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Toxic equivalency factors (TEFs) after acute oral exposure of azaspiracid 1, −2 and −3 in mice

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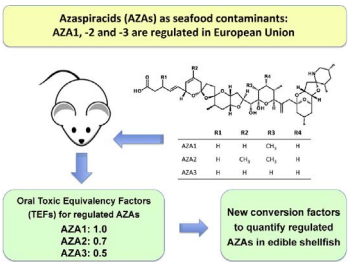
GRAPHICAL ABSTRACT



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ABSTRACT

Azaspiracids (AZAs) are marine algal toxins that can be accumulated by edible shellfish to cause a foodborne gastrointestinal poisoning in humans. In the European Union, only AZA1, −2 and −3 are currently regulated and their concentration in shellfish is determined through their toxic equivalency factors (TEFs) derived from the *intraperitoneal* lethal potency in mice. Nevertheless, considering the potential human exposure by oral route, AZAs TEFs should be calculated by comparative *oral* toxicity data. Thus, the acute oral toxicity of AZA1, −2 and −3 was investigated in female CD-1 mice treated with different doses (AZA1: 135–1100 μg/kg; AZA2 and AZA3: 300–1100 μg/kg) and sacrificed after 24 h or 14 days. TEFs derived from the median lethal doses (LD_{50}) were 1.0, 0.7 and 0.5, respectively for AZA1, −2 and −3. In fact, after 24 h from gavage administration, LD_{50s} were 443 μg/kg (AZA1; 95% CL: 350 − 561 μg/kg), 626 μg/kg (AZA2; 95% CL: 430 − 911 μg/kg) and 875 μg/kg (AZA3; 95% CL: 757 − 1010 μg/kg). Mice dead more than 5 h after the treatment or those sacrificed after 24 h (doses: ≥175 μg AZA1/kg, ≥500 μg AZA2/kg and ≥600 μg AZA3/kg) showed enlarged pale liver, while increased serum markers of liver alteration were recorded even at the lowest doses. Blood chemistry revealed significantly increased serum levels of K^+ ions (≥500 mg/kg), whereas light microscopy showed tissue changes in the gastrointestinal tract, liver and spleen. No lethality, macroscopic, tissue or haematological changes were recorded two weeks post exposure, indicating reversible toxic effects. LC–MS/MS analysis of the main organs showed a dose-dependency in gastrointestinal absorption of these toxins: at 24 h, the highest levels were detected in the stomach and, in descending order, in the intestinal content, liver, small intestine, kidneys, lungs, large intestine, heart as well as detectable traces in the brain. After 14 days, AZA1 and AZA2 were still detectable in almost all the organs and intestinal content.

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1. Introduction

Azaspiracids (AZAs) are cyclic polyethers produced by marine dinoflagellates of the genera *Azadinium* and *Amphidoma* (Tillmann et al., 2009, 2017; Krock et al., 2012) that can accumulate in edible filter-feeding shellfish. Consumption of AZAs contaminated shellfish causes a severe acute gastrointestinal poisoning (Azaspiracid Shellfish Poisoning, AZP), characterized by nausea, vomiting, diarrhoea and stomach cramps (Furey et al., 2010). The first documented episode ascribed to these toxins occurred in 1995 in The Netherlands, after consumption of mussels harvested in Ireland (McMahon and Silke, 1996). Since then, other cases were documented in Ireland or in other countries after consumption of exported Irish mussels, while different AZA analogues were progressively detected in marine organisms from different geographic areas (Alvarez et al., 2010; Bacchiocchi et al., 2015; James et al., 2002; Krock et al., 2014; López-Rivera et al., 2010; Magdalena et al., 2003; Percopo et al., 2013; Rossi et al., 2017; Taleb et al., 2006; Tillmann et al., 2016; , 2017; Torgersen et al., 2008; Trainer et al., 2013; Turner and Goya, 2015; Twiner et al., 2008a; Ueoka et al., 2009; Zamorano et al., 2013), suggesting a worldwide distribution of these toxins.

AZAs include more than 40 analogues that are either produced by *Azadinium* and/or *Amphidoma* dinoflagellates, through biotransformation in shellfish, or as by-products formed during storage or cooking of AZAs-contaminated shellfish (Jauffrais et al., 2013; Krock et al., 2012; McCarron et al., 2009; Rehmann et al., 2008; Satake et al., 1998). However, only AZA1, −2 and −3, differing in their methylation degree, are currently regulated in European Union due to the occurrence and toxicity: the maximum toxin level in edible shellfish has been set at 160 μg AZA1 equivalents/kg shellfish meat (European Commission, 2004; EFSA, 2008). The concentration of these toxins is calculated converting the amounts of individual toxins to AZA1 equivalents (EFSA, 2008; European Commission, 2011), using their toxic equivalency factors (TEFs: 1.0, 1.8 and 1.4 for AZA1, −2 and −3, respectively) derived from the lethal *intraperitoneal* doses in mice (200, 110 and 140 μg/kg for AZA1, −2 and −3, respectively) (Ofuji et al., 1999; Satake et al., 1998). However, due to the potential human exposure to AZAs by oral route, the Scientific Opinion of the European Food Safety Authority Panel on Contaminants in the Food Chain recommended the establishment of robust TEFs on the basis of comparative oral toxicity data (EFSA, 2008), which are still lacking.

Oral toxicity studies on this group of toxins are limited to AZA1 and variable toxic potency was recorded in mice: 24 h after single AZA1 oral administration in mice, Aune et al. (2012) recorded lethal effects at 600 μg/kg and an LD_{50} (lethal dose for 50% of the treated mice) of 775 μg/kg, while previous studies showed lethality at doses ranging from 250 to > 700 μg/kg (Aasen et al., 2010, 2011; Ito et al., 2000, 2002, 2006). The toxin, detected in almost all the main organs and tissues, affected mainly the gastrointestinal tract, liver and lymphoid organs (Aasen et al., 2010, 2011; Aune et al., 2012; Ito et al., 2000, 2002, 2006). In particular, single administration of oral doses ranging from 300 to 900 μg/kg was shown to induce material retention in the stomach and upper small intestine, erosion and necrosis of the lamina propria in the latter, as well as liver alterations visible by pale colour of the organ and microscopically by fatty changes in the hepatocytes. Necrotic lymphocytes in the lymphoid organs were also recorded (Aune et al., 2012; Ito et al., 2000, 2006). At sub-lethal doses (100–300 μg/kg), pathological changes were observed in the duodenum of mice within 24 h from AZA1 administration (cellular detachment in the tips of villi, expansion of the crypts, necrotic/apoptotic changes in lamina propria and neutrophilic infiltration), which recovered within 7 days (Aasen et al., 2010, 2011).

Other studies evaluated the effects of AZA1 after repeated oral administration in mice. After two oral doses of AZA1 in mice of different ages (250–450 μg/kg, two days apart), Ito et al. (2002) observed serious gastrointestinal, lung and liver injuries, some of which persisting up to more than 3 months. Repeated oral administration of lower doses (20–50 μg/kg, twice a week for 18–40 times) was shown to cause interstitial pneumonia, liver changes, gastrointestinal erosions and lethal effects. Lung tumours were also recorded (4/20 mice) at 20 or 50 μg/kg. At doses as low as 1 or 5 μg/kg (twice a week for 40 times followed by a recovery period of three months), intestinal or liver changes were noted (Ito et al., 2002).

To assess the acute oral toxicity of the three AZAs regulated in the European Union and calculate their toxic equivalency factors (TEFs) after oral exposure to be used for regulatory purposes, a comparative oral toxicity study on AZA1, −2 and −3 was carried out in mice. The toxins distribution in the main organs and tissues of mice was also assessed by liquid chromatography-tandem mass spectrometry (LC–MS/MS).

2. Materials and methods

2.1. Toxins, animals and other materials

2.1.1. Toxins and other materials

AZAs were isolated from *Mytilus edulis*, as reported by Kilcoyne et al. (2012). The purity (> 95%) of each toxin (including the 37-epimers) was confirmed by LC–MS/MS (AZA1–10 quantitation) and NMR analyses as reported by Kilcoyne et al. (2014) (Supplementary material: Figs. S1–S6; Tables S1–S3). All solvents (pestican grade) were purchased from Labscan (Dublin, Ireland). If not otherwise specified, other chemicals were from Sigma Aldrich (Milan, Italy and Steinheim, Germany). AZA Certified Reference Materials (CRMs) were obtained from the National Research Council of Canada (Halifax, NS, Canada). Laboratory Reference standard Materials (LRMs) of AZA4 − AZA10 were prepared as described by Kilcoyne et al. (2015).

