Toxicon 55 (2010) 692–701

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

# 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

A. López-Rivera [a], K. O'Callaghan [b], M. Moriarty [b], D. O'Driscoll [b], B. Hamilton [b], M. Lehane [c], K.J. James [b,d], A. Furey [b,*]

[a] Marine Toxins Laboratory, Biomedical Sciences Institute, Faculty of Medicine, University of Chile, Santiago, Chile
[b] PROTEOBIO (Mass Spectrometry Centre), Cork Institute of Technology, Cork, Ireland
[c] Department of Applied Sciences, Limerick Institute of Technology, Moylish Park, Limerick, Ireland
[d] Environmental Research Institute, University College Cork, Lee Road, Cork, Ireland

ARTICLE INFO

*Article history:*
Received 15 July 2009
Received in revised form
12 October 2009
Accepted 14 October 2009
Available online 21 October 2009

*Keywords:*
AZP
Azaspiracid shellfish poisoning
Shellfish toxin
*Mytilus chilensis*
*Argopecten purpuratus*
Food safety
LC-MS/MS

ABSTRACT

Azaspiracids are a family of lipophilic polyether marine biotoxins that have caused a number of human intoxication incidents in Europe since 1995 following the consumption by consumers of intoxicated shellfish (*Mytilus edulis*). These azaspiracids have now been identified in mussels (*Mytilus chilensis*) and scallops (*Argopecten purpuratus*) from two Chilean locations. This is the first report of the occurrence of azaspiracid toxins in these species (*Mytilus chilensis* and *Argopecten purpuratus*) from Chile. The areas studied were Bahía Inglesa (III Region, 27° SL) and Chiloé Archipelago, both important scallop and mussels farming areas. Separation of azaspiracid (AZA1), azaspiracid isomer (AZA6) and its analogues, 8-methylazaspiracid (AZA2) and 22-demethylazaspiracid (AZA3), was achieved using reversed-phase LC and toxins were identified using a turbo electrospray ionisation (ESI) source, to a triple quadrupole mass spectrometer.

In mussels, AZA1 was the predominant toxin in mussel hepatopancreas with AZA2, AZA3 and AZA6 present in approximate equivalent amounts in the remaining tissues, 20–30% of the AZA1 level. AZA2 predominated in the scallop samples with the toxin almost entirely present in the hepatopancreas (digestive gland). AZA1 was only observed in some of the scallop samples and was present at 12–15% of the AZA2 levels.

Whilst the levels of AZAs in Chilean samples are below the EU regulatory limit of 160 µg/kg, it is significant that this toxin is present in Pacific Ocean species. Consequently measures should be taken by regulatory authorities to implement regular seafood monitoring to ensure safety of harvested product.

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## 1. Introduction

Azaspiracid poisoning (AZP) is the most recently discovered human toxic syndrome associated with shellfish (Ito et al., 1997; James et al., 2000; Ofuji et al., 1999a; Satake et al., 1997, 1998). A toxic event, in which individuals ($n = 8$) consumed shellfish and had to seek medical treatment, occurring in the Netherlands in 1995 was attributed to mussels (*Mytilus edulis*) harvested from Killary Harbour in Ireland. Initial symptoms resembled diarrheic shellfish poisoning (DSP), this diagnosis was discounted when only low levels of okadaic acid and other DSP toxins were observed in the sample. A new family of toxins was implicated, azaspiracids (AZA) (Satake et al., 1997, 1998).

* Corresponding author. Tel.: +353 21 432 6701; fax: +353 21 434 5191.
*E-mail address:* ambrose.furey@cit.ie (A. Furey).

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doi:10.1016/j.toxicon.2009.10.020

Nicolaou and co-workings synthesised and proposed the definitive structure of AZA1 (Fig. 1) (Nicolaou et al., 2004).

Subsequently, following a second toxic incident, AZA2 and AZA3 were structurally elucidated by NMR (Fig. 1) (Ofuji et al., 1999a, 2001). The first LC-MS method for the AZAs was developed in 1999, based on selected ion monitoring (SIM) experiments for each molecular ion (Ofuji et al., 1999b). Draisci and co-workers developed the first tandem mass spectrometry methods for the azaspiracid toxins, based on the facile water loss transitions commonly seen in polyether toxins (Draisci et al., 2000). Analytical methods for the detection and quantitation of azaspiracids were improved when ion-trap LC-$MS^3$ methods for azaspiracids were developed and validated (Furey et al., 2002). Unique fragmentation patterns for each azaspiracid ion were utilised to characterise the toxins found in samples tested (Furey et al., 2002; Lehane et al., 2002). Liquid chromatography coupled with mass spectral methods developed for 10 azaspiracids (AZA1–10) allowed selective and sensitive profiling of toxic shellfish. (Lehane et al., 2004). The advances in UPLC technology coupled triple quadrupole mass spectrometry enhances the determination of AZAs (Fux et al., 2007; Rehmann et al., 2008).

Toxicological studies conducted using mice demonstrate that AZA targets the liver, lung, pancreas, thymus, spleen and the small intestine and may be carcinogenic and cytotoxic (Ito et al., 2000, 2002; Twiner et al., 2005). The source of AZA was originally thought to be the predator dinoflagellate *Protoperidinium crassipes,* now it is postulated that *Azadinium spinosum,* which *P. crassipes* grazes on, is the progenitor (James et al., 2003; Jeong and Latz, 1994; Krock et al., 2009; Leising et al., 2005; Osleng et al., 2002; Tillman et al., 2009). Recently, AZA2 has been isolated from a marine sponge *Echinoclathria* sp. collected off the Japanese coastline (Ueoka et al., 2009).

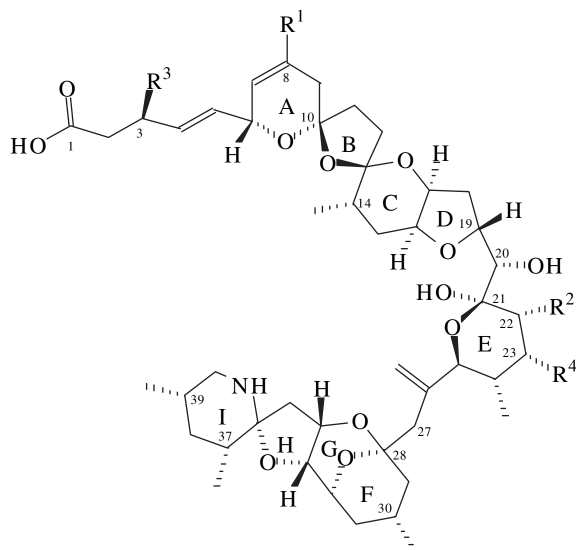


**Fig. 1.** Structure of six predominant azaspiracid toxins. AZA1 ($R_1 = H$, $R_2 = CH_3$, $R_3 = H$, $R_4 = H$); AZA2 ($R_1 = CH_3$, $R_2 = CH_3$, $R_3 = H$, $R_4 = H$); AZA3 ($R_1 = H$, $R_2 = H$, $R_3 = H$, $R_4 = H$); AZA4 ($R_1 = H$, $R_2 = H$, $R_3 = OH$, $R_4 = H$); AZA5 ($R_1 = H$, $R_2 = H$, $R_3 = H$, $R_4 = OH$); AZA6 ($R_1 = CH_3$, $R_2 = H$, $R_3 = H$, $R_4 = H$).

