AZA23	0.0	0.0	0.0	0.1	0.4	0.5	0.6	0.7	0.8
AZAs metabolites (µg/kg)		93.0	141.0	163.3	180.7	313.9	258.9	176.6	176.7	151.5
AZA total (µg/kg)		376.1	310.5	332.4	429.0	622.8	477.7	297.2	271.2	256.6
Regulated AZAs (AZA1–3) (µg/kg AZA1 TEQ)		336.9	209.3	213.5	307.8	387.2	277.4	158.7	123.3	131.7
AZA1, -2, -3, -6, -17 and -19 (µg/kg AZA1 TEQ)		441.6	377.6	409.4	512.5	704.5	534.2	344.3	285.9	249.6

This study[74] confirmed the findings of the previous feeding study that was performed earlier in the project.[42] It further showed that blue mussels can accumulate AZAs to levels above the regulatory limit in less than 6 h. Detoxification was slow (half life ~11 days); with detoxification rates in the same order as other lipophilic toxins.

5.5. Accumulation of AZAs from the dissolved phase

Another study performed over the course of the project investigated whether mussels were capable of accumulating dissolved AZA1 and -2.[75] The toxin profiles of these mussels at 24 h were compared with profiles of those exposed to live or lysed *A. spinosum*. The possibility of preparative production of AZA metabolites by exposing mussels to semi-purified AZA1 was also assessed.

Mussels were exposed to similar concentrations of AZAs: dissolved AZA1 and -2 (crude extract) at 7.5 and 0.75 µg/L, dissolved AZA1 and -2 (7.5 µg/L) in combination with T-Iso, and lysed and live *A. spinosum* cells at 1×10^5 and 1×10^4 cells/mL. Subsequently, the mussels were dissected (digestive glands, gills and remaining flesh) and analysed. Mussels (whole flesh) accumulated AZAs above the regulatory limit except at the lower levels of dissolved AZAs. The toxin profile of the mussels varied significantly with treatment (Figure 26). The gills contained 42–46% and the digestive glands 23–24% of the total toxin load using dissolved AZAs, compared to 3–12% and 75–90%, respectively, in mussels exposed to live *A. spinosum*.

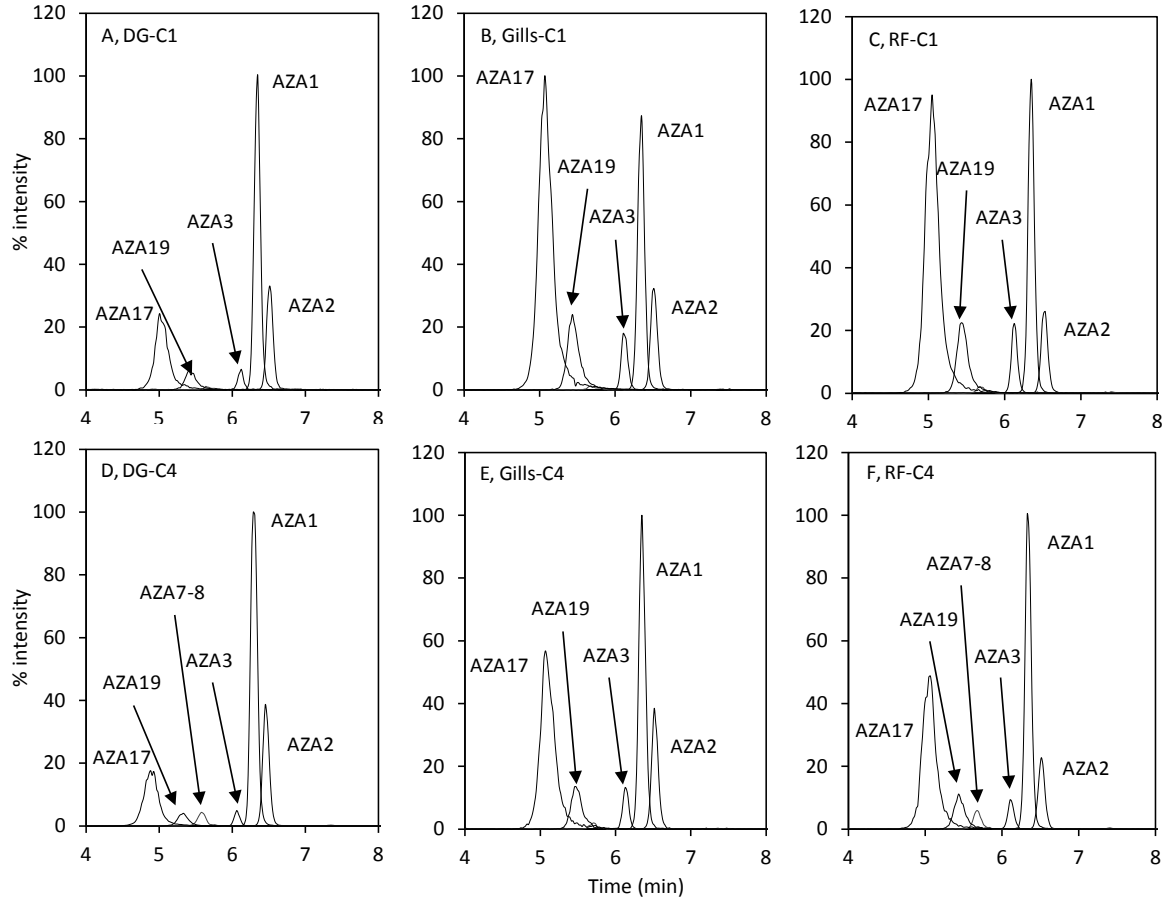


Figure 26. LC-MS/MS comparison of metabolite profiles in mussel tissues after exposure to dissolved AZAs (7.5 µg/mL): A) DGs, B) Gills, and C) RF and after exposure to live A. *spinosum* cells (1×10^5 cells/mL): D) DG, E) Gills, and F) RF.

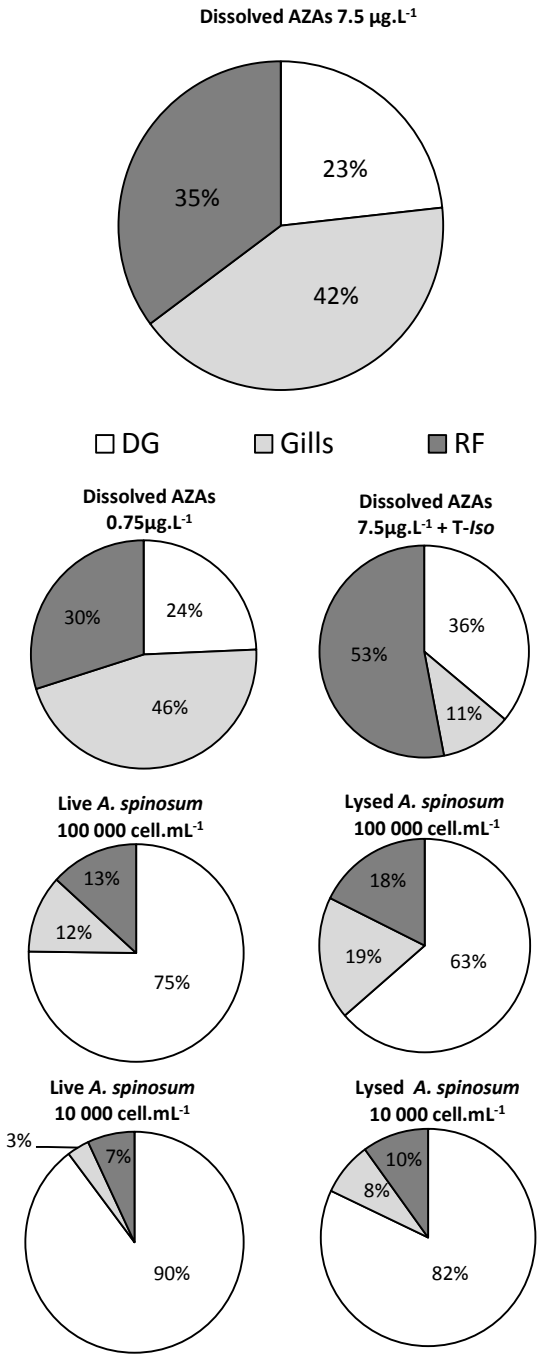


Figure 27. Total AZA tissue distribution in mussels (DG, □, RF, ■, and Gills ▒) after 24 h of exposure to dissolved AZAs, live *A. spinosum* or lysed *A. spinosum* at different concentrations.

The results show that dissolved AZAs were bioavailable for mussels and that AZA accumulation may reach concentrations above the regulatory limit and thus might be considered as a food safety issue. However, differences observed in the distribution of toxins in the shellfish depended on whether the shellfish were exposed to dissolved AZAs or to live or lysed *A. spinosum* (Figure 27).

This finding tended to decrease the importance of dissolved AZAs as a source of contamination relevant for human health as the AZA distribution found *in situ* reflect results observed with live *A. spinosum*. This differing AZA distribution between tissues indicates that AZA uptake can occur in the digestive gland during feeding and also through the gills during respiratory and filtration activities in the presence of dissolved AZAs. This last observation raises questions as to the mechanisms involved in the uptake of AZAs through the gills and about the potential for subsequent re-distribution to other organs. The bioavailability of dissolved AZAs shown for mussels also raises questions regarding possible effects on other aquatic organisms.

An additional experiment on the preparative production of AZA metabolites by exposure of mussels to semi-purified AZA1 resulted in formation of AZA1 metabolites in mussels (AZA17 (16.5%) and AZA3 (1.7%) after 4 days), but the conversion efficiency and the recovery was too low to justify using this procedure for preparative isolation (Table 5). Hence, further studies should investigate these losses and the existence of unknown metabolic transformations in mussels and seawater.

Table 5. AZA budget after exposure of mussels to semi-purified AZA1.

	Amount (µg)				Recovery[b] (%)
	AZA1	AZA17	AZA3	Total AZAs[a]	
Dissolved AZA (day 0)	98.4	-	-	98.4	100
Dissolved AZA (day 1)	55.1	1.0	1.1	57.2	58.1
Dissolved AZA (day 2.5)	37.8	1.7	1.7	41.2	41.9
Dissolved AZA (day 4)	26.3	2.3	2.0	30.6	31.1
Mussels (day 4)	10.3	16.8	1.6	28.7	29.2
Total recovered	36.6	19.1	3.6	59.3	**60.3**

[a]Total AZAs = AZA1 + AZA3 + AZA17
[b]Recovery (%) = (Total AZAs / AZA1 (day0)) × 100

5.6. Effect of *A. spinosum* on the feeding behaviour of mussels

In order to determine whether *A. spinosum* has adverse effects on mussels, feeding experiments were performed[76] as detailed below.

- Mussels were first exposed to a non-toxic diet (T-Iso) for 4 days (20,000 cells/mL). After this period, mussels were fed *A. spinosum* (5,000 cells/mL) for another 4 days
- AZA accumulation and biotransformation in mussels were followed using LC-MS/MS
- Individual assessment of mussel feeding time activity (FTA), clearance rate (CR), filtration rate (TFR), absorption rate (AR), faeces (Fs) and pseudofaeces (Ps) production were carried out on mussels fed either toxic (*A. spinosum*) or non-toxic diets

Fast AZA biotransformation was observed; with AZA1 and -2 metabolites found after 3 h (Table 6). 73% of the AZAs were found in the hepatopancreas. AZA17 reached a maximum proportion of 35% and AZA19 reached 6% after two days of contamination. Other AZAs found were AZA3–10 and AZA21, with proportions below 3% for each analogue. However, although fast initial toxin accumulation was observed (regulatory limit reached after 6 h), the rate of uptake stabilised with little or no increase in concentration levels over a longer time period.

Table 6. AZA accumulation and biotransformation in mussels fed *A. spinosum*.

	Time (days)	0.125	0.25	0.5	1	2	3	4
%	AZA1	66.9	65.5	51.0	47.5	38.6	40.2	45.1
	AZA2	14.3	15.1	13.4	15.0	15.6	14.8	14.1
	AZA3	0.0	0.0	0.0	0.5	0.6	0.7	0.6
	AZA4	0.0	0.0	0.0	0.3	0.2	0.6	0.3
	AZA5	0.0	0.0	0.0	0.3	0.9	0.6	0.8
	AZA6	0.0	0.0	0.1	0.1	0.1	0.1	0.1
	AZA7–10	2.5	2.6	4.1	2.2	1.8	2.2	2.2
	AZA17	13.7	14.0	26.7	29.2	35.0	33.1	29.4
	AZA19	2.5	2.7	4.6	4.9	5.9	5.8	5.2
	AZA21	0.0	0.0	0.0	0.1	1.3	2.1	2.2
µg/kg	AZAs	123.7	242.8	180.3	225.6	173.3	250.4	161.2
µg/kg TEQ	AZA1–3	114.7	225.2	135.6	169.7	116.9	169.5	115.2
	AZA1–3, -6, -17, -19	137.3	270.8	181.9	231.9	165.0	235.7	156.6

A. spinosum had a significant effect on mussel feeding behaviour compared to T-Iso: CR was lower by a factor of 6, FTA by a factor of 5, TFR by a factor of 3 and AR even decreased to negative values for the last day of exposure. The feeding studies performed showed a negative effect of *A. spinosum* on the shellfish in terms of mortality rates (were higher), thickness of digestive tubules (reduced, Figure 28) and on feeding responses and a possible regulation of AZA uptake by decreasing filtration and increasing pseudofaeces production. Consequently, additional experiments are required to explain why AZA concentrations in mussels can get so high (>8,000 µg/kg) in Irish waters compared to the "low" concentration found in these laboratory experiments (200 µg/kg).

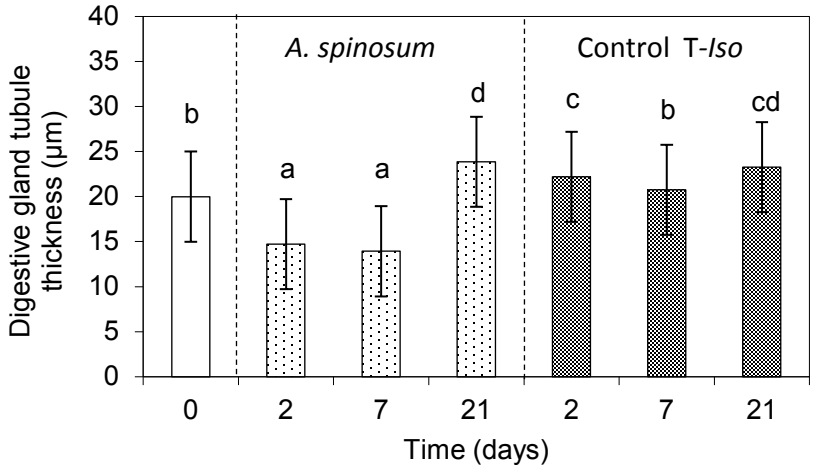


Figure 28. Evolution of the digestive gland tubule thickness (µm) in toxic (*A. spinosum*) and non-toxic control (T-Iso) diets during contamination (days 2 and 7) and at the end of the detoxification period (day 21). The error bars represent the standard deviation (n ≥150 digestive tubules). Values with different letters are statistically different ($P < 0.05$).

6. Isolation of AZAs

6.1. Introduction

The scarcity of high purity AZAs was a severe constraint on AZA research activity. Purification of these compounds is necessary:

1) To enable the preparation of CRMs which are essential to ensure monitoring laboratories can produce accurate results;
2) For use in toxicology studies so that regulators can implement appropriate closure limits, and;
3) To understand the chemistry of these compounds and their behaviour in biological environments.

The initial isolation of AZA1 was performed with contaminated mussel tissue (20 kg whole flesh) from Killary Harbour, Ireland, and involved 7 purification steps yielding 2 mg of AZA1.[2] AZA2–5 were subsequently isolated from contaminated whole flesh using a procedure which included additional clean up steps (8 steps in total).[3,77] Purification in both studies was guided by LC-MS/MS and MBA.

Further AZA1, -2 and -3 isolations performed as part of the ASTOX project[11] employed a modified 7-step procedure with recoveries of ~23% reported.[12] Purification was guided by LC-MS/MS and ultra violet (UV) detection (210 nm).

Up to this point, all the AZA isolation procedures used acidic mobile phases. In 2008, a study evaluating the stability of AZAs at various pH values found that significant degradation (due to the formation of re-arrangement isomers) occurred even under slightly acidic conditions in solution. Under neutral and slightly alkaline conditions, AZAs were more stable.[78]

In 2009 isolation of AZA2 from a marine sponge collected in Japan was reported. Purification was performed using 7 clean up steps without using acidic or inorganic additives in the mobile phase.[10]

6.2. Isolation from shellfish

Sufficient supplies of contaminated *Mytilus edulis* tissue, collected from Bruckless, Donegal during the AZA outbreak in 2005 were available for isolation purposes. Efforts were made to improve on the previously reported isolation methods.

Initially the effect of extraction solvent was investigated. Small scale tests with methanol and ethanol showed that both solvents were equivalent in terms of extraction efficiency. Ethanol was chosen primarily to minimize the formation of methyl derivatives, which can be significant when methanol is used as extractant.[33,68]

Freeze-drying of shellfish prior to extraction had been successfully employed in the isolation of pinnatoxins from Australian oysters.[79] This has many advantages: such as avoiding the necessity of water-miscible extraction solvents; complete control of extractant composition; and low water content in the extract (thus minimising difficulties during evaporation and potential toxin stability problems). The effect of freeze-drying the mussel hepatopancreas prior to extraction of AZAs was therefore explored. Higher extraction efficiencies were achieved for the freeze-dried samples after the first and second extractions, with 12% and 2% more AZAs being extracted respectively. No difference was observed in clean-up efficiency (94.2% for both freeze-dried and wet tissues), but the extracts from the freeze-dried samples evaporated more quickly with little or no foaming in the subsequent vacuum-evaporation step. The subsequent two liquid-liquid partitioning steps resulted in only minor losses of toxin (~95% recovery) with an overall clean up efficiency of 67% (Table 7). The next step in the isolation procedure used in the ASTOX project was elution on a silica column which employed acidic mobile phases.[11] Previous studies have shown that AZAs are unstable in acidic environments, but that shellfish tissue appears to have a protective effect.[78] As the sample at this stage of the isolation was still quite crude, and there appeared to be no degradation of the AZAs during small-scale trials, it was deemed appropriate to use acetic acid in the eluent at this point of the procedure. Of all the steps, silica gel chromatography (step 4) gave the greatest efficiency in terms of clean up (93%) and recovery (~95%) (Table 7).

In the next chromatography step, on sephadex LH20, the AZAs eluted together after ~64 min and were collected in 11 fractions. A clean-up efficiency of 66% was achieved with a recovery of 85%.

In the subsequent flash chromatography step, previous isolation procedures used an acidic mobile phase with a RP-8 stationary phase.[11,12] However, the sample is significanly cleaner at this point so there is a risk of acid-promoted degradation of AZAs during storage or evaporation. The use of triethylamine proved to be a safer alternative, with the toxins being stable whilst stored in the freezer as a dry sample (after evaporation of the mobile phase containing 0.1% triethylamine).

A comparison between RP-8 and phenyl-hexyl (which proved effectual by Ueoka *et al* in the isolation of AZA2 from a marine sponge[10]) stationary phases was additionally performed. Both

phases performed similarly in terms of clean-up efficiency and recovery however the phenyl-hexyl proved to be much more efficient at separating the AZA analogues than the RP-8 stationary phase (Table 7). Separation of the AZA analogues at this stage in the procedure improved recoveries and purities in the final semi-preparative HPLC step (step 7), so the phenyl-hexyl stationary phase was chosen for flash chromatography. This step resulted in a clean-up of 64% and a recovery of ~90%.

Final clean-up was performed using semi-preparative chromatography (C8 or C18) with UV detection (210 nm). An acidic mobile phase was previously used for this step[11,12] but to prevent any stability issues, a neutral mobile phase was chosen. Acceptable chromatography was obtained for AZA1 and -2 using the neutral mobile phase, but broad, fronting peaks were observed for those analogues which lack a methyl group at the C22 position e.g., AZA3 and -6. Fractions from the semi-preparative HPLC purification were diluted with water and recovered on SPE cartridges to remove any buffer trace contaminants introduced via the LC eluents. This also reduced the water content and volume of the AZA fractions prior to evaporation. This SPE recovery resulted in very little loss of toxin, with recoveries of >95% being achieved, and greatly facilitated evaporation of the purified AZA-fractions to dryness.

Overall recoveries improved 2-fold from ~23% in the ASTOX project[12] to ~52% for this work[64] (Table 7).

Table 7. Batch summary table for purification of AZA1–3 and AZA6 from *Mytilus edulis*.

Step No	Step	AZA1 (mg)	AZA2 (mg)	AZA3 (mg)	AZA6 (mg)	Weight (g)
	Subsampling (HP)	14.1	4.0	4.8	0.78	505.0
1	1st crude extract	14.0	3.9	4.7	0.77	26.9
2	1st partitioning	13.3	3.7	4.4	0.73	23.9
3	2nd partitioning	12.6	3.5	4.2	0.69	8.9
4	Silica gel	11.9	3.3	4.0	0.65	0.6
5	LH20	10.1	2.8	3.4	0.55	0.2
6	Flash (phenyl-hexyl)*	9.2	2.5	2.4	0.49	-
7	Prep HPLC (C8/C18)	7.3	1.7	2.0	0.30	-
	% Recovery	52	43	43	38	
	% Purity	>95	>95	>95	>95	

* AZA1–3 and AZA6 were separated from each other in this step.

Using this method 11 AZAs (AZA1–10)[64,80] and 37-*epi*-AZA1[81] were purified and their structures confirmed by NMR (Table 11).

6.3. Isolation from phytoplankton

Marine biotoxins are ideally isolated from cultures of the producing organism due to the initial extract being significantly cleaner than from shellfish, and therefore requiring fewer purification steps.[59–61] Once the causative organism was identified[40] significant efforts (see Section 4) were successfully made as part of ASTOX 2 to bulk culture the *A. spinosum*.

6.3.1. Isolation from *A. spinosum*

The *A. spinosum* extracts obtained using HP-20 were considerably purer (Table 8) than the shellfish extracts (Table 7). Hence, it was expected that fewer purification steps would be required. As with the isolation from shellfish, the crude extract was initially partitioned between ethyl acetate and 1 M sodium chloride. The step resulted in a clean-up of 57% and a recovery of 90%. The sample was further purified by chromatography on a silica gel column giving a very high efficiency in terms of clean up (87%) and recovery (~91%, Table 8).

Table 8. Batch summary table for purification of AZA1 and -2 from *A. spinosum*.

Step No	Step	AZA1 (mg)	AZA2 (mg)	Weight (g)	Purity (%)†
	HP-20 resin extract	12.5	3.2	3.04	0.5
1	Partitioning	11.2	3.0	1.32	1.1
2	Silica gel	10.2	2.8	0.17	7.6
3	Flash (phenyl-hexyl)*	9.7	2.4	0.01	>90
4	Prep HPLC (C8/C18)	9.3	2.2	-	>95
	% Recovery (steps 1–4)	75	70		

*AZA1 and -2 were separated from each other in this step
†For total AZA1 + AZA2, based on w/w

The third step was flash chromatography using a phenyl-hexyl stationary phase and a weakly alkaline mobile phase, resulting in separation of AZA1 and -2. This step resulted in the highest clean-up (93%) and recovery (95%) (Table 8).

Final purification was achieved by semi preparative HPLC using a neutral mobile phase. Fractions were collected based on UV detection (210 nm) to facilitate removal of non-AZA components. This step resulted in recoveries of ~93%.

Finally, SPE cartridges were used to remove buffer remaining in the sample, as well as to reduce the water content in, and volume of, the AZA fractions prior to evaporation, and as an additional final clean-up step to remove trace contaminants introduced via the LC eluents. The ^{1}H NMR spectra of AZA1 and -2 were compared to published NMR data and found to be identical. Examination of the spectra indicated purities of >95%. Six successive culture lots (1,200 L harvested in total) enabled the recovery of 9.3 mg of purified AZA1 and 2.2 mg of purified AZA2.

Overall recoveries (steps 1–4) were 75% for AZA1 and 70% for AZA2. This recovery is a significant improvement compared to isolations from shellfish with recoveries increasing by a factor of ~1.5. Furthermore, the procedure is easier to perform with two less clean-up steps after extraction being required to achieve sufficient purities (Figure 29).

6.3.2. Isolation from *A. poporum*

Two strains (North sea and Korean) of *A. poporum* were bulk cultured (see section 4.4.2) for purification using the procedure described in section 6.3.1. From the pellet produced from the North Sea strain, an AZA with a *m/z* of 858 (and a fragment *m/z* of 348) was purified and subsequently named AZA36 (Table 9).

Table 9. Batch summary table for purification of AZA36.

Step No	Step	AZA36 (mg)	Weight (g)	Purity (%)†
	HP-20 resin extract	1.70	1.03	0.2
1	Silica gel	1.60	0.25	0.6
2	Flash (phenyl-hexyl)	1.57	0.01	20.0
3	Prep HPLC (C18)	1.28	-	>95
	% Recovery (steps 1–3)	75		

† based on w/w

From the pellet produced from the Korean strain, an AZA with a *m/z* of 846 (and a fragment *m/z* of 348) was purified and subsequently named AZA37 (Table 10).

Table 10. Batch summary table for purification of AZA37.

Step No	Step	AZA37 (mg)	Weight (g)	Purity (%)†
	HP-20 resin extract	1.30	8.30	<0.1
1	Ethyl acetate partitioning	1.20	0.74	0.2
2	Silica gel	1.05	0.18	0.6
3	Flash (phenyl-hexyl)	0.92	<0.01	40.0
4	Prep HPLC (C18)	0.89	-	>95
	% Recovery (steps 1–4)	69		

† based on w/w

Six AZAs (AZA1 and -2[70], AZA33 and -34[82] and AZA36 and -37[83]) were purified using this method.

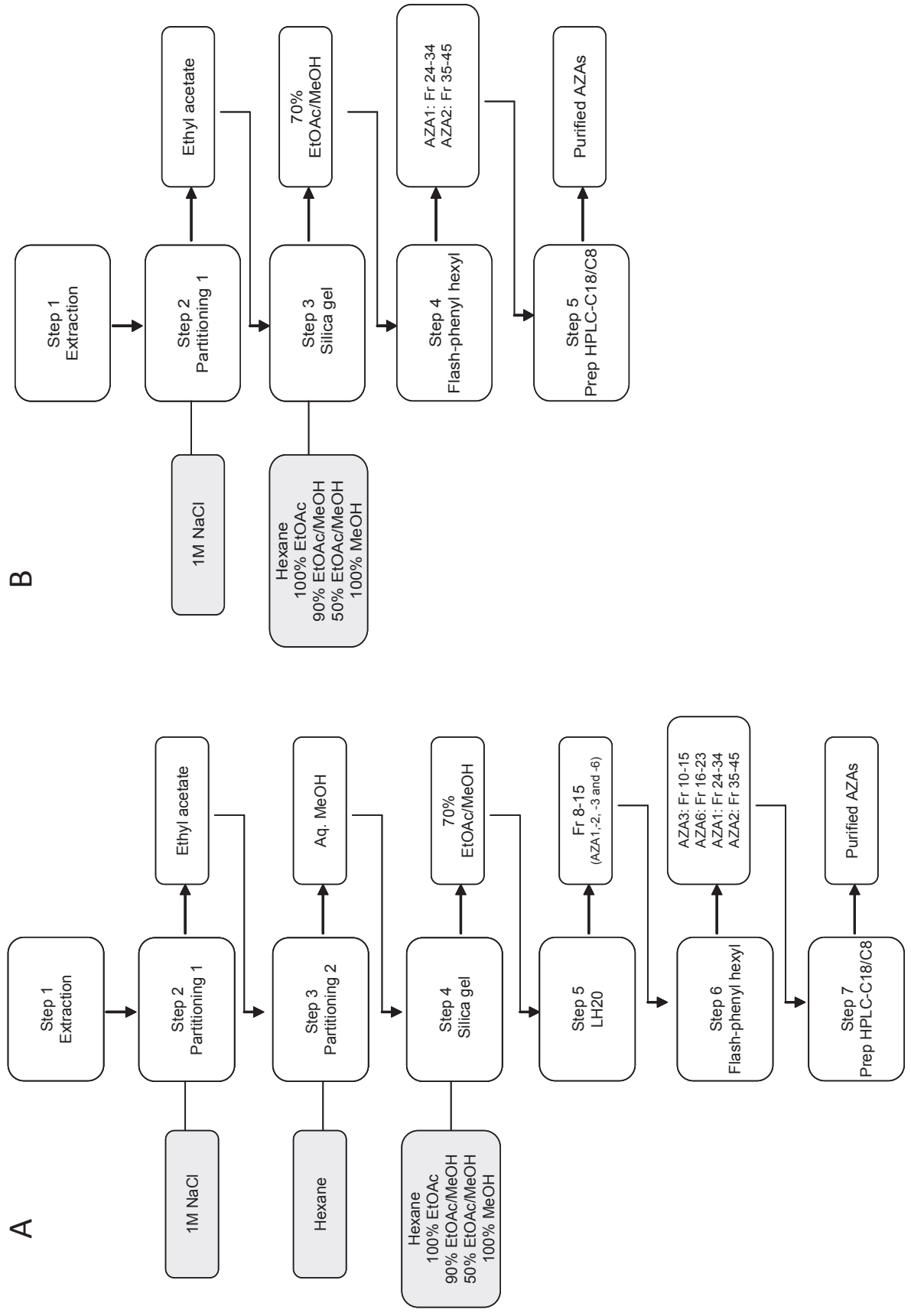


Figure 29. Isolation schemes from A) shellfish (*Mytilus edulis* hepatopancreas) and B) phytoplankton (*A. spinosum* and *A. poporum*).

6.4. Purity assessment

An LC-MS scan was performed in the range *m/z* 100–1000, followed by LC-MS/MS (SIM) analysis for all the known AZA analogues as well as for any additional masses picked up in the MS scan. The sample was also analyzed using the LC-PDA semi-preparative method to ensure that no additional peaks were observed in the UV trace. To determine whether strongly retained compounds, such as phthalates, were present in the sample, isocratic LC-PDA was performed. NMR analysis had shown the presence of a phthalate in some fractions that was detectable by LC-PDA (λ_{max} 205, 225 and 275 nm). The contaminant was conveniently removed by partitioning with hexane. Once samples were sufficiently pure (LC-MS/MS and LC-PDA), they were prepared for NMR spectroscopy. The ^{1}H NMR spectra of AZA1–3 were compared to published NMR data and found to be essentially identical, and examination of the spectra indicated purities of >95%.

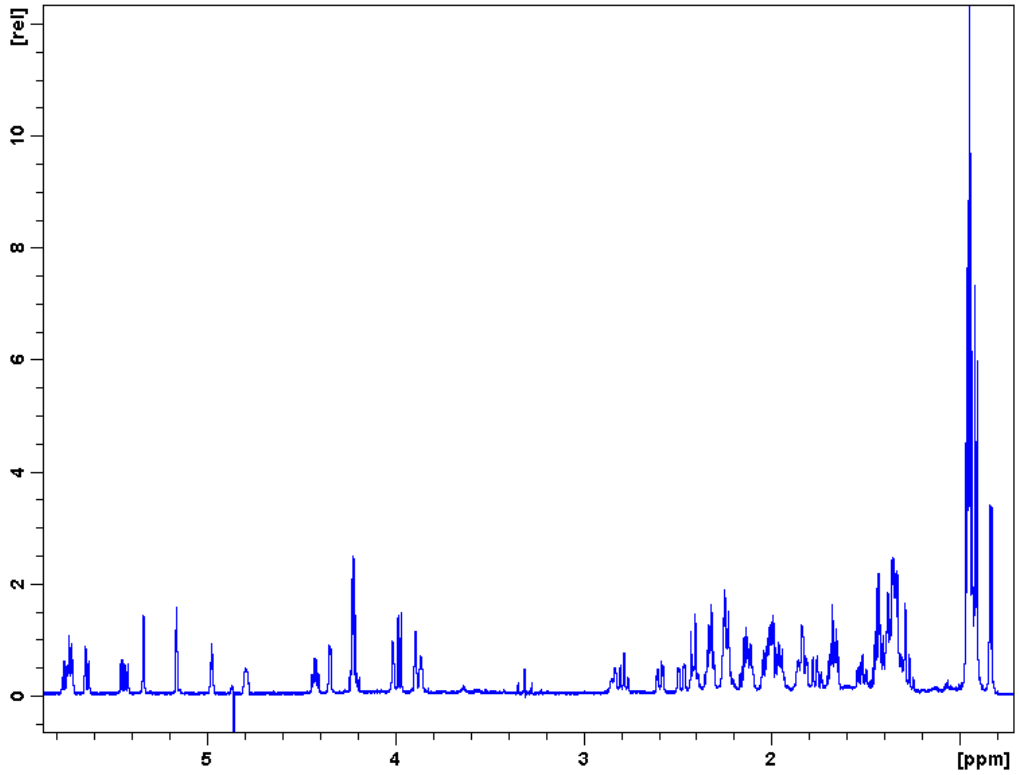


Figure 30. ^{1}H NMR spectrum of AZA1 in CD_3OD.

6.5. Minor analogues and novel AZAs

Over the course of ASTOX up to 30 AZA analogues were identified in shellfish extracts.[33] Further studies revealed how many of these analogues are formed – via metabolism.[75,84] Apart from AZA1–3, other known and novel analogues (13) were isolated to determine their relevance in terms of food safety, but also to study SARs.

AZA4–10 were isolated from shellfish in sufficient quantities to enable structural determination by NMR, the preparation of reference standards and for toxicity testing. Structures for AZA6–10, previously proposed by LC-MS/MS, were confirmed by NMR spectroscopy. The relative toxicities of these analogues are detailed in Table 11.

The development of an LC-MS/MS method for lipophilic toxin analysis using a neutral mobile phase led to the discovery of unidentified isomers of AZA1, -2 and -3 in tissue and calibrant CRMs.[15] These isomers were resolved using a neutral eluent, but co-eluted with the parent toxin under acidic[85] and basic[86] conditions. The proportion of these isomers for AZA1–3 in the tissue CRM ranged from 2–15% of their parent analogues. This finding is significant due to the potential of these isomers to interfere with the accuracy of analytical results. These isomers were found to be C37 epimers following the isolation of 37-*epi*-AZA1 (from shellfish) and subsequent structural elucidation using LC-MS/MS and NMR[81] (Table 11). Studies revealed that the epimers are spontaneous epimerization products whose formation is accelerated with the application of heat and most likely exist for all the AZA analogues. Interestingly, toxicity assessment using the Jurkat T lymphocyte cell assay indicated that the epimers are more toxic than the parent analogues[81] (Table 11).