2.1.2. Animals

Female CD-1 mice (18–20 g) were purchased from Harlan Laboratories (Udine, Italy). Animals were acclimatized for 2 weeks before the experiments, at controlled temperature (21 ± 1° C) and humidity (60–70 %), under a fixed artificial light cycle (07.00–19.00). Animals experiments were carried out at the University of Trieste in compliance with the Italian Decree (n. 116/1992, the EU Directive 2010/63/EU) and the European Convention ETS 123.

2.2. Experimental design

AZAs, dissolved in phosphate buffered saline (PBS) pH 7.0 containing 1.8% (v/v) ethanol, were administered by gavage (volume: 10 mL/kg) to mice fasted for 3 h, at the following dose ranges: from 135 to 1100 μg/kg (AZA1; nine groups of 8 or 16 mice/dose) or from 300 to 1100 mg/kg (AZA2 or AZA3; respectively six groups of 16 mice/dose). Control mice were administered with the vehicle alone (10 mL/kg; 16 mice). Signs of toxicity and lethality were recorded up to 24 h and up to 14 days for subgroups of surviving mice (3 or 6/dose, which body weight and food consumption were daily recorded in the morning). At the scheduled times of sacrifice (24 h after treatment and 14 days after treatment for the subgroup of surviving mice), animals were weighed, anesthetized by intraperitoneal injection of tiletamine/zolazepam (Zoletil®; Virbac; Milan, Italy; 20 mg/kg) and xylazine (Virbac; Milan, Italy; 5 mg/kg) and exsanguinated to collect blood from the abdominal aorta. Half of the mice from each group of treatment were used to evaluate the acute toxicity of AZA1, −2 and −3, while the others were used to evaluate the toxin distribution in the main organs and intestinal content, as specified below.

2.2.1. Acute oral toxicity

Collected blood was allowed to clot for 15 min at room temperature, and centrifuged at 2,000*g* for 10 min at 4° C. Serum was collected and stored at − 80 °C until the hematochemical analyses were carried out

(see Section 2.3). Exsanguinated animals were then necropsied, the main organs and tissues were removed and fixed in 10% neutral buffered formalin for the histological analysis (see Section 2.4). Similarly, animals that died during the observation were immediately weighed and necropsied to collect blood for hematochemical analyses and remove/fix the main organs and tissues for the histological examination. Mice eventually found dead were not considered for gross pathology examination and other analyses.

2.2.2. AZAs distribution

Exsanguinated mice were necropsied to collect and weigh the main organs (heart, lungs, liver, stomach, small intestine, large intestine, kidneys, brain and intestinal content), which were stored at −80 °C until LC–MS/MS analysis to assess the toxins concentration and the recovery of the relevant administered dose (see Section 2.5). Similarly, spontaneously died animals during the observation were immediately weighed and necropsied to remove and weigh the main organs and intestinal content for toxin quantitation.

2.3. Hematochemistry

Using an automatized analyzer (AU400 Olympus with Beckman Coulter reagents), the following parameters were quantified in the serum of mice: aspartate aminotransferase (AST), alanine aminotransferase (ALT), glutamate dehydrogenase (GLDH), creatine phosphokinase (CPK), creatinine, Na^+, K^+, Cl^-, Ca^{2+} and inorganic phosphate (P_i).

2.4. Histological analysis

Heart, liver, lungs, kidneys, spleen, stomach, duodenum, jejunum, colon, rectum, pancreas, thymus, cerebrum, cerebellum, uterus, ovaries and skeletal muscle (soleus) were fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin. Sections (thickness: 5 μm) were stained with hematoxylin-eosin and blind histopathological examination was carried out. Images were obtained with a Nikon eclipse *i* 50 microscope equipped with a DS-Vi1 digital camera and NIS-Elements Microscope Imaging Software.

2.5. AZAs distribution in the main organs and intestinal content

Organs or intestinal content (~0.2 g) were extracted with methanol (1 mL) by mashing the tissues up with a steel rod followed by vortex mixing for 1 min. Samples were centrifuged at 3,000*g* for 5 min and the extract decanted. The remaining pellet was extracted a second time as described above. The two combined extracts were filtered through a glass pipette plugged with cotton wool into HPLC vials for analysis by LC–MS/MS.

Analysis was performed on an Acquity UPLC coupled to a Xevo G2-S QToF (Waters, Manchester, UK) operated in positive MS^e mode, scanning from 100 to 1200 *m/z*. Leucine encephalin was used as the reference compound. The cone voltage was 40 V, collision energy was ramped from 40 to 70 eV, the cone and desolvation gas flows were set at 0 and 600 L/h, respectively, and the source temperature was 120 °C.

A binary gradient elution was used, with phase A consisting of water and phase B of 95% acetonitrile in water (both containing 2 mM ammonium formate and 50 mM formic acid). The column used was a 50 mm × 2.1 mm i.d., 1.7 μm, Acquity UPLC BEH C18 (Waters, Wexford, Ireland). The gradient was from 30 to 90% B over 5 min at 0.3 mL/min, held for 0.5 min, and returned to the initial conditions and held for 1 min to equilibrate the system. The injection volume was 2 μL and the column and sample temperatures were 25 °C and 6 °C, respectively.

2.6. Statistical analysis

Data are expressed as mean ± standard deviation (S.D.) or median (interquartile range) for survival times. Significant differences between control and experimental groups were calculated by one-way analysis of variance, followed by the Dunnett's test for multiple comparisons of unpaired data, accepting $p < 0.05$ as significant. LD_{50} (lethal dose for 50% of the treated mice), based on 24 h mortality data, was calculated according to the Finney method at a 95% confidence level (Finney, 1971).

Table 1
Lethality and survival times of mice after acute oral AZAs administration.

Toxin	Dose (μg/kg)	Lethality at 24 h*	Survival times (h:min) of mice that died within 24 h	Lethality after 24 h*	Survival times (h:min) of mice that died after 24 h
AZA1	135	0/16	–	0/16	–
	175	0/16	–	0/16	–
	230	2/16	23:42–23:49	0/16	–
	300	4/16	11:55 – 14:54-17:56-22:44-24:26-24:44	2/16	24:26–24:44
	390	11/16	12:08 – 12:55-10:27-11:41-19:00-20:45-20:50-20:51-21:36-22:03-22:48	2/16	26:48–29:12
	500	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	0/16	–
	650	5/8	08:22 – 08:38-09:47-10:26-10:57	2/8	24:15–24:23
	850	5/8	00:23 – 00:30-09:21-10:44-13:19	2/8	25:59–26:31
	1100	8/8	00:56 – 01:19 – 01:35-03:19-03:36-06:39-08:33-09:01	0/8	–
AZA2	300	0/16	–	0/16	–
	390	0/16	–	0/16	–
	500	3/16	09:47 – 21:05 – 21:50	0/16	–
	650	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	0/16	–
	850	11/16	07:45 – 10:24-12:39-14:25-15:10-16:09-17:31-19:40-19:44-22:10-23:11	0/16	–
	1100	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	0/16	–
AZA3	300	0/16	–	0/16	–
	390	0/16	–	0/16	–
	500	0/16	–	0/16	–
	650	1/16	01:37	0/16	–
	850	11/16	00:54 – 00:58-01:09-01:23-01:44-02:05-02:10-02:23-02:23-22:06-23:40	0/16	–
	1100	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	0/16	–

* Died animals/treated animals.

3. Results

3.1. Mortality

The incidence of death and the median survival times of mice after oral administration of AZA1, −2 or −3 are reported in Table 1 and in the box plot in Supplementary material (Fig. S7). Within 24 h from administration, AZA1 (doses ranging from 135 to 1100 μg/kg) was lethal at the dose of 230 mg/kg (2/16 mice; median survival time: 23.5 h; interquartile range: 23.4–23.5 h) and above, inducing the death of all the mice administered with the highest dose (1100 μg/kg; median survival time: 3.2 h; interquartile range: 1.2–7.8 h). In the subgroups of mice kept alive for 14 days, lethality was recorded within 30 h at 300 and 850 μg/kg (2 mice/dose). After AZA2 administration (300–1100 μg/kg), deaths occurred within 24 h at the dose of 500 μg/kg (3/16 mice; median survival time: 21.0 h; interquartile range: 9.5–21.5 h) and above, and 100% lethality was recorded at 1100 μg/kg (median survival time: 12.8 h; interquartile range: 9.8–17.2 h). No deaths occurred in the subgroups of mice observed up to 14 days. AZA3 (300–1100 μg/kg) induced lethal effects within 24 h at 650 mg/kg (1/16 mice; survival time: 1.4 h) and above, with 75% mortality at 1100 μg/kg (12/16 mice; median survival time: 1.1 h; interquartile range: 0.5–1.5 h). No deaths occurred in the subgroups of mice observed up to 14 days (Table 1).