The occurrence of diarrhetic shellfish poisoning (DSP) has been found in Chile for nearly 40 years (Guzmán et al., 2002; Lembeye et al., 1993; Muñoz et al., 1992; Uribe et al., 2001; Zhao et al., 1993). Presently, DSP is endemic in the three most austral regions (X, XI and XII) (Lagos, 1998, 2002; Uribe et al., 2001). To date, six important human illnesses associated with microalgae toxins have been described (Hallegraeff, 1993; Lagos, 1998; Yasumoto et al., 1995; Yasumoto and Murata, 1993). Of these, two are endemically present in the southern of Chile (Patagonian fjords): paralytic shellfish poisoning (PSP) and diarrhetic shellfish poisoning (DSP), both very well documented (Compagnon et al., 1998; Lagos, 2002; Lagos et al., 1996; Lembeye et al., 1975; Muñoz et al., 1992; Uribe, 1993; Uribe et al., 2001; Zhao et al., 1993). In Chile the mouse bioassay is used for DSP detection, so the true toxin levels and human consequences are never known. (Suganuma et al., 1998). The harvesting areas studied in this manuscript were along Bahía Inglesa (III Region, 27° SL) and Chiloe archipelago, important scallop (*Argopecten purpuratus*) and mussels (*Mytilus chilensis*) farming areas respectively (Fig. 2).

## 2. Materials and methods

### 2.1. Materials

Purchased chemicals included trifluoroacetic acid, ammonium acetate (Aldrich, Dorset, UK) and formic acid (Merck, Darmstadt, Germany). All solvents (acetonitrile, water) were HPLC grade and were purchased from Labscan (Dublin, Ireland). AZA authentic standard toxins, AZA1, AZA2 and AZA3 were isolated from toxic mussels (*Mytilus edulis*) as described previously (Furey et al., 2002; Ofuji et al., 1999b; Satake et al., 1998). Azaspiracid reference materials were prepared from toxic mussels harvested in Bruckless Co. Donegal, Ireland. These reference materials were quantitatively standardised against authentic standard AZA1 toxin that was kindly donated by Professors Yasumoto and Satake of Tohoku University, Japan. Cultivated shellfish, scallops (*Pecten purpuratus*) and mussels (*Mytilus chilensis*) were obtained both from coasts of the Bahía Inglesa (III Region, 27° SL) and Chiloé archipelago, respectively (Fig. 2).

### 2.2. Mouse bioassay sample extraction

The mussel and scallops extracts were obtained from shellfish samples collected on November 2005 and March 2206. Forty grams of the digestive glands were removed from mussels (*Mytilus chilensis*) and scallops (*Argopecten purpuratus*), and homogenised. The bioassay following the procedure developed by Yasumoto et al. (1984). A sample of homogenised hepatopancreas (20 g) was extracted twice with acetone (50 ml). The extracts are filtered, then the filtrate was collected and the solvent removed by rotary evaporation. The residue was made up with water (20 ml) and the suspension is extracted with dichloromethane (60 ml; 3 × 20 ml). The combined organic layers are back-washed twice with water (20 ml) and evaporated to dryness. The residue was re-suspended in 1% Tween 60 solution to a concentration of 5 g hepatopancreas/ml