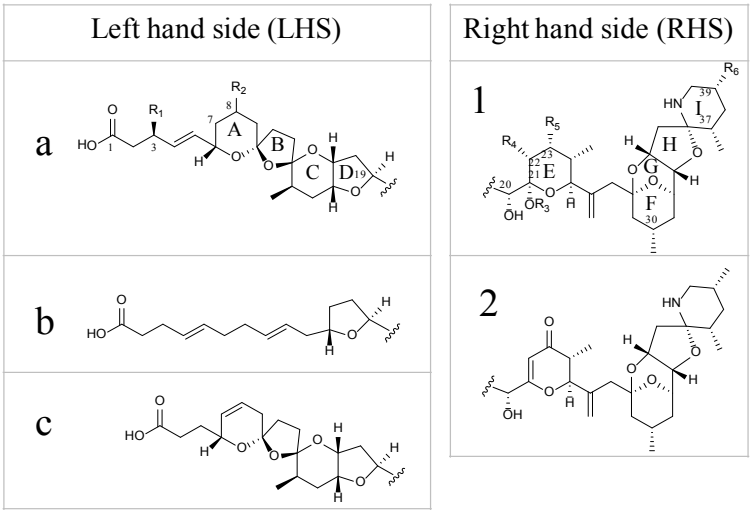
An additional compound was isolated from shellfish with a mass of 823 Da, which had previously been proposed as dehydro AZA1 by Rehmann *et al.*[33] The compound, named AZA26, was found not to be dehydro AZA1 but instead had a ketone functionality at C23.[87] Very low levels were detected in shellfish and it is not considered of relevance to human health, however, the fact that it was found to be significantly less toxic than AZA1 (Table 11) is of interest in terms of structure activity.

LC-MS/MS analysis of *A. spinosum* extracts revealed the presence of other AZA analogues with masses of 715 and 815 Da, subsequently named AZA33 and -34, respectively. Sufficient quantities were available in bulk cultures of the *A. spinosum* to enable isolation for NMR structural elucidation and toxicity testing.[82] Both compounds were found to differ from AZA1 at the carboxyl end of the molecule (Table 11) and although they were not found in shellfish at significant levels their structural and toxicity differences provided valuable information.[82]

A further two previously unknown compounds were isolated from a bulk culture of *A. poporum* and were named AZA36 and -37. These compounds differed in that they lacked a methyl group at C39 compared to all the other isolated AZAs and that they have a 3-hydroxy substitution, that so far had only been observed in shellfish metabolites of AZA1 and -2, (Table 11). Toxicity testing revealed that both AZAs were less toxic than AZA1, with a relative potency of 0.33 for AZA36 and 0.16 for AZA37 in comparison to AZA1.[83]

In total, 16 AZA analogues were purified in ASTOX 2 from shellfish and phytoplankton, 11 of which were fully characterised by LC-MS/MS and NMR for the first time.

Table 11. Structural variants of AZAs, their protonated masses, origin and toxicity.



	Type§	R_1	7,8	R_2	R_3	R_4	R_5	R_6	$[M+H]^+$	Origin	Status	Toxicity (Jurkat) EC_{50}
AZA1	a1	H	Δ	H	H	CH_3	H	CH_3	842.5	*A. spinosum*	phycotoxin	1.1
37-*epi*-AZA1	a1	H	Δ	H	H	CH_3	H	CH_3	842.5	*A. spinosum*	phycotoxin	0.2
AZA2	a1	H	Δ	CH_3	H	CH_3	H	CH_3	856.5	*A. spinosum*	phycotoxin	0.3
AZA3	a1	H	Δ	H	H	H	H	CH_3	828.5	shellfish	metabolite	0.6
AZA4	a1	OH	Δ	H	H	H	H	CH_3	844.5	shellfish	metabolite	2.0
AZA5	a1	H	Δ	H	H	H	OH	CH_3	844.5	shellfish	metabolite	3.0
AZA6	a1	H	Δ	CH_3	H	H	H	CH_3	842.5	shellfish	metabolite	0.2
AZA7	a1	OH	Δ	H	H	CH_3	H	CH_3	858.5	shellfish	metabolite	-
AZA8	a1	H	Δ	H	H	CH_3	OH	CH_3	858.5	shellfish	metabolite	0.3
AZA9	a1	OH	Δ	CH_3	H	H	H	CH_3	858.5	shellfish	metabolite	1.9
AZA10	a1	H	Δ	CH_3	H	H	OH	CH_3	858.5	shellfish	metabolite	3.1
AZA26	a2	H	Δ	H	-	-	-	-	824.5	shellfish	metabolite	36.6
AZA33	b1	-	Δ	-	H	CH_3	H	CH_3	716.5	*A. spinosum*	phycotoxin	5.2
AZA34	c1	-	Δ	-	H	CH_3	H	CH_3	816.5	*A. spinosum*	phycotoxin	0.2
AZA36	a1	OH	Δ	CH_3	H	CH_3	H	H	858.5	*A. poporum*	phycotoxin	5.0
AZA37	a1	OH	-	H	H	CH_3	H	H	846.5	*A. poporum*	phycotoxin	6.3

§ The type refers to variations of the LHS and RHS parts of the molecule.

6.6. Structure confirmation/elucidation

6.6.1. LC-MS/MS spectra

CID mass spectra of AZAs are characterized by specific fragmentations of the molecule, which are followed by several water losses. The molecular ion usually cleaves off up to six molecules of water (group 1 fragments). The smallest cleavage (apart from a water loss) is caused by a retro Diels Alder (RDA) reaction of ring A (A-ring cleavage), where the bonds between C6 and the ether bridge and between C9 and C10 are cleaved. This results in the loss of C1–C9 with its substituents, followed by several water losses resulting in the group 2 fragments. The second fragmentation of the carbon skeleton is the C-ring cleavage, where the bonds between C15 and C16 as well as the bond between C17 and the ether bridge of the C-ring are cleaved. The remaining fragment, with its water losses, gives the group 3 fragments. By the C-ring cleavage, the C1–C15 part of the molecule is eliminated. The group 4 fragments are formed by the E-ring cleavage (RDA), where C1–C23 is eliminated. Before identification of the AZA-producing organisms, all published AZAs had diagnostic substituents located at C1–C23, and all shared the remaining *m/z* 362 fragment. The 39-desmethyl AZA variants form *m/z* 348 as the major ion in the group 4 fragments. Other fragmentations of less diagnostic value are the cleavage between C27 and C28 resulting in the group-5 fragments, with *m/z* 262/248 and the G-ring cleavage giving the group-6 fragments with *m/z* 168/154.[47–49]

Apart from these general fragmentation patterns, a specific fragmentation pathway exists for 3-hydroxy-AZAs, which can be observed within the molecular ion cluster. In addition to the subsequent water losses from the pseudo-molecular ion, which is common among all AZAs, 3-hydroxy-AZAs also eliminate CO_2 followed by several water losses. The fragments resulting from the CO_2 elimination are not very abundant (typically they make up to ~20% of the highest peak), but are clearly visible. Another fragmentation pathway unique for 3-hydroxy-AZAs is the elimination of a 78 Da fragment equivalent to $C_2H_6O_3$, which probably is caused by a cleavage between C2 and C3 combined with one water loss. Like the CO_2 elimination, this fragmentation pathway is not dominant, but the combination of a CO_2 cleavage and a 78 Da-elimination is characteristic for 3-hydroxy-AZAs.

The only differences in the LC-MS/MS spectra between the parent analogue and its 37-epimer were minor changes to the ratio of the RDA (*m/z* 672/654 and 462) fragment ions.[81]

The mass spectrum of the AZA33 showed typical AZA type structure water loss fragments (*m/z* 698 and 680). Additionally a fragment peak for the *m/z* 362 and 462 ions indicated that the amine end of the AZA1 compound was present. No RDA fragment was present, suggesting that the A ring

system of AZA1 was different in AZA33. AZA34 also displayed water loss fragments (*m/z* 798 and 780), a RDA fragment, in addition to the typical *m/z* 362 and 462 ions.[82]

AZA36 and AZA37 display the same group fragments as the other AZAs, except that all fragments are shifted *m/z* 14 lower masses, because of the missing methyl group at C39, i.e., *m/z* 658 instead of *m/z* 672 for group 2, *m/z* 448 instead of *m/z* 462 for group 3, *m/z* 348 instead of *m/z* 362 for group 4, etc. It is also noteworthy that the saturation of the 7,8 doublebond in ring A of AZA37 results in a different fragmentation of ring A: instead of the fragment *m/z* 658 a bigger fragment *m/z* 686 is formed.[83] This modified fragmentation pathway will help to identify structural characteristics of new AZAs by LC-MS/MS.

The mass spectrum of AZA26 showed typical AZA type structure water loss (*m/z* 806 and 788) and RDA (*m/z* 672) fragments. Additionally a fragment peak for the *m/z* 362 and 462 ions indicated that the amine end of the AZA compound was present.[87]

6.6.2. NMR studies

AZAs consist of chains of carbon atoms substituted with hydrogen atoms with and connected with ether links and an amino link. The ^{1}H and ^{13}C nuclei are particularly amenable to NMR spectroscopy, and it is this method, supplemented by LC-MS/MS, that has primarily been used for AZA structure determination. The structure of AZA1 was established in this way and full ^{1}H and ^{13}C NMR assignments were reported in 1998.[2] Subsequent isolation of two other AZAs led to structural elucidation of AZA2 and -3 and presentation of their full ^{1}H- and ^{13}C NMR assignments.[3] AZA4 and AZA5 are hydroxylated analogues and were isolated in smaller amounts, such that only ^{1}H NMR was obtained.[77] It should be noted that these NMR studies did not reveal the absolute stereochemistry of the AZAs, nor even their complete relative stereochemistries. From 2000, the first syntheses of AZA substructures began to be reported. This synthetic activity[88–91] eventually led to the definition of the absolute stereochemistry of AZAs, which is not accessible from normal NMR analyses.

Establishing the 2- and 3-dimensional structures of complex molecules by NMR spectroscopy requires the presence of useful through-bond ^{1}H–^{1}H and ^{1}H–^{13}C connectivities, through-space ^{1}H–^{1}H NoE interactions, and coupling constant information. In complex organic molecules, some of these correlations can be absent or hard to observe due to overlapping signals or unfavourable coupling constants, preventing full structure determination from NMR spectra alone. A series of synthetic studies by Nicolaou *et al* revealed that the originally proposed structure of AZA1 was incorrect, and

eventually established the absolute stereochemistry and correct structures of AZA1–3 as shown in Table 11.[88–91] The structures of the remaining AZAs have been revised accordingly (Table 11), with a consequent re-assignment of the original NMR assignments for resonances associated with C7 and C9.

AZA6 was isolated and its structure, proposed originally from MS data, was established by NMR.[64] AZA7–10 have also been isolated, and their structures originally proposed from MS studies, were confirmed by NMR[80] (Table 11). However, NMR analysis of AZA26[87] showed a different structure to that originally proposed from MS studies.[33] Most of the remaining AZAs (i.e., AZA11–23 and AZA29–32) have not yet been characterised by NMR, and their structures therefore remain tentative. Additional structural information was derived for AZA17, -19, -21 and -23 following the discovery that these AZAs convert to analogues with established structures (AZA3, -6, -4, and -9 respectively).[84]

6.6.3. Characterisation reactions

In addition to NMR a number of different approaches were used to support structural hypotheses. All AZAs have a carboxyl group at the C1 position. Treatment of a sample with diazomethane, which specifically reacts with a carboxyl moiety to produce the methyl ester, was used to confirm the presence of the carboxyl group for some AZAs.[47] This method has also been used to confirm the identity of AZA methyl derivatives.[64,68] In addition, derivatisation of the free carboxyl group on AZAs with 9-anthryldiazomethane (ADAM)[92] was used to differentiate between methyl esters of AZAs and methyl ketals.[68]

The presence of a diol at C21–C22 can also be confirmed by reaction with sodium periodate, which cleaves the *cis* diol on AZAs to produce a lactone derivative containing only C21–C40 with appended functional groups. This method was used to support structural evidence for dehydro-AZA3[33] and for structural confirmation (C21–C40) of AZA6[64] and AZA33 and -34.[82]

6.6.4. Relative molar response study

Reference standards were prepared for the majority of the isolated analogues. Accurate quantitation of these standards by qNMR facilitated their use in relative molar response studies. The molar responses of AZA4–10 were assessed against AZA1. A number of LC-MS/MS experiments were performed. Only slight differences were observed between the gradient and isocratic results in SIM mode with the isocratic conditions giving somewhat higher results. The response factor of AZA6 matched most closely that of AZA1 under isocratic conditions in both SIM and SRM modes. All the other hydroxylated analogues gave lower response factors, in particular for AZA7 and -9 (Table 12).

Table 12. Results for AZA analogue relative molar response (to AZA1) by LC-MS/MS using acidic HPLC conditions.

AZA	SIM		SRM isocratic				SRM gradient			
	isocratic	gradient	Water loss	RDA	462	362	Water loss	RDA	462	362
37-*epi*-AZA1	0.93	-	0.88	0.91	0.75	1.1	0.86	0.91	0.75	1.1
4	0.88	0.75	0.80	0.81		0.86	0.96	0.96		1.03
5	0.77	0.70	0.55	0.47		0.47	0.61	0.54		0.53
6	0.95	0.93	1.06	1.21		1.17	1.05	1.21		1.13
7	0.45	0.40	0.37	0.33		0.37	0.40	0.37		0.40
8	0.84	0.81	0.58	0.41		0.47	0.59	0.47		0.51
9	0.53	0.49	0.45	0.48		0.61	0.50	0.53		0.68
10	0.69	0.67	0.47	0.46		0.49	0.48	0.49		0.51
33	0.97	1.03	1.05		0.80	0.90	1.03		0.77	0.91
34	1.05	1.01	1.17	0.39		0.87	1.22	0.41		0.90

6.6.5. Toxicity testing

The analogues were assessed for toxicity using the Jurkat T lymphocyte cell assay. Issues with impurities for AZA7 due to the presence of other AZA contaminants prevented it from being subjected to the Jurkat cell assay. All other AZA analogues tested were cytotoxic to Jurkat T lymphocyte cells in a time- and concentration-dependent manner. However, there were distinctive differences in the relative potencies of each analogue as determined by their respective EC_{50} values (Table 11). Relative to AZA1 (used to normalize all potencies based on EC_{50} values) the potency order was:

AZA2 > AZA6 > AZA34 ≈ 37-*epi*-AZA1 > AZA8 ≈ AZA3 > AZA1 > AZA4 ≈ AZA9 > AZA5 ≈ AZA10 > AZA33 ≈ AZA36 > AZA37 > AZA26.

The results indicate AZA potency is gained by methylation of C8 and/or C22 (AZA2, -6) while AZA potency is reduced by C3 and/or C23 hydroxylation (AZA4, -5). The *A. poporum* toxins (AZA36 and -37) that each have a hydroxyl group at C3, and lack a methyl group at C39, were found to be less toxic.

AZA33 (AZA1 missing A/B/C rings) was less potent than AZA1 (~5-fold), whereas AZA34 (AZA1 missing C4/C5 alkene) was 5.5-fold more potent. Similarly, 37-*epi*-AZA1 was 5.1-fold more potent than AZA1.

Semi-synthetic hydrogenated AZA1 analogues, 4,5-dihydroAZA1 and 4,5,7,8-tetrahydroAZA1, were also tested. Both compounds were found to be approximately equipotent to AZA1 suggesting that

the C4/C5 alkene and the C7/C8 olefin bonds are not necessary for toxicological activity (see also section 11.6).

Interestingly AZA26 was the least potent (~30–40-fold less toxic) of all the analogues tested. This compound differs from the other analogues by having a ketone functionality at C23 and lacking the C20–21 diol. These results suggest that it is this middle part of the molecule which confers the potency. However, a previous study by Ito *et al*[11] in which truncated analogues were synthesised and tested in mice for toxic effects concluded that the whole molecule was necessary for toxicity. One of the truncated analogues contained the diol at C20–21 and the methyl group at C23 but lacked the F, G, H and I rings and it exhibited no measurable toxicity. The same study also found that altering the orientation of the functional groups from C1–C20 (in the AZA1 structure) had a significant effect – resulting in little or no toxicity.

6.6.6. Proportions of AZA analogues

To determine the proportions of the analogues (relative to AZA1), raw shellfish contaminated with AZAs were cooked so that AZA3, -4, -6 and -9 could be determined. These analogues are heat induced decarboxylation products of AZA17, -21, -19 and -23 respectively.[84] The analysis of cooked shellfish reflects what is typically ingested by the consumer. Additional differences have been reported between the analysis of raw and cooked shellfish in terms of concentration levels.[17] Figure 31 shows that AZA1–3 and -6 are the predominant toxins. The levels of the remaining analogues relative to AZA1 are low, with AZA5 comprising ~7% while AZA4, AZA7–10, AZA33 and -34 are all below 3%.

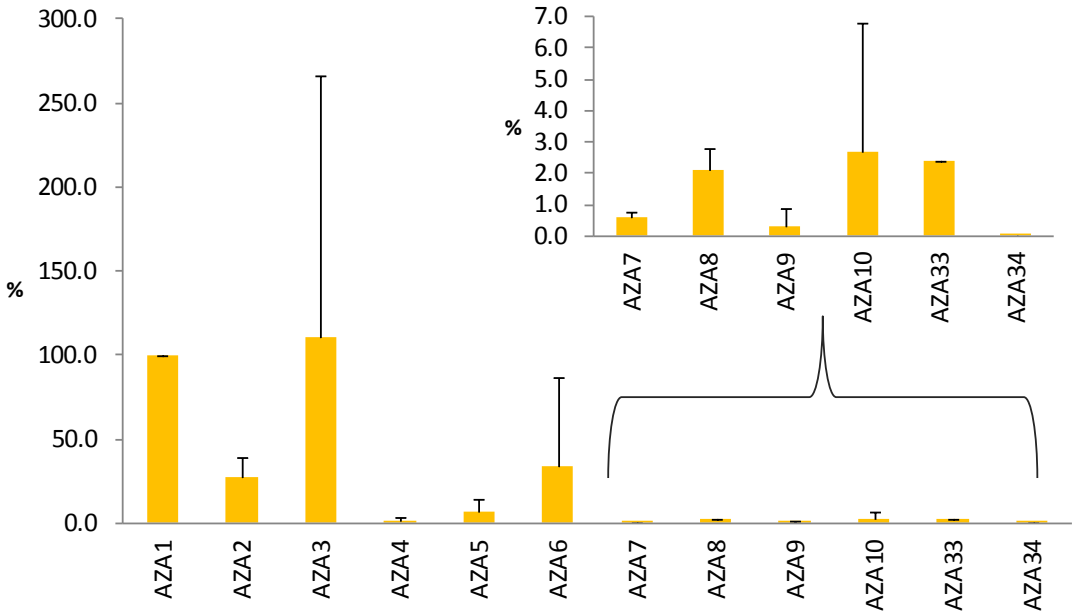


Figure 31. Proportions of AZA2–10, AZA33 and -34 relative to AZA1 in cooked shellfish (n=20).

Table 13. Distribution of purified AZAs to project partners.

Compound	Amount (mg)	Sent to	Purpose
AZA1	1.70	UM	Jurkat T lymp. cell assay
AZA2	0.43	UM	Jurkat T lymp. cell assay
AZA3	0.40	UM	Jurkat T lymp. cell assay
AZA4–10, 37-*epi*-AZA1, AZA33 and -34	~0.05	UM	Jurkat T lymp. cell assay
AZA1	0.27	NSVS	*ip* mouse study (LD_{50})
AZA2	0.15	NSVS	*ip* mouse study (LD_{50})
AZA3	0.23	NSVS	*ip* mouse study (LD_{50})
AZA6	0.22	NSVS	*ip* mouse study (LD_{50})
AZA1	1.5	University of Trieste	Oral mouse study
AZA2	1.5	University of Trieste	Oral mouse study
AZA3	1.5	University of Trieste	Oral mouse study
AZA1	3.5	NRCC	CRMs
AZA2	3.5	NRCC	CRMs
AZA3	3.5	NRCC	CRMs
AZA1	0.22	NVI	ELISA
AZA2	0.01	NVI	ELISA
AZA3	0.01	NVI	ELISA
AZA6	0.003	NVI	ELISA
AZA1	2.0	Perkin Elmer	Tritiation
AZA1	0.22	MI	Mussel feeding
AZA2	0.28	MI	Mussel feeding
AZA3	0.13	MI	Mussel feeding
AZA6	0.015	MI	Mussel feeding
AZA1	2.1	QUB	Antibody work
AZA2–10	~0.005	QUB	Antibody work

Biotoxin monitoring programs within the EU only analyse raw shellfish and typically low levels of AZA3 are detected in such samples.[5] In cooked shellfish, levels of AZA3 and -6 ranged widely, probably due to the different rates of AZA1 and -2 metabolism (oxidation of methyl at C22 to produce the carboxy analogues AZA17 and -19 respectively) in the shellfish tested. These results highlight the degree to which AZA equivalents are underestimated in routine monitoring programs where uncooked shellfish are tested, and suggest that AZA6 should be included in the AZA regulation. Previously, levels of AZA analogues other than AZA1–3 were reported to comprise less than 5% of the total AZA equivalents[33], however, this study indicates that the AZA analogues 4–10, as well as AZA33 and -34, comprise ~17% of the total AZA content with AZA6 being the most significant (~12%). Different toxin profiles have been reported from other countries whereby AZA2 is more predominant than AZA[10,17,93,94], suggesting that shellfish from these locations are more likely to contain higher levels of AZA6 and -10, and to a lesser extent AZA9. In such circumstances these analogues may have greater significance.

6.7. Analytical methods and CRMs

With the shift towards chemical monitoring methods (away from the MBA) for lipophilic toxins, including AZAs, it is important that appropriate RMs are available. RMs are essential for the development, validation and quality control of analytical methods[66] and can generally be classified in two categories: pure standard RMs are necessary for instrument calibration while matrix RMs are designed to assess the performance of entire analytical methods, including: testing of extraction methods; assessing matrix effects; and evaluating the accuracy of measurement or determination steps.

As part of the ASTOX project significant work was performed on the isolation and purification of AZAs for the preparation of AZA calibrant RMs.[11] Work was also carried out by the NRCC in collaboration with the UK Food Safety Authority.[95] These efforts led to the production of CRMs for AZA1, -2 and 3[11], which are commercially available from the NRCC (Table 14).[96] Certified values were assigned using qNMR[97], with confirmation through a relative molar response study based on LC-FLD analysis following ADAM derivatisation.[92] Isolation procedures for AZAs have been further improved to ensure continued supply of pure AZAs for production of calibrant CRMs.[64] AZA matrix RMs were also developed as part of the ASTOX project.[11] Studies were aimed at the production of homogeneous and stable materials, primarily prepared from mussel tissues. A variety of stabilisation techniques were studied, including gamma irradiation and freeze-drying.[98,99] This extensive feasibility work led to the production and certification of a mussel matrix CRM with assigned values for AZA1–3.[13] Certified values were assigned using direct analysis by LC-MS/MS, and confirmed by LC-FLD following ADAM derivatisation.[92] Recently, as part of an international collaboration, a multi-toxin CRM containing AZAs was prepared as a freeze-dried mussel powder[14], and this has been utilised in method validation exercises.[100]

Table 14. List of AZA CRMs available from the NRCC.

Product	CRM type	Certified analyte	Concentration	Uncertainty
CRM-AZA1	Calibration solution	AZA1	1.47 µmol/L	0.08
CRM-AZA2	Calibration solution	AZA2	1.50 µmol/L	0.06
CRM-AZA3	Calibration solution	AZA3	1.25 µmol/L	0.05
CRM-AZA-Mus *	Mussel matrix (wet homogenate)	AZA1	1.16 mg/kg	0.10
		AZA2	0.273 mg/kg	0.024
		AZA3	0.211 mg/kg	0.023
CRM-FDMT1 *	Mussel matrix (freeze-dried powder)	AZA1	4.07 mg/kg	0.26
		AZA2	1.13 mgkg	0.07
		AZA3	1.00 mgkg	0.06

* Certified values are traceable to the International System of Units (SI) through the use of NRCC CRMs.

7. MINI PIG STUDY

7.1. Introduction

Little is known about the toxicokinetics and toxicodynamics of AZAs. To date, few animal trials have been carried out to assess the oral toxicity of AZAs and in all of the studies only mice have been used. These studies have shown AZA1 to cause extensive damage (Section 9).

Pigs are often used in toxicology studies as they possess similar anatomic and physiological characteristics to humans; their cardiovascular, urinary, integumentary and digestive systems being similar. These animals were therefore chosen to further investigate the oral toxicity of these compounds.

7.2. Preparaton of pig feed

An initial (pilot) mini pig feeding trial was carried out in the NSVS using one mini pig. Feed was prepared using naturally contaminated *Mytilus edulis* tissue that was further concentrated using semi isolated AZA1, -2 and -3. It was necessary to prepare the feed using semi isolated AZAs as it was not possible to obtain AZA contaminated shellfish at high enough concentrations to allow for the direct mixing of shellfish with commercial pig feed. To obtain the semi isolated AZA material, AZAs were isolated from contaminated mussels using the first four steps of the isolation process (Figure 29). In order to ensure that the mini pigs would consume the feed, it was necessary for it to resemble the commercial feed as much as possible. High proportions of AZA contaminated hepatopancreas could make the feed unpalatable.

The feed was prepared by adding semi-isolated AZA1, -2 and -3 to ~20 g of homogenised and sieved pig feed pellets. This was achieved by dissolving the AZAs in 20 mL of methanol in a round bottomed flask, adding the feed and removing the methanol on a rotary evaporator at room temperature. To this dried feed, an additional 90 g of dry pig feed was added and homogenized, followed by the addition of 22 mL of water. Using a 5 mL syringe, cylindrical rows of feed were extruded and cut into pellets using a scalpel. These were allowed to air dry overnight. Moisture content analysis was carried out on the pellets to ensure they were similar to the pig acclimatization feed. Four portions of feed (~1 g) were weighed out and placed in an oven at 104 °C for 24 h. The moisture content was determined to be 10.5±0.5%, which compares well to the moisture content (11%) of the commercial pig feed to which the pigs are acclimatised prior to the trial. The contaminated pig feed also had a similar appearance and texture to the uncontaminated pig feed (Figure 32).

The mean AZA1, -2 and -3 combined concentration of the feed was 16.6 µg/g (Table 15). Sub-samples (n=12) were analysed and the feed shown to be homogenous (Table 15).

Table 15. AZA (AZA1–3) concentration in pig feed prepared for initial pig feeding trial.

	AZA1	AZA2	AZA3	AZA Total (No TEF)	AZA Total (TEF)
Mean (µg/120g feed)	857.5	710.8	428.7	1997.1	2762.8
stdev				162.7	222.9
%CV				8.1	8.1

Figure 32. Pig feed pellets purchased from Special Dietary Services (left) and AZA1, -2 and -3 contaminated pellets prepared in the MI (right).

The mini pig weight was ~8.3 kg, therefore the feed equated to 240.6 µg of AZA equivalents per kg of mini pig. EFSA base their exposure assessment on a 60 kg adult consuming a 400 g portion of shellfish. Based on the regulatory limit of 160 µg AZA1 equivalents/kg shellfish meat, a 60 kg adult would be exposed to 64 µg AZA1 equivalents in a single shellfish portion of 400 g. The mini pig received 2,762.8 µg AZA1 equivalents in a 120 g food portion, which equates to a concentration of 23 µg/g (144 times the EU regulatory limit). Once the contaminated feed was consummed the pig had access to AZA free feed and water until the end of the 24 h trial. Blood was sampled throughout the trial.

The initial trial demonstrated that the technique for preparing the contaminated feed was suitable as all the feed was consumed. Analysis of tissues confirmed uptake and distribution of AZA1–3.

Based on these results, it was decided to prepare the feed for the subsequent mini pig trial (using four mini pigs) at a level of 3,200 µg AZA equivalents. The feed was prepared as in the initial trial. Analysis of the feed was carried out by LC-MS/MS and shown to be homogenous (Table 16).

Table 16. AZA (AZA1–3) concentration in pig feed prepared for second pig feeding trial.

	AZA1	AZA2	AZA3	AZA Total
Mean (µg/g)	17.1	4.6	3.0	24.7
stdev				1.7
%CV				6.8

The vacuum packed feed was forwarded to NSVS for the mini pig trial. Portions of the feed were also maintained frozen for future analysis if required. Each mini pig was dosed with 267 µg AZA1–3/kg pig (Table 17).

Table 17. Dose rate of AZAs used in the second mini pig trial.

Pig	Pig weight (kg)	Feed weight (g)	AZA1 (µg)	AZA2 (µg)	AZA3 (µg)	Total AZA1–3 No TEF(µg)	Dose µg AZA1–3/kg pig
1	9.0	97.4	1660.7	451.9	289.3	2401.9	266.9
2*	8.8	93.9	0.0	0.0	0.0	0.0	0.0
3	8.7	94.2	1606.1	437.1	279.8	2323.0	267.0
4	8.6	93.1	1587.4	432.0	276.5	2295.9	267.0

*Mini pig number 2 was used as the control.

In addition to AZA1, -2 and -3, the feed contained other AZA analogues (Figure 33) such that the concentration for AZA1–12 was 29.6 µg/g.

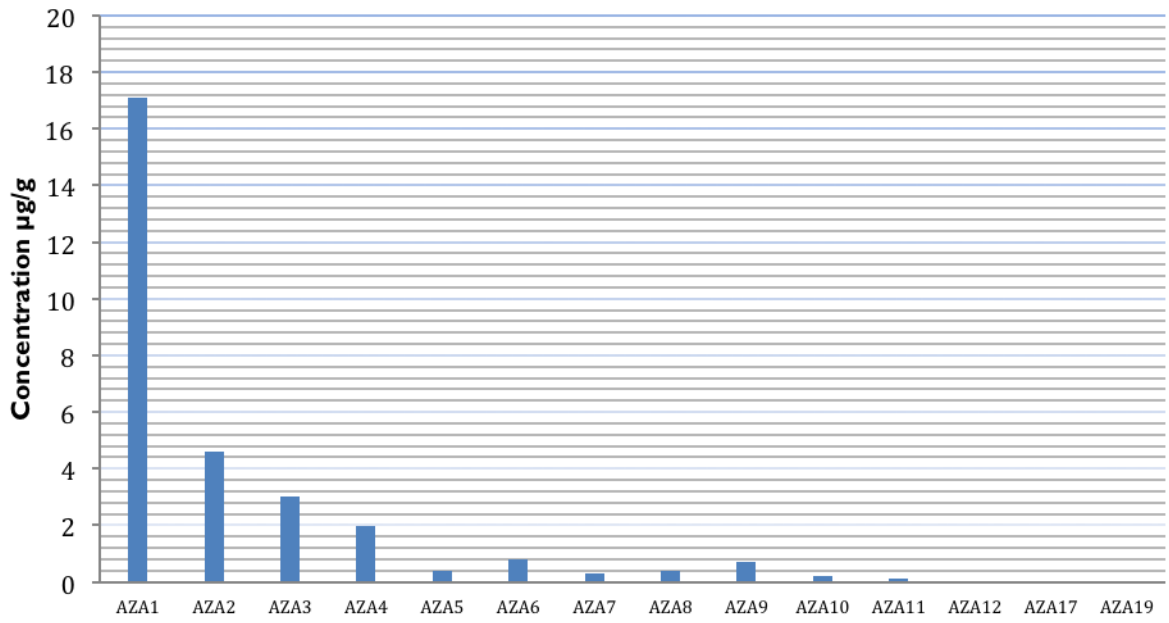


Figure 33. Concentration of AZA analogues in pig feed.

7.3. Toxicology study

In the first trial (pilot) no sickness (diarrhea, vomiting, etc) was observed in the one mini pig that was dosed (333 µg/kg). In the second trial three pigs and a control received the same dose. The only clinical observation was that the pigs were more sedate after 24 h compared with the control minipig.

Pathalogical changes were evaluated based on general histological descriptions on normal tissue structure and comparisons with the data from the pilot study, in addition to the histology in the control minipig. In general, the results showed that the second trial, in many aspects, was similar to the findings in the pilot study but there were also some differences.

-Duodenum: The most obvious change was the presence of aggregates of multiple dark, round structures, interpreted as apoptotic bodies, in the lamina propria of the villi (Figure 34A, arrows). The apoptotic bodies seemed to be localised in large cells, probably macrophages. There were also an increased number of neutrophils in the lamina propria, some of which had disrupted nuclei, and thus a possible source for the apoptotic bodies. The epithelium was sitting on the basal lamina but on the villi the epithelial cells had vacuoles in the apical cytoplasm with eosinophilic round "bodies" (Figure 34C, arrowheads). There appeared to be a reduced number of goblet cells on the villi or the goblet cells were "empty". The observed changes were noted in both trials.

-Jejunum, ileum and colon: In the pilot study, the jejunum and ileum were unaffected. In the second trial, these segments displayed similar changes to those described for the duodenum. However, in the colon, the apoptotic bodies (Figure 34D, arrows) and increased number of neutrophils were localised in the outer lamina propria. In the ileum, the amount of apoptotic bodies in the lymphoid follicles also increased compared to the control but this needs confirmation (since there is ongoing apoptotic activity in these follicles in normal animals).

-Stomach: In one animal, apoptotic bodies were found in the most distal part (pyloric part) of the outer lamina propria. The upper areas appeared to be normal.

-Liver: In the pilot minipig, the hepatocytes showed mild perilobular vacuolisation interpreted as hydropic degeneration. Liver sections were evaluated blind with changes recorded in 15 microscopic fields using a ×40 lens. The results showed that in the exposed minipigs (in the second trial), a multifocal distribution of hepatocytes with condensed, hypereosinophilic cytoplasm and dark, condensed nucleus was found (Figure 34F and H, asterisk). In the 15 fields, the number of cells varied between 3 and 47 in the three exposed animals. In the liver of the pilot, no such cells were recorded.

In the second trial, the hepatocytes of the exposed group also appeared to have large vesicular nuclei with more prominent nucleolus than in normal animals. The cells described are interpreted as multifocal, single cell degeneration/necrosis or apoptosis. Although differences were observed, the results from both studies indicated that the liver cells had been exposed to a harmful substance.

-Spleen: In the second trial, changes were found that consisted of cell depletion and hemorrhages of the marginal zone with an increased number of neutrophils in the red pulp. Some of these cells showed apoptotic changes. In the pilot study observations consisted of a bloody marginal zone and ellipsoids. The ellipsoids did not contain erytrocytes in the second trial.

-Lungs: In the lungs an increased number of neutrophils including apoptotic bodies, were found in the alveolar septa of the exposed animal.

-Pancreas: In one exposed animal, scattered single cells of the exocrine cells were rounded and small with a condensed nucleus, present inside a vacuole. This was interpreted as single cell death/ apoptosis.

In the other organs, histopathological changes were not noted or the possible changes need to be further investigated.

The haematology/clinical chemistry analysis showed a moderate increase in the number of white blood cells of the exposed minipigs. The number of neutrophils increased after 4 h, and after 24 h the number increased by ~5-fold. The percentage of neutrophils (of all leucocytes) had increased from ~20 to 80%, while the number of lymphocytes lowered from ~60 to 20%.