Based on 24 h lethality data, the LD_{50} of AZA1, −2 and −3 was 443 μg/kg (95% confidence limits, CL: 350–561 μg/kg), 626 μg/kg (95% CL: 430–911 μg/kg) and 875 mg/kg (95% CL: 757–1010 μg/kg), respectively. Considering AZA1 as the reference compound, the *oral* toxic equivalency factors (TEFs) derived from LD_{50} values are 1.0 (AZA1), 0.7 (AZA2) and 0.5 (AZA3) (Table 2).

3.2. Signs and symptoms

The signs and symptoms of mice administered with the toxins (immobility, tremors, abdominal breathing, hypothermia and cyanosis) were noted at lethal AZAs doses (≥230, ≥500 and ≥650 μg/kg for AZA1, −2 and −3, respectively). Furthermore, jumping was recorded in some mice administered with the highest dose of AZA1 (1100 μg/kg) or doses ≥850 μg/kg of AZA3, while no visible evidence of diarrhoea was noted in any mouse.

No signs or symptoms of toxicity were observed in the subgroups of mice kept alive up to 14 days from the treatment. In these animals, food consumption and body weight were similar to those of control mice (data not shown).

3.3. Hematochemistry

A marked increase in the serum levels of transaminases and glutamate dehydrogenase as indices of possible hepatotoxicity was observed both in spontaneously dead mice and in those sacrificed 24 h after AZA administration. In particular, AZA1-treated mice showed an ALT increase ranging from 423 to 3510%, while AST and GLDH were increased by 93–1600% and 595–7263%, respectively. In AZA2 treated mice, the serum levels of these enzymes were increased by 376-2416% (ALT), 314–3498% (AST) and 356–5379% (GLDH). Serum ALT, AST and GLDH levels of AZA3 administered mice were increased by 235–487%, 82–430% and 140–418%, respectively (Fig. 1). In addition, the serum concentration of K^+ ions was significantly increased in mice administered with AZA1, −2 or −3 at doses ≥500 mg/kg (18–80%, 31–53% and 11–22%, respectively). No significant changes in other electrolytes or creatinine serum levels were recorded in spontaneously died mice or in those sacrificed at 24 h (Fig. 2).

At the end of 14-day recovery period, no significant differences in the blood chemistry parameters were recorded between controls and AZAs treated mice (data not shown).

3.4. Necropsy

Necropsy showed macroscopic alterations (i.e. liver and gastrointestinal tract) in spontaneously dead mice and in those sacrificed 24 h after the treatment. At AZA1 doses ≥175 mg/kg, AZA2 doses ≥500 mg/kg and AZA3 doses ≥650 mg/kg, mice that died at approximately 5 h or more after the treatment and those sacrificed after 24 h presented an enlarged pale liver. The liver was weighed and a significant increase of the organ weight was recorded also at lower doses (≥135 μg AZA1/kg, ≥300 μg AZA2/kg and ≥390 μg AZA3/kg), within the following ranges: 29–70% (AZA1), 22–58% (AZA2) and 17–61% (AZA3) (Table 3).

Mice administered with lethal toxins doses (≥230 mg AZA1/kg, ≥500 mg AZA2/kg, ≥650 mg AZA3/kg) also showed increased gas and food material in the stomach, sometimes accompanied with redness of the gastric wall. In addition, the small intestine was dilated and contained fluid material, whereas redness of the intestinal wall and bloody lumen content were recorded in some mice (data not shown).

No macroscopic changes were observed in the subgroups of mice sacrificed 14 days after the treatment (data not shown).

3.5. Histological analysis

Light microscopy analysis showed tissue changes in the small intestine, spleen and liver of mice administered with each toxin. In particular, alterations in the duodenum were recorded in spontaneously dead mice or those sacrificed 24 h after the administration of each AZA dose: slightly contracted and blunt villi, decreased thickness of the brush border of the enterocytes and minimal to moderate increase of apoptotic cells and infiltrated polymophonuclear cells in the lamina propria of the mucosa were noted (Fig. 3). At spleen level, minimal to severe white pulp lymphoid depletion and lymphoid cells necrosis were recorded in mice administered with doses ≥500 μg/kg of AZA1 or AZA2. At doses ≥650 μg/kg, mice showed also liver alterations visible as slight single hepatocytes necrosis (data not shown).

No tissue changes were recorded at the end of 14-day recovery period (data not shown).

3.6. Distribution of AZAs

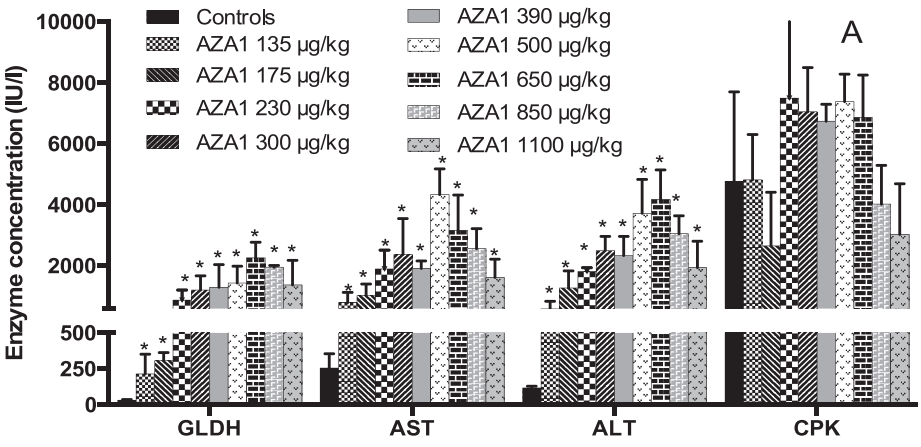
LC–MS/MS analysis of the main organs and intestinal content from spontaneously died mice and those sacrificed at 24 h showed that AZAs were more concentrated in the gastric tissues and, in descending order, in the intestinal content, liver, small intestine, kidneys, lungs, large intestine, heart as well as detectable traces in the brain. The toxins concentrations were highly variable in relation to the large differences of mice survival times, when the organs and intestinal content were collected. Less variable concentrations were detected within 20–24 h post-administration of doses ≤390 μg/kg (AZA1) or ≤650 μg/kg (AZA2 and AZA3), which values are presented in Fig. 4(A–C).

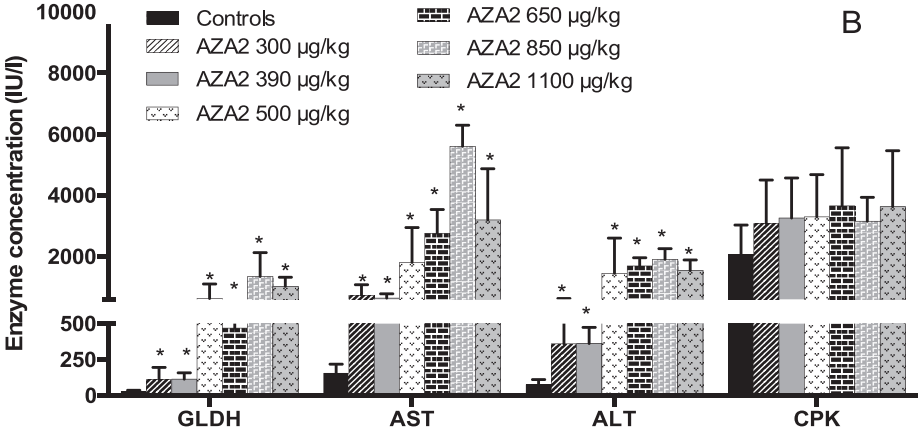
Considering the recovery of the administered doses, AZA1 and AZA2 were recovered mainly from the liver and intestinal content, followed by stomach, small intestine, kidneys, large intestine, lungs, heart and only traces in the brain. AZA3 doses were recovered mainly from the stomach, followed by small intestine, intestinal content, liver,

Table 2
LD_{50} and TEF of AZA1, −2 and −3 based on lethality wihin 24 h from the oral toxins administration.

Toxin	LD_{50} (μg/kg)	95% CL (μg/kg)	Toxic Equivalency Factor
AZA1	443	350–561	1.0
AZA2	626	430–911	0.7
AZA3	875	757–1010	0.5

CL = confidence limits.