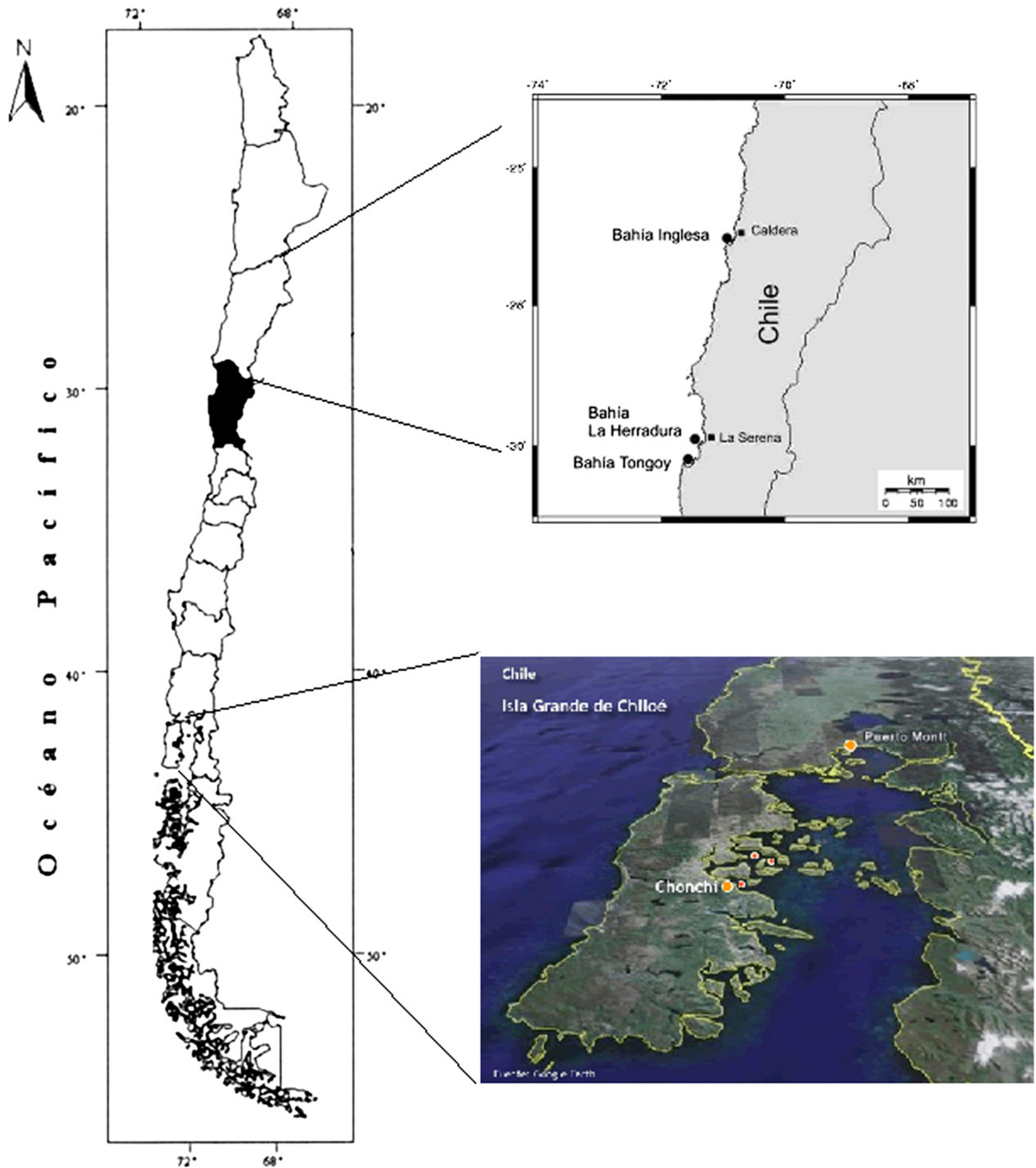


**Fig. 2.** Map of Chile showing two different Chilean locations: Bahía Inglesa and Chiloé Archipelago, important shellfish farming areas where AZAs was detected.

Tween 60 prior to intraperitoneally injection into each of three mice (CF-l) weighting 18 g each. Initially, 1 mouse unit (MU) was defined as the minimum dose of toxin required to kill a mouse within 48 h. All shellfish samples indicating mouse positive bioassay for diarrhetic shellfish poisoning (DSP) toxins, were retained for further investigation to determine whether they could also contain the lipophilic toxins azaspiracids (Sections 2.3 and 2.4).

*2.3. Sample preparation*

Fresh uncooked shellfish (*Mytilus chilensis* and *Argopecten purpuratus*) samples (50 g) were first carefully dissected to remove digestive glands from the remaining tissues. Each sample compartment (hepatopancreas and remaining tissue) were homogenised and approximately 5 g of this homogenate was accurately weighed. Acetone

(15 ml) was added and the samples were re-homogenised (3 min), the resulting slurry was then centrifuged at 3000 × g for 10 min and the supernatant was filtered (0.45 mm, Phenomenex, Macclesfield, UK). The final supernatant was made up to volume in a volumetric flask (25 ml) using acetone as diluent. One fifth of the solution (corresponding to 1 g of the original meat sample) was blown to dryness under a stream of nitrogen gas. Samples were stored in amber glass vials and frozen (−80 °C). Prior to analysis the samples were reconstituted in methanol (500 µl) and an aliquot (5 µl) was injected.

### 2.4. Liquid chromatography-multiple tandem mass spectrometry (LC-MS/MS)

An LC-MS/MS method previously developed by James and co-workers was used for the quantification of AZA toxins. In order to ensure unambiguous identification, more than one multiple reaction monitoring (MRM) experiment was conducted on each peak, i.e. two product ions (Q3) were selected for each precursor ion (Q1). The loss of water transition $[M+H]^+/[M+H–H_2O]^+$ was used to quantify analogues and the A-ring loss transition $[M+H]^+/[M+H–H_2O–A\text{-ring}]^+$ was used for confirmatory purposes. Analysis were performed on an Agilent 1100 series HPLC system (Agilent, Palo Alto, CA, USA) coupled to an API 3000 (Applied Biosystems, Warrington, UK) triple quadrupole mass spectrometer equipped with Turbo-assisted Ionspray (ESI) ionisation source (Sciex, Toronto, Canada) in positive mode. Analyst 1.3 software was used for instrument control, data acquisition and data analysis. The sample vials were maintained at 4 °C in the thermostatic autosampler. Chromatographic separation was obtained using isocratic elution on a reverse phase LC column (Luna C18(2), 5 µm, 150 × 2.0 mm, Phenomenex, Macclesfield, UK) at 35 °C and at a flow rate of 200 µl/min. The mobile phase used was acetonitrile/water (58/42% *v/v*) containing 0.05% trifluoroacetic acid and 0.5 mM ammonium acetate. The injection volume was 5 µl and the eluent flow diverted to waste for 1 min after sample injection using a Valco switching valve. MS detection was carried out between 1 and 15 min of the chromatography. The mass spectrometer was operated in positive mode. Q1/Q3 ion pairs outlined previously by Lehane and co-workers were used to allow simultaneous detection of AZA1-10 (Lehane et al., 2004). Instrument parameters were Declustering Potential 60, Collision Energy 45–70, Focusing Potential 300, Nebuliser Gas 10, Curtain Gas 15, CAD gas 5, Ion Source Voltage 4250 V, Ion Source Temp 450 °C.

## 3. Results and discussion

Chile is a country with a Pacific Ocean coastline of over 3500 miles and has an extensive industry in shellfish mariculture. Cases of gastrointestinal disorders following human consumption of bivalves were first observed in Chile in 1970 and 1971, apparently associated with blooms of *Dinophysis* spp. (IPCS, 1984). A substantial DSP intoxication was reported in January 1991 with approximately 120 people becoming ill after consuming fresh mussels. *D. acuminata* was identified was identified as the culprit dinoflagellate (Aune and Yndestad, 1993; Zhao et al., 1993). Other species have being associated with toxic DSP, the *Dinophysis acuta* (Lembeye et al., 1993), *D. acuminata*, *D. argus*, *D. caudata*, *D. operculata* and *D. Ovum* (Muñoz et al., 1992).

### 3.1. Determination of AZA in shellfish using LC-MS/MS

Triple quadrupole mass spectrometry was used to confirm AZA toxins in shellfish extracts. Two Q1/Q3 ion pairs were monitored for each toxin. The first corresponds to $[M+H]^+/[M+H–H_2O]^+$ water loss transition, while the second corresponds to the loss of the A-ring portion of the azaspiracid structure $[M+H]^+/[M+H–H_2O–C_9H_{10}O_2R_1R_3]^+$. This second fragmentation allows characteristic identification of each azaspiracid through their unique precursor/product ions combination. Figs. 3 and 4 show typical chromatograms obtained in this study for azaspiracid spiked standards (mussel and scallop samples respectively). Fig. 3 shows a chromatogram for AZA1–6 obtained when analyzing the spiked standard extract with this method. This methodology was validated for AZA1–3, the only AZAs available as purified standards which were spiked into blank shellfish extracts. The concentration ranges, correlation coefficients ($r^2$), limit of detection (signal: noise = 3) and limit of quantitation (signal:-noise = 5) are outlined in Table 1. The correlation coefficients obtained from standard calibrations curves were satisfactory (0.996) and were reproducible from day to day. Percentage RSD values for azaspiracid reference shellfish material were below 4.5% ($n = 3$) for AZA1 (197 µg/l) AZA2 (335 µg/l) and AZA3 (220 µg/l), for both MRM transitions.