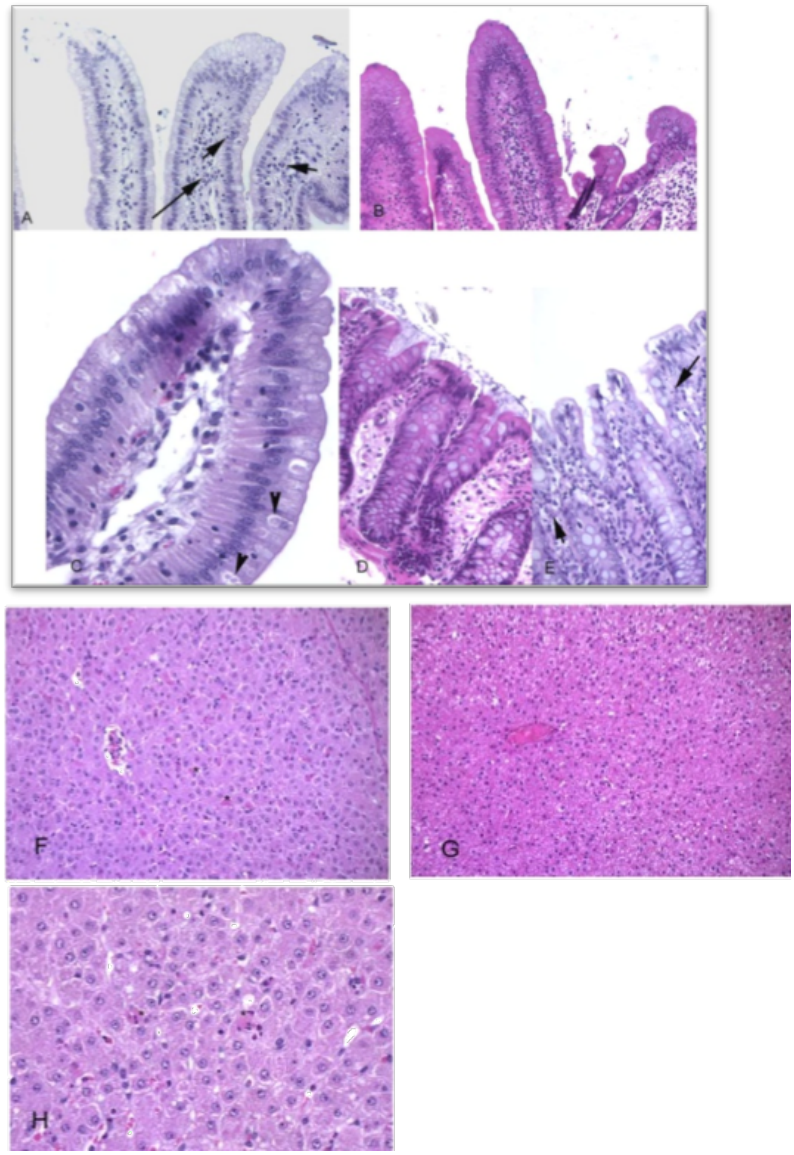


Figure 34. A/C) duodenum from exposed minipig, B) duodenum from control minipig, D) colon from exposed minipig, E) colon from control minipig, F/H) liver from exposed minipig, G) liver from control minipig.

The clinical chemistry analysis showed an increase in the level of several enzymes, especially AST, ALT, AP and GGT in the exposed minipigs compared to the controls (Figure 35). The data support the changes observed in the liver, as these enzymes are more or less specific for liver cell damage. However, damage to other tissues, such as the intestine, could also be a source for one or more of these enzymes. The increase in total bilirubin after 24 h in two of the exposed animals might indicate that the liver function is reduced. The reason for the marked increase in triglycerides in two of the exposed minipigs is not readily explained but some disturbance in the pancreatic function or hyperadrenocortisism ("stress") might be possible causes.

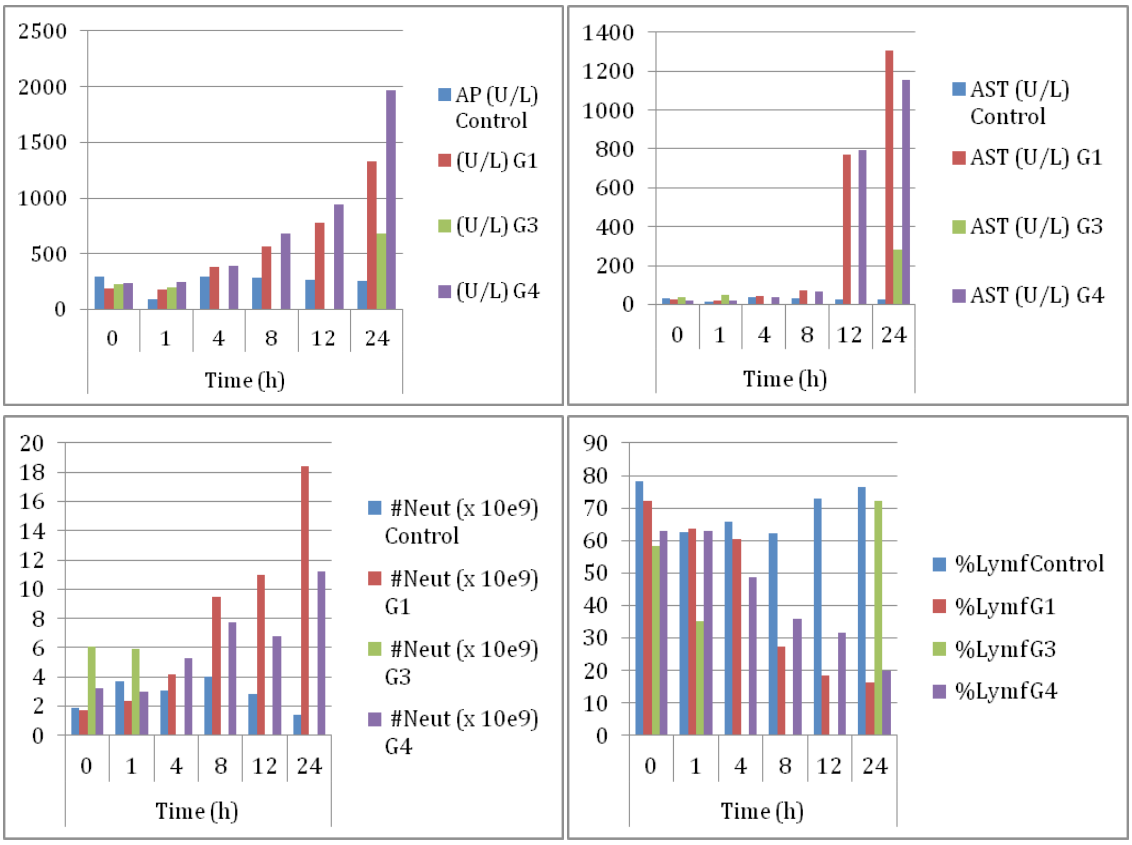


Figure 35. Levels of enzymes detected in minipig samples over the 24 h exposure period. (G3, was the animal with the hematoma).

7.4. Validation of method for pig feed tissue extraction and analysis

7.4.1. LC-MS/MS matrix effects

Pig tissues that were free from AZAs were extracted and spiked with AZA standards. Different types (20) of pig tissues were extracted using a double methanolic extraction to investigate the extent of matrix effects using LC-MS/MS. Results for tissues that were found to give recoveries that were outside the limits of 100±15% were deemed to be enhanced or suppressed due to matrix effects. The tissues types that were worst effected by matrix effects (enhancement or suppression), were the buffy layer, brain tissue and jejunum (Figure 36).

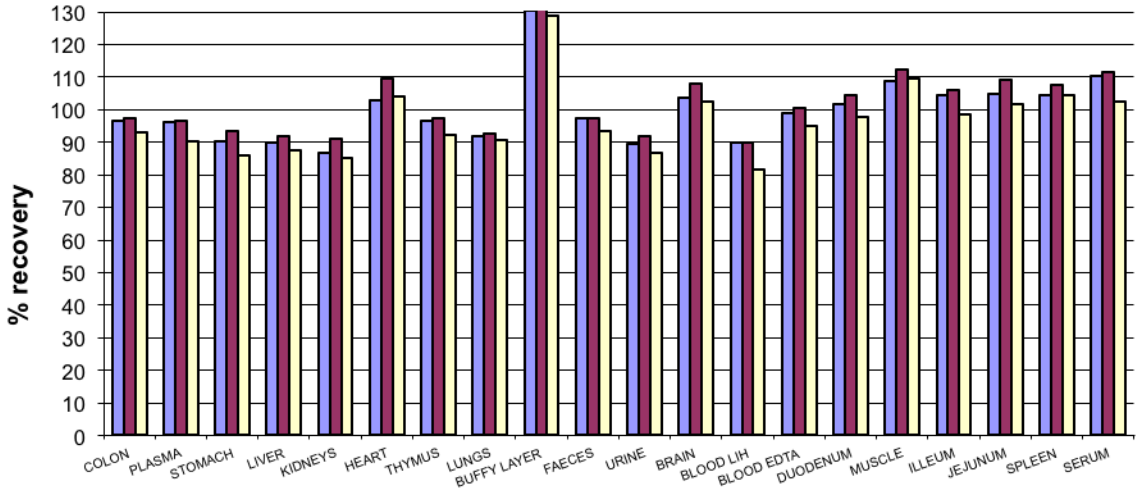


Figure 36. LC-MS/MS matrix effects for AZA1, -2 and -3 in pig tissue extracts.

7.4.2. Toxin extraction from animal tissues

Four different extraction methods were evaluated (Figure 37) using the brain tissue; a double methanolic extraction; double methanolic extraction with an ethyl acetate/water cleanup; double methanolic extraction with an SPE cleanup and an acetone extraction with an ethyl acetate/water cleanup. The acetone extraction with an ethyl acetate clean up proved to give the least amount of matrix interferences. This extraction technique also has the advantage of not producing AZA methyl derivatives.[68]

The acetone extraction was then assessed for extraction efficiency. The first two extractions removed ~95% of AZA equivalents.

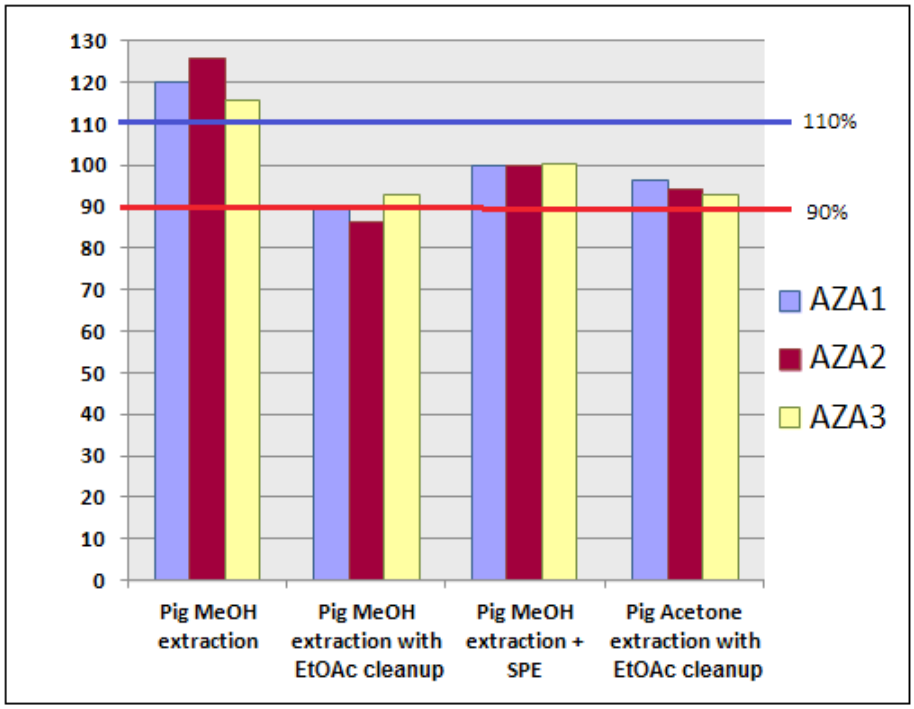


Figure 37. LC-MS/MS matrix effects for AZA1, -2 and -3 using different extraction techniques.

7.4.3. Toxin extraction from wet blood

A method was developed for the extraction of AZA1 from spiked mouse plasma that yielded recoveries of ~100%. A similar method, employing acetonitrile followed by ethyl acetate extractions (three extractions; combining/drying supernatants) and a reverse-phase SPE clean-up was applied to AZA1 spiked mouse whole blood giving recoveries of ~100%. This method was used to extract AZAs from the minipig blood samples (see Section 7.5.2).

Attempts to develop an extraction protocol for use with dried blood spots (i.e., blood spot cards), were only moderately successful, with maximum efficiencies of 50–70% achieved. Future efforts should investigate use of protein digests (e.g., pepsin), manipulation of physical variables (e.g., sonication), and tests of additional solvent systems.

7.5. Tissue analysis

7.5.1. LC-MS/MS analysis

7.5.1.1. Initial trial

Triplicate analysis was performed on each tissue type (~2 g tissue) using a double acetone extraction with a triple ethyl acetate/water partition. The organic layer was dried under nitrogen and re-suspended in methanol for LC-MS/MS analysis.

Twenty five tissue samples were analysed for fourteen AZA analogues, AZA1–12, AZA17 and -19. AZAs were found in many tissues of the mini pig indicating that AZAs entered the body and were absorbed into the blood stream. Interestingly, relatively small quantities (<0.75 ng/g) of the known AZA analogues were found in the faeces suggesting that absorption and metabolism had occurred. (Figure 38).

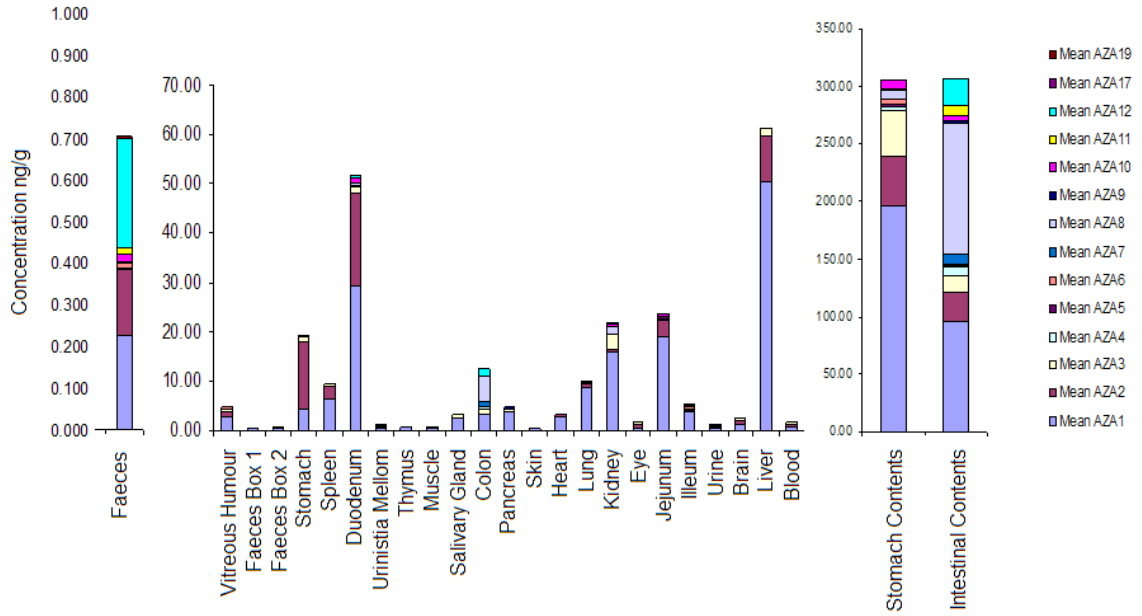


Figure 38. Concentration of AZAs in mini pig tissues from initial trial.

The mini pig in the initial trial displayed no AZA related symptoms (nausea, vomiting, diahorrea, etc). The information gathered from the trial was used to define the levels for the second trial and it was decided to use a triplicate dose at a higher AZA concentration, instead of three incremental doses.

7.5.1.2. Second trial

The second mini pig trial used 4 mini pigs (3 dosed and one control). Due to complications with the catheter used for blood collection, Pig 3 did not consume the AZA contaminated feed as quickly as Pig 1 or Pig 4 (Pig 2 was the control).

AZAs were distributed throughout the tissues of the GI tract. After 24 h there was absorption of AZAs into the stomach (Figure 39) and to a lesser extent, the intestines (Figures 40 and 41). AZA1 and -2 hydroxylates were found in the colon contents. These hydroxylates could be due to the alkaline conditions found in the small intestines and colon.

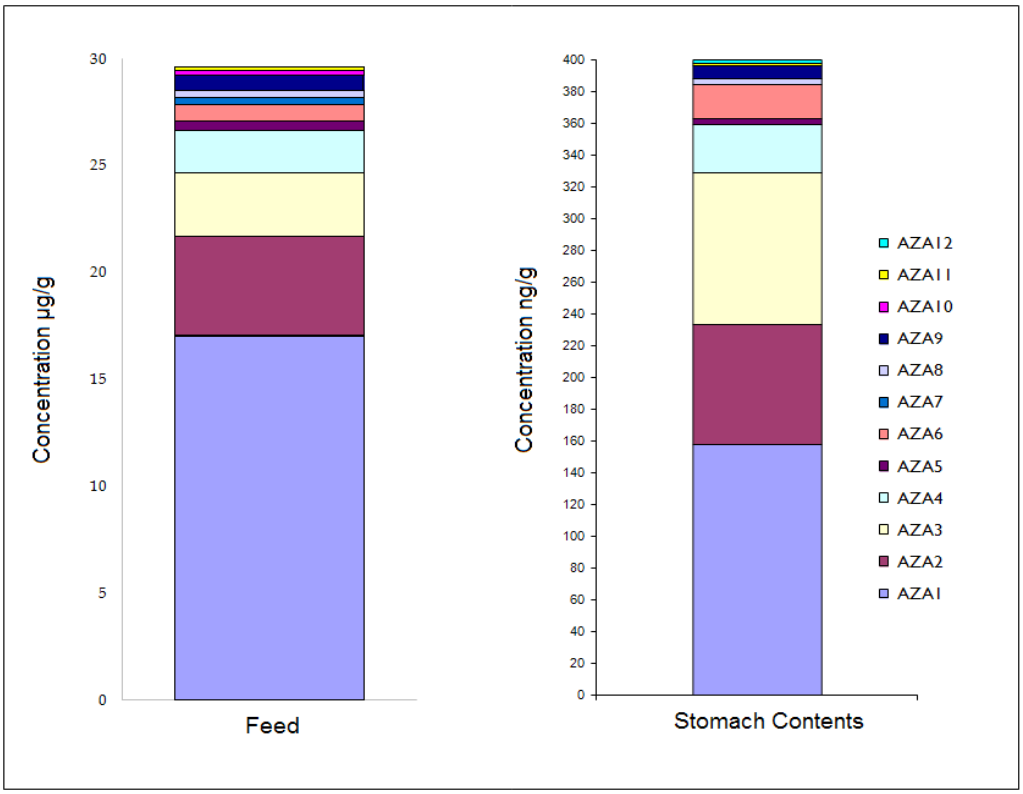


Figure 39. Concentration of AZAs in the feed and in the stomach (following 24 h).

AZAs found in other tissues indicate that AZAs have crossed the intestinal barrier, entered the bloodstream and have been transported throughout the body (Figure 41). The total AZA concentration recovered from the tissues was 14% of the administered dose. This indicates that the AZAs are metabolised or degraded in the process of digestion.

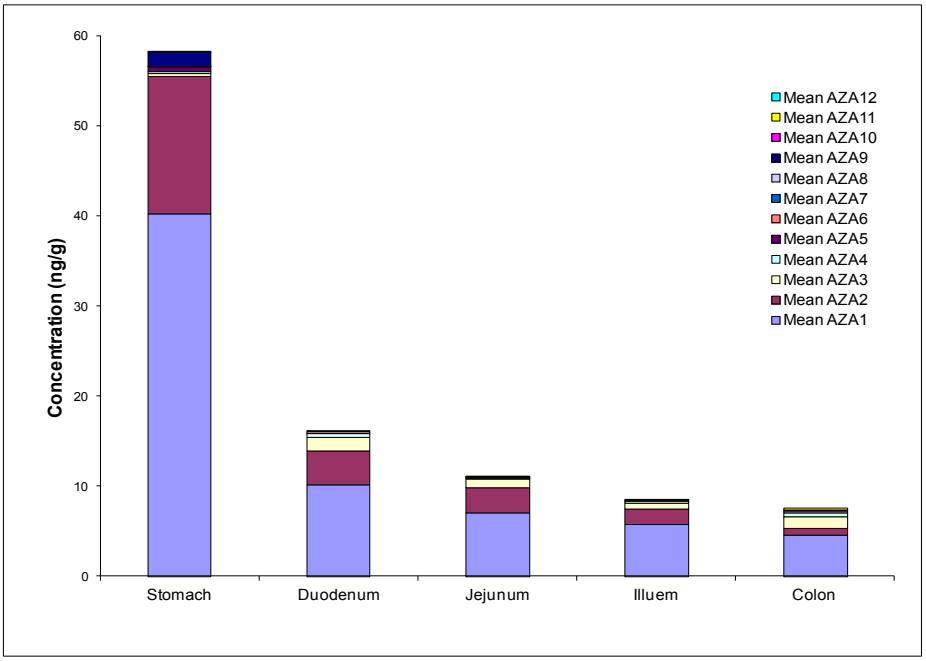


Figure 40. Concentration of AZAs in the tissues of the GI tract.

Liver samples also showed a relatively high concentration of AZA1, -2 and -3 (~75 ng/g total), but interestingly, only showed very low concentrations of the other AZA analogues (Figure 41).

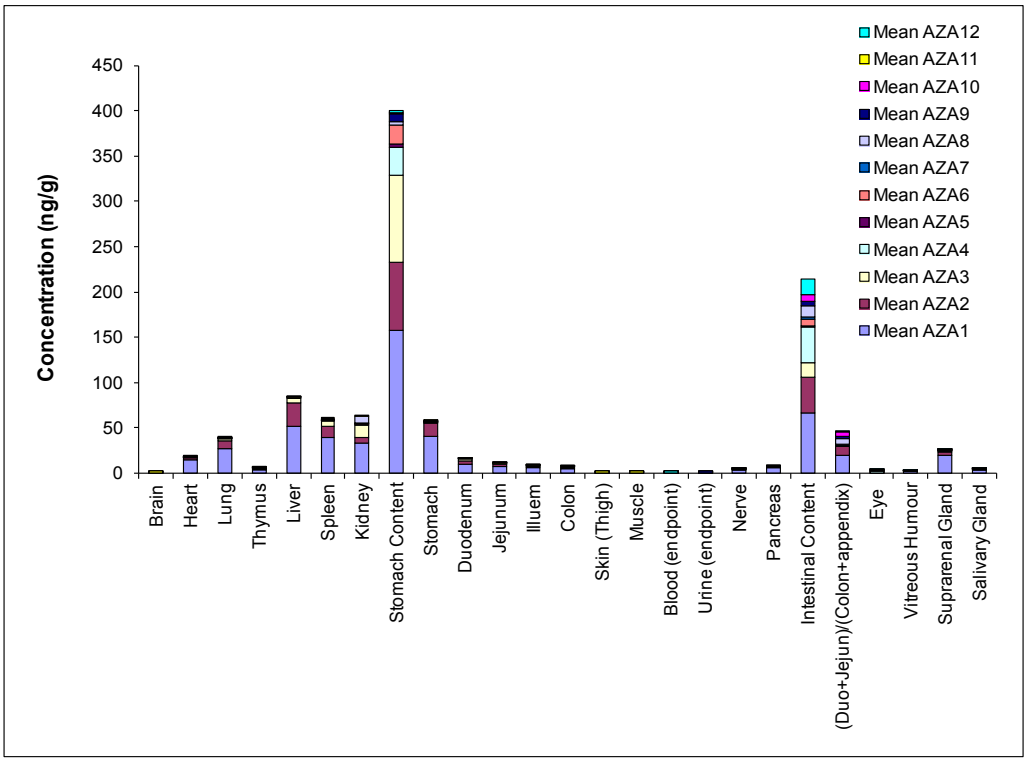


Figure 41. Concentration of AZAs in pig tissues from the second pig trial.

The second trial contained animals dosed at the same level so it is possible to obtain information on the intra animal variability. Pig 1 and Pig 4 show good correlation for the majority of tissue types tested but due to the complications with Pig 3, there was a time delay with absorption of the AZAs. This can clearly be seen from the lower concentration of AZAs obtained (Figure 42).

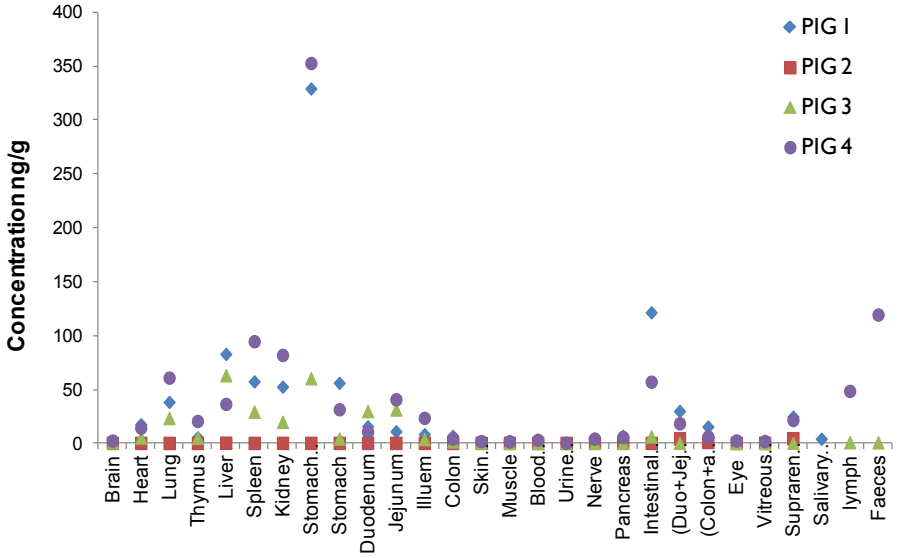


Figure 42. Comparison of total AZA concentration in each of the four mini pigs per tissue.

7.5.2. Blood analysis

Complete, 24 h time course results were obtained from only two of three experimental animals due to sampling issues with the third. Interestingly, there was a clear difference between these two minipigs in the overall pattern of AZA uptake and elimination (Figure 43). One animal (Pig 1) showed a very rapid appearance of AZAs in the blood within 0.5 h (0.44 ng/mL AZA1–3), whereas the other (Pig 4) showed less than 20% of this amount (0.08 ng/mL AZA1–3) by the 1 h time point. Moreover, the highest AZA1–3 concentrations in the blood was attained within 3 h in the former, but not until 2 h later (i.e., 5 h) in the latter minipig. Notably, the peak concentration in both animals was ~3- to 4-fold higher than that recorded in the pilot study (from one pig).

AZA1:AZA2 ratios were elevated initially (~5 to 6) compared to that measured in the contaminated feed (feed = 3.7) (Figure 43), suggesting that AZA1 was taken up faster into blood at the outset. Although the AZA1:AZA2 ratios declined to levels at or slightly below that in the feed as highest concentrations were reached, these ratios increased over the remainder of the experiment, indicating that either AZA2 was eliminated at a faster rate than AZA1, or AZA1 was remobilized into blood from other tissues.

Compared with the other two analogues, AZA3 entered the blood at levels far below its availability in the feed. In terms of elimination, both animals showed a generally consistent loss (albeit much slower than the uptake rate) of toxin after the highest levels were reached followed by a more gradual decline, with measurable levels of AZA remaining in the blood at the final 24 h time point (~2–3 ng/mL AZA1–3).

The differences in AZA uptake observed between these two minipigs likely reflected inter-animal variability; however, the overall pattern for Pig 4 was most similar to that described for the pig used in the pilot study.

Preliminary toxicokinetic/toxicodynamic modelling was performed, taking into consideration the exact dosage and starting ratios of the AZA analogues. Using a one compartment first order uptake/elimination model, it was estimated that the rate of AZA entry into the blood was ~10-fold the rate of toxin elimination, with a half-life of elimination from blood of ~12–24 h.

Overall, AZAs are taken up into the minipig blood rapidly but at different rates: AZA1 > AZA2 > AZA3. Importantly, this is the same order of relative potency (*ip* LD_{50}) determined in the ASTOX 2 project (*in vivo* mouse), implying that the most toxic analogue (AZA1) is taken up more rapidly and remains elevated longer in the bloodstream than less toxic AZA2 and -3. It remains to be determined whether this results in preferred delivery of AZA1 to other tissues and contributes to its higher relative potency.

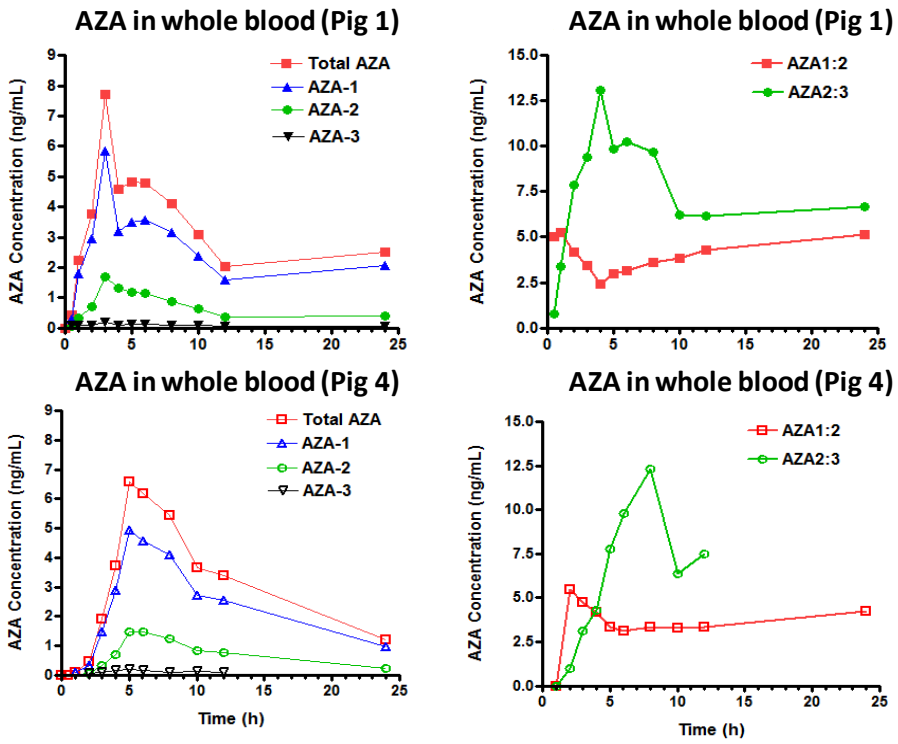


Figure 43. Time course of individual AZAs and AZA_{total} (left panel), and ratios of AZA analogues (right panel) in minipig blood following exposure to contaminated feed. Results are shown for animals G1 (top graphs) and G4 (bottom graphs).

7.6. Digestive simulation study

This study investigated what happens to AZAs when they are exposed to the chemical and physical conditions of the mammalian digestive system. The study was carried out by an *in-vitro* digestive simulation using AZA naturally contaminated mussels *(Mytilus edulis)*, which mimics what is actually consumed as part of a meal. The investigation used the change in concentration of AZA1, -2 and -3 to indicate how AZAs change or degrade throughout the digestive process when they are consumed. The difference between cooked and uncooked mussel tissue was also investigated. Digestive simulated fluids (salivary, gastric and intestinal) were prepared in accordance with Naim *et al*, 2004.[101]

The three stage digestion simulation (salivary, gastric and intestinal) was monitored at eight time points and each time point was represented by three samples. To prepare the samples, aliquots of homogenised shellfish tissue (2 g) were weighed into 50 mL polypropylene centrifuge tubes. The 24 samples to be cooked were placed in a closed centrifuge tube and placed in a water bath at 90 °C for 10 min.

Three of each of the cooked and uncooked homogenates were extracted (double methanolic extraction) at t=0. Artificial saliva (AS) (2 mL at 37 °C) was added to two of the 2 g portions of homogenised cooked and uncooked tissue samples and the samples slowly shaken for 1 min to simulate mastication. Simulated gastric juices (SGF) (16 mL) were then added to 18 homogenised cooked and uncooked tissue samples that had undergone the AS addition. These 36 samples were then placed in an incubated shaker for 1 hour at 37 °C with gentle shaking. After 1 hour, three cooked and uncooked samples were removed. The remaining 30 samples were left to incubate at 37 °C for another 1 h to mimic an overall 2 h gastric simulation. The pH was adjusted to 8 for twelve of the remaining samples with 2 M NaOH. The simulated intestinal fluids (SIF) were added and allowed to incubate at 37 °C for 4 h with three samples being removed for analysis every h.

Once the allotted time for each sample was reached, the reaction was stopped by placing the sample in an ice bath. The sample was centrifuged at 4500 rpm for 5 min at 4 °C and the supernatant decanted into a centrifuge tube. The pH was adjusted to 7 and extracted three times with 5 mL portions of ethyl acetate. The ethyl acetate layers were combined and dried under nitrogen. The samples were re-suspended in methanol (10 mL) and analysed by LC-MS/MS. The pellets that remained after centrifugation were extracted by double methanolic extraction before analysis by LC-MS/MS.

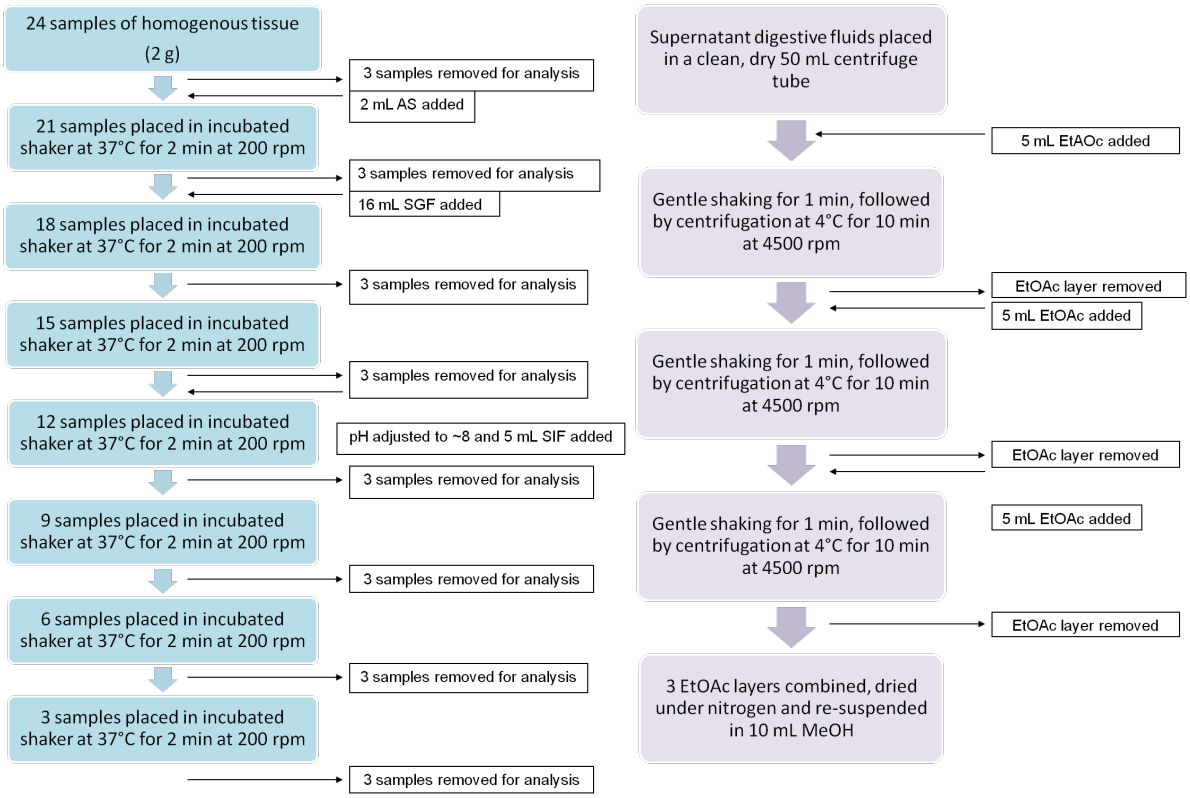


Figure 44. Schematic of the procedure for the digestive simulation.