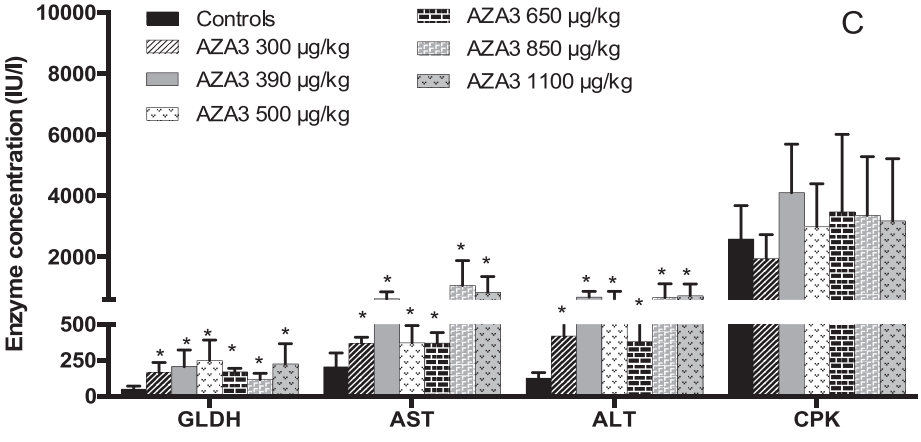


Fig. 1. Mice serum levels of transaminases (ALT and AST), glutamate dehydrogenase (GLDH) and creatine phosphokinase (CPK) at 24 h from the acute oral administration of AZA1 (A), AZA2 (B) or AZA3 (C). Data are the mean ± S.D. from 5 mice; *$p < 0.05$ at the analysis of variance with respect to controls.

kidneys, large intestine, lungs, and traces from the heart and brain. Fig. 5 shows the toxins recovery after 20–24 h from the toxins administration at doses ≤390 µg/kg (AZA1) or ≤650 µg/kg (AZA2 and AZA3): the total recovery of the administered doses was within the ranges 3–23% (AZA1), 4–19% (AZA2) and 1–8% (AZA3).

After 14 days from the administration of AZA1 (175, 300 or 390 µg/kg), AZA2 (300, 500 or 850 µg/kg) or AZA3 (300, 500 or 850 µg/kg), only AZA1 and AZA2 were still detectable in almost all the organs and intestinal content although in very low amounts (total dose recovery: < 3%), with the exception of the brain and the heart where the toxins were detectable only in some mice (data not shown).

4. Discussion

Azaspiracids include more than 40 cyclic polyether analogues but, due to their occurrence and toxicity, only AZA1, −2 and −3 are currently regulated in the European Union and their concentration in shellfish is determined using TEFs derived from their intraperitoneal lethal doses in mice (EFSA, 2008; European Commission, 2011; Ofuji et al., 1999; Satake et al., 1998). Nevertheless, considering the human dietary exposure to these toxins, new TEFs should be derived using comparative oral toxicity data. Thus, a single comparative oral toxicity study on AZA1, −2 and −3 has been carried out in mice. The study showed AZA1 > AZA2 > AZA3 as rank order of lethal potency: based on 24 h lethality data, AZAs oral LD_{50} values were 443 µg/kg (95% confidence limits, CL: 350–561 µg/kg), 626 µg/kg (95% CL:

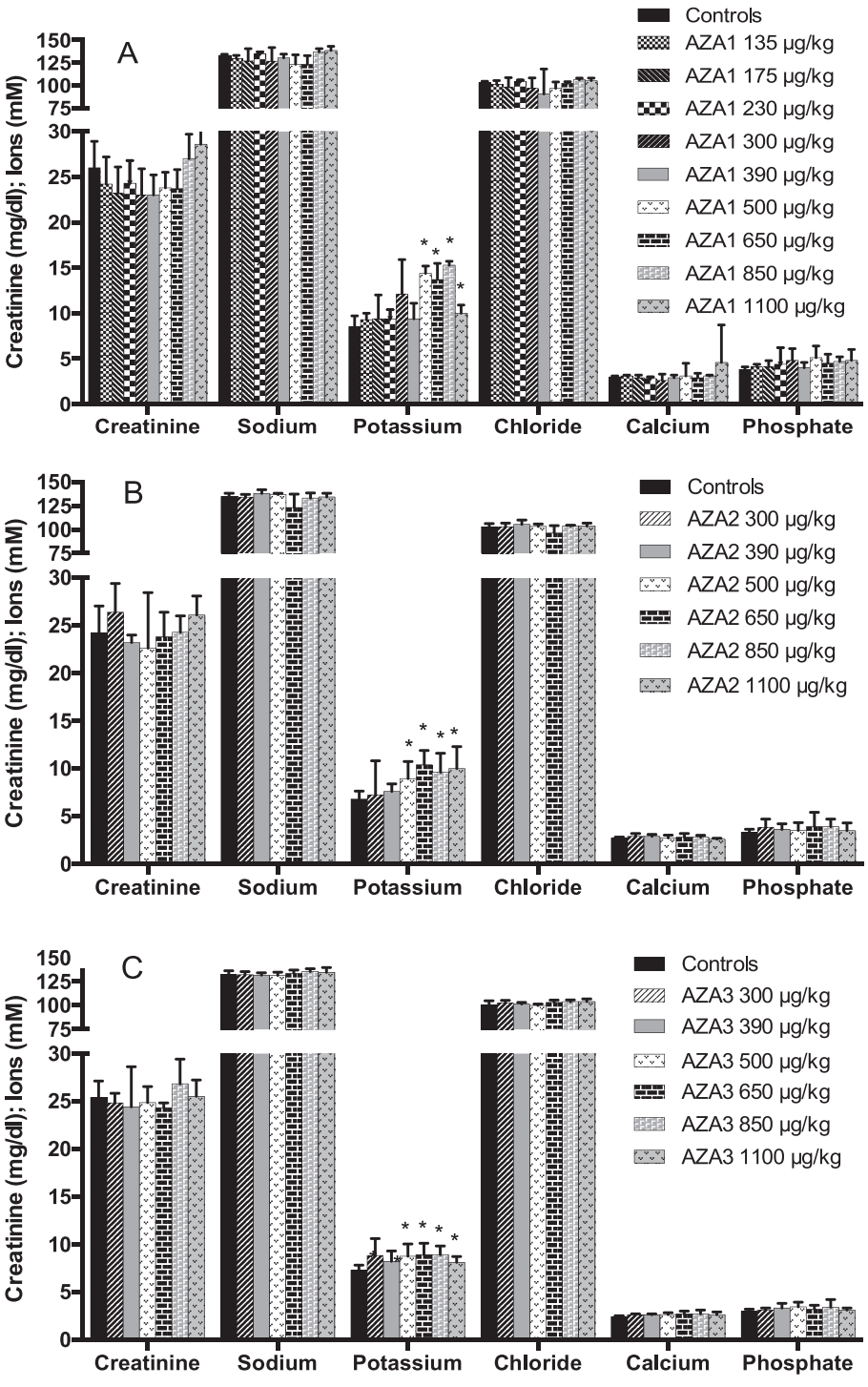


Fig. 2. Mice serum levels of creatinine and electrolytes (Na^+, K^+, Ca^{2+}, Cl^- and inorganic phosphate, P_i) at 24 h from the acute oral administration of AZA1 (A), AZA2 (B) or AZA3 (C). Data are the mean ± S.D. from 5 mice; *$p < 0.05$ at the analysis of variance with respect to controls.

430–911 µg/kg) and 875 mg/kg (95% CL: 757–1010 µg/kg), respectively. Assuming AZA1 as the reference toxin, the TEFs calculated from oral LD_{50} values are 1.0 (AZA1), 0.7 (AZA2) and 0.5 (AZA3), different from those previously obtained after intraperitoneal injection (1.0, 1.8 and 1.4, for AZA1, −2 and −3, respectively). Since, the TEFs derived by intraperitoneal injection indicate AZA2 > AZA3 > AZA1 as rank order of toxic potency, they do not correlate to those obtained by oral administration in this study. From a toxicological point of view, the new TEFs can be suitable for regulatory purposes to quantify toxic equivalents of AZA2 and −3 from the concentrations in edible shellfish due to the following reasons: (1) the dietary human exposure to these toxins, (2) these TEFs are derived within a single comparative oral toxicity study; (3) the administered toxins had the same purity.

The study also showed that the oral lethal potency of AZA1 in mice (LD_{50} = 443 µg/kg) is higher than that recorded by Aune et al. (LD_{50} = 775 µg/kg; Aune et al., 2012), and that the lethal effects of this toxin occurred at a dose (230 µg/kg) lower than those (250–600 or > 700 µg/kg) previously reported (Aasen et al., 2011; Aune et al., 2012; Ito et al., 2000, 2002, 2006). These differences can possibly be ascribed to a slightly differing purity of the toxins, to different strains/age of mice used, or a combination of these factors.