### 3.2. Comparison of azaspiracid toxic profiles and tissue distribution within Chilean shellfish (Mytilus chilensis; Argopecten purpuratus)

The concentrations of AZA toxins (AZA1, 2, 3 and 6) in the hepatopancreas (HP, digestive glands) and remaining

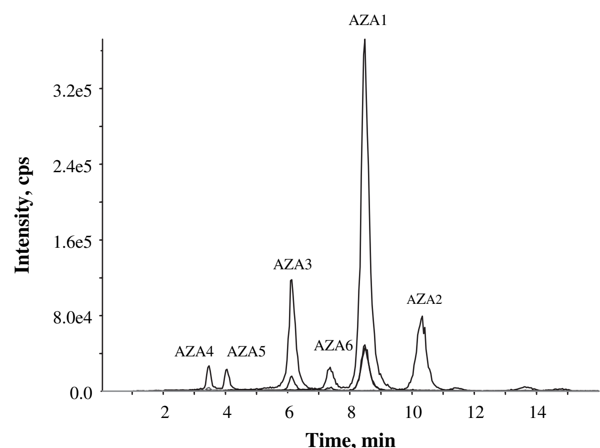


**Fig. 3.** LC-MS/MS chromatogram of azaspiracid standards showing azaspiracid 1–6. (AZA1, 1950 µg/kg, 8.8 min; AZA2, 620 µg/kg, 11.0 min; AZA3, 500 µg/kg, 6.2 min; AZA4, 99 µg/kg, 3.2 min; AZA5, 81 µg/kg, 3.9 min; AZA6, 119 µg/kg, 7.6 min). Chromatographic conditions: acetonitrile:water (58%:42%) containing 0.05% TFA and 0.5 mM $NH_4^+$ OAc, flow rate 200 µl/min, $C_{18}$ Luna(2) column 150 × 2.0 mm, 5 µm.

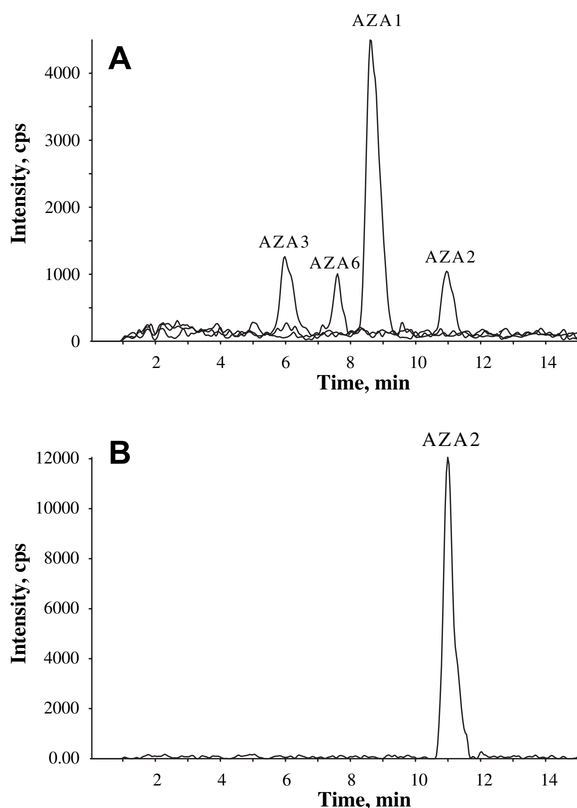


**Fig. 4.** (A) LC-MS/MS chromatogram of a Chilean mussel, hepatopancreas (HP) extract. (AZA1, 44 µg/kg, 8.7 min; AZA2, 9 µg/kg, 11.0 min; AZA3, 12 µg/kg, 6.1 min; AZA6, 7 µg/kg, 7.6 min). (B) LC-MS/MS chromatogram of a Chilean scallop hepatopancreas (HP) extract (AZA2, 86 µg/kg, 11.0). Chromatographic conditions as outlined in Fig. 3.

tissue were compared for both shellfish species (*Mytilus chilensis* and *Argopecten purpuratus*). For the mussel samples, all four azaspiracid toxins were present in both the HP and remaining tissues subsamples. In Figs. 5A,B, sample #3 contained the highest amount of azaspiracids (HP: 70 µg/kg; remaining total tissue: 30 µg/kg) from all the five sample locations. The percentage of azaspiracids in the HP portion was AZA1 (61%) followed by AZA3 (16%), then AZA2 (13%) and finally AZA6 (10%), in the remaining tissue (i.e. total tissue minus the HP) portion the percentage of azaspiracids were AZA1 (50%), AZA3 (19%), AZA2 (18%) and AZA6 (13%). One noticeable result was that the total azaspiracid concentration levels in the remaining tissue portion for the five sample locations were fairly consistent with an RSD of 20% (Fig. 5B). A high variability in the total azspiracid concentration was noticed in the HP portions for the five sample locations with an RSD of 56% (Fig. 5A).

For the Chilean scallop sample, none of the azaspiracids (AZA1, 2, 3 and 6) were detected in the remaining tissue portion. AZA1 and AZA2 were only detected in the HP of the scallops, with AZA2 being the predominant toxin (86%) and consistently present in all HP samples. AZA1 was only present in five of the nine samples locations and was present at levels of one-seventh of that of AZA2 (Fig. 6).

### 3.3. *Comparison of AZA Chilean results with previously published worldwide data*

Table 2 outlined all previously reported azaspiracid occurrences worldwide including shellfish species affected, toxin profiles and concentrations for comparison purposes. Since 1995, all human azaspiracid intoxication incidents to date were related to shellfish harvested along the west coast of Ireland with mussels (*M. edulis*) being the only bivalve mollusc responsible for these intoxications and AZA1 being the predominant toxins present (James et al., 2004). What initially was reported as an Irish problem with harvesting bays being closed for periods of 6–8 months quickly became a European problem when mussels from the UK and Norway were discovered to contain AZA toxins (James et al., 2002a). Toxin profiles were similar to that of mussels found in Ireland with AZA1 being the predominant toxin followed by AZA2 and AZA3. In the Norwegian mussels, the toxins were confined mainly to the HP tissue (James et al., 2002a). In the Chilean mussels, AZA1, 2, 3 and 6 were distinctively present in both the HP and remaining tissues (Fig. 5). This correlates with results from Irish mussels where AZAs were detected in both HP and remaining tissue samples. However, for Irish mussels, the main toxin profiles were significantly different between the mussel HP and the remaining shellfish tissues. AZA1 was usually the predominant toxin in mussel HP whereas AZA3, and an isomer of AZA1 (AZA6), were the predominant toxins in the remaining shellfish tissues (James et al., 2002b). This profile within tissue compartments is not annually consistent as report by Hess and co-workers who found that the ratio of AZA toxins in the HP compared to whole mussel was on average ca 5, however a large relative standard deviation of 41% was reported for this ratio (Hess et al., 2005). However in another study by