The samples were extracted in triplicate at t=0 after cooking by double methanolic extraction. No changes were observed in the AZA1 and -2 concentrations between the cooked and uncooked tissues, while the levels of AZA3 increased by a factor of 3.6 from 0.06 µg/g to 0.22 µg/g. This increase is due to the heat induced decarboxylation of AZA17.[84]

7.6.1. Effects of gastric juices on AZA concentrations in uncooked mussels

AZA1 levels in the uncooked tissue decreased from 0.80 µg/g in the pellet to ~0.29 µg/g (Figure 45), while 36% remained in the tissue. This represents the portion that would be excreted. A further 38.6% is bioaccessible (i.e., it is available for absorption) and the remaining 25.4% may be degraded, transformed or lost through some other means. AZA2 behaved similarly with 43.7% remaining in the pellet (Figure 45). Only 30% was present in the supernatant (representing the portion that was bioaccessible). 26% of AZA2 is transformed/broken down by the digestive process. 46% of AZA3 (Figure 45) was found in the pellet, while ~54% was extracted from the supernatant.

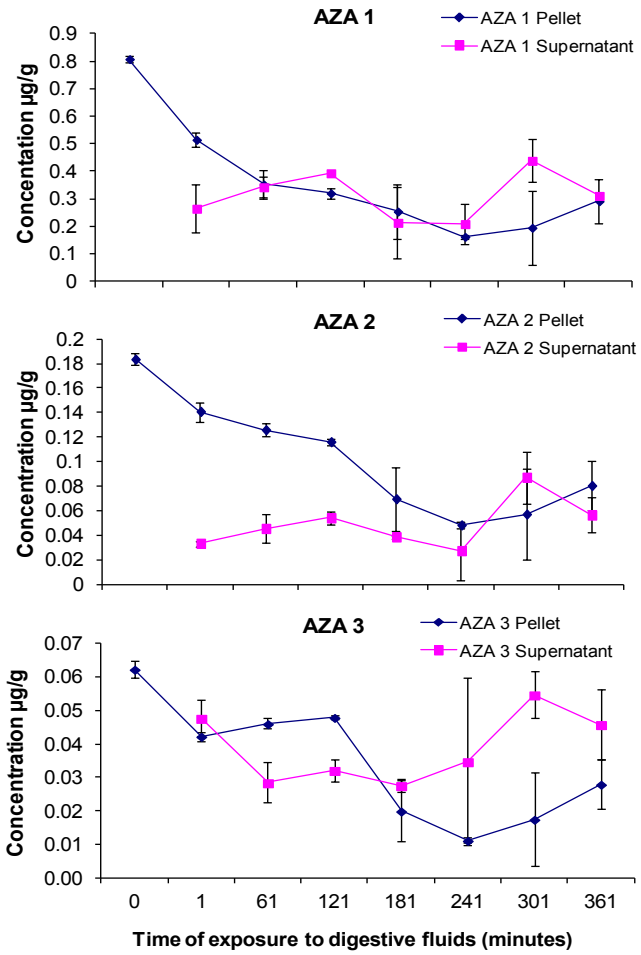


Figure 45. Concentration of AZA1, -2 and -3 remaining in the pellet (blue) and the supernatant digestive fluids (pink) at various time points throughout the digestive simulation using uncooked shellfish tissue.

7.6.2. Effects of gastric juices on AZA concentrations in cooked mussels

Following completion of the digestive simulation on the cooked tissues ~90% of AZA1, -2 and -3 remained in the undigested tissue (pellet) (Figure 46). A possible hypothesis for this is that thermal denaturation of the proteins in the mussel tissue had occurred. As proteins are heated, the hydrogen bonds responsible for the native state of the protein can be disrupted, i.e., the tertiary structure of the protein is disrupted. An interaction between the amino and carboxyl groups during heat denaturation might diminish the lyophobic or polar properties of natural protein, whereas an unfolding of the peptide chains by surface denaturation might expose lyophobic groups to the surface, which in the native state, are buried in the interior.[102] AZAs are lipophilic and weakly bound to a 45 kDa protein, it is possible therefore that AZAs are held in the tissue when cooked.[103]

It is difficult to make assumptions on the bioavailability of a toxin based on *in vitro* testing because the relationship between bioaccessibility and bioavailability is unclear. However, the bioaccessibility of AZAs, as demonstrated by *in vitro* testing, can be used as a maximum value (worse case scenario) for bioavailability. This study demonstrates that the bioaccessibility of AZAs is greater in uncooked shellfish. Ultimately, processing of mussels involving a heating step may lead to an increase in toxin levels due to interconversion of analogues of AZA and loss of water, but this heating may also render the AZAs less toxic due to inaccessibility. As a result, this may have implications for the recent evaluation of the current regulatory limit.

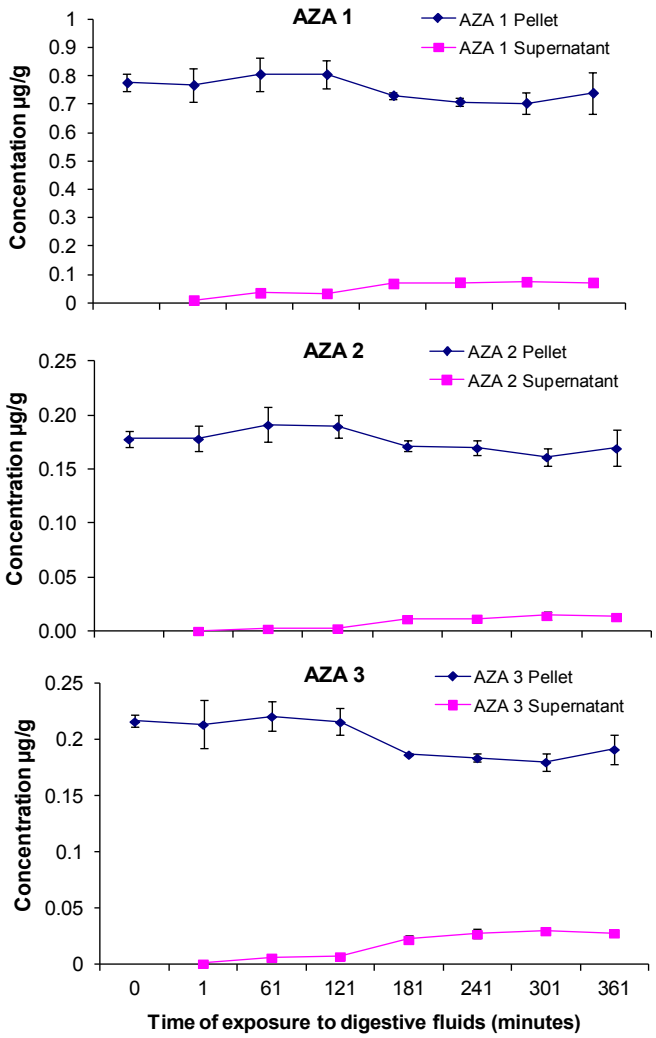


Figure 46. Concentration of AZA1, -2 and -3 remaining in the pellet (blue) and the supernatant digestive fluids (pink) at various time points throughout the digestive simulation using cooked shellfish tissue.

7.6.3. Effect of duration of cooking on availability of AZAs

The heat study was carried out on 2 g aliquots of raw mussel tissue naturally contaminated with AZA1, -2 and -3. The tissue aliquots were exposed to between 1 and 10 min of heating at 90 °C. At each of the time points the reactions were stopped by cooling the samples in an ice bath. These were then subjected to a full digestive simulation, i.e., the tissues were exposed to simulated saliva for 1 min, followed by gastric juice for 2 h and pancreatic intestinal juices for 4 h. After the allotted time all samples were cooled in an ice bath, followed by separation of the digestion fluids from the remaining pellet by centrifugation. Extraction of the pellet was performed immediately.

Following a cooking time of 2 min, <90% of AZA1 and -2 remain in the pellet (Figure 47). The pellet represents the meat that would remain in the intestines and be excreted i.e., any AZAs contained in this portion would not be available for absorption in the body. This indicates that after 2 min of cooking at 90 °C, there is a reduction in the bioaccessability of AZA1 and -2. Similarly, the graph for AZA3 (Figure 47) indicates a reduction in bioavailabilty after cooking. Figure 47 also shows an increase in AZA3 due to the cooking process due to heat induced decarboxylation of AZA17.[84]

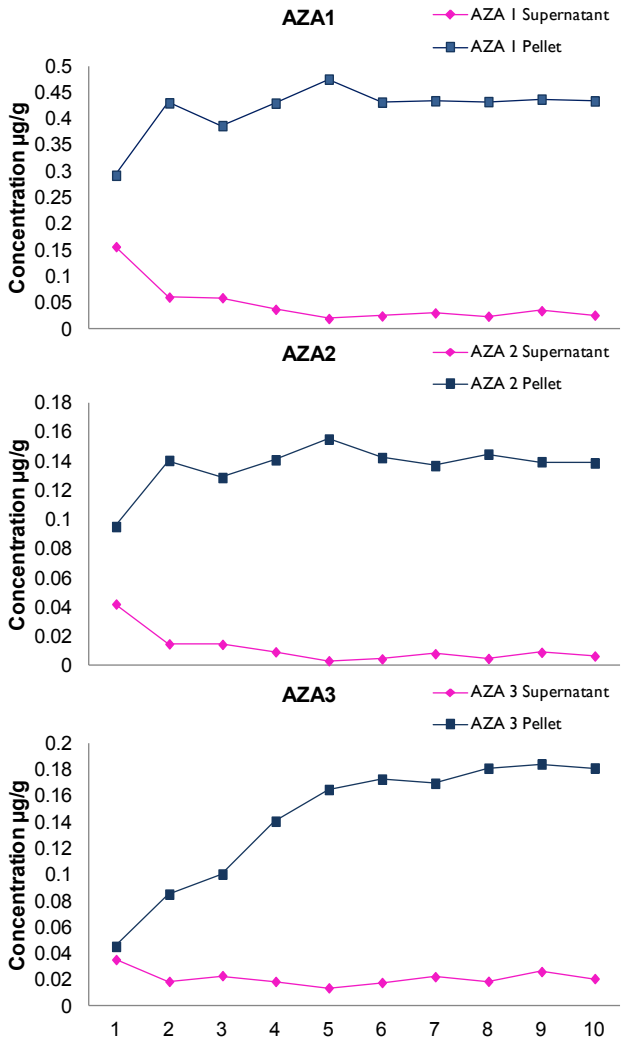


Figure 47. Concentration of AZA1, -2 and -3 remaining in the pellet (blue) and the supernatant digestive fluids (pink) at various time points throughout the heat study.

8. MOUSE *IP* STUDY

8.1. Introduction

Mice exposed to mussel extracts containing AZA via *ip* injection exhibited "neurotoxin-like" symptoms characterized by sluggishness, respiratory difficulties, spasms, progressive paralysis, and death within 20–90 min.[1,30,104]

The *ip* minimum lethal dose of partially purified AZA1 was originally determined to be 150 µg/kg[104], while from the first purified AZA1 a lethal dose was identified to be 200 µg/kg.[2] The *ip* minimum lethal doses of AZA2 and -3 were 110 and 140 µg/kg, respectively[3], suggesting higher potency relative to AZA1. These results have since been used for TEF determination and application for regulatory purposes.[32]

The more polar AZA4 and -5 (hydroxylated versions of AZA3) were less potent with lethal dose values of 470 and <1,000 µg/kg, respectively.[77]

Ip injection of mice with a lethal dose (>150 µg/kg) caused swelling of the stomach and liver concurrent with reduction in size/weight of the thymus and spleen.[105] There was vacuole formation and fatty acid accumulation in the hepatocytes, parenchymal cell pyknosis in the pancreas, dead lymphocyte debris in the thymus and spleen, and erosion and bleeding in the stomach. The pathological changes induced by AZA were stated to be unique from those induced by diarrhetic, paralytic and amnesic shellfish poisoning toxins.[105] This was an important observation at the time before the AZAs were identified as a new toxin class.

8.2. Toxicology study – AZA1, -2, -3 and -6

The LD_{50} of AZA1, -2, -3 and -6 toxins was estimated by using both response surface analysis and survival analysis. Female NMRI mice were acclimatised for a week prior to the trial. Experiments were performed at the NSVS. Mice between 15 and 22 g were used and given adjusted dosages for 20 g mice. The first level dose was administered to 3 mice, second level to 5, third level to 7 and fourth level to 9 mice.[106] Between each dosage level the numbers of dead and live mice were used for adjustment of the dose for the next step.

Clinical observations showed distinct symptoms; jumping, cramps and heavy breathing 3–5 min prior to death at the highest dosages. At the lower doses the animals survived but appeared to be ill; sweating and moving slowly, before recovering again.

AZA1 gave an unsuspected result in that the lethality dosage described in the literature had to be reduced (from 200 µg/kg bw to 74 µg/kg bw). The initial dosage for AZA2 was set at 110 µg/kg bw and the LD_{50} was found to be 117 µg/kg bw. The starting dosage for AZA3 was 140 µg/kg bw with the LD_{50} determined to be 164 µg/kg bw (Table 18).

Table 18. Data on *ip* LD_{50} on NMRI-female mice and comparison with data currently applied.

Compound	*Present		New study	
	LD_{50} (µg/kg)	TEF	LD_{50} (µg/kg)	TEF
AZA1	200	1.0	74	1.0
AZA2	110	1.8	117	0.6
AZA3	140	1.4	164	0.5
AZA6	-	-	100	0.7

*Satake *et al*[2] (using 2 male ddY mice) for AZA1. Ofuji *et al*[3] for AZA2 and -3.

To confirm the results for AZA1 the experiment was repeated using male mice (Table 19).

Table 19. Data on *ip* LD_{50} on NMRI-male mice for AZA1.

Compound	New study	
	LD_{50} (µg/kg)	TEF
AZA1	75	1.0

8.3. Pathology

A range of organs were collected in formaldehyde, either at time of death, the next day (for mice that died during the night) or after 24 h (for mice that survived for 24 h). Pathological changes were evaluated in HE-stained paraffin section. Control mice were used to verify that the changes observed were associated with exposure to AZAs. The pathological changes were similar for both AZA1 and -6.

-Liver: In the liver, single-cell necrosis (apoptosis) was observed in single cells multifocally or in small groups of cells. A few neutrophils were detected in relation to the necroses (Figure 48). There were a reduced number of mitotic figures in the liver tissue of mice exposed to AZA1 or -6.

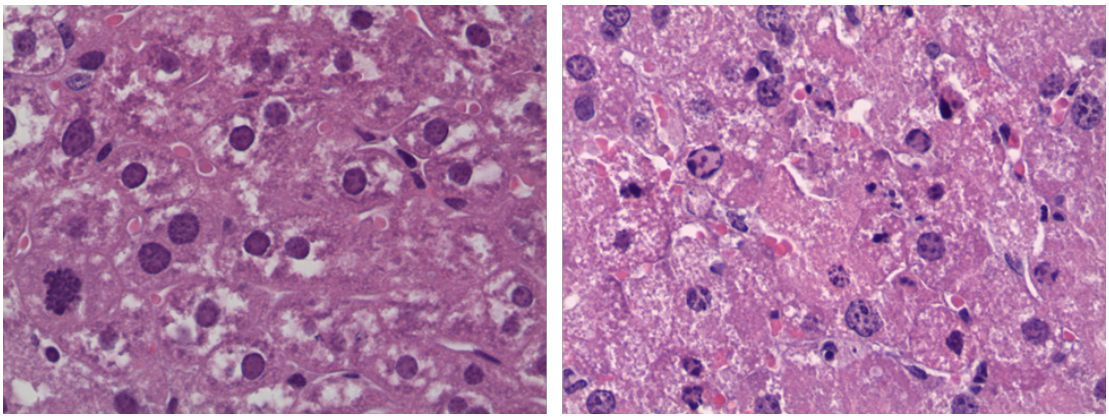


Figure 48. A) Liver, control. Mitotic cell bottow left, × 40 and B) Liver, AZA6. Apoptosis: cells with apoptotic nuclei.

-Thymus: An increased number of apoptotic bodies were observed in all areas of the thymus. In the controls, some apoptosis occured, but significantly less than in AZA exposed animals (Figure 49).

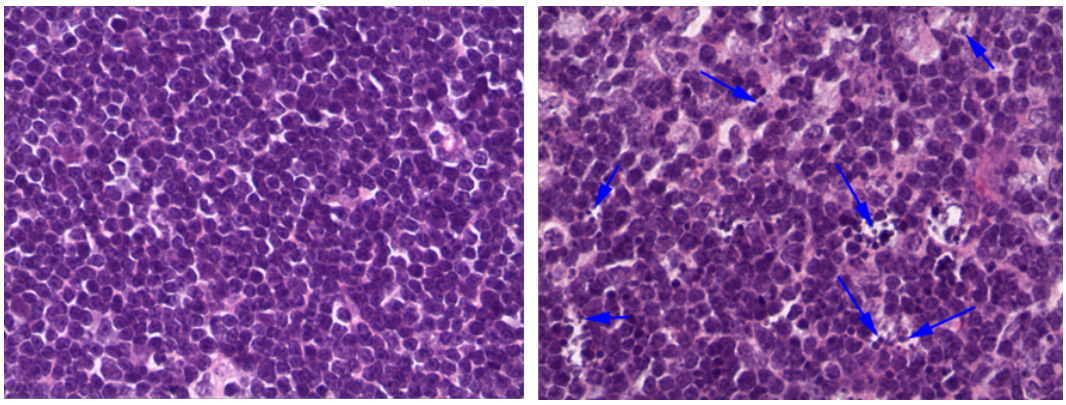


Figure 49. A) Thymus, control. Low apoptotic activity, × 40 and B) Thymus, AZA6. Many apoptotic cells (arrows).

-Spleen: In the lymphoid tissues of the spleen, an increased level of apoptotic activity was observed (Figure 50).

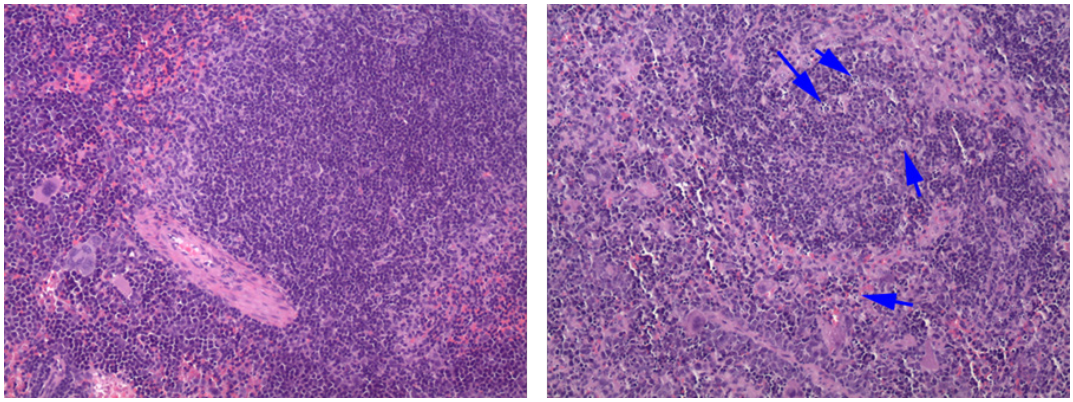


Figure 50. A) Spleen, control. Normal lymphoid tissue, × 10 and B) Spleen, AZA6. Increased apoptosis (arrows).

-Intestine (duodenum, jejunum): In lymphoid tissues and in the lamina propria, exposure to AZAs resulted in some increased apoptosis. There was also single-cell necrosis/apoptosis in epithelial cells, both in the tips of villi and in crypts/glands deep in the mucosa. Additionally, there was accumulation of dead cells and possibly neutrophils in the crypt abscesses (Figure 51).

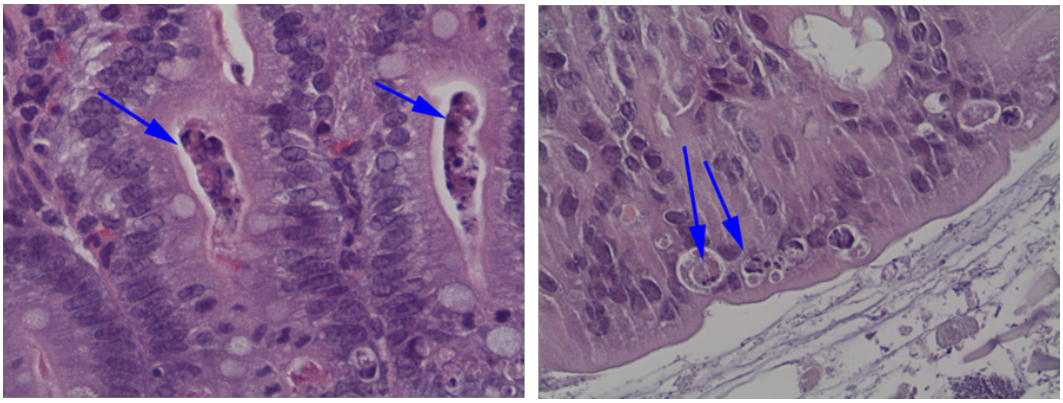


Figure 51. A) Duodenum, AZA1. Cryptabscesses, × 40 and B) Duodenum, AZA6. Apoptosis of epithelial cells.

Heart muscle, lungs, kidney, skin, skelatal muscle, stomach and brain did not show any obvious changes in mice exposed to AZA1 or -6 by *ip*.

8.4. Tissue analysis (LC-MS/MS)

The tissue samples were doubly extracted with 100% methanol. Organs (~0.2 g) were extracted with methanol (1 mL) initially by mashing with a steel rod followed by vortex mixing for 1 min. Samples were centrifuged at 3,000 *g* for 5 min and the extracts decanted into pre-weighed

centrifuge tubes. The remaining pellet was extracted a second time as described above. The extract was combined with the extract from the first extraction, with the weight of the extract and centrifuge tube being recorded. Extracts were filtered through a glass pipette plugged with cotton wool into HPLC vials for analysis by LC-MS/MS. Matrix effects were assessed on two instruments; a Waters 2795 coupled to a Micromass time of flight (QTof) Ultima using an acidic mobile phase in both isocratic and gradient modes; and on a Waters 2695 coupled to a Micromass triple stage quadrapole (TSQ) using the same acidic gradient method employed on the QTof. Previous studies have shown that matrix effects can differ between instruments.[85] Both matrix suppression and enhancement were observed depending on the tissue type, with the most significant interferences occurring on the TSQ instrument (Figure 52). The least amount of interferences were obtained using the isocratic method on the QTof. Hence this method was used to quantitate the toxin concentrations in the various tissues.

AZAs were detected in all tissues, with low levels recorded in the brain, urine and some of the feaces samples. The highest levels were detected in the lung, liver, spleen and kidney (Figure 53). Recoveries ranged from 12–37%, with the exception of AZA3 (2.8 µg dose, mouse still alive following 24 h) which gave a recovery of 2%. This may be due to the fact that AZA3 is significantly less stable than AZA1 and -2[12] and may therefore be more prone to metabolic processes.

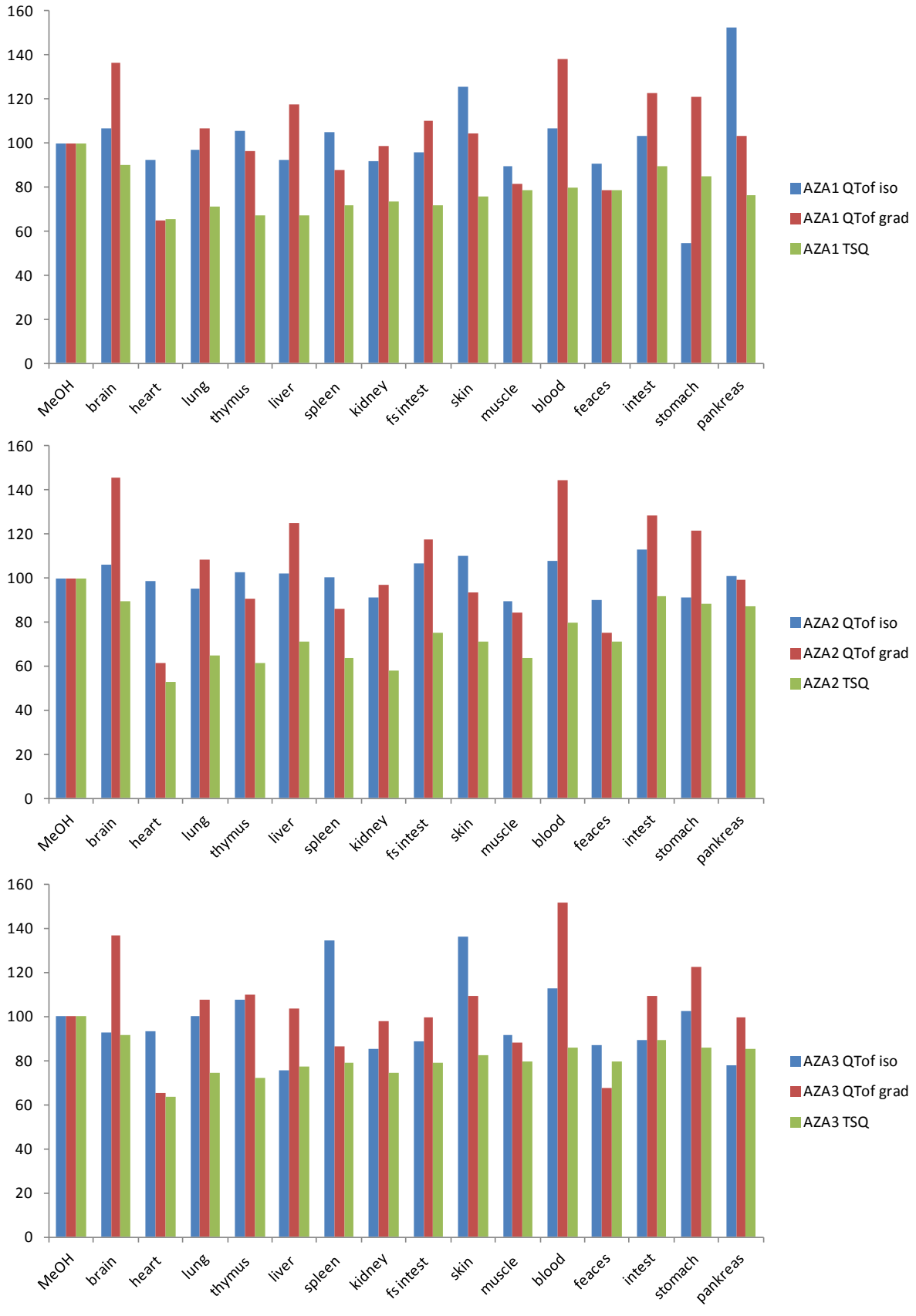
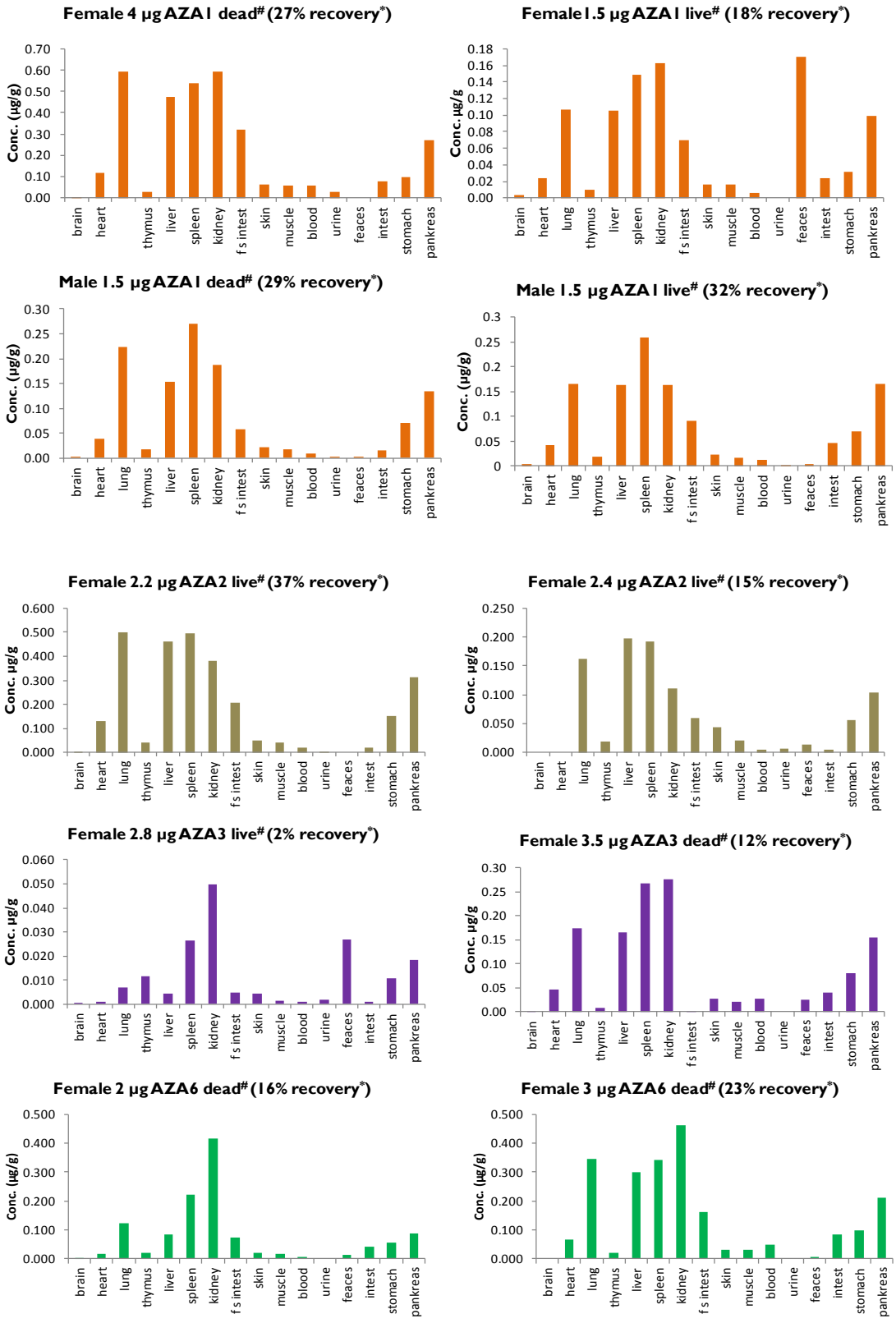


Figure 52. Matrix effects observed for AZA1, -2 and -3 in mouse tissues (SSR 10).



#condition of mouse (live or dead) following 24 h.
* % of toxin detected in tissues relative to dose amount.

Figure 53. Distribution of toxins in mouse *(ip)* tissues.

9. Mouse oral study

9.1. Introduction

Due to the lack of purified AZAs, limited studies on their acute oral toxicity have been performed. Previous studies focused only on AZA1. These studies found that the GI tract, liver, spleen and thymus were the main organs affected.[21–24,29,107]

In the original studies, crude extracts of AZA1 (>900 µg/kg) were given orally to mice via gastric intubation.[105] Mice receiving moderate oral doses of AZA1 (100–300 µg/kg) generally display little to no signs of toxicity.[22,23] However, mice surviving higher doses (420–780 µg/kg) developed dose- and time-dependent signs and symptoms of toxicity (i.e., depression and reduced movement) but without indications of diarrhea. After 24 h, all surviving mice were greatly depressed and did not move from sternal recumbency (i.e., remained lying down on chest).[29] Similarly, many mice receiving low (20 or 50 µg/kg), repeated (>30) doses of AZA1 exhibited extreme weakness that necessitated them being sacrificed in order to prevent further suffering. Repeated doses of 1 or 5 µg/kg did not induce weakness or illness.[24]

At 6 times the *ip* injected dose that induced 100% mortality, all mice survived with no clinical signs after 24 h. Subsequent studies using purified material have further detailed the effects of AZA1 on mice following oral exposure.[21–23,29] Studies by Ito *et al.*[21] using male ICR mice orally administered single doses of AZA1 ranging from 300 to 900 µg/kg demonstrated the lethal nature of AZA1, whereby all mice receiving 900 µg/kg AZA1 had to be sacrificed prior to the end of the 24 h experiment. Although there was not a clear dose-response, likely due to insufficient experimental replicates, an approximate oral minimum lethal dose with purified AZA1 was estimated at 500 µg/kg. Similarly, separate studies by Aasen *et al.*[22] and Aune *et al.*[29] using female NMRI mice demonstrated that doses of 100–540 µg/kg were insufficient to kill any of the tested animals but doses above 600 µg/kg resulted in some mortality. The experimentally determined LD_{10} and LD_{50} levels (with 95% confidence intervals) were 570 (435–735) and 775 (596–1,055) µg/kg, respectively.[29]

In another series of *in vivo* exposure studies, mice were orally administered low, repeated doses of AZA1 (1–50 µg/kg) and then monitored for recovery.[24] Ten percent of the mice survived the 40 repeated injections at the highest dose (50 µg/kg), as the other 90% were sacrificed due to extreme weakness, and 30% of the mice in the 20 µg/kg treatment group were also sacrificed early.

In separate experiments, severe injuries were induced by two repeated doses of 250, 300, 350, or 450 µg/kg, two days apart, and recovery was monitored for up to 90 days. Of the 16 mice receiving 450 µg/kg, 11 died prior to the second dose, suggesting a revised minimum oral lethal dose of <450 µg/kg. In fact, some mice died at 250 and 300 µg/kg, but only two replicate mice were available for each dose.

The most common pathological effect of AZA1 following oral exposure is degradation of the lining surrounding the upper small intestine.[21,22,24,29] Single dose studies in mice examined 24 h following oral intubation of AZA1 (100–300 µg/kg) clearly demonstrated shortened villia, elongated crypts, and exfoliation of epithelial layer in villi along the lumen of the duodenum.[22] In addition, there were indications of edema, hyperemia, infiltration of neutrophils (>5-fold more than controls), and single cell necrosis/apoptosis with apoptotic bodies in the lamina propria.[22,29] These pathological effects were dose-dependent but were completely healed after 7 days recovery.