After each toxin administration, the recorded systemic signs and symptoms included immobility, tremors, abdominal breathing, hypothermia and cyanosis, which was coherent to previous findings after

Table 3
Weight of mouse livers at 24 h from acute AZAs oral administration.

Dose (μg/kg)	AZA1		AZA2		AZA3	
	N° mice	Liver weight (g) Mean ± S.D.	N° mice	Liver weight (g) Mean ± S.D.	N° mice	Liver weight (g) Mean ± S.D.
0	10	0.8639 ± 0.1486	10	0.8721 ± 0.1338	10	0.8824 ± 0.1059
135	10	1.1126 ± 0.1423* *(29%)*	–	–	–	–
175	10	1.1416 ± 0.1527* *(32%)*	–	–	–	–
230	10	1.2286 ± 0.2551* *(42%)*	–	–	–	–
300	10	1.4716 ± 0.1768* *(70%)*	10	1.0648 ± 0.1938* *(22%)*	10	0.9118 ± 0.2682 *(3%)*
390	11	1.3031 ± 0.1284* *(51%)*	10	1.0823 ± 0.1300* *(24%)*	10	1.0312 ± 0.2334* *(17%)*
500	12	1.3423 ± 0.1968* *(55%)*	10	1.3035 ± 0.1445* *(49%)*	10	1.1457 ± 0.1872* *(30%)*
650	5	1.3574 ± 0.2120* *(57%)*	13	1.3791 ± 0.2052* *(58%)*	10	1.4175 ± 0.2125* *(61%)*
850	5	1.3612 ± 0.1605* *(58%)*	11	1.3604 ± 0.2428* *(56%)*	11	1.1656 ± 0.2640* *(32%)*
1100	8	1.2534 ± 0.1703* *(45%)*	16	1.2449 ± 0.2948* *(43%)*	12	1.2503 ± 0.2013* *(42%)*

* $p < 0.01$ at the analysis of variance, with respect to controls; % difference with respectto controls in brackets.

acute oral AZA1 administration (Aune et al., 2012). Jumping was also observed in mice administered with high doses of AZA1 (1100 μg/kg) or AZA3 (≥850 μg/kg), whereas no visible signs of diarrhoea were noted despite this being the main sign of AZA poisoning in humans.

Macroscopic and/or microscopic changes were observed in the liver, gastrointestinal tract and spleen. At lethal doses (≥230 μg AZA1/kg, ≥500 μg AZA2/kg and ≥650 μg/kg AZA3) spontaneously dead mice or those sacrificed after 24 h showed macroscopic gastrointestinal alterations visible as increased gas and food material in the stomach, redness of the gastric wall and/or redness and dilation of the small intestine, which contained abundant and sometimes bloody fluid. Alterations in the small intestine were noted also by light microscopy.

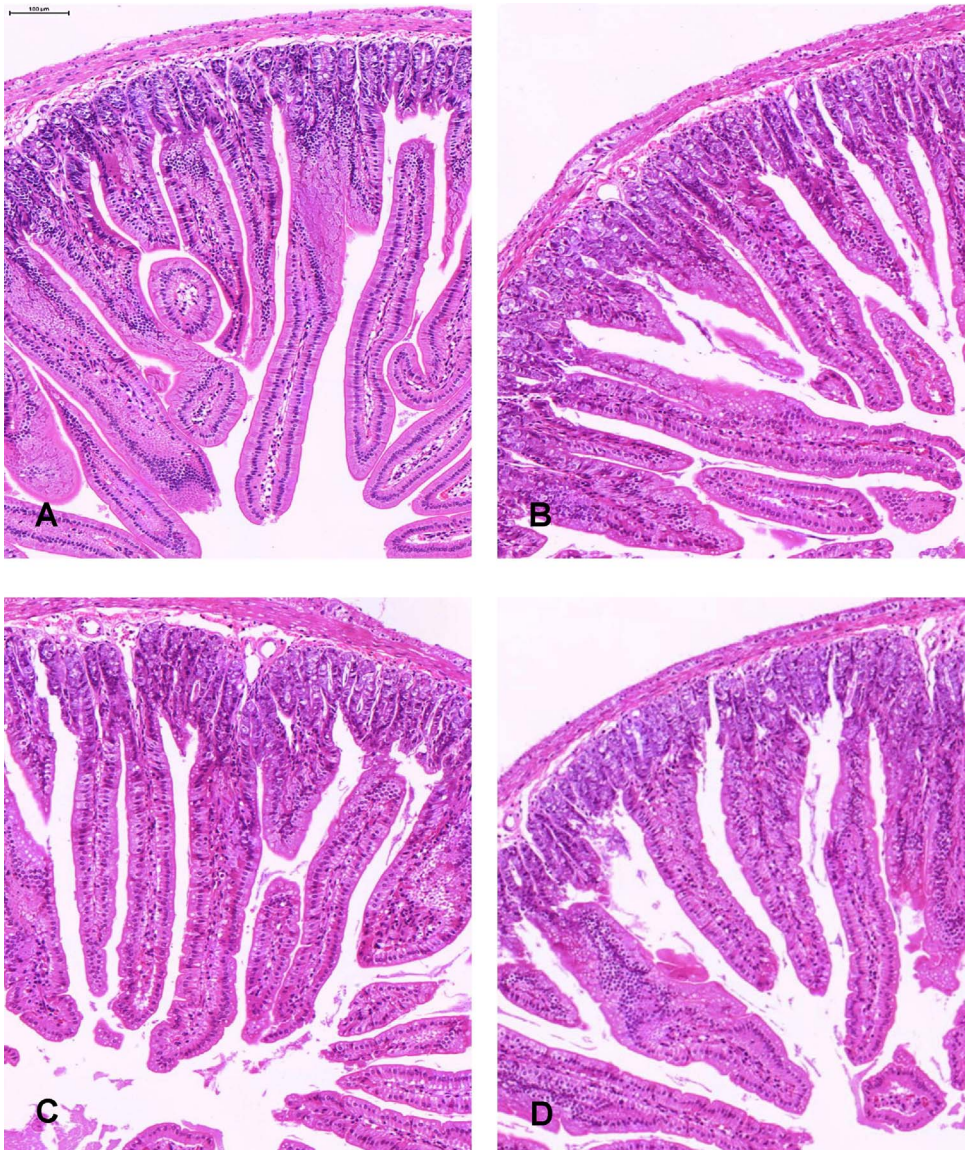


Fig. 3. Light micrographs of duodenum from a control mouse (A) and mice treated with 1100 μg/kg of AZA1 (B), AZA2 (C) or AZA3 (D). Haematoxylin–eosin stain; scale mark: 200 μm.