**Table 1**
MRM calibration data for AZA1, AZA2 and AZA3.

| MRM transitions | Linear range (µg/l) | Correlation coefficient ($r^2$) ($n = 8$) | LOQ (µg/l) | LOD (µg/l) | LOQ (µg/kg) | LOD (µg/kg) |
|---|---|---|---|---|---|---|
| AZA1 | | | | | | |
| $[M+H]^+/[M+H–H_2O]^+$ | 0.985–1970 | 0.9968 | 5.3 | 3.2 | 2.1 | 1.3 |
| $[M+H]^+/[M+H–H_2O–A\text{-ring}]^+$ | 0.985–1970 | 0.9973 | 5.5 | 3.3 | 2.2 | 1.3 |
| AZA2 | | | | | | |
| $[M+H]^+/[M+H–H_2O]^+$ | 0.62–620 | 0.9918 | 1.9 | 1.1 | 0.7 | 0.4 |
| $[M+H]^+/[M+H–H_2O–A\text{-ring}]^+$ | 0.31–620 | 0.9974 | 1.7 | 1.0 | 0.7 | 0.4 |
| AZA3 | | | | | | |
| $[M+H]+/[M+H–H_2O]^+$ | 0.25–500 | 0.9979 | 1.4 | 0.9 | 0.6 | 0.3 |
| $[M+H]^+/[M+H–H_2O–A\text{-ring}]^+$ | 0.25–500 | 0.9981 | 1.7 | 1.0 | 0.7 | 0.4 |

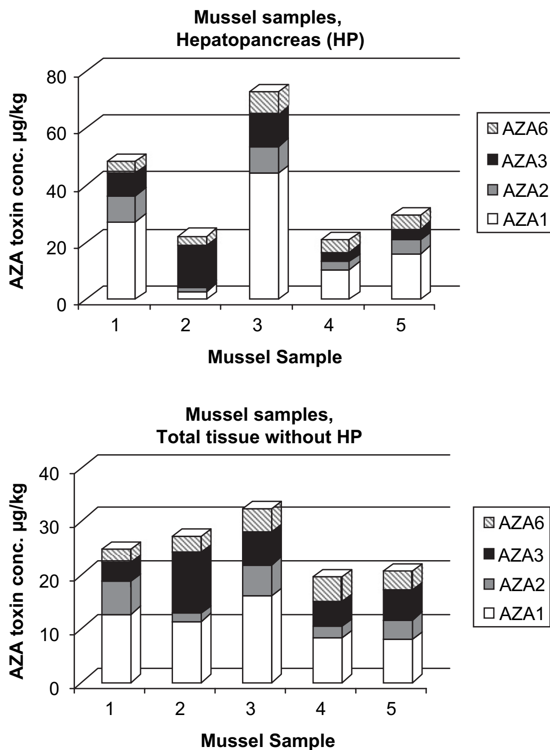


**Fig. 5.** Bar chart indicating azaspiracid concentrations and profiles in Chilean mussels (*M. edulis*). (A) mussel samples, hepatopancreas (HP) only; (B) mussel samples, total tissue without hepatopancreas (HP).

Furey and co-workers AZA1, AZA2 and AZA3 was reported to be routinely present in both mussel HP and remaining mussel tissue (Furey et al., 2003).

Screening of mussels and scallops in Spain and France respectively showed the presence of azaspiracid (Magdalena et al., 2003a). Toxin profiles found in these mussels were similar to that found in the Irish mussels. In the scallops tested however only AZA1 and 2 were observed with AZA1 being the predominant toxins, similar to those found in equivalent shellfish in Ireland (Furey et al., 2003). Differences in the AZA toxin profile have been observed in

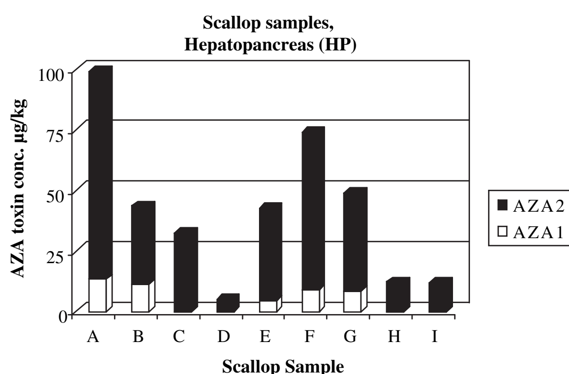


**Fig. 6.** Bar chart indicating azaspiracid concentrations and profiles in Chilean scallops, hepatopancreas (HP) only. No toxins were detected in remaining tissues.

mussels, but with King scallops the ratio of AZA1/AZA2 is fairly consistent within the tissue compartments with 85% of AZA toxins residing in the scallop HP (Magdalena et al., 2003b). Similar AZA profiles and ratios (AZA1/AZA2, 80/20%) was reported in Queen scallops (*Aequipecten opercularis*) from France in 2006 with HP containing the majority of the toxins (95%) (Amzil et al., 2008).

A survey of Portuguese shellfish (*Mytilus galloprovincialis*) showed no quantifiable azaspiracid levels over a 2-year period. However, some samples contained AZA2 but at levels below the current regulatory limit of 160 µg/kg (Vale, 2004). The presence of azaspiracid was confirmed in mussels harvested from the north Atlantic coast of Morocco which contained significant levels of the toxin; exceeding the current EU limit. Unlike the previous toxin profiles seen in northern Europe, in the Moroccan case AZA2 was the predominant toxin found in the mussels (Taleb et al., 2006). Only the digestive glands were extracted in this study, whereas in the previous study from the Portuguese incident (Vale, 2004), total tissue homogenate extracts were analysed as recommended by Furey and co-workers (Furey et al., 2002). The highest toxin levels for mussels harvested near the Moroccan coast were reported in July, and a correlation between declining DSP toxin levels and increasing AZP levels was observed (Taleb et al., 2006).

Portuguese shellfish were once again sampled for azaspiracid in 2006, whole tissue was homogenised and extracted using a new extraction method. Again levels of azaspiracid were below the EU limit but toxin concentrations were higher than on the previous occasion; AZA1 (0.004 µg/g), AZA2 (0.003 µg/g) and AZA3 (<0.001 µg/g), with AZA1 being the predominant toxin (average 60%). The clams, cockles, scallops and oysters that were tested contained only AZA1 and AZA2, with the relative amounts varying by species from 20 to 40% AZA2; no AZA3 was observed in species other than mussels (Vale et al., 2008).