Similarly, Ito *et al.*[21] observed sporadic degeneration and erosion of the small intestinal microvilli, vacuole degeneration in epithelial cells, and atrophy of the lamina propria at 4 h following exposure to 300 µg/kg AZA1. Higher doses of AZA1 (500–700 µg/kg) revealed progressive intestinal erosion at 8 h and continued atrophy of the lamina propria at 24 h. However, at 24 h there were fewer degenerating epithelial cells in the microvilli, suggesting some signs of recovery. Compared to mice orally exposed to OA, the damage elicited by AZA1 was slower in onset with much longer times required for recovery.[21]

Despite known uptake and systemic distribution of AZA1 following oral exposure, only limited and less severe histopathological changes were observed in other internal organs/tissues. The stomach, which has consistently been shown to contain the highest AZA concentrations following oral exposure[22], was pathologically normal. However, there was a dramatic increase in food retention suggesting localized constipation.[22,29] Moderate doses of AZA1 (100–300 µg/kg) resulted in the liver being abnormally pale in coloration[29], which may be the result of fatty acid droplet accumulation.[21] Higher doses (500–700 µg/kg) increased liver weight by 38%. There were time- and dose-dependent effects on the number of necrotic lymphocytes in the thymus, spleen, and the Peyer's patches of the small intestine, which was supported by quantitation of the number of non-granulocytes (lymphocytes, monocytes, macrophages) in the spleen. AZA1 treatments of 600 and 700 µg/kg resulted in a 33% decrease in the number of non-granulocytes, which were primarily T and B lymphocytes.[21] There were no reported histological changes associated with the kidney, heart, lung, and brain.[21,22,29]

9.2. Toxicology study – AZA1, -2, -3

9.2.1. Experimental method

Female CD-1 mice (18–20 g) were acclimatized for 2 weeks prior to the trial. Experiments were carried out at the University of Trieste. AZA1, -2 and -3, dissolved in PBS pH 7.0 containing 1.8 % ethanol, were administered by gavage to groups of 8 mice fasted for 3 h (administered volume: 10 mL/kg) at the following dose ranges: 135–1,100 µg/kg for AZA1; 300–1,100 µg/kg for AZA2 and -3. Control mice were administered with the vehicle alone (10 mL/kg). Signs of toxicity and lethality were recorded for 24 h (time of sacrifice), and up to 14 days for subgroups of surviving mice. After 24 h, animals were weighed and anesthetized by *ip* injection of tiletamine/zolazepam (Zoletil®; Milan, Italy; 20 mg/kg) and xylazine (Virbac, Milan, Italy; 5 mg/kg). Blood samples were collected from the abdominal aorta for hematochemical determinations (AST; ALT; GLDH; CPK; creatinine; sodium ions; potassium ions; calcium ions; chloride ions; inorganic phosphate (P_i)). Animals were then exsanguinated and the main organs were removed and fixed in neutral buffered 10% formalin for the histological evaluation. Similarly, animals that died during the observation period were immediately weighed and necropsied; the main organs and tissues were removed and fixed for the histological evaluation, while blood was collected for hematological analyses. The LD_{50} values based on 24 h mortality data, were calculated according to the Finney method[108] at a 95% CL.

9.2.2. Lethality

At 24 h, lethality was noted starting at 230 µg/kg (2/16 mice; AZA1), 500 µg/kg (3/16 mice, AZA2) and 650 µg/kg (1/16 mice, AZA3). Furthermore, only 8 mice administered with AZA1 (300–850 µg/kg) died between 24 and 48 h (Tables 20–22). At 24 h, the LD_{50} values were 443 µg/kg (95% CL: 350–561 µg/kg), 626 µg/kg (95% CL: 430–911 µg/kg) and 875 µg/kg (95% CL: 757–1,010 µg/kg), respectively. The toxic potency is AZA1 > AZA2 > AZA3 and TEFs, based on LD_{50}, are 1.0 (AZA1), 0.7 (AZA2) and 0.5 (AZA3) (Table 23).

Symptoms observed in mice treated with each toxin were immobility, tremors, abdominal breathing, hypothermia and cyanosis. Jumping was observed at doses ≥850 µg/kg of AZA3; no mouse developed diarrhoea. No symptoms were recorded in mice that survived for 14 days following the treatment. In these animals, food consumption and body weight were recorded daily and were similar to those of control mice.

Table 20. Lethality and survival times of mice treated with AZA1.

Dose (μg/kg)	At 24 h	After 24 h	Total	Survival times (h:min)
135	0/16	0/16	0/16	--
175	0/16	0/16	0/16	--
230	2/16	0/16	2/16	23:42-23:49
300	4/16	2/16	6/16	11:55-14:54-17:56-22:44-24:26-24:44
390	11/16	2/16	13/16	12:08-12:55-10:27-11:41-19:00-20:45-20:50-20:5121:36-22:03-22:48-26:48-29:12
500	12/16	0/16	12/16	08:30-09:26-10:16-11:49-11:54-12:44-12:56-13:33-14:12-20:46-20:47-22:54
650	5/8	2/8	7/8	08:22-08:38-09:47-10:26-10:57-23:24:15-24:23
850	5/8	2/8	7/8	00:23-00:30-09:21-10:44-13:19-25:59-26:31
1,100	8/8	0/8	8/8	00:56-01:19-01:35-03:19-03:36-06:39-08:33-09:01

Table 21. Lethality and survival times of mice treated with AZA2.

Dose (μg/kg)	At 24 h	After 24 h	Total	Survival times (h:min)
300	0/16	0/16	0/16	--
390	0/16	0/16	0/16	--
500	3/16	0/16	3/16	09:47-21:05-21:50
650	13/16	0/16	13/16	13:20-16:20-16:30-17:23-18:55-19:23-20:43-20:47-21:00-21:56-22:40-22:48-23:07
850	11/16	0/16	11/16	07:45-10:24-12:39-14:25-15:10-16:09-17:31-19:40-19:44-22:10-23:11
1,100	16/16	0/16	16/16	01:38-05:03-09:15-09:45-11:03-11:40-11:58-12:40-13:17-15:05-16:10-17:20-17:22-20:37-20:55-23:42

Table 22. Lethality and survival times of mice treated with AZA3.

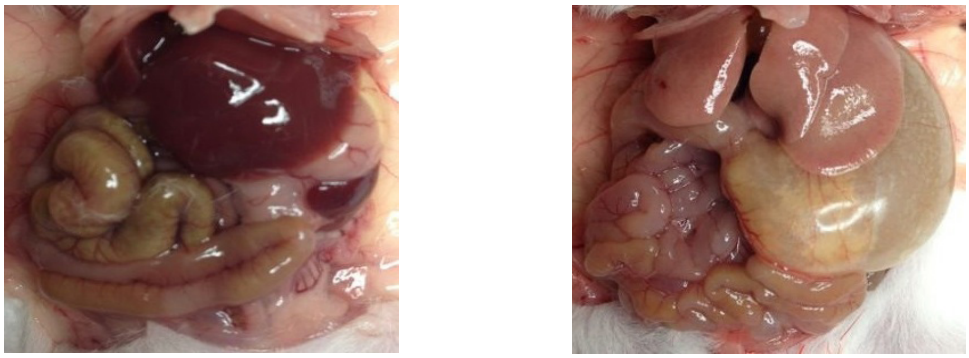
Dose (μg/kg)	At 24 h	After 24 h	Total	Survival times (h:min)
300	0/16	0/16	0/16	--
390	0/16	0/16	0/16	--
500	0/16	0/16	0/16	--
650	1/16	0/16	1/16	01:37
850	11/16	0/16	11/16	00:54-00:58-01:09-01:23-01:44-02:05-02:10-02:23-02:23-22:06-23:40
1,100	12/16	0/16	12/16	00:28-00:29-00:45-00:46-00:55-00:58-01:09-01:17-01:45-11:12-14:35-19:06

Table 23. LD_{50} of AZA1, -2 and -3 in CD-1-female mice at 24 h after acute oral administration.

Compound	LD_{50} (μg/kg)	TEF
AZA1	443	1.0
AZA2	626	0.7
AZA3	875	0.5

9.3. Pathology and hematochemistry

Necropsy showed the presence of macroscopic alterations only in spontaneously dead mice or in those sacrificed at 24 h. Pale liver was noted in mice that died more than 5 h after the treatment, or sacrificed after 24 h, at the doses ≥175 μg/kg (AZA1), ≥500 μg/kg (AZA2) and ≥650 μg/kg (AZA3). At lethal doses, increased gas and gastric content in the stomach, sometimes associated with redness of the gastric wall, was observed. Furthermore, at these doses, dilation of the small intestine and fluid content, sometimes associated with redness of the intestinal wall and bloody fluid content, were also observed (Figure 54). In mice that survived after AZAs administration and sacrificed after 14 days, gross pathology did not reveal the presence of macroscopic alterations in comparison to controls.



Control AZA1 230 µg/kg

Figure 54. Liver and GI tract of a control mouse in comparison to those observed in a mouse treated with AZA1 (230 µg/kg).

Hematochemical analysis results are reported in Tables 24–29. Mice that died within 24 h or sacrificed at 24 h showed a marked increase in serum levels of transaminases (ALT and AST) and GLDH indicating hepatic damage (also evident by necropsy). This effect was recorded at all the administered toxin doses.

AZA1 induced a 423% increase of ALT at the lowest dose (135 µg/kg) and up to 3,510% at the dose of 650 µg/kg. The increase of AST level ranged from 93% (135 µg/kg) and 1,600% (500 µg/kg), while GLDH ranged from 595% (135 µg/kg) and 7,263% (650 µg/kg).

AZA2 (300–1,100 µg/kg) induced ALT serum level increases from 376% (300 µg/kg) to 2,416% (850 µg/kg); the increase of AST and GLDH levels were in the ranges of 314–3,498 % (390–850 µg/kg) and 356–5,379% (300–850 µg/kg), respectively.

AZA3 (300–1,100 µg/kg) increased serum levels of ALT, AST and GLDH up to 487% (1,100 µg/kg), 430% (850 µg/kg) and 418% (500 µg/kg), respectively (Tables 24–26).

In addition, a moderate to marked increase in serum levels of K^+ ions was recorded in mice administered with all the three AZAs. In AZA1 treated mice, a significant increase (18 to 80%) was recorded at the dose of 500 µg/kg and above. At the same dose range, an increase from 31% to 53% was observed for AZA2, while AZA3 treated mice showed a slightly lower increase (from 11 to 22%).

No significant changes in other hematochemical parameters were recorded within 24 h after AZA administration (Tables 24–26). At the end of the recovery period (14 days) no significant differences in hematochemical parameters were recorded compared with the controls (Tables 27–29).

In conclusion, single oral administration of AZA1, -2 and -3 to mice induced lethality, with a potency AZA1 > AZA2 > AZA3: from the LD_{50} values, the relevant TEFs were 1.0 (AZA1), 0.7 (AZA2) and 0.5 (AZA3). Moreover, although macroscopic alterations were observed starting from the doses of 175 µg/kg (AZA1), 500 µg/kg (AZA2) and 650 µg/kg (AZA3), hematological examination showed significant changes in some enzymes indicative of liver injury at the lowest administered dose of each toxin (135 µg/kg of AZA1 and 300 µg/kg of AZA2 or -3).

Table 24. Hematochemical parameters of mice within 24 h after AZA1 administration.

Parameter	Controls Mean ± S.E. (n = 4)	AZA1 135 μg/kg Mean ± S.E. (n = 5)	AZA1 175 μg/kg Mean ± S.E. (n = 5)	AZA1 230 μg/kg Mean ± S.E. (n = 4)	AZA1 300 μg/kg Mean ± S.E. (n = 4)	AZA1 390 μg/kg Mean ± S.E. (n = 4)	AZA1 500 μg/kg Mean ± S.E. (n = 4)	AZA1 650 μg/kg Mean ± S.E. (n = 3)	AZA1 850 μg/kg Mean ± S.E. (n = 4)	AZA1 1,100 μg/kg Mean ± S.E. (n = 7)
ALT (IU/l)	115.8 ± 5.9	605.4 ± 106.7* *(423 %)*	1,276.6 ± 280.0* *(1002 %)*	1,815.8 ± 65.3* *(1468 %)*	2,503.3 ± 270.0* *(2062 %)*	2,342.5 ± 361.3* *(1923 %)*	3,723.8 ± 555.3* *(3116 %)*	4,181.0 ± 562.0* *(3510 %)*	3,062.0 ± 583.1* *(2544 %)*	1,945.7 ± 326.7* *(1580 %)*
AST (IU/l)	255.3 ± 49.8	492.4 ± 153.4 *(93 %)*	1,024.2 ± 190.0* *(301 %)*	1,894.5 ± 311.4* *(642 %)*	2,368.8 ± 683.1* *(828 %)*	1,921.8 ± 142.3* *(653 %)*	4,340.3 ± 422.1* *(1600 %)*	3,166.7 ± 666.7* *(1140 %)*	2,560.8 ± 659.6* *(903 %)*	1,622.1 ± 226.3* *(535 %)*
GLDH (IU/l)	30.8 ± 2.3	214.1 ± 61.1* *(595 %)*	304.8 ± 29.0* *(890 %)*	882.8 ± 162.3* *(2766 %)*	1,207.6 ± 269.3* *(3821 %)*	1,303.3 ± 424.9* *(4131 %)*	14,38.8 ± 275.7* *(4571 %)*	2,267.7 ± 293.2* *(7263 %)*	1,956.3 ± 57.4* *(6252 %)*	1,377.2 ± 303.5* *(4371 %)*
CPK (IU/l)	4,772.8 ± 1469.1	4817.8 ± 665.1 *(1 %)*	2,658.2 ± 877.8 *(- 44 %)*	1,782.7 ± 649.8 *(- 63 %)*	7,056.3 ± 833.5 *(48 %)*	6,816.7 ± 379.3 *(41 %)*	6,743.5 ± 321.0 *(44 %)*	6,871.0 ± 803.0 *(44 %)*	4,024.3 ± 1270.7 *(- 16 %)*	3,031.3 ± 627.9 *(- 36 %)*
Creatinine (mg/dl)	26.0 ± 1.5	24.2 ± 1.4 *(- 7 %)*	23.2 ± 1.4 *(- 11 %)*	24.3 ± 1.3 *(- 7 %)*	23.0 ± 1.7 *(- 12 %)*	23.0 ± 1.2 *(- 12 %)*	23.8 ± 0.9 *(- 8 %)*	23.7 ± 1.2 *(- 9 %)*	27.0 ± 2.7 *(4 %)*	28.6 ± 0.8 *(10 %)*
Ca^{2+} (mM)	3.0 ± 0.1	3.1 ± 0.1 *(3 %)*	2.9 ± 0.2 *(- 3 %)*	2.8 ± 0.1 *(- 7 %)*	2.6 ± 0.4 *(- 13 %)*	2.8 ± 0.2 *(- 7 %)*	3.1 ± 0.7 *(3 %)*	2.9 ± 0.2 *(- 3 %)*	3.1 ± 0.1 *(3 %)*	4.6 ± 1.5 *(53 %)*
Na^{+} (mM)	133.3 ± 0.6	129.9 ± 1.5 *(- 3 %)*	127.1 ± 6.7 *(- 5 %)*	134.9 ± 1.1 *(1 %)*	126.9 ± 8.6 *(- 5 %)*	130.6 ± 2.4 *(- 1 %)*	123.3 ± 5.4 *(- 8 %)*	123.4 ± 5.6 *(- 7 %)*	137.1 ± 3.5 *(3 %)*	138.5 ± 1.8 *(4 %)*
K^{+} (mM)	8.5 ± 0.6	9.3 ± 0.3 *(9 %)*	9.4 ± 1.3 *(11 %)*	9.4 ± 0.5 *(11 %)*	12.1 ± 2.2 *(42 %)*	9.4 ± 1.0 *(11 %)*	14.4 ± 0.4* *(69 %)*	13.7 ± 1.0* *(61 %)*	15.3 ± 0.4* *(80 %)*	10.0 ± 0.3* *(18 %)*
Cl^{-} (mM)	103.2 ± 0.8	101.3 ± 1.8 *(2 %)*	98.5 ± 5.1 *(- 5 %)*	104.8 ± 0.6 *(2 %)*	97.1 ± 6.5 *(- 6 %)*	90.9 ± 15.8 *(- 12 %)*	97.0 ± 3.5 *(- 6 %)*	102.5 ± 1.1 *(- 1 %)*	106.6 ± 1.7 *(3 %)*	105.6 ± 1.0 *(2 %)*
Pi (mM)	3.8 ± 0.1	4.1 ± 0.2 *(8 %)*	4.1 ± 0.4 *(8 %)*	4.4 ± 0.9 *(16 %)*	4.8 ± 0.8 *(26 %)*	4.0 ± 0.4 *(5 %)*	5.1 ± 0.7 *(34 %)*	4.5 ± 0.6 *(18 %)*	4.7 ± 0.5 *(24 %)*	4.8 ± 0.5 *(26 %)*

*p<0.05 at the analysis of variance with respect to controls; in brackets the percent difference with respect to controls.

Table 25. Hematochemical parameters of mice within 24 h after AZA2 administration.

Parameter	Controls Mean ± S.E. (n = 5)	AZA2 300 µg/kg Mean ± S.E. (n = 5)	AZA2 390 µg/kg Mean ± S.E. (n = 5)	AZA2 500 µg/kg Mean ± S.E. (n = 5)	AZA2 650 µg/kg Mean ± S.E. (n = 4)	AZA2 850 µg/kg Mean ± S.E. (n = 4)	AZA2 1,100 µg/kg Mean ± S.E. (n = 7)
ALT (IU/l)	75.6 ± 15.9	360.2 ± 118.5* *(376 %)*	361.8 ± 50.3* *(379 %)*	1,456.4 ± 513.5* *(1826 %)*	1,690.3 ± 133.5* *(2136 %)*	1,902.0 ± 176.5* *(2416 %)*	1,535.4 ± 132.6* *(1931 %)*
AST (IU/l)	155.8 ± 28.6	723.4 ± 159.2* *(364 %)*	645.6 ± 59.3* *(314 %)*	1,809.2 ± 508.3* *(1061 %)*	2,763.3 ± 386.8* *(1674 %)*	5,606.0 ± 340.9* *(3498 %)*	3,201.0 ± 629.2* *(1955 %)*
GLDH (IU/l)	24.6 ± 3.0	112.2 ± 37.5* *(356 %)*	114.6 ± 19.5* *(366 %)*	628.3 ± 211.8* *(2454 %)*	470.1 ± 51.8* *(1811 %)*	1,347.8 ± 389.0* *(5379 %)*	1,025.0 ± 113.6* *(4067 %)*
CPK (IU/l)	2,068.8 ± 429.9	3,073.8 ± 637.3 *(49 %)*	3,262.0 ± 584.8 *(58 %)*	3,301.2 ± 616.0 *(60 %)*	3,659.5 ± 947.2 *(77 %)*	3,155.8 ± 387.7 *(53 %)*	3,625.7 ± 690.8 *(75 %)*
Creatinine (mg/dl)	24.2 ± 1.2	26.4 ± 1.3 *(9 %)*	23.2 ± 0.4 *(4 %)*	22.6 ± 2.6 *(- 7 %)*	23.8 ± 1.3 *(- 2 %)*	24.3 ± 0.9 *(1 %)*	26.1 ± 0.7 *(8 %)*
Ca^{2+} (mM)	2.7 ± 0.1	2.9 ± 0.1 *(7 %)*	2.9 ± 0.1 *(7 %)*	2.7 ± 0.1 *(0 %)*	2.8 ± 0.2 *(4 %)*	2.8 ± 0.1 *(4 %)*	2.6 ± 0.1 *(- 4 %)*
Na^{+} (mM)	134.5 ± 1.6	134.1 ± 1.3 *(- 1 %)*	138.0 ± 1.8 *(3 %)*	136.5 ± 0.7 *(1 %)*	123.1 ± 7.1 *(- 8 %)*	133.1 ± 2.7 *(- 1 %)*	1,334.4 ± 1.5 *(- 1 %)*
K^{+} (mM)	6.8 ± 0.4	7.2 ± 1.6 *(6 %)*	7.6 ± 0.4 *(12 %)*	8.9 ± 0.8* *(31 %)*	10.4 ± 0.8* *(53 %)*	9.6 ± 1.0* *(41 %)*	10.0 ± 0.9* *(47 %)*
Cl^{-} (mM)	102.5 ± 1.5	102.9 ± 1.7 *(1 %)*	105.6 ± 2.0 *(3 %)*	103.2 ± 1.1 *(1 %)*	96.3 ± 3.9 *(- 6 %)*	103.9 ± 0.4 *(1 %)*	103.5 ± 1.2 *(1 %)*
Pi (mM)	3.3 ± 0.1	3.8 ± 0.4 *(15 %)*	3.6 ± 0.2 *(9 %)*	3.5 ± 0.4 *(6 %)*	4.0 ± 0.7 *(21 %)*	3.9 ± 0.4 *(18 %)*	3.5 ± 0.3 *(6 %)*

*p<0.05 at the analysis of variance with respect to controls; in brackets the percent difference with respect to controls.

Table 26. Hematochemical parameters of mice within 24 h after AZA3 administration.

Parameter	Controls Mean ± S.E. (n = 5)	AZA3 300 µg/kg Mean ± S.E. (n = 5)	AZA3 390 µg/kg Mean ± S.E. (n = 5)	AZA3 500 µg/kg Mean ± S.E. (n = 5)	AZA3 650 µg/kg Mean ± S.E. (n = 4)	AZA3 850 µg/kg Mean ± S.E. (n = 6)	AZA3 1,100 µg/kg Mean ± S.E. (n = 7)
ALT (IU/l)	124.8 ± 17.7	418.0 ± 63.2* *(235 %)*	686.0 ± 86.5* *(450 %)*	549.0 ± 130.2* *(340 %)*	378.5 ± 69.6* *(203 %)*	673.2 ± 182.9* *(439 %)*	733.2 ± 172.0* *(487 %)*
AST (IU/l)	202.0 ± 43.8	367.0 ± 19.4* *(82 %)*	632.2 ± 105.4* *(213 %)*	371.2 ± 53.7* *(84 %)*	369.5 ± 36.7* *(83 %)*	1071.2 ± 332.2* *(430 %)*	848.4 ± 230.1* *(320 %)*
GLDH (IU/l)	47.7 ± 9.9	161.3 ± 32.7* *(238 %)*	205.2 ± 51.4* *(330 %)*	246.9 ± 64.3* *(418 %)*	168.3 ± 12.7* *(253 %)*	114.7 ± 18.3* *(140 %)*	223.5 ± 70.6* *(369 %)*
CPK (IU/l)	2,583.6 ± 489.9	1,947.7 ± 351.7 *(- 25 %)*	4,103.8 ± 707.5 *(59 %)*	2,990.8 ± 626.1 *(16 %)*	3,472.0 ± 1134.7 *(34 %)*	3,357.8 ± 785.4 *(30 %)*	3,186.7 ± 828.1 *(23 %)*
Creatinine (mg/dl)	25.4 ± 0.7	24.8 ± 0.4 *(- 2 %)*	24.4 ± 1.9 *(4 %)*	24.8 ± 0.8 *(- 2 %)*	24.3 ± 0.2 *(- 4 %)*	26.8 ± 1.0 *(6 %)*	25.4 ± 0.9 *(0 %)*
Ca^{2+} (mM)	2.4 ± 0.1	2.6 ± 0.1 *(8 %)*	2.6 ± 0.1 *(8 %)*	2.6 ± 0.1 *(8 %)*	2.7 ± 0.2 *(12 %)*	2.7 ± 0.2 *(12 %)*	2.6 ± 0.1 *(8 %)*
Na^{+} (mM)	132.5 ± 1.7	132.5 ± 1.3 *(0 %)*	131.7 ± 1.1 *(- 1 %)*	131.3 ± 1.5 *(1 %)*	133.6 ± 1.6 *(1 %)*	135.6 ± 1.2 *(2 %)*	134.7 ± 2.1 *(2 %)*
K^{+} (mM)	7.3 ± 0.2	8.8 ± 0.8 *(21 %)*	8.2 ± 0.5 *(12 %)*	8.7 ± 0.6* *(19 %)*	8.9 ± 0.6* *(22 %)*	8.9 ± 0.4* *(22 %)*	8.1 ± 0.3* *(11 %)*
Cl^{-} (mM)	100.6 ± 1.8	102.6 ± 1.2 *(2 %)*	101.4 ± 0.7 *(1 %)*	99.7 ± 0.6 *(- 1 %)*	103.1 ± 1.1 *(2 %)*	103.9 ± 0.7 *(3 %)*	104.1 ± 1.0 *(3 %)*
Pi (mM)	3.0 ± 0.1	3.1 ± 0.1 *(3 %)*	3.3 ± 0.2 *(10 %)*	3.5 ± 0.4 *(17 %)*	3.2 ± 0.2 *(7 %)*	3.4 ± 0.3 *(13 %)*	3.1 ± 0.1 *(3 %)*

*$p<0.05$ at the analysis of variance with respect to controls; in brackets the percent difference with respect to controls.

Table 27. Hematochemical parameters of mice at 14 days after AZA1 administration.

Parameter	Controls Mean ± S.E. (n = 3)	AZA1 135 μg/kg Mean ± S.E. (n = 3)	AZA1 175 μg/kg Mean ± S.E. (n = 3)	AZA1 230 μg/kg Mean ± S.E. (n = 3)	AZA1 300 μg/kg (n = 1)	AZA1 390 μg/kg (n = 1)	AZA1 500 μg/kg (n = 1)
ALT (IU/l)	26.7 ± 4.6	35.3 ± 6.4 *(32 %)*	37.3 ± 8.1 *(40 %)*	43.0 ± 10.4 *(61 %)*	32.0 *(20 %)*	33.0 *(24 %)*	51.0 *(91 %)*
AST (IU/l)	118.7 ± 44.8	129.0 ± 24.9 *(9 %)*	165.3 ± 17.9 *(39 %)*	150.3 ± 8.2 *(27 %)*	146.0 *(23 %)*	98.0 *(- 17 %)*	108.0 *(- 9 %)*
GLDH (IU/l)	20.4 ± 7.8	18.3 ± 5.3 *(- 10 %)*	22.5 ± 7.2 *(10 %)*	21.5 ± 2.0 *(5 %)*	19.2 *(- 6 %)*	20.2 *(- 1 %)*	18.3 *(- 10 %)*
CPK (IU/l)	1492.7 ± 370.3	1213.7 ± 485.0 *(- 19 %)*	1903.3 ± 125.6 *(28 %)*	1547.7 ± 306.6 *(4 %)*	1907.0 *(28 %)*	1235.0 *(- 17 %)*	1276.0 *(- 15 %)*
Creatinine (mg/dl)	28.0 ± 1.2	26.7 ± 1.5 *(- 5 %)*	27.7 ± 0.3 *(1 %)*	29.0 ± 0.6 *(4 %)*	26.0 *(- 7 %)*	28.0 *(0 %)*	24.0 *(- 14 %)*
Ca^{2+} (mM)	2.5 ± 0.1	2.4 ± 0.1 *(- 4 %)*	2.4 ± 0.1 *(4 %)*	2.5 ± 0.1 *(0 %)*	2.5 *(0 %)*	2.5 *(0 %)*	2.5 *(0 %)*
Na^{+} (mM)	128.9 ± 1.3	132.0 ± 1.2 *(2 %)*	133.6 ± 2.0 *(4 %)*	133.8 ± 2.6 *(4 %)*	128.0 *(- 1 %)*	128.5 *(- 1 %)*	134.4 *(4 %)*
K^{+} (mM)	6.0 ± 0.6	6.3 ± 0.4 *(5 %)*	6.9 ± 0.8 *(15 %)*	7.2 ± 0.8 *(20 %)*	7.3 *(22 %)*	6.3 *(5 %)*	7.1 *(18 %)*
Cl^{-} (mM)	100.6 ± 1.6	104.9 ± 1.5 *(4 %)*	102.6 ± 1.8 *(2 %)*	104.5 ± 1.8 *(4 %)*	98.6 *(- 2 %)*	99.5 *(- 1 %)*	105.2 *(5 %)*
Pi (mM)	2.9 ± 0.3	2.8 ± 0.3 *(- 3 %)*	2.7 ± 0.3 *(- 7 %)*	2.9 ± 0.1 *(0 %)*	3.2 *(10 %)*	2.9 *(0 %)*	2.6 *(- 10 %)*

In brackets: percent differences with respect to controls (no significant differences at the analysis of variance).

Table 28. Hematochemical parameters of mice at 14 days after AZA2 administration.

Parameter	Controls Mean ± S.E. (n = 3)	AZA2 300 μg/kg Mean ± S.E. (n = 3)	AZA2 390 μg/kg Mean ± S.E. (n = 3)	AZA2 500 μg/kg Mean (n = 2)	AZA2 650 μg/kg Mean ± S.E. (n = 3)	AZA2 850 μg/kg (n = 1)	AZA2 500 μg/kg (n = 0)
ALT (IU/l)	112.3 ± 27.4	161.0 ± 64.0 *(43 %)*	127.7 ± 30.7 *(14 %)*	78.0 *(- 31 %)*	166.0 ± 33.7 *(48 %)*	165.0 *(47 %)*	–
AST (IU/l)	184.7 ± 46.2	266.0 ± 59.2 *(44 %)*	209.7 ± 67.8 *(14 %)*	114.0 *(- 38 %)*	221.0 ± 38.8 *(20 %)*	235.0 *(27 %)*	–
GLDH (IU/l)	31.4 ± 4.0	41.6 ± 15.2 *(32 %)*	18.7 ± 3.2 *(- 40 %)*	24.5 *(- 22 %)*	29.9 ± 4.9 *(5 %)*	34.6 *(10 %)*	–
CPK (IU/l)	1,389.3 ± 351.9	1,380.0 ± 380.8 *(- 1 %)*	1,342.0 ± 286.7 *(- 3 %)*	783.0 *(- 44 %)*	1,717.3 ± 398.9 *(24 %)*	1,405.0 *(1 %)*	–
Creatinine (mg/dl)	25.0 ± 1.2	26.0 ± 1.5 *(4 %)*	27.0 ± 2.1 *(8 %)*	26.0 *(4 %)*	28.0 ± 1.7 *(12 %)*	26.0 *(4 %)*	–
Ca^{2+} (mM)	2.8 ± 0.1	2.6 ± 0.2 *(- 7 %)*	2.6 ± 0.1 *(- 7 %)*	2.6 *(- 7 %)*	2.6 ± 0.1 *(- 7 %)*	2.7 *(4 %)*	–
Na^{+} (mM)	141.6 ± 0.9	138.9 ± 1.1 *(- 2 %)*	138.2 ± 2.3 *(- 2 %)*	140.8 *(- 1 %)*	138.7 ± 1.4 *(- 2 %)*	139.8 *(- 1 %)*	–
K^{+} (mM)	6.4 ± 0.3	6.6 ± 0.4 *(3 %)*	7.1 ± 0.5 *(11 %)*	6.4 *(0 %)*	6.5 ± 0.2 *(2 %)*	6.7 *(5 %)*	–
Cl^{-} (mM)	108.0 ± 1.2	108.7 ± 0.5 *(1 %)*	105.1 ± 1.7 *(- 3 %)*	106.9 *(- 1 %)*	106.3 ± 0.6 *(- 2 %)*	106.8 *(- 1 %)*	–
Pi (mM)	3.0 ± 0.1	2.9 ± 0.1 *(- 3 %)*	2.9 ± 0.1 *(- 3 %)*	2.8 *(- 7%)*	2.8 ± 0.1 *(- 7 %)*	3.0 *(0 %)*	–

In brackets: percent differences with respect to controls (no significant differences at the analysis of variance).

Table 29. Hematochemical parameters of mice at 14 days after AZA3 administration.

Parameter	Controls Mean ± S.E. (n = 3)	AZA3 300 μg/kg Mean ± S.E. (n = 3)	AZA3 390 μg/kg Mean ± S.E. (n = 3)	AZA3 500 μg/kg Mean ± S.E. (n = 3)	AZA3 650 μg/kg Mean ± S.E. (n = 3)	AZA3 850 μg/kg Mean ± S.E. (n = 3)	AZA3 500 μg/kg (n = 1)
ALT (IU/l)	112.3 ± 27.4	112.3 ± 42.9 *(0 %)*	187.3 ± 105.2 *(67 %)*	192.3 ± 79.1 *(71 %)*	162.0 ± 38.7 *(44 %)*	128.5 ± 17.5 *(14 %)*	85.0 *(- 24 %)*
AST (IU/l)	184.7 ± 46.2	211.7 ± 53.1 *(15 %)*	183.0 ± 40.3 *(- 1 %)*	271.0 ± 46.7 *(47 %)*	218.7 ± 47.0 *(18 %)*	209.0 ± 12.0 *(14 %)*	147.0 *(- 20 %)*
GLDH (IU/l)	31.4 ± 4.0	22.2 ± 5.7 *(- 29 %)*	35.4 ± 10.0 *(11 %)*	45.3 ± 13.1 *(44 %)*	45.9 ± 19.0 *(46 %)*	35.6 ± 11.9 *(13 %)*	23.4 *(- 25 %)*
CPK (IU/l)	1,389.3 ± 351.9	1,914.0 ± 589.6 *(38 %)*	1,491.3 ± 749.5 *(7 %)*	1,701.0 ± 268.2 *(86 %)*	769.0 ± 320.8 *(- 45 %)*	1,310.5 ± 312.5 *(- 6 %)*	998.0 *(- 28 %)*
Creatinine (mg/dl)	25.0 ± 1.2	27.0 ± 1.5 *(8 %)*	27.7 ± 0.9 *(11 %)*	28.3 ± 2.2 *(13 %)*	27.3 ± 2.2 *(9 %)*	26.0 ± 2.0 *(4 %)*	28.0 *(12 %)*
Ca^{2+} (mM)	2.8 ± 0.1	2.7 ± 0.1 *(- 4 %)*	2.8 ± 0.1 *(0 %)*	2.7 ± 0.1 *(1 4 %)*	2.6 ± 0.1 *(- 7 %)*	2.8 ± 0.1 *(0 %)*	2.9 *(4 %)*
Na^{+} (mM)	141.6 ± 0.9	136.3 ± 2.8 *(- 4 %)*	140.1 ± 1.2 *(- 1 %)*	137.0 ± 2.5 *(- 3 %)*	138.8 ± 1.8 *(- 2 %)*	138.0 ± 1.1 *(- 3 %)*	134.7 *(- 5 %)*
K^{+} (mM)	6.4 ± 0.3	7.4 ± 0.8 *(16 %)*	7.9 ± 1.1 *(23 %)*	6.9 ± 0.1 *(8 %)*	7.5 ± 0.7 *(17 %)*	7.3 ± 0.4 *(14 %)*	6.8 *(6 %)*
Cl^{-} (mM)	108.0 ± 1.2	105.1 ± 1.7 *(- 3 %)*	105.4 ± 0.8 *(- 2 %)*	105.3 ± 2.3 *(- 2 %)*	105.4 ± 0.6 *(- 2 %)*	105.7 ± 0.8 *(- 2 %)*	102.6 *(- 5 %)*
Pi (mM)	3.0 ± 0.1	2.8 ± 0.3 *(- 7 %)*	2.8 ± 0.1 *(- 7 %)*	2.9 ± 0.2 *(- 3 %)*	2.6 ± 0.2 *(- 13 %)*	3.1 ± 0.1 *(3 %)*	3.2 *(7 %)*

In brackets: percent differences with respect to controls (no significant differences at the analysis of variance).