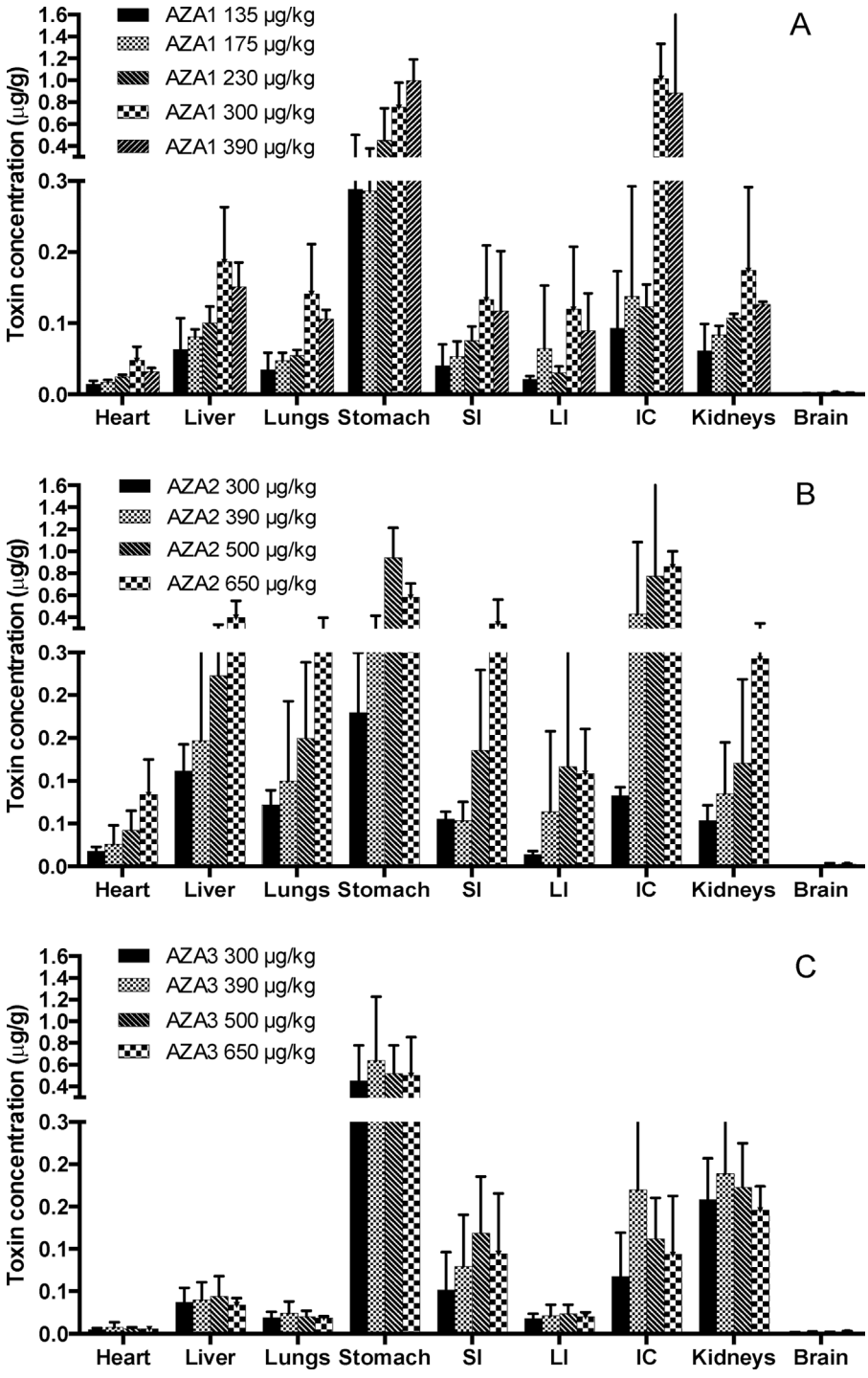


Fig. 4. Concentrations (µg toxin/g) of AZA1 (A), AZA2 (B) and AZA3 (C) in the main organs and intestinal content of mice at 20–24 h from the toxins oral administration, determined by LC–MS/MS. Data are the mean ± S.D. from 3 mice.

Minimal to moderate increase of apoptotic cells and infiltration of polymophonuclear leukocytes in the lamina propria was noted at all the doses of AZA1. In addition, the duodenum of mice administered with the highest dose of each toxin (1100 µg/kg) presented contracted and blunt villi, with a slightly decreased thickness of enterocytes brush border, while the small intestine of mice treated with AZA2 (1100 µg/kg) showed also slight-moderate mucosa degeneration, often with increased apoptotic cells in the lamina propria. Similar alterations were previously recorded in mice orally administered with AZA1, also at doses as low as 100–200 µg/kg (Aasen et al., 2010, 2011; Aune et al., 2012; Ito et al., 2000).

The most evident macroscopic changes were noted in the liver of mice spontaneously died after ≥5 h from the toxins administration or those sacrificed after 24 h (doses: ≥175 mg AZA1/kg, ≥500 mg AZA2/kg and ≥650 mg AZA3/kg). The enlarged organ assumed a pale pinkish colour and significantly increased in weight (up to 70% weight increase at doses ≥135 µg AZA1/kg and up to about 60% increase at doses ≥300 or 390 µg/kg of AZA2 or AZA3, respectively). Even though these findings were not clearly related to the histopathological changes, a hepatic involvement had been confirmed also by the increased serum levels of glutamate dehydrogenase and/or transaminases, recorded both in spontaneously dead mice and in those sacrificed 24 h after exposure all the doses of AZA1, −2 or −3. In addition, records of pale livers were also previously reported (Aune et al., 2012; Ito et al., 2000, 2002), and had been linked to alterations of lipid metabolism in cellular studies (Twiner et al., 2008b).

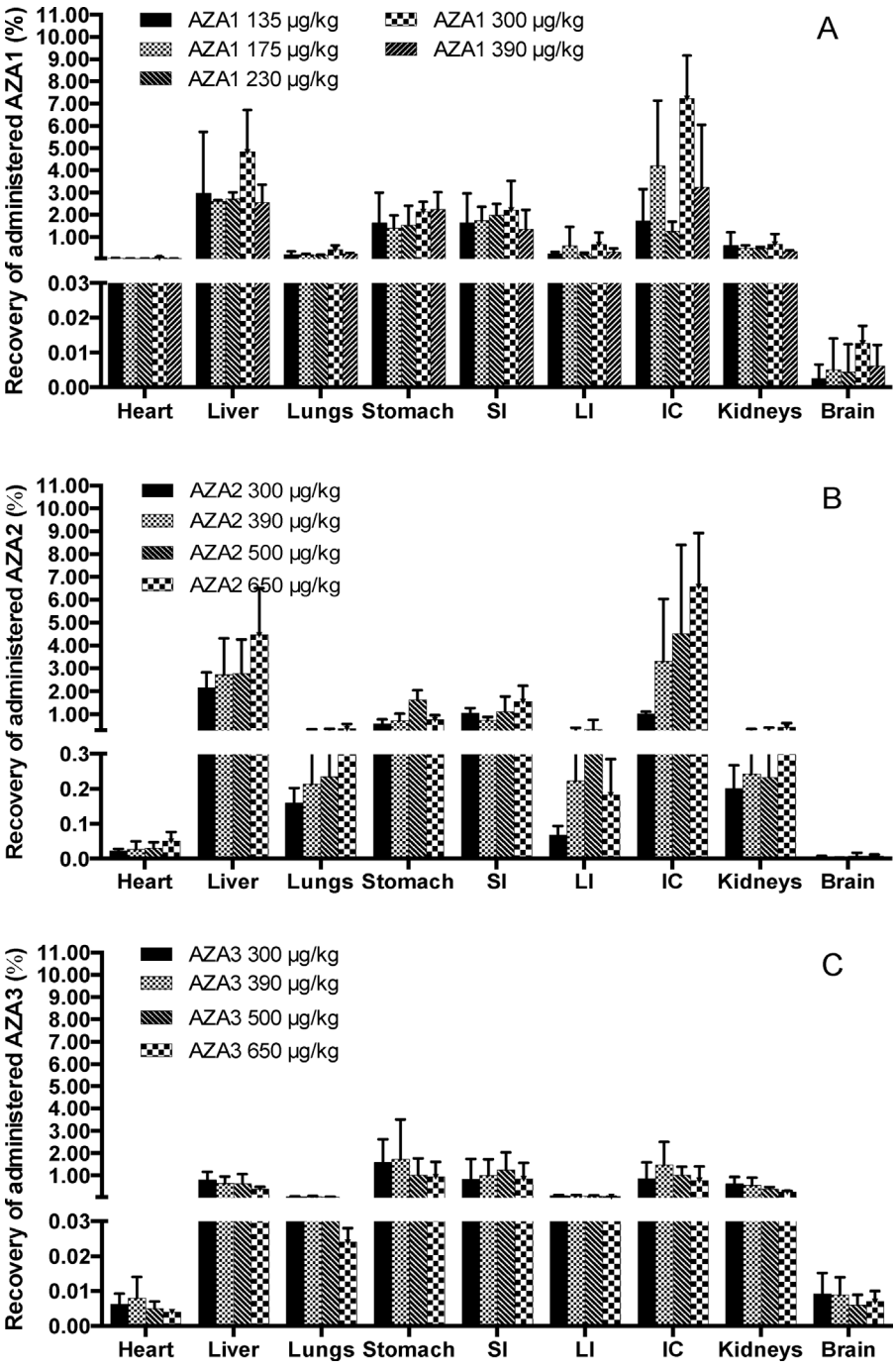


Fig. 5. Recovery (%) of the administered doses of AZA1 (A), AZA2 (B) and AZA3 (C) from internal organs and intestinal content of mice at 20–24 h from the toxins oral administration. Data are the mean ± S.D. from 3 mice.

Spleen alterations, visible only at histopathology, consisted in white pulp lymphoid depletion and lymphoid cells necrosis, were also noted in mice treated with AZA1 or AZA2 at doses ≥500 mg/kg, similarly to previous records after single or repeated oral administration of AZA1 in mice (Ito et al., 2000, 2002, 2006).