Previously believed to be confined to bivalve molluscs, azaspiracid toxicity has recently been reported in Scandinavian Brown Crabs. As with previous findings the digestive glands were reported to contain the highest concentrations of the toxin. Azaspiracid profiles observed were similar to that of mussels found in Northern Europe. Concentrations greatly varied from 1.4 to 733 µg/kg, although most samples fell below the EU regulatory limit of 160 µg/kg (Table 2). Mussels, a probable food source for the crabs, were collected at the location simultaneously with crab sampling. Little or no azaspiracid toxicity was found in these mussels, and neither were culprit algae found in the region. This suggests bioaccumulation of azaspiracid toxins in the digestive gland of crabs is perhaps due to poor clearance of the toxin *in vivo* (Torgersen et al., 2008).

In 2009 azaspiracids (AZA1, AZA2) and spirolides were detected in two commercially important bivalves; macha (*Mesodesma-donacium*) and clams (*Mulinia edulis*) harvested from Coquimbo Bay, Chile (Table 2). Unfortunately that study did not apply the previously published LC-MS/MS confirmatory compound specific transitions as reported in this manuscript (Section 2.4). Also, the levels of AZA toxins reported in each sample were below the LC-MS/MS quantitation limits (Alvarez et al., 2010).

**Table 2**
Previously reported azaspiracid occurrences worldwide including marine species affected, toxin profiles and concentrations.

| Shellfish | Country | Sampling location | Year sampled | Species | AZA1 µg/kg | AZA2 µg/kg | AZA3 µg/kg | Total AZA µg/kg (TT) | Reference |
|---|---|---|---|---|---|---|---|---|---|
| Mussels | Ireland | Killary Harbour | 1995 | *Mytilus edulis* | 1140 | 230 | 60 | 1430 | (Ofuji et al., 1999b; Satake et al., 1997) |
| Mussels | Ireland | Arranmore Island | 1997 | *Mytilus edulis* | 865 | 250 | 240 | 1355 | (Ofuji et al., 1999a, 2001) |
| Mussels | Ireland | Bantry Bay | 1998 | *Mytilus edulis* | 1008 | 630 | 462 | 2100 | (James et al., 2002a) |
| Mussels | England | Craster | 1998 | *Mytilus edulis* | 100 | 8 | 22 | 130 | (James et al., 2002a) |
| Mussels | Norway | Sognefjord | 1998 | *Mytilus edulis* | 500[a] | 180[a] | 140[a] | 820* | (James et al., 2002a) |
| Mussels | Ireland | West coast | 1999 | *Mytilus edulis* | 2200 | 1400 | 600 | 4200 | (Furey et al., 2003) |
| Clams | Ireland | West coast | 1999 | *Tapes phillipinarum* | 460 | 150 | nr | 610 | (Furey et al., 2003) |
| Cockles | Ireland | West coast | 1999 | *Cardium edule* | 80 | 120 | nr | 200 | (Furey et al., 2003) |
| Scallops | Ireland | West coast | 1999 | *Pecten maximus* | 200 | 200 | nr | 400 | (Furey et al., 2003) |
| Phytoplankton | Ireland | South-west | 1999–2001 | *Protoperidinium crassipes* | 1.25 pg/cell | 0.15 pg/cell | 0.12 pg/cell | 1.52 pg/cell | (James et al., 2003) |
| Mussels | Spain | Ria de Vigo | 2001 | *Mytilus galloprovincialis* | 130 | 70 | 40 | 240 | (Magdalena et al., 2003a) |
| Scallops | France | Brittany | 2001 | *Pecten maximus* | 140[a] | 109[a] | 1[a] | 250[a] | (Magdalena et al., 2003a) |
| Mussels | Scotland | Loch Striven | 2003 | *Mytilus edulis* | 80 | <LOQ | nr | 80 | (Stobo et al., 2008) |
| Scallops | Scotland | Loch Striven | 2003 | *Pecten maximus* | 20 | <LOQ | nr | 20 | (Stobo et al., 2008) |
| Cockles | Scotland | Loch Striven | 2003 | *Cerastoderma edule* | <LOQ | <LOQ | nr | | (Stobo et al., 2008) |
| Mussels | Portugal | Faro | 2003 | *Mytilus galloprovincialis* | | 16 | nr | 16 | (Vale, 2004) |
| Mussels | Morocco | Oulad Ghanem | 2004 | *Mytilus galloprovincialis* | 200[a] | 650[a] | 50[a] | 900[a] | (Taleb et al., 2006) |
| Mussels | Canada | Nova Scotia | 2005 | *Mytilus edulis* | <LOQ | nr | nr | | (Twiner et al., 2008) |
| Crabs | Norway/Sweden | Tjarno Island | 2005 | *Cancer pagurus* | 733 | nr | nr | 733 | (Torgersen et al., 2008) |
| Scallops | France | Brittany | 2006 | *Aquepectin opercularis* | 220 | 50 | nr | 270 | (Amzil et al., 2008) |
| Oysters | Portugal | Aveiro | 2006 | *Crassotrea* spp | 4 | 1 | nr | 5 | (Vale et al., 2008) |
| Mussels | Portugal | Aveiro | 2006 | *Mytilus galloprovincialis* | 3 | 1 | <1 | 5 | (Vale et al., 2008) |
| Clams | Portugal | Aveiro | 2006 | *Spisula solido* | <20 | 20 | nr | 20 | (Vale et al., 2008) |
| | | | | *Venerupis senegalensis* | 2 | 3 | | 3 | |
| | | | | *Solen marginatus* | 3 | 3 | | 6 | |
| Cockles | Portugal | Aveiro | 2006 | *Cerastoderma edule* | 4 | 1 | | 5 | (Vale et al., 2008) |
| Plankton | North Sea | Many | 2008 | *Dinoflagellate 3D9* | 30 pg/l | nr | nr | 30 pg/l | (Krock et al., 2009) |
| Plankton | North Sea | Many | 2008 | *Azadinium gen.* et sp nov | 0.40 pg/cell | nr | nr | nr | (Tillman et al., 2009) |
| Sponge | Japan | Amami–Oshima | 2004 | *Echinoclathria* sp | | 250 | | | (Ueoka et al., 2009) |
| Clams | Chile | Coquimbo Bay | 2008 | *Mulinia edulis* | <LOQ | <LOQ | nr | <LOQ | (Alvarez et al., 2010) |

TT = total tissue; * based on µg isolated from 40 kg; a = µg/g hepatopancreas; nr = not reported.