9.4. Tissue analysis (LC-MS/MS)

AZA1, -2 and -3 mouse tissues for each dose were extracted and analysed as outlined in section 8.4. Preliminary results (n=2) show that AZAs were detected in all tissues with very low levels or none at all detected in the brain. The results for the dose of 300 µg/kg are shown in Figure 55. A preliminary evaluation of the AZA distribution in the main internal organs of mice was performed 24 h after gavage (or at the time of death for some mice) and after 14 days of recovery. At 24 h, the highest levels of AZAs were found in the stomach, and in descending order, in the intestinal content, liver, small intestine, kidneys, lungs, large intestine, heart and brain. Recoveries ranged from ~6–23% for AZA1, ~4–19% for AZA2 and were lower for AZA3 (~2–8%) after 24 h (Table 30). A dose-dependency in AZAs absorption from the GI tract was observed. After 14 days (at doses of 300 or 500 µg/kg), only AZA1 and -2 levels in the internal organs were still detectable, with a total recovery lower than 3% (Table 30).

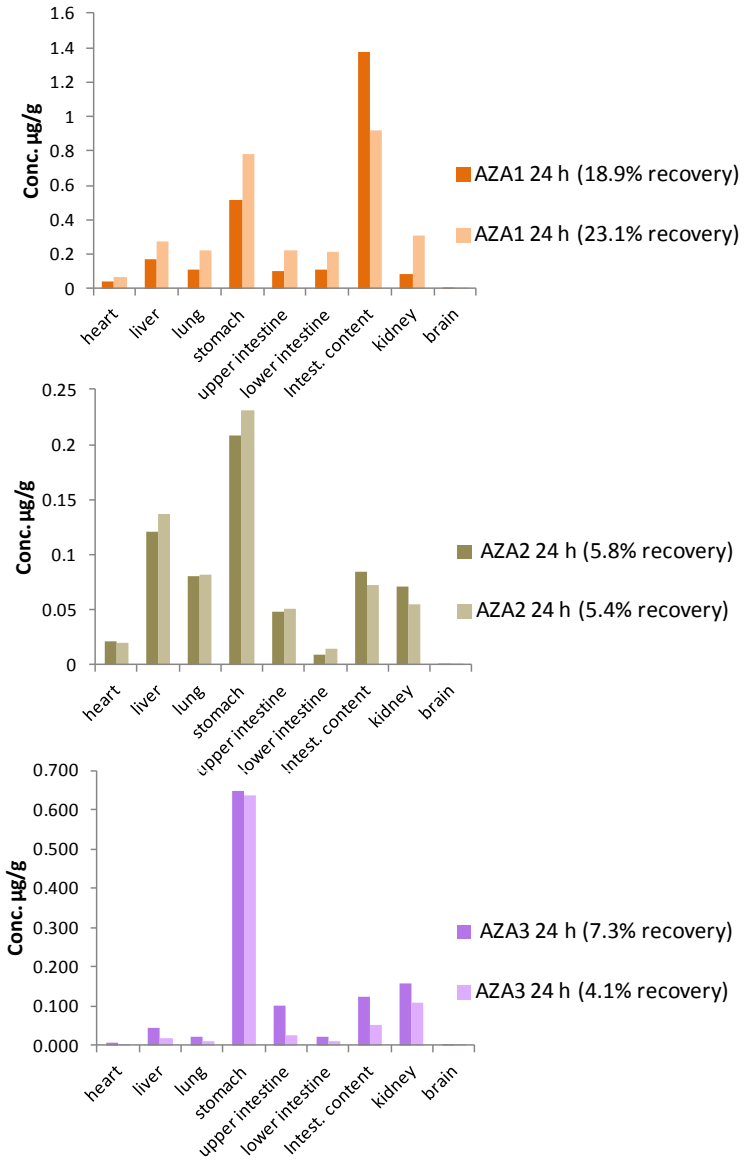


Figure 55. Distribution of AZA1, -2 and -3 in mouse tissues (n=2) after 24 h following oral administration at a dosage of 6 µg (300 µg/kg).

Table 30. LC-MS analysis (recoveries) of AZAs in mouse tissues following oral administration.

Replicate	Dose (µg/kg)	135	175	230	300	390	500	AZA1	Dose (µg/kg)
	Toxin	AZA1	AZA1	AZA1	AZA1	AZA1	AZA1		
	Death time	24 h	24 h	24 h	24 h	20.5 h	14.2 h	14 d	
1	% recovery	6.5	7.7	10.5	18.9	14.9	15.2	1.3	300
	Death time	24 h	24 h	24 h	24.4 h	23.5 h	12.6 h	14 d	
2	% recovery	16.3	7.2	7.0	23.1	5.8	14.3	2.2	390

Replicate	Dose (µg/kg)	300	390	500	650	850	1100	AZA2	Dose (µg/kg)
	Toxin	AZA2	AZA2	AZA2	AZA2	AZA2	AZA2		
	Death time	24 h	24 h	24 h	24 h	22.1 h	5.03 h	14d	
1	% recovery	5.8	18.5	18.7	19.3	14.8	19.5	1.6	500
	Death time	24 h	24 h	24 h	20.5 h	23.1 h	20.5 h	14d	
2	% recovery	5.4	4.1	8.6	15.4	11.3	14.2	0.7	300

Replicate	Dose (µg/kg)	300	390	500	650	850	1100	AZA3	Dose (µg/kg)
	Toxin	AZA3	AZA3	AZA3	AZA3	AZA3	AZA3		
	Death time	24 h	24 h	24 h	24 h	24 h	0.5 h	14d	
1	% recovery	7.3	8.1	3.5	5.2	2.3	16.3	0	500
	Death time	24 h	24 h	24 h	20.5 h	1.2 h	0.5 h	14d	
2	% recovery	4.1	6.3	7.2	3.6	15.9	33.8	0	300

10. Combined toxicities (oral mouse)

10.1. Introduction

When assessing toxin profiles in shellfish, it is not uncommon to find the presence of multiple algal toxin classes. As such, there is increasing concern about the potential toxicological effects of mixtures of these various toxins towards human and environmental health - particularly if these toxin combinations prove to behave synergistically. Regarding the AZA class, it is not uncommon to find these toxins in shellfish that are also contaminated with PTX, YTX, and/or the OA group toxins.

The first attempts to address these questions were performed *in vivo* using mice orally exposed to AZA1 and PTX2. Although combined exposures of PTX2 (5 mg/kg) and AZA1 (200 µg/kg) did not elicit any clinical symptoms in mice, there was a substantial increase in the absorption/distribution of PTX2 in mice exposed to PTX2/AZA1 versus PTX2 alone. This was particularly evident in the lung, blood, spleen, kidney, and liver.[109] The combination also induced a slightly greater degree of pathological change in the small intestine (i.e., enhanced edema in the lamina propria and fusion of villi) but not in any other tissue/organ.

10.2. AZA1 and OA combined study

Among the lipophilic marine algal toxins in shellfish currently regulated in the EU, toxins from the OA and AZA groups pose the largest threat to consumers.[32,110] These toxins regularly co-occur in Irish shellfish.[5] They cause considerable negative health effects in consumers when present in shellfish above certain levels. The main symptoms, dominated by diarrhoea, are caused by damage to the GI tract. Even though OA and AZAs exert toxicity via different mechanisms, it is important to find out whether they may enhance the health effects if present together, since they act on the same organs and are regulated individually.

The main issue in this study was the possibility of enhanced lethality in mice upon combined oral exposure to OA and AZA, which had not been studied previously. The pathological effects in several organs and effects on absorption from the GI tract were also studied.

The experiment was performed in two parts. The first part was designed to determine the LD_{10} and LD_{50} of both OA and AZA1 individually. The second part focused on combined toxicity with the three doses 0, LD_{10} and LD_{50} for each of the two toxins OA and AZA (Table 31).

Table 31. Combinations of dosing and ideal number of mice needed. Bold number indicates the combinations that were carried out in the current study and numbers in italics indicate previously covered.

		OA Toxin		
		0	LD_{10}	LD_{50}
AZA1 Toxin	0	**3**	**3**	*3*
	LD_{10}	**3**	**4**	**4**
	LD_{50}	*3*	*3*	*3*

The LD_{10} and LD_{50} for OA were determined to be ~780 mg/kg bw and 880 mg/kg bw respectively while for AZA1 they were ~570 mg/kg bw and 775 mg/kg bw, respectively.

No increases in pathological changes were observed in mice exposed to combinations of AZA1 and OA. The combination influenced absorption from the GI tract; absorption was reduced considerably for both toxins. This may be caused by competition for the simple diffusion of weak organic acids across the membranes of the GI tract. The lack of pathological effects outside the GI tract corresponds well to the low degree of absorption observed for OA and AZA1 when administered separately.

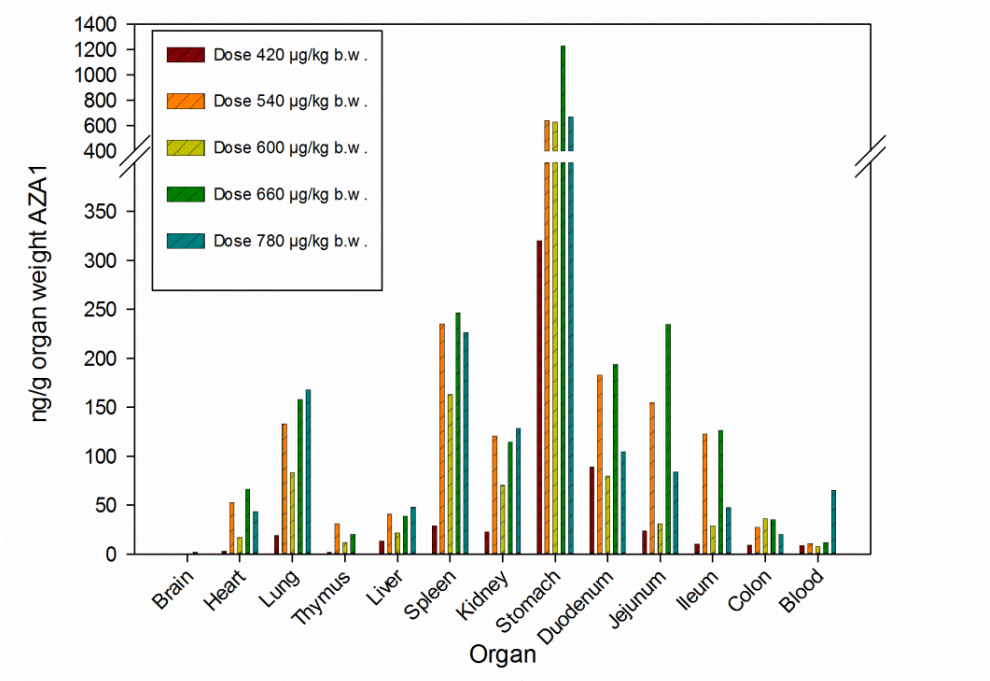


Figure 56. Concentrations and distribution of AZA1 (by LC-MS/MS) in ng/g in tissues of female NMRI mice, after oral exposure at different dose levels of AZAs.

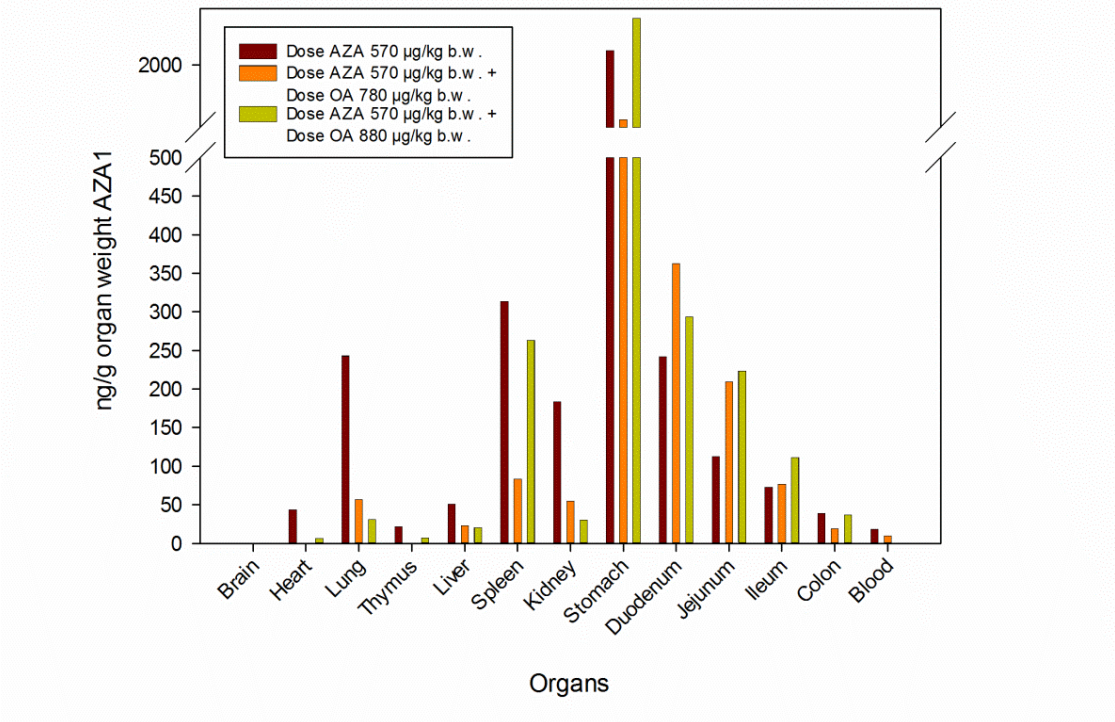


Figure 57. Concentrations of AZA1 (by LC-MS/MS) in ng/g in tissues of female NMRI mice, when given alone at 570 µg/kg and in combination with 780 and 880 µg/kg OA.

In conclusion, the present results indicate that combined exposure to AZA1 and OA does not lead to enhanced acute oral toxicity compared to the toxic effects from exposure to the same toxins when administered separately. Consequently, regulation of toxins from these two groups, based on maximum tolerance levels for each group, appears to be valid.

10.3. AZA1 and YTX combined study

YTX and its analogues are produced by the dinoflagellate algae *Protoceratium reticulatum*[111,112], *Lingulodinium polyedrum*[113] and *Gonyaulax spinifera*.[114] Although they have not been reported in Irish shellfish[5] they have been found in shellfish from many other countries, including Japan, New Zealand, Australia, Canada, Italy, France, Norway and the United Kingdom.[115,116] For many years, the presence of YTXs in shellfish has resulted in positive mouse bioassays leading to the closure of shellfisheries. Since YTXs do not appear to cause diarrhoea in humans and exert low oral toxicity in animal experiments, it has been suggested that they should be removed from regulation. Before doing so, it is important to determine whether the oral toxicity of YTXs is enhanced when present together with shellfish toxins known to cause damage to the GI tract.

Yessotoxin was given by gavage to mice at 1 or 5 mg/kg, either alone or in combination with AZA1 at 200 mg/kg (a level that causes damage to the small intestine[22]). One group of mice received 200 mg/kg AZA1 alone while a group of control mice were given vehicle only. Each mouse was dosed a volume of 250 mL per 20 g bw. The groups consisted of three mice except the control group which consisted of six mice. All mice were sacrificed after 24 h.

In the study YTX levels in internal organs were very low, suggesting that only a small proportion of YTX is transported across the intestinal barrier. The total fraction of YTX in the internal organs measured by LC-MS/MS accounted for less than 0.1% of the administered dose. Most of the YTX was recovered from the lower intestine and the faeces, while only trace amounts were found in blood, urine and tissues (R. Munday, AgResearch, Hamilton, New Zealand, unpublished results). The level of YTX in the stomach was ~2-fold higher when YTX was given in combination with AZA1, compared to when YTX was given alone.[23] Since the stomach contents were removed before the toxin level was measured, a distinction between toxin levels in the stomach contents and the stomach wall could not be made. The observed increase in retention of the stomach contents, suggest an inhibitory effect of AZA1 on the motility of the stomach and duodenum, which would extend the period of high YTX levels in the stomach. Another possible explanation is that the combination induced absorption of YTX into the stomach wall, but this is less likely since such an absorption ought to have resulted in higher toxin levels in the liver and/or other internal organs. However, since the YTX concentration in the distal parts of the intestines was similar to when YTX was given alone (Figure 58), it seems that the motility of the stomach and intestines is not severely inhibited due to AZA1 but rather slowed. It may be assumed that the part of the intestine with pathological changes has been exposed to a substantial amount of YTX. The concentrations of AZA1 found in the different tissues when given in combination with YTX were similar to the levels when AZA1 was given alone at 200 mg/kg (Figure 59).

These results were also similar to those obtained in Aasen *et al.* (2010) for AZA1 at 200 mg/kg.[22] Consequently, YTX does not seem to influence the absorption of AZA1. In mice exposed to a combination of AZA1 and YTX, the pathological changes in the intestine were similar to the changes caused by AZA1 alone. The damage to the intestine did not appear to increase the absorption of YTX as shown by the quantitation of YTX in blood and internal organs by LC-MS/MS. The levels found in the blood by LC-MS/MS corresponded with the levels reported by Tubaro *et al.* (2008).[117] The low YTX levels in the internal organs were consistent with the histological investigations by light microscopy showing normal morphology in the same organ.

In conclusion, 5 mg/kg YTX, either alone or together with AZA1 at 200 mg/kg does not lead to enhanced absorption of YTX nor to toxic effects in the heart or other internal organs.

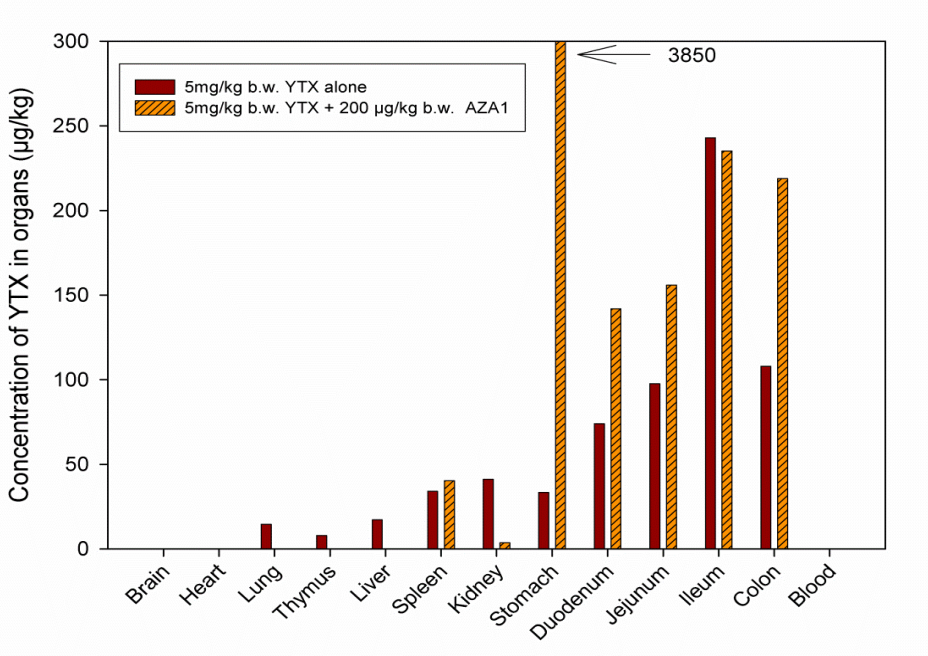


Figure 58. Concentrations of YTX (by LC-MS/MS) in µg/kg in tissues of female NMRI mice, when given alone at 5 mg/kg and in combination with 200 µg/kg AZA1.

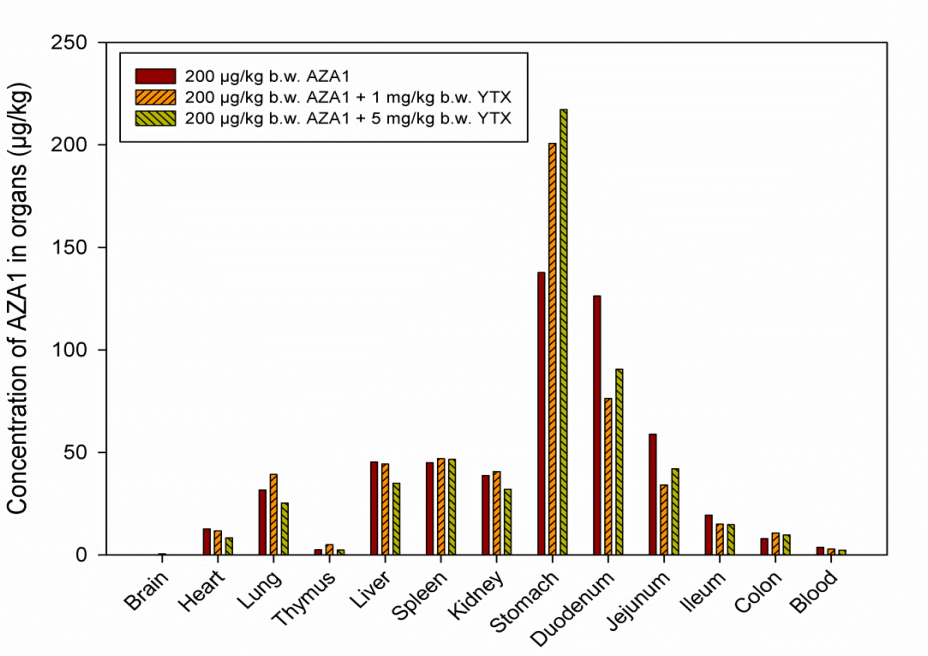


Figure 59. Concentrations of AZA1 in µg/kg in tissues of female NMRI mice after dosing of 200 µg/kg AZA1 alone and in combination with 1 mg/kg and 5 mg/kg YTX.

11. Cellular and molecular studies of AZA toxicology

11.1. Introduction

Preliminary experiments performed by Flanagan *et al.*[118–120] using HepG2 hepatoblastoma cells and human bladder carcinoma cells (ECV-304) exposed to contaminated crude mussel extracts showed AZAs to have a cytotoxic effect. In the ASTOX project, cellular and molecular studies were designed to investigate the mode of action underlying the toxicity of AZAs. Studies conducted on seven different mammalian cell lines showed that AZAs strongly affect most cell types. A functional assay was developed in the ASTOX project for the specific detection of AZAs using morphological changes of pseudopodia in lymphocyte T cells (Jurkat). It was subsequently found to be the most sensitive for AZAs of all the assays tested.[11]

During the many cytotoxicity experiments used to assess AZA toxicity, a variety of morphological effects were observed. In T lymphocytes, cells initially responded to AZA1 by a reduction in membrane integrity, organelle protrusion concurrent with flattening of cells, and a retraction of their pseudopodia or lamellipodia.[27] This was followed by protracted cell lysis. In leukemia cells, AZA2 caused DNA synthesis phase arrest.[10] In neuroblastoma cells, AZA1 induced cell rounding and detachment from adjacent cells.[26] At the subcellular level, disruption of the Golgi complex and an accumulation of vesicles have been reported.[121] At the cellular level, AZA1, -2, and two other semi-synthetic analogues of AZA2 all induced gross morphological changes.[122] AZA1 is a potent cytotoxin towards primary CGCs[123], neocortical cells[124], and spinal cord neurons.[31] In CGCs, AZA-induced cytotoxicity was related to the activation of the c-Jun-N-terminal (JNK) kinase[125,126], whereby AZA1 exposure resulted in decreased neuronal volume that was protected by pre-incubation of the neurons with a JNK inhibitor (SP 600125), a chloride channel blocker (4,4-diisothiocyanatostilbene-2,2-disulfonic acid; DIDS), and a Na+-K+-ATPase blocker (amiloride).[125,127] The effects of AZA1 and -2 on cytotoxicity (and other cellular indices) appear to be irreversible.[26,31,122,128] Experiments using human breast cancer cells and mouse fibroblasts exposed to AZA1 have also demonstrated that the reductions in cellular proliferation and density are not unlike the actions elicited by YTX, raising the possibility of similar mechanisms of action for these two phycotoxin classes.[25]

Other phycotoxins such as the DSP toxins are known inducers of apoptosis.[130] *In vivo*, there is an indication that mice orally exposed to AZA1 exhibited pyknosis (chromatin condensation indicative

of apoptosis) in dead and dying lymphocyte cells within the spleen and thymus.[107] However, initial *in vitro* studies were inconclusive and suggestive of necrotic lysis. These observations were made based on cytotoxic morphological observations[27], the absence of mitochondrial membrane potential changes in neuroblastoma cells[129], and the absence of a sub-G1 populuation in leukemia cells exposed to AZA2.

There are ample data available that suggest the AZA toxins alter ion flux in various cell types. The AZAs have been shown to alter intracellular calcium flux[124,129,131,132], proton homeostasis[133], and membrane hyperpolarization.[127] In CGCs, anion channel blockers and ouabain greatly ameliorated the cytotoxic effect of AZA1 in immature neurons and completely eliminated it in older cultures.[127] Furthermore, short exposures of cultured neurons to AZA1 caused a significant decrease in neuronal volume that was reduced by pre-incubation of the neurons with 4,4-diisothiocyanatostilbene-2,2-disulfonic acid (DIDS; a chloride channel blocker) or amiloride (Na^+-K^+-ATPase blocker).[126] In neocortical neurons, VGSCs, NMDA, glutamate receptors, and L-type Ca^{2+} channels were ruled out as potential targets for AZA1-induced neurotoxicity, which is consistent with earlier reports that demonstrated AZA1 did not affect VGSC or voltage-gated calcium channel currents.[31] Although AZA1 did not alter membrane potential in SH-SY5Y neuroblastoma cells, a plethora of proteins related to ion/anion channels were differentially expressed.[121]

11.2. AZA1, -2 and -3 relative potencies using the Jurkat T lymphocyte cell assay

The relative potencies of various AZA analogues were assessed using the Jurkat T lymphocyte cell assay. This cell line exhibited suitable sensitivity to AZA1[11,27], hence, was the natural choice for a model system to perform these comparative experiments.

Results of the metabolically-based cytotoxicity assays for the Jurkat cells exposed to AZA1, -2, and -3 are shown in Figure 60 where all exposures were performed for 24 h, 48 h, and 72 h. AZA1, -2, and -3 each exhibited a cytotoxic response that was both concentration- and time- dependent, with EC_{50} values in the low or sub-nanomolar range (Table 11). Based on curve shift analysis and EC_{50} comparisons, the order of *in vitro* potency is as follows: AZA2 > AZA3 > AZA1. Relative to AZA1, -2 and -3 are 8.3-fold and 4.5-fold more potent, respectively. The order of relative *in vitro* potencies is the same as the corresponding *in vivo* results reported by Satake *et al* (1998)[2] and Ofuji *et al* (1999)[3] (based on minimal lethal concentrations); however, the *in vivo* studies suggest less drastic

potency differences between these three AZA analogues (up to 1.5-fold). The potencies of other AZA analogues are detailed in Section 6.6.5, see also Table 11.

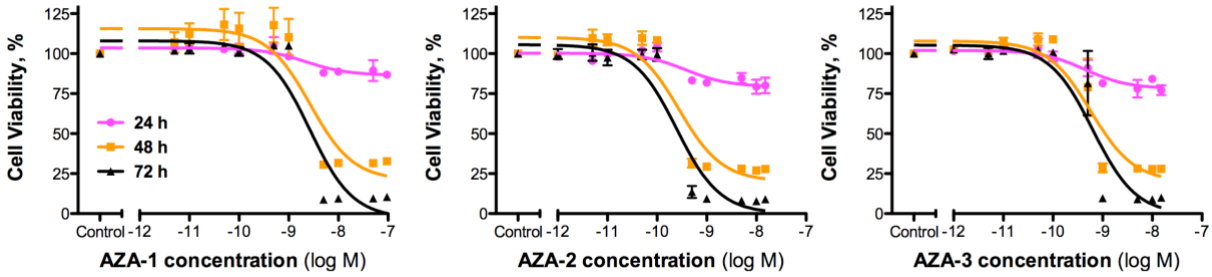


Figure 60. Effect of various AZA analogues on cell viability. Lymphocyte T cells were exposed to A) AZA1, B) AZA2 and C) AZA3 for 24, 48 and 72 h and viability was assessed using the MTS assay. All data (mean ± SE; n=3) were normalized to the control (0 M AZA1). Non-linear three parameter dose response (variable slope) analysis with calculated EC_{50} values are shown in Table 11.

The data generated by the above cytotoxicity assays effectively confirm the sensitivity of the Jurkat T lymphocyte cell line to AZA1, -2, and -3 at low nanomolar to sub-nanomolar concentrations. In order to further characterize the mechanism(s) of action of AZAs, the morphological effects of these three AZA analogues on Jurkat cells were assessed.

Results of these experiments are illustrated in Figure 61. Corroborating the metabolic-based cytotoxicity data, these images suggest that AZA1, -2, and -3 are each cytotoxic to Jurkat T lymhocyte cells resulting in time-dependent cell lysis. Similar to previous findings and published reports for AZA[127,134] time-dependent withdrawl/retraction of pseudopodia with a concurrent rounding of the outer cell surface was observed. However, exposure of Jurkat cells to AZA2 and -3 did not have these same specific effects on pseudopodia. These outcomes have also been confirmed at higher concentrations (data not shown). For AZA2 and -3, cells appeared to expand in size (particularly when imaged using Nomarski differential interference contrast optics: Figure 61 (left panel), while retaining their normal morphological characteristics (i.e., cell shape, pseudopodia extensions) over the duration of the exposure (72 h). These data may represent evidence for differences between the AZA analogues in the cell signaling pathways and the mechanism(s) of action involved in their induction of cytotoxicity and other downstream effects.

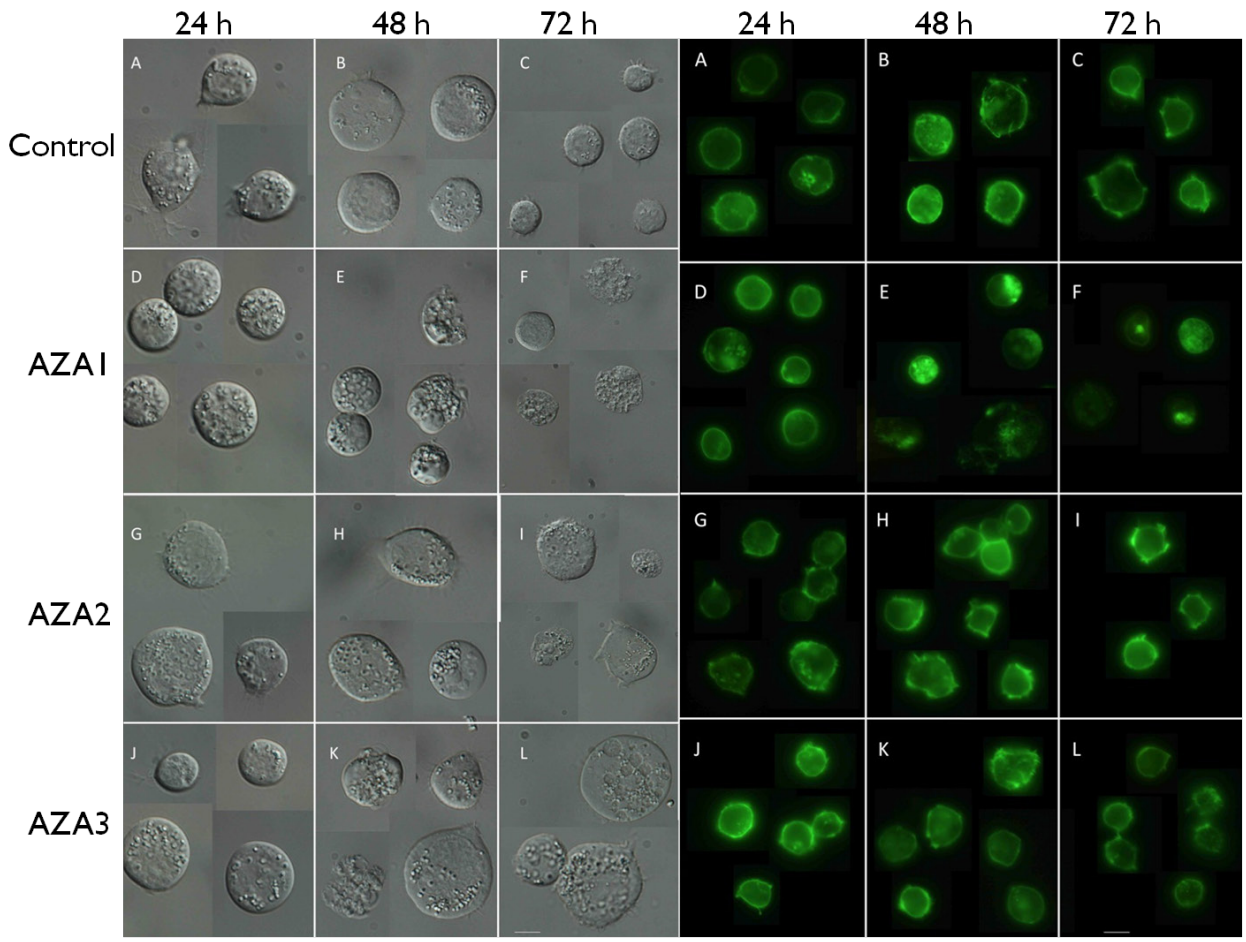


Figure 61. Effects of AZA1, -2 and -3 on the morphology of Jurkat lymphocyte T cells. Cells were exposed to methanol (control), AZA1 (5 nM), AZA2 (0.5 nM) and AZA3 (1 nM) for 24, 48 and 72 h. Cells were then imaged using Nomarksi differential interference contrast (DIC).

11.3. AZA induced cell lysis via apoptosis

Experiments focusing on the mechanism of cell lysis by assessing the effects of AZA1 on caspase activity in Jurkat lymphocyte T cells were performed. Caspases are endonuclease suicidal enzymes involved in lysing DNA during PCD. The data illustrate that AZA1 up-regulates the effector caspase, caspase 3/7, at 48 and 72 h for all concentrations tested (Figure 63). Caspase 2 is also up-regulated at 48 h following treatment with 10 and 95 nM. There is also an indication that caspase 10 is slightly up-regulated under these same conditions. Collectively, these enzyme activity data suggest that apoptosis is an active process in Jurkat T lymphyocyte cells exposed to AZA1.

To confirm the effects of AZA1 on the up-regulation of caspases, the effects of AZA1, -2, and -3 on DNA fragmentation were assessed. Fragmentation of DNA is a hallmark of PCD that is observed

towards the latter part of the process. It is clear in Figure 62 that AZA1, -2, and -3 each cause a laddering effect on the DNA fragementation pattern of Jurkat cells exposed to AZAs for 48 and 72 h. Positive controls DTX2 and camptothecin also cause a similar fragmentation pattern starting at earlier time points. The effects of the AZAs, DTX2, and camptothecin on DNA fragmentation contrast the control cells (methanol control) where no DNA fragmentation was observed across all time points.