At doses ≥500 mg/kg, the toxins significantly increased serum levels of K^+ ions within 24 h from their administration (AZA1: 18–80%, AZA2: 31–53%, AZA3: 11–22%), without affecting the levels of other electrolytes (Na^+, K^+, Cl^-, Ca^{2+} or inorganic phosphate). An elevated serum level of K^+ ions could result in an impaired membrane potential of cells with significant impact on excitable tissues. One of the most sensitive tissues to variations in serum K^+ levels is myocardium due to the ion involvement in the generation and conduction of cardiac impulses (Lehnhardt and Kemper, 2011; Weiner and Wingo, 1998). Thus, cardiac electrophysiological changes in mice orally treated with AZA1, −2 or −3 related to the increased serum K^+ ions cannot be excluded, as previously observed after intravenous or intraperitoneal injection of AZA2 to rats. Indeed, single intravenous AZA2 injection to rats (11 or 15 µg/kg) induced arrhythmia and prolonged electrocardiogram PR intervals, without structural myocardial damages (Ferreiro et al., 2014a). On the other hand, multiple intraperitoneal injections of AZA1 to rats (1–55 µg/kg, four injections over 15 days) induced functional signs of heart failure and structural myocardial alterations (Ferreiro et al., 2016), with evidence of apoptotic processes in cardiomyocytes at 10 µg/kg (Ferreiro et al., 2017). Other *in vitro* studies showed that AZA1, −2 and −3 are low/moderate blockers of K^+ ions current through hERG (human *ether-à-go-go* related gene) channels, known to conduct the rapid component of the delayed rectifier potassium current, which is crucial for repolarization of cardiac action potentials (Twiner et al., 2012). Inhibition of hERG channels, manifested by a prolonged

electrocardiogram QT interval due to a delayed repolarization of the heart following a heartbeat, can lead to arrhythmia, ventricular fibrillation and sudden death (Sanguinetti and Tristani-Firouzi, 2006). However, *in vivo* studies on AZA2 showed that its single intravenous injection to rats did not affect the electrocardiogram QT interval, in spite of AZA2 *in vitro* inhibitory activity on cardiac hERG K^+ channels (Ferreiro et al., 2014a). Other *in vitro* studies showed that cells exposure to AZA2 up to 12 h increased hERG levels in the plasma membrane, probably through hERG retrograde trafficking impairment, but this change was not accompanied by an increased hERG channel-related current (Ferreiro et al., 2014b). The possible cardiac effects of AZAs recorded by previous *in vivo* and *in vitro* studies and the increased serum levels of K^+ ions recorded in this study suggest potential adverse effects of AZAs at cardiac level. Thus, the potential cardiotoxicity of AZAs should be considered in the risk assessment of these toxins as seafood contaminants, particularly for at risk subjects, with pre-existing cardiovascular dysfunctions.

The present study evaluated also AZA1, −2 and −3 distribution in the main organs and intestinal content of mice after their single oral administration. Within 24 h after treatment, LC–MS/MS analysis showed gastrointestinal absorption of the three toxins, with their detection in all the examined organs. The total recovery of the administered doses of AZA1 (3–23%) and AZA2 (4–19%) was higher than that of AZA3 (2–8%). The highest fractions of the administered doses of AZA1 and AZA2 were recovered from the liver and intestinal content, followed by stomach, small intestine, kidneys, large intestine, lungs, heart and only traces in the brain, similarly to previous records after AZA1 oral administration in mice (Aasen et al., 2010, 2011; Aune et al., 2012). On the other hand, AZA3 showed a slightly different pattern of distribution: the highest amount of the administered dose was recovered from the stomach, followed by slightly lower and comparable amounts from the small intestine, liver and intestinal content, whereas low amounts were recovered from the kidneys, large intestine, lungs and only trances from the heart and brain. These data suggest a different toxicokinetic pathway between AZA1 or AZA2 and AZA3. This may be related to lower stability of AZA3 as previously observed in accelerated stability studies (McCarron et al., 2011).

After a 14-day withdrawal period from AZAs administration, no macroscopic, histological or biochemical alterations were recorded, indicating a complete recovery of AZAs-induced adverse effects in surviving mice.

In conclusion, the acute oral toxicity of AZA2 and −3 was studied in mice for the first time, within a single comparative study with AZA1. The lethal potency of these toxins is AZA1 > AZA2 > AZA3 and their main targets at the histological level are the liver, gastrointestinal tract and spleen. The new toxic equivalency factors (TEFs) of AZA1, −2 and −3 are 1.0, 0.7 and 0.5, respectively, and should be the suitable correction factors for regulatory purposes to estimate AZA1 equivalents in edible shellfish.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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References

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

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

Alvarez, G., Uribe, E., Avalos, P., Mariño, C., Blanco, J., 2010. First identification of azaspiracid and spirolides in *Mesoderma donacium* and *Mulinia edulis* from Northern Chile. Toxicon 55, 638–641.

Aune, T., Espenes, A., Aasen, J.A., Quilliam, M.A., Hess, P., Larsen, S., 2012. Study of possible combined toxic effects of azaspiracid-1 and okadaic acid in mice via the oral route. Toxicon 60, 895–906.

Bacchiocchi, S., Siracusa, M., Ruzzi, A., Gorbi, S., Ercolessi, M., Cosentino, M.A., Ammazzalorso, P., Orletti, R., 2015. Two-year study of lipophilic marine toxin profile in mussels of the North-central Adriatic Sea: first report of azaspiracids in Mediterranean seafood. Toxicon 108, 115–125.

EFSA (European Food Safety Authority), 2008. Opinion of the scientific panel on contaminants in the food chain on a request from the European Commission on marine biotoxins in shellfish—azaspiracids. EFSA J. 723, 1–52. Available online: www.efsa.europa.eu.

European Commission, 2004. Regulation No 853/2004 of 29th April 2004 laying down specific hygiene rules for on the hygiene of foodstuff. Off. J. Eur. Union L139, 55–205.

European Commission, 2011. Regulation No 15/2011 of 10th January 2011 amending regulation (EC) No 2074/2005 as regards recognised testing methods for detecting marine biotoxins in live bivalve molluscs. Off. J. Eur. Union L6, 3–6.

Ferreiro, S.F., Vilariño, N., Carrera, C., Louzao, M.C., Santamarina, G., Cantalapiedra, A.G., Rodríguez, L.P., Cifuentes, J.M., Vieira, A.C., Nicolaou, K.C., Frederick, M.O., Botana, L.M., 2014a. In vivo arrythmogenicity of the marine biotoxin azaspiracid-2 in rats. Arch. Toxicol. 88, 425–434.

Ferreiro, S.F., Vilariño, N., Louzao, M.C., Nicolaou, K.C., Frederick, M.O., Botana, L.M., 2014b. In vitro chronic effects on hERG channel caused by the marine biotoxin azaspiracid-2. Toxicon 91, 69–75.

Ferreiro, S.F., Vilariño, N., Carrera, C., Louzao, M.C., Cantalapiedra, A.G., Santamarina, G., Cifuentes, J.M., Vieira, A.C., Botana, L.M., 2016. Subacute cardiovascular toxicity of the marine phycotoxin azaspiracid-1 in rats. Toxicol. Sci. 151, 104–114.

Ferreiro, S.F., Vilariño, N., Carrera, C., Louzao, M.C., Santamarina, G., Cantalapiedra, A.G., Cifuentes, J.M., Crespo, A., Botana, L.M., 2017. In vivo cardiomyocyte response to YTX- and AZA1-induced damage: autophagy versus apoptosis. Arch. Toxicol. 91, 1859–1870.

Finney, D.J., 1971. Probit Analysis, 3rd ed. Cambridge University Press, Cambridge, UK.

Furey, A., O'Doherty, S., O'Callaghan, K., Lehane, M., James, K.J., 2010. Azaspiracid poisoning (AZP) toxins in shellfish: toxicological and health considerations. Toxicon 56, 173–190.

Ito, E., Satake, M., Ofuji, K., Kurita, N., McMahon, T., James, K., Yasumoto, T., 2000. Multiple organ damage caused by a new toxin azaspiracid, isolated from mussels produced in Ireland. Toxicon 38, 917–930.

Ito, E., Satake, M., Ofuji, K., Higaschi, M., Harigaya, K., McMahon, T., Yasumoto, T., 2002. Chronic effects in mice caused by oral administration of sublethal doses of azaspiracid, a new marine toxin isolated from mussels. Toxicon 40, 193–203.

Ito, E., Frederick, M.O., Koftis, T.V., Tang, W., Petrovic, G., Ling, T., Nicolaou, K.C., 2006. Structure toxicity relationships of synthetic azaspiracid-1 and analogs in mice. Harmful Algae 5, 586–591.