**Table 3**
DSP mouse bioassay results.

| Shellfish | Sample | Total AZA conc. μg/kg | VDM results (mouse death) | Chile locations (Third region) |
|---|---|---|---|---|
| Scallop (A) | Whole tissue | <LOD | Neg | Bahia Tongoy |
| | Digestive glands | 96 | Neg | |
| Scallop (B) | Whole tissue | <LOD | Neg | Bahia Tongoy |
| | Digestive glands | 42 | Neg | |
| Scallop (C) | Whole tissue | <LOD | Pos 2/3 | Bahia Salada |
| | Digestive glands | 30 | Pos 3/3 | |
| Scallop (D) | Whole tissue | <LOD | Neg | Bahia Salada |
| | Digestive glands | 4 | Neg1/3 | |
| Scallop (E) | Whole tissue | <LOD | Neg | Bahia Tongoy |
| | Digestive glands | 41 | Neg1/3 | |
| Scallop (F) | Whole tissue | <LOD | Pos 2/3 | Bahia Tongoy |
| | Digestive glands | 72 | Pos 2/3 | |
| Scallop (G) | Whole tissue | <LOD | Pos 2/3 | Bahia Tongoy |
| | Digestive glands | 47 | Pos 2/3 | |
| Scallop (H) | Whole tissue | <LOD | Pos 2/3 | Bahia Inglesa |
| | Digestive glands | 10 | Pos 2/3 | |
| Scallop (I) | Whole tissue | <LOD | Pos 2/3 | Bahia Inglesa |
| | Digestive glands | 10 | Pos 3/3 | |
| Shellfish | Sample | Total AZA conc. μg/kg | VDM results (mouse death) | Chile locations (Chiloe Archipiélago) |
| Mussel (1) | Whole tissue | 24 | Neg 1/3 | Huyar Bajo |
| | Digestive glands | 45 | Neg 1/3 | |
| Mussel (2) | Whole tissue | 27 | Neg | Changuitad |
| | Digestive glands | 21 | Neg | |
| Mussel (3) | Whole tissue | 31 | Neg 1/3 | Punta Pinto |
| | Digestive glands | 72 | Neg 1/3 | |
| Mussel (4) | Whole tissue | 18 | Neg 1/3 | Canal Caicaen |
| | Digestive glands | 18 | Neg 1/3 | |
| Mussel (5) | Whole tissue | 19 | Neg | Estero Tubildad |
| | Digestive glands | 26 | Neg | |

Comparison between DSP mouse bioassay results (Table 3) with AZA concentrations determined for each sample, did not show a clear trend or correlation. The highest AZAs concentration sample gave a negative result when tested by mouse bioassay, while some of the less concentrated samples indicated positive results by mouse bioassay. Triplicate analysis by mouse bioassay showed some variations in outcome for the same sample. This points to a high level of inaccuracy in the mouse bioassay and the need for quantitative LC-MS/MS analysis of shellfish samples.

In summary, azaspiracids have now been identified in mussels (AZA1, AZA2, AZA3) and scallops (AZA1, AZA2) from Chile (Bahía Inglesa (III Region) and Chiloé archipelago). AZA1 was the predominant toxin in mussel HP and remaining tissue with AZA2, AZA3 and AZA6 present in approximate equivalent amounts whilst, AZA2 was the predominate toxin in the scallop samples with the toxin almost entirely present in the HP. The findings of low levels of azaspiracids toxins in this study suggest a low risk level when compared with the high risks posed by paralytic shellfish poisoning (PSP) (Compagnon et al., 1998; Lagos et al., 1996; Uribe, 1993). However, a more detailed study into the variation in azaspiracid profile in several different bivalve mollusc species must be carried out in Chile annually in order to generate predictive data.

## 4. Conclusion

This is the first report of Azaspiracid toxicity in bivalve molluscs in Chile, originated in two different locations Bahía Inglesa (III Region) and Chiloé archipelago. The affected bivalve mollusc species showed trace levels of AZP and it was found predominantly in the digestive glands. The average concentration reported in this study for AZP was 40 μg/kg in the HP of scallop and mussel samples and 250 μg/kg AZP in the remaining mussel tissue. Toxic profiles of the mussels and scallops tested in this study varied in their tissue distribution as well as between species. Scallops contained predominantly AZA2 (86% of total toxin) and it was entirely contained in the hepatopancreas for all of the samples tested. Mussels showed less inter-compartment variability suggesting the toxin distribution is more widespread in this species.

Based on insights gained during the azaspiracid intoxication episodes in Ireland and in Europe, it would be prudent for the Chilean regulatory authorities to implement the following recommendations to protect consumers and the shellfish industry: the undertaking of

- Monitoring by LC-MS/MS to determine the presence of algal toxins in shellfish cultivation regions.
- The conduction of long term temporal, spatial, geographic and multiple species studies to facilitate the accumulation of data for the identification of intoxication trends from AZP.

## Acknowledgments

We gratefully acknowledge funding from the Higher Education Authority (Programme for Research in Third-Level

Institutions 4) is gratefully acknowledged. The Science Foundation Ireland Basic Research Grant Programme 2004 awarded to Dr A. Furey funding the PhD program (K. O'Callaghan) is also acknowledged.

## Conflict of interest

The authors declare that there are no conflicts of interest.

## References


Alvarez, G., Uribe, E., Avalos, P., Marino, C., Blanco, J., 2010. First Identification of azaspiracid and spirolides in Mesodesma donacium and Mulinia edulis from Northern Chile. Toxicon 55 (2–3), 638–641.

Amzil, Z., Sibat, M., Royer, F., Savar, V., 2008. First report of azaspiracid and yessotoxin groups detection in French shellfish. Toxicon 52, 39–48.

Aune, T., Yndestad, M., 1993. Chapter 5: Diarrhetic shellfish poisoning. In: Falconer, I.R. (Ed.), Algal Toxins in seafood and Drinking Water London, UK. Academic Press, pp. 87–104.

Compagnon, D., Lembeye, G., Marcos, N., Ruiz-Tagle, N., Lagos, N., 1998. Accumulation of paralytic shellfish poisoning toxins in the bivalve Aulacomya ater and two carnivorous gastropods Conchalepas conchalepas and Argobuccinum ranelliformes during an Alexandrium catenella bloom in southern Chile. Journal of Shellfish Research 17, 67–73.

Draisci, R., Palleschi, L., Ferretti, E., Furey, A., James, K.J., Satake, M., Yasumoto, T., 2000. Development of a method for the identification of azaspiracid in shellfish by liquid chromatography-tandem mass spectrometry. Journal of Chromatography A 871, 13–21.

Furey, A., Brana-Magdalena, A., Lehane, M., Moroney, C., James, K.J., Satake, M., Yasumoto, T., 2002. Determination of azaspiracids in shellfish using liquid chromatography/tandem electrospray mass spectrometry. Rapid Communications in Mass Spectrometry 16, 238–242.

Furey, A., Moroney, C., Magdalena, A.B., Saez, M.J.F., Lehane, M., James, K.J., 2003. Geographical, temporal, and species variation of the polyether toxins, azaspiracids, in shellfish. Environmental Science & Technology 37, 3078–3084.

Fux, E., McMillan, D., Bire, R., Hess, P., 2007. Development of an ultra-performance liquid chromatography-mass spectrometry method for the detection of lipophilic marine toxins. Journal of Chromatography A 1157, 273–280.