These data collectively support the finding that AZA-induced cell lysis is via an apoptotic pathway(s).[135]

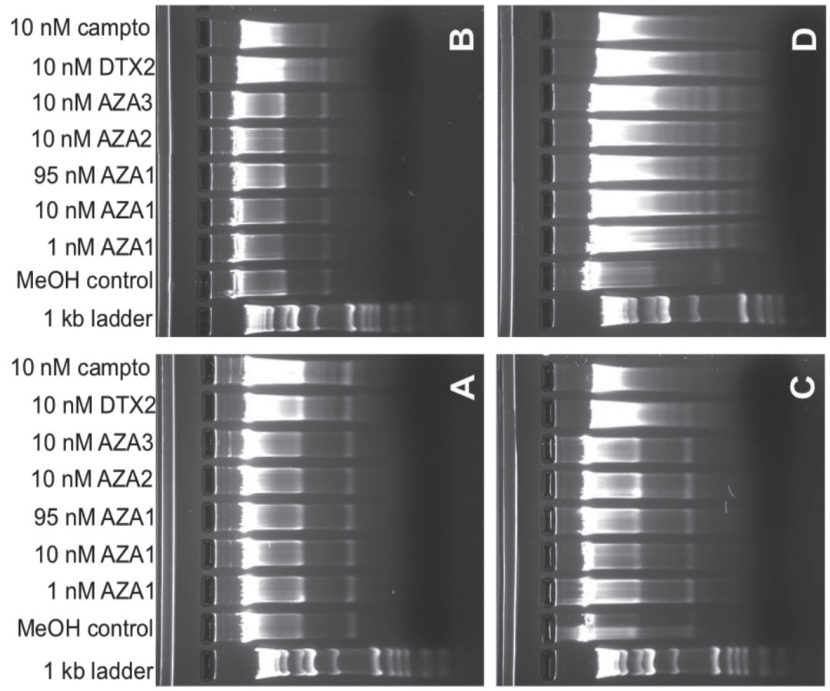


Figure 62. Effects of AZA1, -2 and -3 on DNA fragmentation in Jurkat cells. Cells were exposed for A) 12 h, B) 24 h, C) 48 h and D) 72 h.

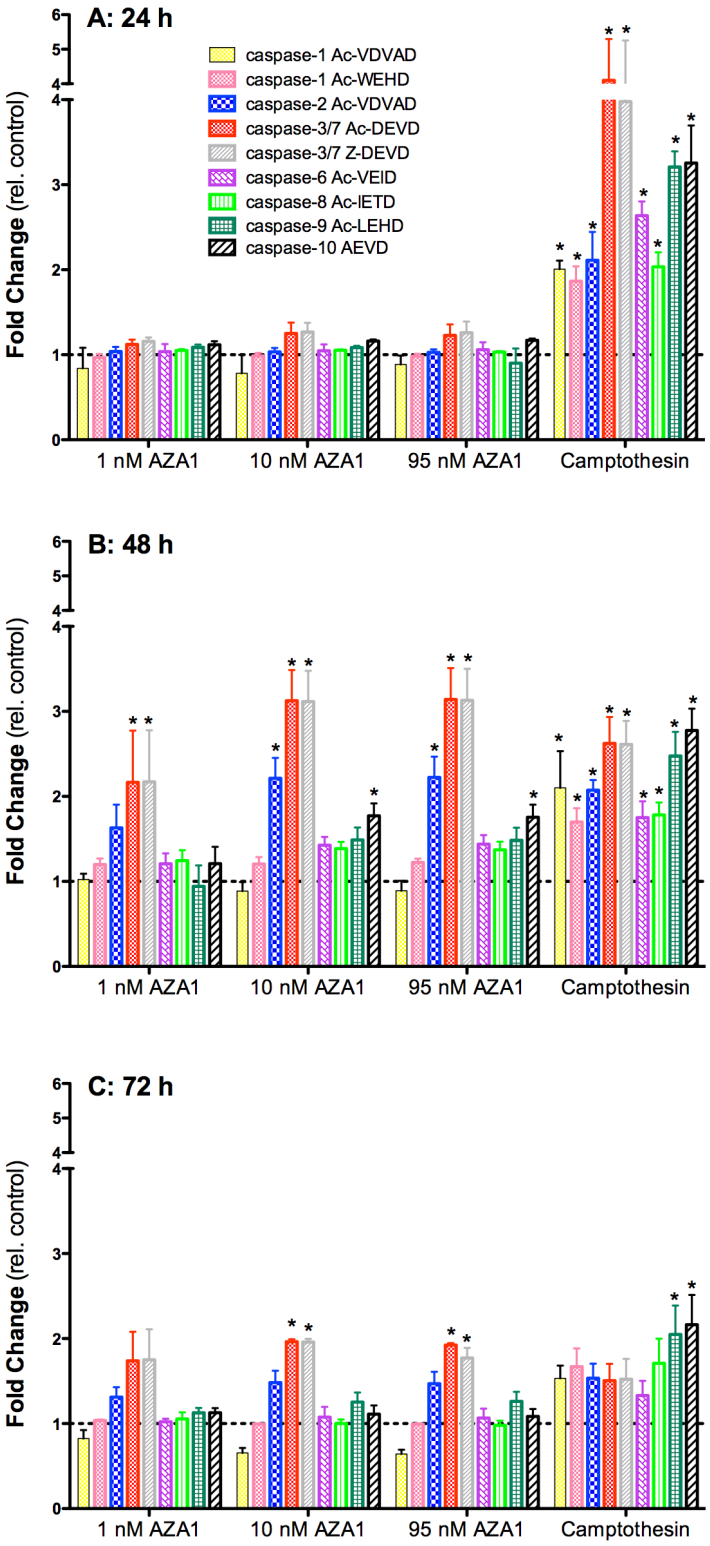
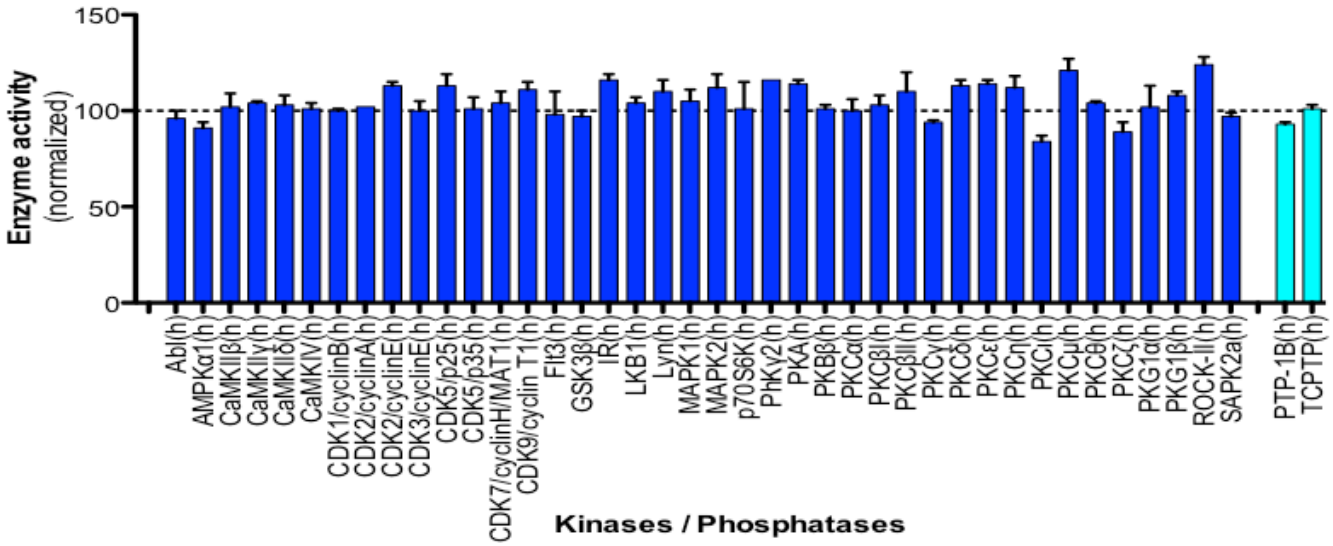


Figure 63. Relative change in the activity of seven different caspases in Jurkat cells exposed to 1, 10, and 95 nM AZA1 concentrations for A) 24 h, B) 48 h, and C) 72 h. Camptothesin (1 µM) serves as positive control.

11.4. Mode of action studies

The proposed mode of action for the AZAs is inhibition of the cholesterol synthesis pathway.[134] It was sought to test this mechanism by supplying extracellular cholesterol and other cholesterol biosynthetic substrates to Jurkat T lymphocyte cells concurrently exposed to AZA1. If the toxicological mechanism of AZA1 was via inhibition of this pathway, supplying cholesterol to the cells should rescue the cells from AZA-induced cytotoxicity. This hypothesis was extensively tested using various forms of cholesterol as well as four metabolic intermediates and failed to show any indication that the cells were being rescued (data not shown). This finding suggests that AZA1 is not inhibiting cholesterol biosynthesis within T lymphocytes, at least to the extent of causing cytotoxicity.

To further explore AZA's mechanism(s) of action, AZAs were screened against >100 common drug targets. These included 40 kinases, 2 phosphatases, 76 GPCR, and 8 ion channels. To maximize the chances of success (in the event of different targets for the different AZA analogues), these targets were screened using a mixture of the AZAs (51.6% AZA1, 20.6% AZA2, 19.7% AZA3, and 8.0% AZA6). All targets were tested in duplicate at a single AZA_{total} concentration. As illustrated in Figure 64 the activity of all tested kinases was not altered in the presence of 348 nM AZA_{total} and thus did not cause a response/change. Similarly, and in support of the published literature, the two tested phosphatases were also not altered.[8,27,120] Furthermore, the AZA mixture did not have any major effects on the activation or inhibition of GPCRs (Figure 65). In these experiments, the AZA_{total} concentrations were 348 nM and 436 nM, respectively. Although the IP1 GPCR appears to be inhibited by the AZAs, follow-up studies using pure AZA1, -2, and -3 do not support this finding (data not shown).



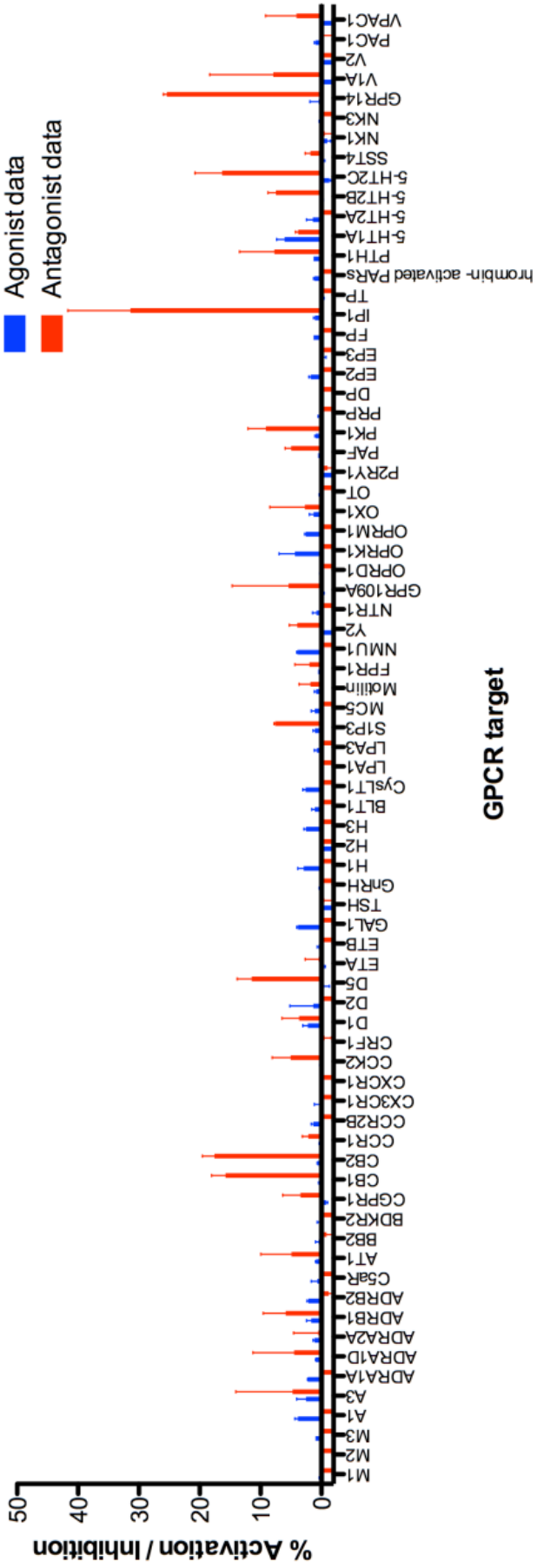


Figure 65. Effects of AZAs on activation or inhibition of GPCR activity.

Finally, the AZA mixture was tested against 8 various ion channels (Figure 66) and there was some indication that the AZAs were inhibiting the activity of the hERG potassium ion channel by 31%. The hERG channel is highly important in the action potentials of cardiomyocytes by mediating the 'rapid' delayed rectifier current (I_{Kr}).[136] The hERG was then tested for channel binding and activity in the presence of purified AZA1, -2, and -3.

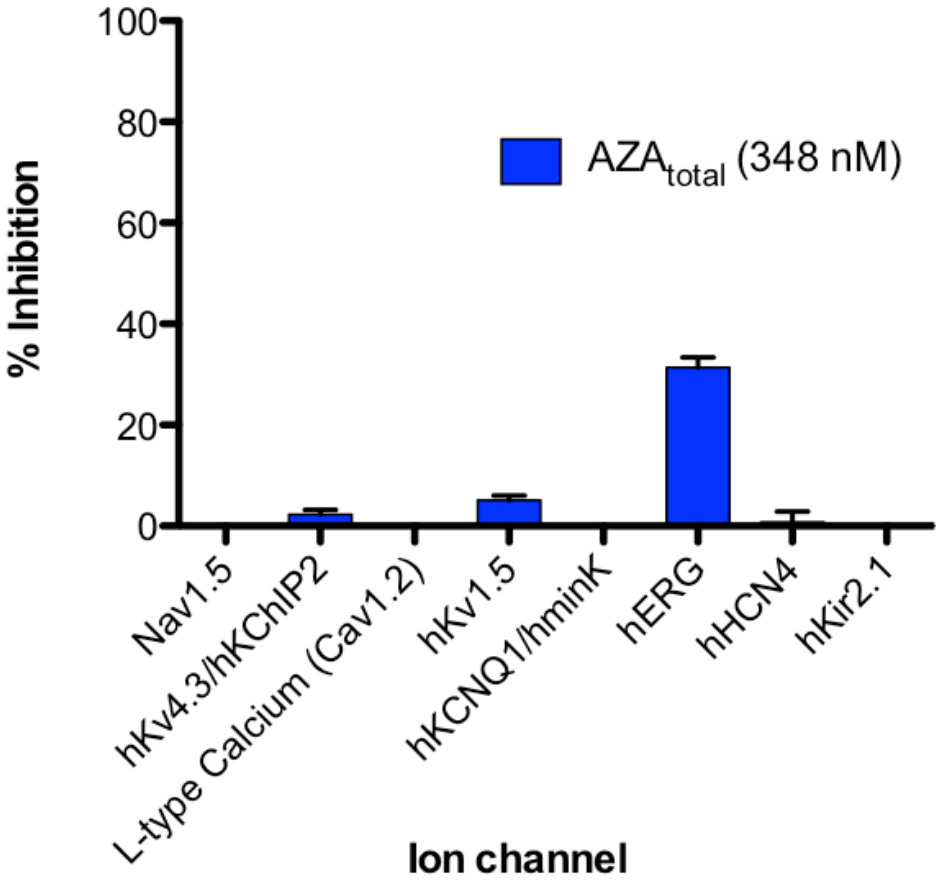


Figure 66. Effects of AZAs on ion channel activity.

In HEK-293 cells transfected with the hERG channel, patch clamp electrophysiology data were collected for cells exposed to various concentrations of AZA1 (Figure 67A), -2, or -3 (Figure 67B). Inhibition of K^+ current for each AZA analogue was concentration-dependent (IC_{50} value range: 640–840 nM) and inhibited the channel while in its open state.[137]

Transected Xenopus oocytes were then used for mutagenesis experiments. Using mutant hERG channels, AZA1 was shown to interact with phenylalanine at position 656 (F656), but not with tyrosine at position 652 (Y652), strongly suggesting that AZA1 interacts with the central pore of the hERG channel (Figure 68).

The interactions between AZAs and the hERG channel were further confirmed using a receptor binding assay and an ion flux assay.[137]

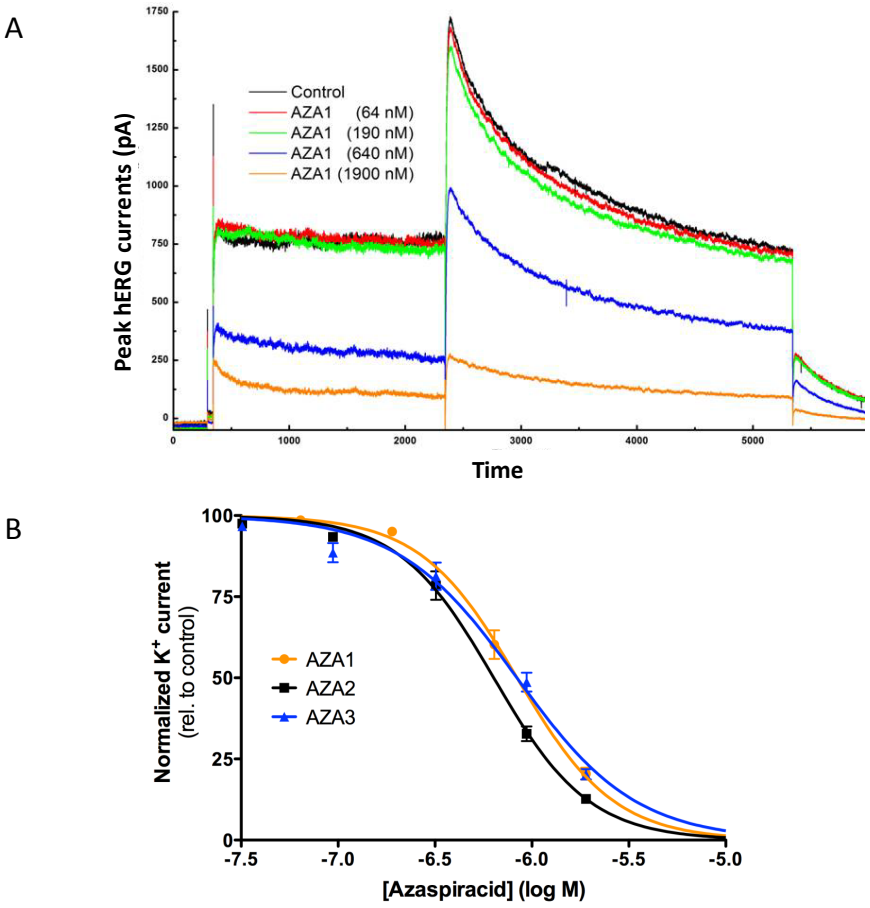


Figure 67. AZAs block hERG1 channels expressed in HEK-293 cells. A) Representative patch clamp traces of potassium ion current through hERG channels stably expressed in HEK-293 cells in the presence of various concentrations of AZA1. B) Concentration dependent inhibition of potassium ion current through hERG channels by AZA1, -2 and -3.

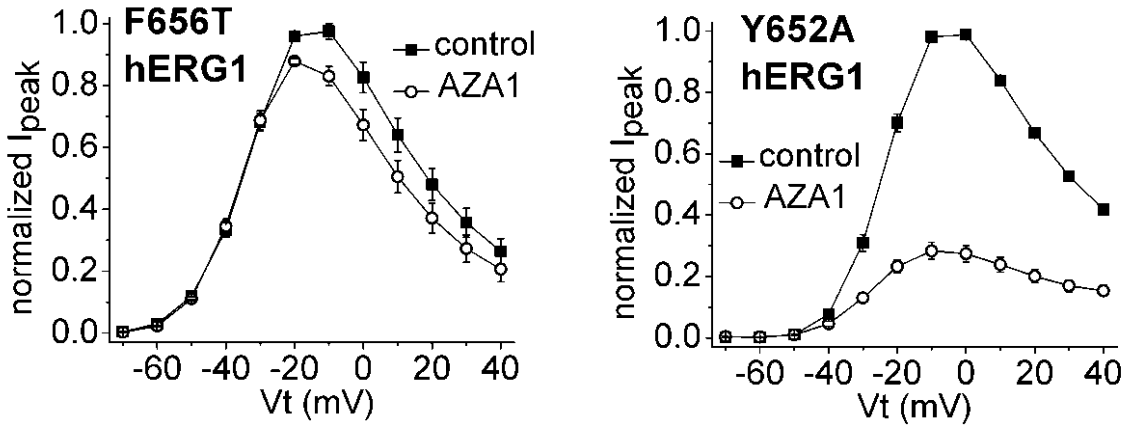


Figure 68. Inhibition of hERG1 by AZA1 (10 μM) is attenuated by mutation of F656.

11.5. Determination of toxin receptors

An AZA1/CM5 (i.e., AZA1 immobilized on a carboxymethyldextran surface) SPR chip was used in experiments to detect toxin receptors in subcellular fractions of Jurkat T lymphocyte cells, BE(2)-M17 neuroblastoma cells, and Caco2 colorectal epithelial cells. Subcellular fractions, including cytoplasm (CE), membrane (ME), nuclear (NE), chromatin bound (CB), and pellet (PE) extracts, were reacted with the AZA1 sensor chip in order to assess binding activity. Specificity of binding was evaluated by comparing samples with and without free AZA1 added, which should reduce or eliminate binding to the chip surface if specific for the toxin. The PE fraction of Caco2 cells, containing cytoskeletal proteins, showed the highest level of specificity with ~70% of the binding activity eliminated by the addition of free AZA1; however, the absolute response was low indicating a weak interaction (Figure 69). This result was consistent with the earlier report of cytoskeletal effects of AZA1 in mammalian cells.[27] The highest absolute response was observed for the NE fraction, yet the binding activity was reduced by only ~15% with the addition of AZA1, which suggests a high level of non-specific binding. The ME fraction showed ~20% inhibition by AZA1 for all three cell lines tested, whereas similar specificity was noted for the CE and CB fractions of BE(2)-17 and Caco2 cells, respectively. Overall, all three cell lines showed fractions that bound AZA1 with a range of specificities. Selected fractions will be further processed using an AZA1 immunoaffinity column in order to isolate specific binding partner(s) for AZA1, followed by identification using LC-MS/MS and de novo sequencing.

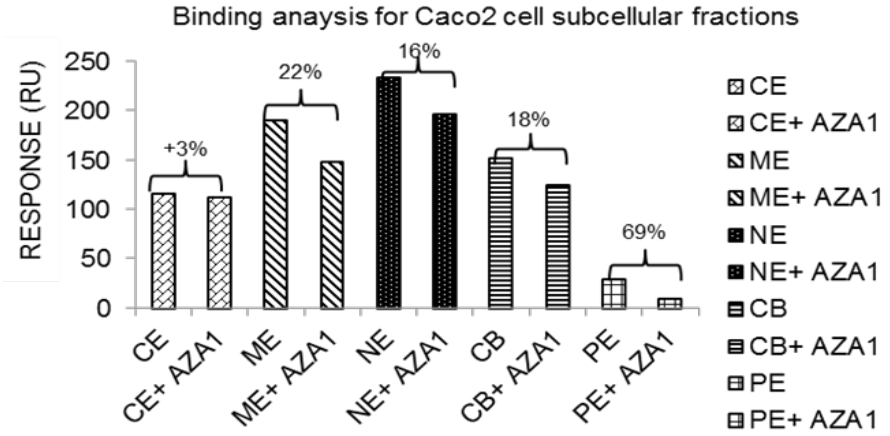


Figure 69. SPR results for Caco 2 cell lysate samples (40 µg/mL) from subcellular fractions with and without AZA1 (100 ng/mL), and containing 2 mg/mL NSB (i.e., non-specific binding) reducer.

11.6. Radiolabelling

Approximately 300 µg of AZA1 was hydrogenated (cold) in a reaction using Brown's catalyst. The singly and doubly hydrogenated products were subsequently purified using semi-preparative chromatography. The predominant product was the doubly hydrogenated AZA1 (*m/z* 846).

Table 32. Quantities of AZA1 reduced products in sample after hydrogenation reaction.

	m/z 842 µg	*m/z* 844 µg	*m/z* 846 µg
AZA1 Hydrogenated sample	2.5	17.2	61.8

The MS spectra for both the singly (*m/z* 844) and doubly (*m/z* 846) hydrogenated compounds lacked the RDA cleavage products indicating that the C7–C8 double bond was reduced. NMR confirmed the doubly hydrogenated compound to be 4,5,7,8-tetrahydroAZA (Figure 70).

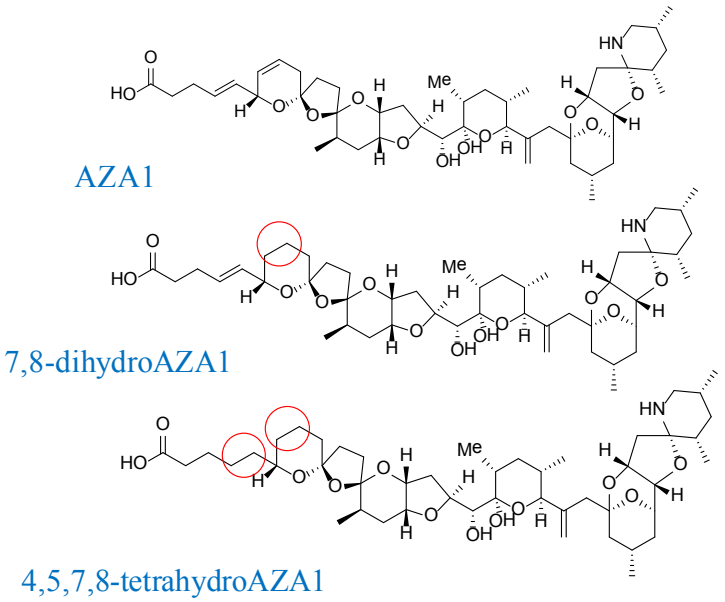


Figure 70. Structures of AZA1 (*m/z* 842), 7,8-dihydroAZA1 (*m/z* 844) and the NMR elucidated structure of 4,5,7,8-tetrahydroAZA1 (*m/z* 846).

Using the Jurkat lymphocyte T cytotoxicity assay both hydrogenated forms of AZA1 induced time- and concentration-dependent cytotoxicity (Figure 71). Mean EC_{50} values were 0.99 nM (7,8-dihydroAZA1) and 4.58 nM (4,5,7,8-tetrahydroAZA1). Relative to AZA1, comparable potencies were 1.4 and 0.3; respectively.

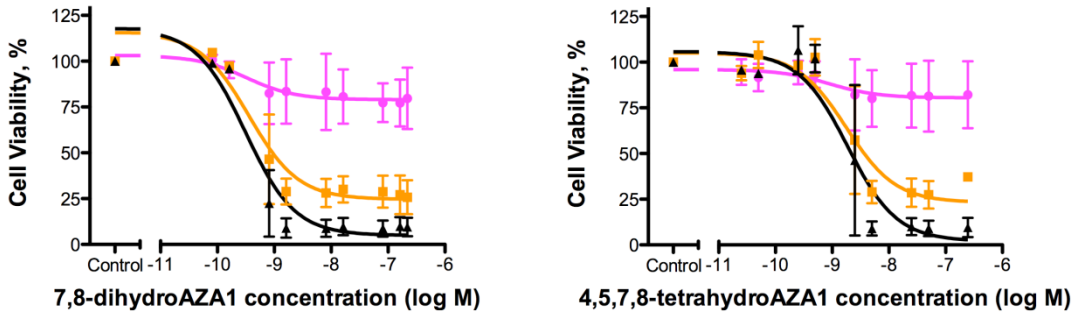


Figure 71. Cytotoxicity of two hydrogenated AZA1 analogues (Jurkat).

The success of the cold reaction and the fact that the products were biologically active permitted the progression to reacting AZA1 with tritium (hot). Purified AZA1 (1 mg) was reacted with tritium resulting in a radioactivity reading of ~60 mCi. However, the reaction did not go to completion (Figure 72).

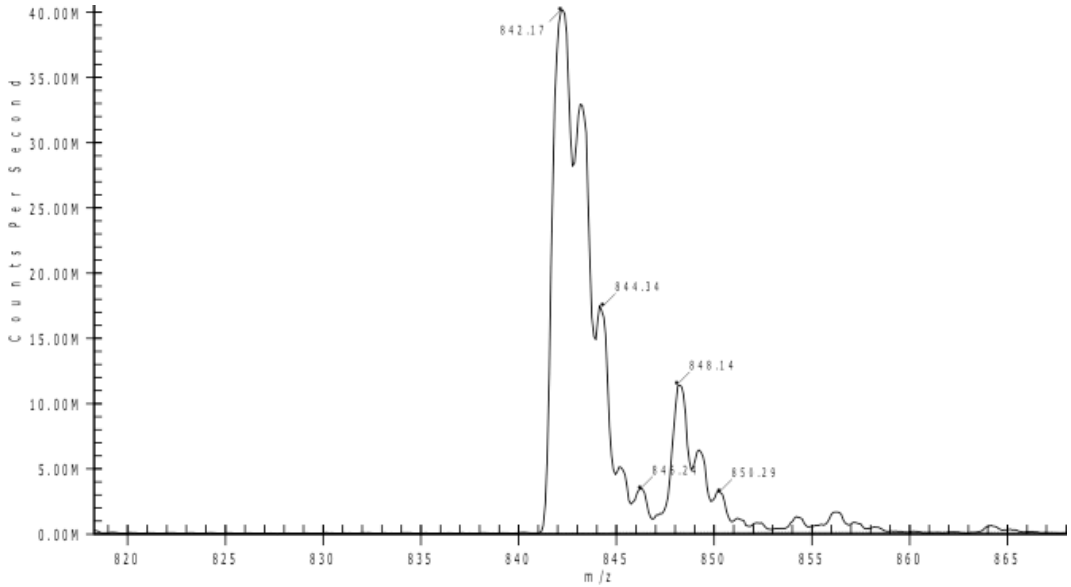


Figure 72. MS peaks of AZA1 sample following tritium reaction.

The reaction was repeated using 1 mg of AZA1, this time reacting for longer with higher levels of hydrogen gas resulting in a radioactivity reading of 98 mCi (Figure 73).

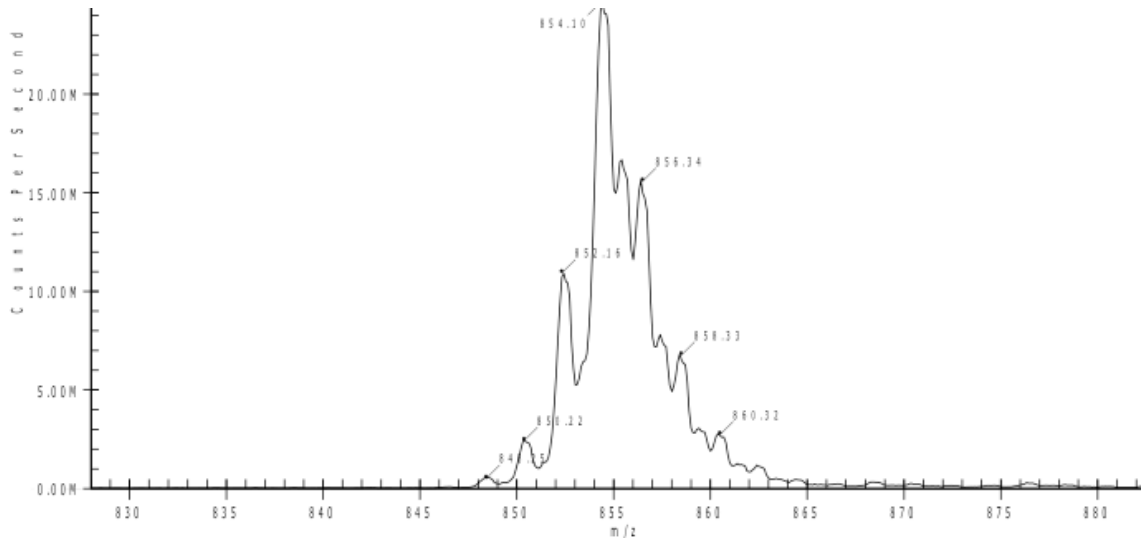


Figure 73. MS peaks of AZA1 sample following 2nd tritium reaction.

The reacted samples require further analysis by LC-MS/MS using a more refined method to better indicate structural changes and purities. Purification will also be required prior to biological testing.

12. Conclusions

The prevelance of AZA accumulation in shellfish appears to be increasing with greater intensity, not only in Ireland, but also globally. There continues to be human poisoning events associated with AZAs and their occurrence has been most problematic for the Irish shellfish industry.

Over the course of the ASTOX 2 project, a diverse and complex profile of the phytoplankton associated with this toxin group has emerged. Apart from *A. spinosum*, 10 additional *Azadinium* and related species were identified, some of which do not appear to produce any AZAs, while others are producers of novel AZAs.

Feeding studies with *A. spinosum* showed that mussels will feed directly on these organisms with no requirement for a vector species. Mussels reached the regulatory limit after 5 days, however, the high concentrations observed in the wild could not be replicated under laboratory conditions. This may indicate: (i) that vectors are required to facilitate shellfish toxin accumulation (e.g., *Favella ehrenbergii* which has been shown to actively feed on *A. spinosum*); (ii) longer term feeding trials are required or (iii) other variables, as yet unknown, contribute to high uptake of toxins. The laboratory studies also showed that *A. spinosum* has a negative effect on shellfish in terms of mortality rates, feeding behaviour and physiology. Additionally, these studies showed that mussels take up AZAs from the dissolved phase and therefore the release of AZAs from decaying blooms may also have consequences for other species, not normally associated with these toxins.

From a monitoring standpoint, the development of a molecular probe capable of distinguishing between *A. spinosum*, *A. obesum* and *A. poporum* will contribute significantly to the toxin monitoring programme at the Marine Institute, enabling more effective observation and forecasting of blooms, which otherwise were difficult to detect. Further benefits for the monitoring programme arising from the project are the production of sustained supplies of CRMs. Bulk culturing of the producing organisms and improvements in isolation procedures have enabled more effective purification of a range of AZA analogues. The availability of these toxins has allowed the relevance of analogues to be established, in terms of proportions and toxicity. However, further research is still required, particulary for the novel analogues.

The heat-induced transformation of AZA17 and -19 into AZA3 and -6, respectively, was confirmed in the project. Using the RMs produced, the relevance of some of the minor and novel AZAs

were determined, highlighting AZA6 as a potential significant contributor to overall toxicty. The other purified analogues for which RMs were prepared were found to be of less significance. The importance of other novel AZA analogues identified in the project, such as those which lack a methyl group in the amino ring, have yet to be established.