James, K.J., Furey, A., Lehane, M., Ramstad, H., Aune, T., Hovgaard, P., Morris, S., Higman, W., Satake, M., Yasumoto, T., 2002. First evidence of an extensive northern European distribution of azaspiracid poisoning (AZP) toxins in shellfish. Toxicon 40, 909–915.

Jauffrais, T., Kilcoyne, J., Herrenknecht, C., Truquet, P., Séchet, V., Miles, C.O., Hess, P., 2013. Dissolved azaspiracids are absorbed and metabolized by blue mussels (*Mytilus edulis*). Toxicon 65, 81–89.

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

Kilcoyne, J., McCarron, P., Twiner, M.J., Nulty, C., Wilkins, A.L., Rise, F., Quilliam, M.A., Miles, C.O., 2014. Epimers of azaspiracids: isolation, structural elucidation, relative LC–MS response, and in vitro toxicity of 37-epi-azaspiracid-1. Chem. Res. Toxicol. 27, 587–600.

Kilcoyne, J., Twiner, M.J., McCarron, P., Crain, S., Giddings, S.D., Wilkins, A.L., Hess, P., Miles, C.O., 2015. Structure elucidation, relative LC-MS and in vitro toxicity of azaspiracids 7–10 isolated from mussels (*Mytilus edulis*). J. Agric. Food Chem. 63, 5083–5091.

Krock, B., Tillmann, U., Voß, D., Koch, B.P., Salas, R., Witt, M., Potvin, É., Jeong, H.J., 2012. New azaspiracids in Amphidomataceae (Dinophyceae). Toxicon 60, 830–839.

Krock, B., Tillmann, U., Witt, M., Gu, H., 2014. Azaspiracid variability of *Azadinium poporum* (Dinophyceae) from the China Sea. Harmful Algae 36, 22–28.

López-Rivera, A., O'Callaghan, K., Moriarty, M., O'Driscoll, D., Hamilton, B., Lehane, M., James, K.J., Furey, A., 2010. First evidence of azaspiracids (AZAs): a family of

lipophilic polyether marine toxins in scallops (*Argopecten purpuratus*) and mussels (*Mytilus chilensis*) collected in two regions of Chile. Toxicon 55, 692–701.
Lehnhardt, A., Kemper, M.J., 2011. Pathogenesis, diagnosis and management of hyperkalemia. Pediatr. Nephrol. 26, 377–384.
Magdalena, A.B., Lehane, M., Krys, S., Fernández, M.L., Furey, A., James, K.J., 2003. The first identification of azaspiracids in shellfish from France and Spain. Toxicon 42, 105–108.
McCarron, P., Kilcoyne, J., Miles, C.O., Hess, P., 2009. Formation of Azaspiracids-3, −4, −6, and −9 via decarboxylation of carboxyazaspiracid metabolites from shellfish. J. Agric. Food Chem. 57, 160–169.
McCarron, P., Emteborg, H., Giddings, S., Wright, E., Quilliam, M.A., 2011. A mussel tissue certified reference material for multiple phycotoxins: part 3: homogeneity and stability. Anal. Bioanal. Chem. 400, 847–858.
McMahon, T., Silke, J., 1996. West coast of Ireland; winter toxicity of unknown aetiology in mussels. Harmful Algae News 14, 2.
Ofuji, K., Satake, M., McMahon, T., Silke, J., James, K.J., Naoki, H., Oshima, Y., Yasumoto, T., 1999. Two analogues of azaspiracid isolated from mussels, *Mytilus edulis*, involved in human intoxication in Ireland. Nat. Toxins 7, 99–102.
Percopo, I., Siano, R., Rossi, R., Soprano, V., Sarno, D., Zingone, A., 2013. A new potentially toxic *Azadinium* species (Dinophyceae) from the Mediterranean Sea *A. dexteroporum* sp. nov. J. Phycol. 49, 950–966.
Rehmann, N., Hess, P., Quilliam, M.A., 2008. Discovery of new analogs of the marine biotoxin azaspiracid in blue mussels (*Mytilus edulis*) by ultra-performance liquid chromatography/tandem mass spectrometry. Rapid Commun. Mass Spectrom. 22, 549–558.
Rossi, R., Dell'Aversano, C., Krock, B., Ciminiello, P., Percopo, I., Tillmann, U., Soprano, V., Zingone, A., 2017. Mediterranean *Azadinium dexteroporum* (Dinophyceae) produces six novel azaspiracids and azaspiracid-35: a structural study by a multi-platform mass spectrometry approach. Anal. Bioanal. Chem. 409, 1121–1134.
Sanguinetti, M.C., Tristani-Firouzi, M., 2006. hERG potassium channels and cardiac arrhythmia. Nature 440, 463–469.
Satake, M., Ofuji, K., Naoki, H., James, K.J., Furey, A., McMahon, T., Silke, J., Yasumoto, T., 1998. Azaspiracid, a new marine toxin having unique spiro ring assemblies, isolated from Irish mussels, *Mytilus edulis*. J. Am. Chem. Soc. 120, 9967–9968.
Taleb, H., Vale, P., Amanhir, R., Benhadouch, A., Sagou, R., Chafik, A., 2006. First detection of azaspiracids in mussels in North West Africa. J. Shellfish Res. 25, 1067–1070.
Tillmann, U., Elbrächter, M., Krock, B., John, U., Cembella, A.D., 2009. *Azadinium spinosum gen. et* sp. nov (Dinophyceae) identified as a primary producer of azaspiracid toxins. Eur. J. Phycol. 44, 63–79.
Tillmann, U., Borel, C.M., Barrera, F., Lara, R., Krock, B., Almandoz, G.O., Witt, M., Trefault, N., 2016. *Azadinium poporum* from the Argentine continental shelf, Southwestern Atlantic, produces azaspiracid-2 and azaspiracid-2 phosphate. Harmful Algae 51, 40–55.
Tillmann, U., Jaénb, D., Fernándezb, L., Gottschlingc, M., Wittd, M., Blancoe, J., Krocka, B., 2017. *Amphidoma languida* (Amphidomatacea, Dinophyceae) with a novel azaspiracid toxin profile identified as the cause of molluscan contamination at the Atlantic coast of southern Spain. Harmful Algae 62, 113–126.
Torgersen, T., Bremnes, N.B., Rundberget, T., Aune, T., 2008. Structural confirmation and occurrence of azaspiracids in Scandinavian brown crabs (*Cancer pagurus*). Toxicon 51, 93–101.
Trainer, V.L., Moore, L., Bill, B.D., Adams, N.G., Harrington, N., Borchert, J., da Silva, D.A., Eberhart, B.T., 2013. Diarrhetic shellfish toxins and other lipophilic toxins of human health concern in Washington State. Mar. Drugs 11, 1815–1835.
Turner, A.D., Goya, A.B., 2015. Occurrence and profiles of lipophilic toxins in shellfish harvested from Argentina. Toxicon 102, 32–42.
Twiner, M.J., Rehmann, N., Hess, P., Doucette, G.J., 2008a. Azaspiracid shellfish poisoning: a review on the chemistry, ecology, and toxicology with emphasis on human health impact. Mar. Drugs 6, 39–72.
Twiner, M.J., Ryan, J.C., Morey, J.S., Smith, K.J., Hammad, S.M., Van Dolah, F.M., Hess, P., McMahon, T., Satake, M., Yasumoto, T., Doucette, G.J., 2008b. Transcriptional profiling and inhibition of cholesterol biosynthesis in human T lymphocyte cells by the marine toxin azaspiracid. Genomics 91, 289–300.
Twiner, M.J., Doucette, G.J., Rasky, A., Huang, X.P., Roth, B.L., Sanguinetti, M.C., 2012. Marine algal toxin azaspiracid is an open-state blocker of hERG potassium channels. Chem. Res. Toxicol. 25, 1975–1984.
Ueoka, R., Ito, A., Izumikawa, M., Maeda, S., Takagi, M., Shin-ya, K., Yoshida, M., van Soest, R.W., Matsunaga, S., 2009. Isolation of azaspiracid-2 from a marine sponge *Echinoclathria* sp. as a potent cytotoxin. Toxicon 53, 680–684.
Weiner, I.D., Wingo, C.S., 1998. Hyperkalaemia: a potential silent killer. J. Am. Soc. Nephrol. 9, 1535–1543.
Zamorano, R., Marín, M., Cabrera, F., Figueroa, D., Contreras, C., Barriga, A., Lagos, N., García, C., 2013. Determination of the variability of both hydrophilic and lipophilic toxins in endemic wild bivalves and carnivorous gastropods from the southern part of Chile. Food Addit. Contam. Part A Chem. Anal. Control. Expo. Risk. Assess. 30, 1660–1677.