The *in vitro* and *in vivo* toxicity studies performed confirmed AZA toxicity. Although the mini pigs were less susceptible to AZAs than humans, the studies showed that AZAs cross the intestinal barrier and distribute throughout the body causing internal organ damage at cellular and tissue levels. The results from the oral and *ip* mice studies correlated very well, contradicting previous reports and showing that AZA1 is more toxic than AZA2 and -3. These results were reflected in the analysis of the mini pig blood samples that showed AZA1 is most rapidly taken up into the blood, followed by AZA2 and then -3. Studies looking at the combined effects of AZA1 with OA and YTX showed that there is no increased toxicity when these groups co-occur. The *in vitro* studies were effectively used to assess the toxicity of the minor analogues and demonstrated the following potencies: AZA2 > AZA6 > AZA34 ≈ 37-*epi*-AZA1 > AZA8 ≈ AZA3 > AZA1 > AZA4 ≈ AZA9 > AZA5 ≈ AZA10 > AZA33 ≈ AZA36 > AZA37 > AZA26.

One of the remaining questions prior to the ASTOX 2 project concerned the mode of action of AZAs. Such knowledge is necessary for confirming or changing the regulatory limits, and also for development of treatments following exposure to AZAs. Experiments performed as part of the project led to the discovery that AZAs are potassium channel blockers, however the concentrations required to induce such effects are 2-fold those required to cause cytotoxicity and therefore this is not considered to be the primary mode of action. Hence other modes of action need to be explored and it is likely that the various analogues may exhibit unique modes of action and/or receptors. Finally, the understanding of the main molecular mechanisms could also lead to the production of rapid, receptor-based toxin screening assays.

13. Implications for monitoring programmes

- The project yielded new knowledge regarding monitoring and forecasting
  - The availability of bulk cultures of the producing organisms and isolation of sufficient stocks of AZA1, -2 and -3 for the preparation of CRMs will ensure that Ireland will maintain effective monitoring programmes.
  - The development of a molecular probe to detect these organisms (*A. spinosum*, *A. poporum* and *A. obesum*) in water samples will ensure effective monitoring and contribute to the provision of an early warning system for Ireland's shellfish industry.
- Toxicity and cooking studies indicated that the current regulatory limit may be sufficiently low and that the EFSA recommendation to reduce the current limit may be unnecessary.
  - *In vivo* studies looking at the combined effects of AZAs with OA and YTXs have shown that there are no synergistic effects.
  - Studies performed in ASTOX and ASTOX 2 have shown that the levels of AZAs can increase significantly after cooking due to a concentration effect[17,18] and toxin conversions.[74,84] As the concentrations of AZA3 and -6 are negligible in raw mussels, yet can increase significantly through decarboxylation of AZA17 and -19 during the cooking of mussels[84], the overall concentrations are underestimated by methods used according to current legislation. However, as no cases of human intoxications have been reported from shellfish, which would have been over the limit had the shellfish been cooked prior to analysis, indicates that the current regulatory limit is appropriate.
  - Only miminal effects were observed in pigs after feeding with high doses of AZAs. However, the results suggest that the pig-model may not be the most appropriate model in which to assess the potential impact of AZAs in humans.
  - Studies mimicking the digestive process and its effects on AZAs indicate that AZAs are less bioavailable when shellfish are cooked thereby rendering them 'less toxic'.

14. Recommendations

Based on the findings of the ASTOX 2 project, it is recommended that a review of the current regulatory method and limit for AZAs is undertaken.

- This will entail the introduction of a cooking step in the method for the analysis of shellfish by LC-MS/MS
  - The knowledge of toxin transformations (e.g., AZA17 to AZA3) suggests that a heating step should be introduced into the analysis of raw shellfish to more accurately reflect toxin profiles, thereby enhancing human health protection and preventing loss of valuable processed product due to rejection by importing countries.
- A change in the current regulation is recommended to provide for the inclusion of AZA6 in the legislation
  - Feeding and cooking studies have shown that AZA6 (after heat induced conversion from AZA19) can reach concentrations more than twice that of AZA2 in some samples and is therefore more significant than previously thought. *In vivo* toxicology analysis indicates that this analogue is slightly less toxic than AZA1, while *in vitro* analysis indicates 5-fold greater potency.
- It is recommended that the TEFs for AZA2 and -3 be amended
  - The results from both the oral and the *ip* mouse studies performed in ASTOX 2 confirmed the toxicity of AZAs with very good correlation. Compared to the previous studies, (Satake *et al*[2] and Ofuji *et al*[3]) on which the current TEFs are based, AZA1 was found to be more toxic than AZA2 and -3.
  - Analysis of blood samples taken from AZA1 dosed mini pigs showed that AZA1 is taken up faster into the blood, followed by AZA2 and then -3.

15. Future research

Impact of novel AZA producers

An assessment of the relevance of the additional species/toxins to the shellfish industry and human health is required. Mapping of these species using gene probes at sea, and development of *in situ* biosensors for temporal studies in aquaculture areas could be performed incorporating some of the approaches of the ASIMUTH[139] project in developing an early warning system.

Adverse effects of *A. spinosum* on shellfish and other species

The adverse effects of *A. spinosum* on mussel larvae/eggs needs to be investigated further. Additionally studies looking at the impact of dissolved AZAs in the water on other species should be performed (e.g., AZAs found in periwinkles and have been shown to have adverse effects on fish embryo development).

Vector/mitigation studies

It is still not fully understood how mussels may become so highly contaminated in the field. ASTOX 2 studies have shown only limited accumulation occurs when mussels are fed *A. spinosum*. Additionally, the shellfish are adversely affected by this species. Knowledge of feed and environmental factors and what additional vectors could be involved would aid in developing mitigation strategies.

Toxicology

AZAs are suspected carcinogens, therefore more long term exposure studies need to be performed. AZA targets other than the potassium channel need to be explored. As part of the ASTOX 2 project a radiolabelled AZA was produced. This product now needs to be used in receptor binding assays which will aid in determining how these toxins interact with human cells.

Biodiscovery

There is the potential for some of the *Azadinium* and related species to be producers of novel compounds with therapeutic effects, potentially with effects antagonistic to those by AZAs.

Metabolism studies

It is important to know how AZAs behave when consumed i.e., only accounting for less than 30% of what had been administered. In particular, verification of the so-called "phase II" metabolites

(i.e., the result of conjugation reactions generally converting lipophilic to more polar compounds more easily excreted as part of the detoxification process) and evaluation of their distribution and toxicity is required. Efforts to implement toxicokinetic/toxicodynamic (i.e., TK/TD) modeling of *in vivo* exposure data will yield a more complete conceptual framework of how organisms process and eliminate AZAs following exposure, with the potential for interspecies extrapolation (including humans). Application of '-omics-based strategies (i.e., genomics, transcriptomics, proteomics, metabolomics) in parallel with *in vivo* experiments (as well as *in vitro* models) will provide additional insights into the response of organisms to AZA exposure and may lead to the identification of biomarkers of toxin exposure, potentially useful in epidemiogical studies.

Sampling

Obtaining representative samples for analysis can be challenging and has been problematic in the past. There is a need to harmonise effective sampling strategies to protect consumers and limit losses for the shellfish industry.

Relevance of novel *Azadinium* species/AZA analogues for the Irish shellfish industry

The identification of new *Azadinium* and related species, some of which produce novel AZAs, warrants further research to fully assess the occurrence and impact of these additional species/AZA analogues in terms of prevalence and toxicity.

Impact of *Azadinium* on the viability of commercial shellfish species

The exposure of mussels to *A. spinosum* has shown several negative effects on the viability and effective feeding of those mussels. Taken together with the initial studies (ASTOX) showing that AZAs have negative effects on the larval development of aquatic animals, these findings suggest that the negative impact of *A. spinosum* on the shellfish resource should be investigated in more depth to estimate the economic impact of this species on the shellfish production sector.

Impact of AZAs on other species

The knowledge of potential uptake from the dissolved phase suggests that the impact and necessity for monitoring of AZAs in species, not normally associated with AZA accumulation, should be investigated.

Epidemiology data

From the standpoint of risk assessment and risk management, there is a clear need for additional epidemiological data given that all of the information available to date is based on only seven human poisoning events. Additionally, it should be noted that all six risk assessments have been based on a single, albeit well-described, poisoning incident—the 1997 incident on Arranmore Island, Ireland. There is a need to have strategies in place in the event of a poisoning outbreak to acquire samples for analysis. Requirement for public education – to retain samples (urine, blood, etc) for analysis and also to retain leftover shellfish (that led to illness).

16. Dissemination

16.1. Journal publications

(1) Aasen, J. A. B.; Espenes, A.; Hess, P.; Aune, T. Sub-lethal dosing of azaspiracid-1 in female NMRI mice. *Toxicon*, **2010**, 56, 1419–1425.

(2) Tillmann, U.; Elbrächter, M.; John, U.; Krock, B.; Cembella, A. D. *Azadinium obesum* (Dinophyceae), a new nontoxic species in the genus that can produce azaspiracid toxins. *Phycologia*, **2010**, *49*, 169–182.

(3) Tillmann, U.; Elbrächter, M.; John, U.; Krock, B. A new nontoxic species in the genus in the dinoflagellate genus *Azadinium*: *A. poporum* sp. nov. *European Journal of Phycology*, **2011**, *46*, 74–87.

(4) Salas, R.; Tillmann, U.; John, U.; Kilcoyne, J.; Burson, A.; Cantwell, C.; Hess, P.; Jauffrais, T.; Silke, J. The role of *Azadinium spinosum* (Dinophyceae) in the production of azaspiracid shellfish poisoning in mussels. *Harmful Algae*, **2011**, *10*, 774–783.

(5) Aasen, J. A. B.; Espenes, A.; Miles, C. O.; Samdal, I. A.; Hess, P.; Aune, T. Combined oral toxicity of azaspiracid-1 and yessotoxin in female NMRI mice. *Toxicon*, **2011**, *57*, 909–917.

(6) McCarron, P.; Giddings, S. D.; Miles, C. O.; Quilliam, M. A. Derivatization of azaspiracid biotoxins for analysis by liquid chromatography with fluorescence detection. *Journal of Chromatography A*, **2011**, *1218*, 8089–8096.

(7) Tillmann, U.; Salas, R.; Gottschling, M.; Krock, B.; O'Driscoll, D.; Elbrächter, M. *Amphidoma languida* sp. nov. (Dinophyceae) reveals a close relationship between *Amphidoma* and *Azadinium*. *Protist*, **2012**, *163*, 701–719.

(8) Potvin, E.; Jeong, H. J.; Kang, N. S.; Tillmann, U.; Krock, B. First report of the photosynthetic dinoflagellate genus *Azadinium* in the Pacific Ocean: morphology and molecular characterisation of *Azadinium cf. poporum*. *Journal of Eukaryotic Microbiology*, **2012**, *59*, 145–156.

(9) Kilcoyne, J.; Keogh, A.; Clancy, G.; LeBlanc, P.; Burton, I.; Quilliam, M. A.; Hess, P.; Miles, C. O. Improved isolation procedure for azaspiracids from shellfish, structural elucidation of azaspiracid-6, and stability studies. *Journal of Agriculture and Food Chemistry*, **2012**, *60,* 2447–2455.

(10) Jauffrais, T.; Herrenknecht, C.; Sechet, V.; Sibat, M.; Tillmann, U.; Krock, B.; Kilcoyne, J.; Miles, C. O.; McCarron, P.; Amzil, Z.; Hess, P. Quantitative analysis of azaspiracids in *Azadinium spinosum* cultures. *Analytical and Bioanalytical Chemistry*, **2012**, *403*, 833–846.

(11) Twiner, M. J.; El-Ladki, R.; Kilcoyne, J.; Doucette, G. J. Comparative effects of the marine algal toxins azaspiracid- 1, -2, and -3 on Jurkat T lymphocyte cells. *Chemical Research in Toxicology,* **2012,** *25*, 747–754.

(12) Twiner, M. J.; Hanagriff, J. C.; Butler, S.; Madhkoor, A. K.; Doucette, G. J. Induction of apoptosis pathways in several cell lines following exposure to the marine algal toxin azaspiracid-1. *Chemical Research in Toxicology*, **2012**, *25*, 1493–1501.

(13) Tillmann, U.; Soehner, S.; Nézan, E.; Krock, B. First record of the genus *Azadinium* (Dinophyceae) from the Shetland Islands, including the description of *Azadinium polongum* sp. nov. *Harmful Algae*, **2012**, *20*, 142–155.

(14) Nézan, E.; Tillmann, U.; Bilien, G.; Boulben, S.; Chèze, K.; Zentz, F.; Salas, R.; Chomérat, N. Taxonomic revision of the dinoflagellate *Amphidoma* caudata: transfer to the genus *Azadinium* (Dinophyceae) and proposal of two varieties, based on morphological and molecular phylogenetic analyses. *Phycologia*, **2012**, *48*, 925–939.

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(17) Twiner, M. J.; Doucette, G. J.; Rasky, A.; Huang, P. X.; Roth, B. L.; Sanguinetti, M. C. The marine algal toxin azaspiracid is an open state blocker of hERG potassium channels. *Chemical Research in Toxicology*, **2012**, *25*, 1975–1984.

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(19) Jauffrais, T.; Kilcoyne, J.; Séchet, V.; Herrenknecht, C.; Truquet, P.; Hervé, F.; Bérard, J. B.; Nulty, C.; Taylor, S.; Tillmann, U.; Miles, C. O.; Hess, P. Production and isolation of azaspiracid-1 and -2 from *Azadinium spinosum* culture in pilot scale photobioreactors. *Marine Drugs*, **2012**, *10*, 1360–1382.

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(21) Töbe, K.; Joshi, A. R.; Messtorff, P.; Tillmann, U.; Cembella, A. D.; John, U. Molecular discrimination of taxa within the dinoflagellate genus *Azadinium*, the source of azaspiracid toxins. *Journal of Plankton Research*, **2013**, *35*, 225–230.

(22) Gu, H.; Luo, Z.; Krock, B.; Witt, M.; Tillmann, U. Morphology, phylogeny and azaspiracid profile of *Azadinium poporum* (Dinophyceae) from the China Sea. *Harmful Algae*, **2013**, *21*–22, 64–75.

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(24) Jauffrais, T.; Séchet, V.; Herrenknecht, C.; Truquet, P.; Véronique, S; Tillmann, U.; Hess, P. Effect of environmental and nutritional factors on growth and azaspiracid production of the dinoflagellate *Azadinium spinosum*. *Harmful Algae*, **2013**, *27*, 138–148.

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(33) Kilcoyne, J.; McCarron, P.; Twiner, M. J.; Nulty, C.; Crain, S., Wilkins, A. L.; Rise, F.; Quilliam, M. A.; Miles, C. O. Epimers of azaspiracids: isolation, structural elucidation, relative LC-MS response, and *in vitro* toxicity of 37-*epi*-azaspiracid-1. *Chemical Research in Toxicology*, **2014**, *27*, 587–600.

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(35) Kilcoyne, J.; Twiner, M. J.; McCarron, P.; Crain, S.; Giddings, S.; Wilkins, A. L.; Hess, P.; Miles, C. O. Isolation, structural determination, relative molar LC-MS responses, and toxicities of AZA7–10. **2014**, manuscript in preparation.

(36) Kilcoyne, J.; Twiner, M. J.; McCarron, P.; Giddings, S. D.; Hess, P.; Miles, C. O. Structural elucidation and toxicity of a unique azaspiracid isolated from shellfish *(Mytilus edulis)*. **2014**, manuscript in preparation.

16.2. Book chapters

Tillmann, U., Salas, R., Jauffrais, T., Hess, P., and Silke, J., **2014**. Azaspiracids; the producing organisms: biology and food web transfer. In: *Seafood and Freshwater toxins, 3rd Ed. Botana, L. M. (Ed.), CRC Press, Boca Raton, FL*., pp 773–796.

Hess, P., McCarron, P., Krock, B., Kilcoyne, J., and Miles, C. O., **2014**. Azaspiracids: chemistry, biosynthesis, metabolism and detection. In: *Seafood and Freshwater toxins, 3rd Ed. Botana, L. M. (Ed.), CRC Press, Boca Raton, FL*., pp 799–821.

Twiner, M. J., Hess, P., and Doucette, G. J., **2014**. Azaspiracids: toxicology, pharmacology, and risk assessment. In: *Seafood and Freshwater toxins, 3rd Ed. Botana, L. M. (Ed.), CRC Press, Boca Raton, FL*, pp 823-855.

16.3. Oral presentations

(1) Twiner, M. J., Hess, P., and Doucette, G. J., 2009. From cells, to transcripts, to proteins, to substrates: the tell-all azaspiracid story.
Gordon conference on mycotoxins and phycotoxins, Colby-Sawyer college, New London, NH, USA.

(2) Krock, B., Tillmann, U. And Cembella, A., 2009. Re-isolation of the azaspiracid producing dinoflagellate *Azadinium spinosum* from the Danish west coast.
7th International conference on molluscan shellfish safety, Nantes, France.

(3) Tillmann, U., 2009. Who the hell produce azaspiracids?
Invited talk, Bequalm workshop, Galway, Ireland.

(4) Twiner, M. J., 2010. Toxicological characterization of emerging algal toxins in US waters.
Invited presentation: Eastern Michigan University, Ypsilanti, Michigan, USA.

(5) Twiner, M. J., Hess, P., El-Ladki, R., Butler, S. C., Doucette, G. J., 2010. Toxicological characterization of emerging algal toxins in US waters.
14th International conference on Harmful Algae, Hersonissos-Grete, Greece.

(6) Silke, J., Salas, R., Kilcoyne, J., Lyons, K., Burson, A., Duffy, C., Tillmann, U., 2010. *Azadinium spinosum* in Irish waters, an azaspiracid producing dinoflagellate.
14th International conference on Harmful Algae, Hersonissos-Crete, Greece.

(7) Jauffrais, T., Séchet, V., Philippe, T., Marcaillou, C., Herrenknecht, C., Salas, R., Silke, J., Kilcoyne, J., Burson, A., Duffy, C., Tillmann, U., Amzil, Z., Hess, P., 2011. Effect of the toxic dinoflagellate *Azadinium spinosum* on azaspiracid accumulation in blue mussels *(Mytilus edulis)*.
International Conference on Molluscan Shellfish Safety (ICMSS), Charlottetown, PEI, Canada.

(8) Tillmann, U., Elbrächter, M., John, U., Krock, B, Salas, R., 2011. The dinoflagellate genus *Azadinium* identified as the planktonic producer of azaspiracid toxins.
Invited talk, National Oceanographic and Atmosperic Administration (NOAA), Seattle (Washington), Seminar at Northwest Fisheries Science Center, Seattle (WA), USA.

(9) Salas, R., Tillmann, U., John, U., Kilcoyne, J., Burson, A., Cantwell, C., Duffy, C., Hess, P., Jauffrais, J., Silke, J., 2011. Culture and isolation of *Azadinium spinosum* (Dinophyceae) and its role in the production of azaspiracid toxins.
Biotoxins and chemical residues in food, annual conference, Queens University, Belfast, N. Ireland

(10) O'Driscoll, D., 2011. ASTOX 2: Azaspiracids, toxicological evaluation, test methods and identification of the source organism.
Biotoxins and chemical residues in food, annual conference, Queens University, Belfast, N. Ireland

(11) Kilcoyne, J., Fux, E., Quilliam, M. A., Miles, C. O., Hess, P., 2011. Meeting the demands for sustainable supply of OA group and AZA certified standards and matrix effects associated with these toxins in the analysis of shellfish by LC-MS/MS.
AOAC, marine and freshwater toxins analysis. Second joint symposium and AOAC task force meeting, Baiona, Spain.

(12) Miles, C. O., McCarron, P., Kilcoyne, J., Jauffrais, T., Quilliam, M. A., Miles, Hess, P., 2011. Biotransformations and chemical reactions of azaspiracids and related analogues.
125th AOAC annual meeting and exposition, New Orleans, USA.

(13) Potvin, E. , Jeong, H. J. , Kang, N. S. , Noh, J. H. , Tillmann, U. and Krock, B., 2011. First Report of the phototrophic dinoflagellate genus *Azadinium* in Korean waters: morphology, DNA sequences and pigments of *A. cf. poporum*.
DINO9, IX International conference on modern and fossil dinoflagellates, Liverpool, UK.

(14) O'Driscoll, D., 2011. ASTOX 2: Azaspiracids, toxicological evaluation, test methods and identification of the source organism.
IFA aquaculture shellfish conference, Cork, Ireland.

(15) Krock, B., Tillmann, U., Jeong, H. J., Potvin, E., Salas, R., Kilcoyne, J., Gu, H., 2012. Novel azaspiracids produced by *Amphidomataceae*.
15th International conferance on Harmful Algae (ICHA), Changwon, Korea.

(16) Tillmann, U., Elbrächter, M., Gottschling, M., Gu, M., Jeong, H. J., Krock, B., Nezan, E., Potvin, E., Salas, R., Soehner, S., 2012. The dinophycean genus *Azadinium* and related species – the algal source of azaspiracid toxins.
15th International conferance on Harmful Algae (ICHA), Changwon, Korea.

(17) Madhkoor, A. K., Doucette, G. J., Twiner, M. J., 2012. Induction of apoptosis pathways in lymphocyte T cells following exposure to the marine algal toxin azaspiracid-1.
The Michigan society of toxicology regional meeting, Ann Arbor, Michigan, USA.

(18) Rasky, A., Foping, D., Hamman, M. T., Doucette, G. J., Twiner, M. J., 2012. Novel identification of the marine algal toxin azaspiracid as a potassium channel inhibitor.
American Chemical Society annual meeting, San Diego, CA, USA.

(19) Jauffrais, T., Kilcoyne, J., Séchet, V., Herrenknecht, C., Truquet, P., Hervé, F., Nulty, C., Tillmann, U., Miles, C. O. And Hess, P., 2012. Production and preparative isolation of azaspiracid-1 and -2 from *Azadinium spinosum* culture in stirred photobioreactors.
IUPAC – mycotoxins and phycotoxins, Rotterdam, The Netherlands.

(20) Jauffrais, T. And Hess, P., 2012. Recent application of algal culture for the production of azaspiracids and recent initiative for the integration of environmental and productive science with commercial stakeholders.
Journées de Concarneau "Where industry meets science", Concarneau, France.

(21) Kilcoyne, J., Twiner, M. J., McCarron, P., Crain, S., Wilkins, A. L., Nulty, C., Quilliam, M. A., Hess, P., Miles, C. O., 2013. AZA analogues – isolation, *in vitro* toxicity and relevance to human health.
Shellfish safety workshop, Marine Institue, Galway, Ireland.

(22) Salas, R., Tillmann, U., Elbrächter, M., Gottschling, M., Gu, H., Jeong, H. J., Krock, B., Nézan, E., Potvin, E., Soehner, S. 2013. The dinophycean genus *Azadinium*: the algal source of azaspiracid toxins.
Shellfish safety workshop, Marine Institue, Galway, Ireland.

(23) Duffy, C., Geraghty, J., Aasen, J., 2013. Azaspiracids – are they as toxic when consumed?
Shellfish safety workshop, Marine Institue, Galway, Ireland.

(24) Kilcoyne, J., Twiner, M. J., McCarron, P., Crain, S., Wilkins, A. L., Nulty, C., Quilliam, M. A., Hess, P., Miles, C. O., 2013. AZA analogues – isolation, *in vitro* toxicity and relevance to human health.
AOAC, marine and freshwater toxins analysis. second joint symposium and AOAC task force meeting, Baiona, Spain.

(25) Kilcoyne, J., Twiner, M. J., McCarron, P., Crain, S., Wilkins, A. L., Nulty, C., Quilliam, M. A., Hess, P., Miles, C. O., 2013. AZA analogues – isolation, *in vitro* toxicity and relevance to human health.
Annual Irish chemistry symposium, Trinity College Dublin, Ireland.

(26) Tillmann, U., Elbrächter, M., Gottschling, M., Gu, M., Jeong, H. J., Krock, B., Nezan, E., Potvin, E., Salas, R., Soehner, S., 2013. The dinophycean genus *Azadinium* and related species – the algal source of azaspiracid toxins.
ASTOX 2 dissemination workshop, Marine Institute, Galway, Ireland.

(27) Clarke, D.,Töebe, K., Joshi, A. R., Messtorff, P., Tillman, U., Cembella, A., John, U., Gosek, K., Brajčić, T., Hunt, K., Salas, R., 2013. Azaspiracid producing organisms in Irish waters – monitoring with molecular probes.
ASTOX 2 dissemination workshop, Marine Institute, Galway, Ireland.

(28) Jauffrais, T., Hess, P., Sechet, V., 2013. *Azadinium spinosum*, toxin production and trophic transfer to shellfish.
ASTOX 2 dissemination workshop, Marine Institute, Galway, Ireland.

(29) Krock, B., 2013. New (putative) azaspiracids in marine dinoflagellates.
ASTOX 2 dissemination workshop, Marine Institute, Galway, Ireland.

(30) Kilcoyne, J., Twiner, M. J., McCarron, P., Crain, S., Wilkins, A. L., Nulty, C., Quilliam, M. A., Hess, P., Miles, C. O., 2013. AZA analogues – isolation, *in vitro* toxicity and relevance to human health.
ASTOX 2 dissemination workshop, Marine Institute, Galway, Ireland.

(31) McCarron, P., 2013. Analytical methods and reference materials for azaspiracids.
ASTOX 2 dissemination workshop, Marine Institute, Galway, Ireland.

(32) Samdal, I., Miles, C.O., 2013. Antibodies and ELISAs for azaspiracids.
ASTOX 2 dissemination workshop, Marine Institute, Galway, Ireland.

(33) Campbell, K., Elliot, C., 2013. Novel approaches for the detection of azaspiracid - biology vs chemistry, targeted and untargeted analysis.
ASTOX 2 dissemination workshop, Marine Institute, Galway, Ireland.

(34) Twiner, M., 2013. The azaspiracid story from A to Z: a mechanism of action revealed.
ASTOX 2 dissemination workshop, Marine Institute, Galway, Ireland.

(35) Sosa, S., 2013. Acute oral toxicity of azaspiracids in mice.
ASTOX 2 dissemination workshop, Marine Institute, Galway, Ireland.

(36) Aasen, J., Espenes, A., Larsen, S., Geraghty, J., Kilcoyne, J., Duffy, C., Hess, P., 2013. Toxicology of AZAs: 1. Bioavailability of AZAs in minipig.
2. Intraperetoneal *(ip)* LD_{50} trial on AZA1, AZA2, AZA3 and AZA6 on NMRI mice.
ASTOX 2 dissemination workshop, Marine Institute, Galway, Ireland.

(37) Doucette, G., Twiner, M., 2013. AZA measurements in blood and subcellular localization of azaspiracid receptors using surface plasmon resonance.
ASTOX 2 dissemination workshop, Marine Institute, Galway, Ireland.

(38) Geraghty, J., 2013. *In vivo* studies of azaspiracids.
ASTOX 2 dissemination workshop, Marine Institute, Galway, Ireland.

(39) Hess, P., Kilcoyne, J., Jauffrais, T., Tillmann, U., Clarke, D., Silke, J., Salas, R., Krock, B., Aasen, J., Geraghty, J., McCarron, P., Sosa, S., Tubaro, A., Twiner, M., Doucette, G., 2013. ASTOX 2 - an example of research in support of legislation.
XVIth workshop of EU-RL/NRLs for marine biotoxins, Split, Croatia.

(40) Hess, P., Kilcoyne, J., Jauffrais, T., Tillmann, U., Clarke, D., Silke, J., Salas, R., Krock, B., Aasen, J., Geraghty, J., McCarron, P., Sosa, S., Tubaro, A., Twiner, M., Doucette, G., 2013. ASTOX 2 - an example of research in support of legislation.
French research network on marine natural products, Lorient, France.

(41) Geraghty, J., Duffy, C., Aasen, J. A. B., Hess, P., Foley, B. 2013. *In vivo* studies of azaspiracids.
Irish Society of Toxicology annual meeting, Dublin, Ireland.

(42) Kilcoyne, J., Twiner, M. J., McCarron, P., Crain, S., Wilkins, A. L., Nulty, C., Quilliam, M. A., Hess, P., Miles, C. O., 2013. Isolation of minor and novel azaspiracids – structure elucidation and toxicology.
Irish Society of Toxicology annual meeting, Dublin, Ireland.

(43) Geraghty, J., Duffy, C., Aasen, J. A. B., Hess, P., Foley, B. 2013. *In vivo* studies of azaspiracids.
21st Meeting of the French Society of Toxinology (SFET), Paris, France.

(44) Kilcoyne, J., Twiner, M. J., McCarron, P., Crain, S., Wilkins, A. L., Nulty, C., Quilliam, M. A., Hess, P., Miles, C. O., 2013. Isolation of minor and novel azaspiracids – structure elucidation and toxicology.
21st Meeting of the French Society of Toxinology (SFET), Paris, France.

16.4. Poster presentations

(1) Kilcoyne, J., Clancy, G., Keogh, A., Duffy, C., LeBlanc, P., Burton, I., Quilliam, M. A., Miles, C. O., 2010. Isolation of AZAs from highly contaminated blue mussel shellfish *(Mytilus edulis)*.
14th International conference on Harmful Algae, Hersonissos-Crete, Greece.

(2) Töbe, K., Joshi, A., Tillmann, U., Cembella, A. and John, U., 2010. Detection of and discrimination within the novel dinoflagellate genus *Azadinium*, the source of azaspiracid toxins.
14th International conference on Harmful Algae, Hersonissos-Crete, Greece.

(3) Tillmann, U., Elbrächter, M., John, U., Krock, B., Messtorff, P., Salas, R. and Schweikert, M., 2010. The dinoflagellate genus *Azadinium*: an update.
14th International conference on Harmful Algae, Hersonissos-Crete, Greece.

(4) Jauffrais, T., Séchet, V., Herrenknecht, C., Tillman, U., Krock, B., Amzil, Z., Hess, P., 2010. Growth and toxin production of *Azadinium spinosum* in batch and continuous culture.
14th International conference on Harmful Algae, Hersonissos-Crete, Greece.

(5) Geraghty, J., Foley, B., O'Driscoll, D., Duffy, C., 2010. *In vitro* digestive simulation to investigate the bioaccessibility of marine biotoxin, azaspiracid, in blue mussels *(Mytilus edulis)*.
14th International conference on Harmful Algae, Hersonissos-Crete, Greece.

(6) Messtorff, P., Tillmann, U., Krock, B., Cembella, A. D., 2010. Fate of *Azadinium spinosum* and associated azaspiracids in marine planktonic food webs.
14th International conference on Harmful Algae, Hersonissos-Crete, Greece.

(7) Geraghty, J., Foley, B., O'Driscoll, D., Duffy, C., 2011. *In vitro* digestive simulation to investigate the bioaccessibility of marine biotoxin, azaspiracid, in blue mussels *(Mytilus edulis)*.
Annual Irish chemistry symposium, Dublin Institute of Technology, Ireland.

(8) Jauffrais, T., Séchet, V., Philippe, T., Herrenknecht, C., Amzil, Z., Hess, P., 2011. Effect of flow rate on *Azadinium spinosum* and azaspiracid productivities in medium scale chemostats in series for azaspiracid-1 and -2 harvests.
International Conference on Molluscan Shellfish Safety (ICMSS), Charlottetown, PEI, Canada.

(9) Giddings, S. D., Miles, C. O., Quilliam, M. A., McCarron, P., 2011. Derivatisation of azaspiracid biotoxins for analysis by liquid chromatography with fluorescence detection.
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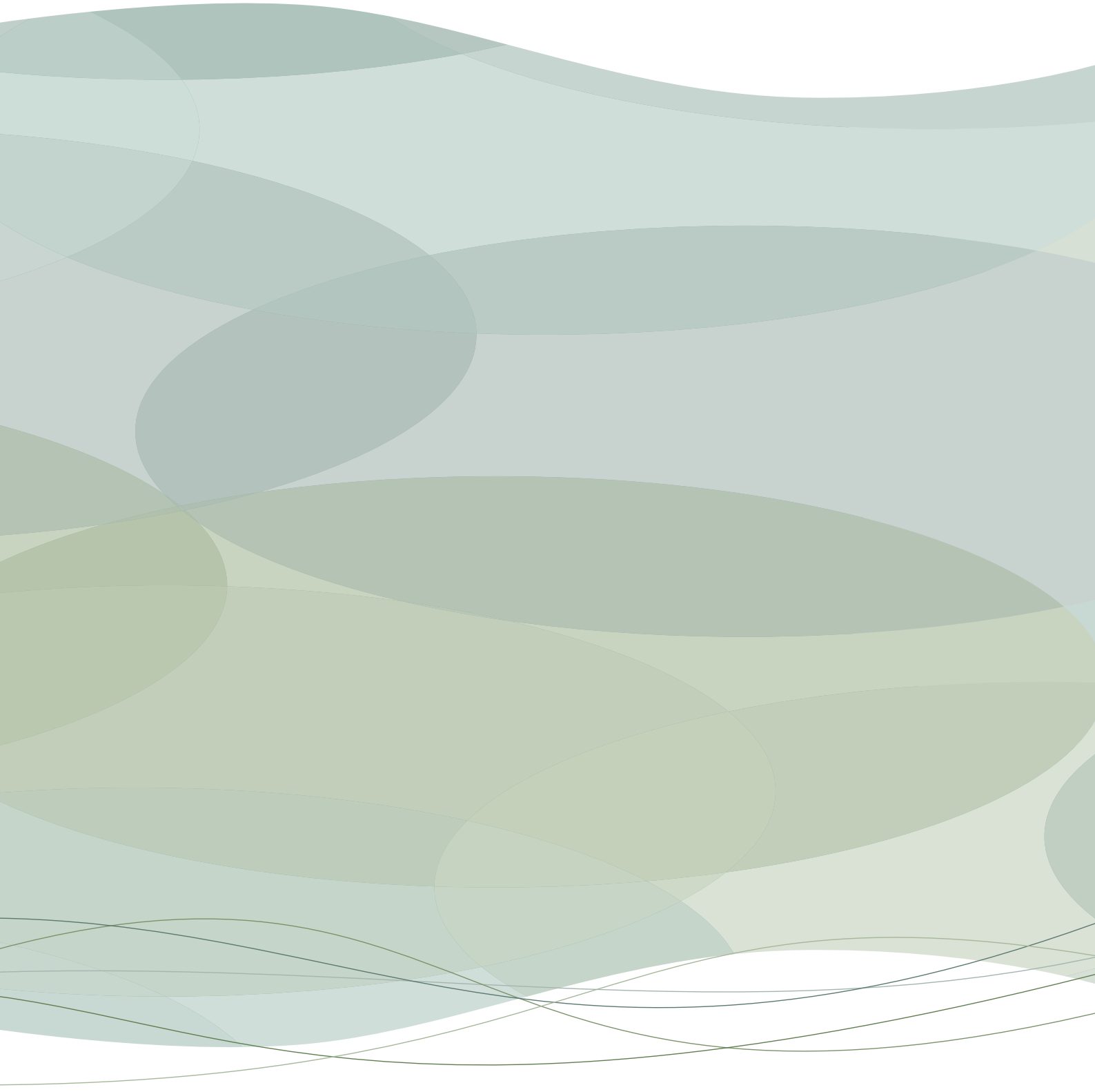

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