Guzmán, L., Pacheco, H., Pizarro, G., Alarcón, C., 2002. Floraciones Algales Nocivas en el Cono Sur Americano. In: Sar, E.A., Ferraro, M.E., Reguera, B. (Eds.), Instituto Español de Oceanografía.

Hallegraeff, G.M., 1993. A Review of harmful algal blooms and their apparent global increase. Phycologia 32, 79–99.

Hess, P., Nguyen, L., Aason, J., Keogh, M., Kilcoyne, J., McCarron, P., Aune, T., 2005. Tissue distribution, effects of cooking and parameters affecting the extraction of azaspiracids from mussels, Mytilus edulis, prior to analysis by liquid chromatography coupled to mass spectrometry. Toxicon 46, 62–71.

IPCS, 1984. International Programme on Chemical Safety. Aquatic (Marine and Freshwater) Biotoxins. Environmental Health Criteria 37. World Health Organization.

Ito, E., Terao, K., McMahon, T., Silke, J., Yasumoto, T., 1997. Acute pathological changes in mice caused by crude extracts of novel toxins isolated from Irish mussels. In: Reguera, B., Blanco, J., Fernandez, M. L., Wyatt, T. (Eds.), Harmful Algae. Intergovernmental Oceanographic Commission of UNESCO, Paris, France, Vigo, Galicia, Spain, pp. 588–589.

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., Higashi, 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.

James, K.J., Fidalgo Sáez, M.J., Furey, A., Lehane, M., 2004. Azaspiracid poisoning, the food-borne illness associated with shellfish consumption: a review. Food Additives and Contaminants 21, 879–892.

James, K.J., Furey, A., Satake, M., Yasumoto, T., 2000. Azaspiracid poisoning (AZP): a new shellfish toxic syndrome in Europe. In: Hallegraeff, G.M., Blackburn, S.I., Bolch, C.J.S., Lewis, R.J. (Eds.), Ninth International Conference on Harmful Algal Blooms. Intergovernmental Oceanographic Commission of UNESCO, Paris, France, Hobart, Tasmania, Australia, pp. 250–253.

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

James, K.J., Lehane, M., Moroney, C., Fernandez-Puente, P., Satake, M., Yasumoto, T., Furey, A., 2002b. Azaspiracid shellfish poisoning: unusual toxin dynamics in shellfish and the increased risk of acute human intoxications. Food Additives and Contaminants 19, 555–561.

James, K.J., Moroney, C., Roden, C., Satake, M., Yasumoto, T., Lehane, M., Furey, A., 2003. Ubiquitous 'benign' alga emerges as the cause of shellfish contamination responsible for the human toxic syndrome, azaspiracid poisoning. Toxicon 41, 145–151.

Jeong, H.J., Latz, M.I., 1994. Growth and grazing rates of the heterotrophic dinoflagellate, Protoperidinium, on red tide dinoflagellates. Marine Ecology Progress Series 106, 173–185.

Krock, B., Tillmann, U., John, U., Cembella, A.D., 2009. Characterization of azaspiracids in plankton size-fractions and isolation of an azaspiracid-producing dinoflagellate from the North Sea. Harmful Algae 8, 254–263.

Lagos, N., 1998. Microalgal blooms: a global issue with negative impact in Chile. Biological Research 31, 375–386.

Lagos, N., 2002. Principales toxinas de origen fitoplanctónico: Identificación y cuantificación mediante cromatografía líquida de alta resolución (HPLC). Floraciones Algales Nocivas en el Cono-Sur Americano Instituto Español de Oceanografía.

Lagos, N., Compagnon, D., Seguel, M., Oshima, Y., 1996. Paralytic Shellfish Toxin Composition: a Quantitative Analysis in Chilean Mussels and Dinoflagellate. Harmful and Toxic Algal Blooms UNESCO Intergovernmental Oceanographic Commission, 121–124.

Lehane, M., Brana-Magdalena, A., Moroney, C., Furey, A., James, K.J., 2002. Liquid chromatography with electrospray ion trap mass spectrometry for the determination of five azaspiracids in shellfish. Journal of Chromatography A 950, 139–147.

Lehane, M., Saez, M.J.F., Magdalena, A.B., Canas, I.R., Sierra, M.D., Hamilton, B., Furey, A., James, K.J., 2004. Liquid chromatography-multiple tandem mass spectrometry for the determination of ten azaspiracids, including hydroxyl analogues in shellfish. Journal of Chromatography A 1024, 63–70.

Leising, A., Horner, R., Pierson, J.J., Postel, J., Halsband-Lenk, C., 2005. The balance between microzooplankton grazing and phytoplankton growth in a highly productive estuarine fjord. Progress in Oceanography 67, 366–383.

Lembeye, G., Guzmán, L., Campodinico, I., 1975. Estudios sobre un florecimiento tóxico causado por Gonyaulax catenella en Magallanes. III. Fitoplancton asociado. An Inst Patagonia Punta Arenas (Chile) 4, 197–208.

Lembeye, G., Yasumoto, T., Zhao, J., Fernández, R., 1993. DSP Outbreak in Chilean Fiords. Toxic Phytoplankton Blooms in the Aea. Elsevier, New York, pp. 525–529.

Magdalena, A.B., Lehane, M., Krys, S., Fernandez, M.L., Furey, A., James, K.J., 2003a. The first identification of azaspiracids in shellfish from France and Spain. Toxicon 42, 105–108.

Magdalena, A.B., Lehane, M., Moroney, C., Furey, A., James, K.J., 2003b. Food safety implications of the distribution of azaspiracids in the tissue compartments of scallops (Pecten maximus). Food Additives and Contaminants 20, 154–160.

Muñoz, P., Avaria, S., Sievers, H., Prado, R., 1992. Presencia de dinoflagelados tóxicos del género Dinophysis en el seno de Aysén, Chile. Revista Biología Marina (Valparaíso) 27, 187–212.

Nicolaou, K.C., Koftis, T.V., Vyskocil, S., Petrovic, G., Ling, T.T., Yamada, Y.M.A., Tang, W.J., Frederick, M.O., 2004. Structural revision and total synthesis of azaspiracid-1, part 2: definition of the ABCD domain and total synthesis. Angewandte Chemie-International Edition 43, 4318–4324.

Ofuji, K., Satake, M., McMahon, T., Silke, J., James, K.J., Naoki, H., Oshima, Y. , Yasumoto, T., 1999a. Two analogs of azaspiracid isolated from mussels, Mytilus edulis, involved in human intoxication in Ireland. Natural Toxins 7, 99–102.

Ofuji, K., Satake, M., Oshima, Y., McMahon, T., James, K.J., Yasumoto, T., 1999b. A sensitive and specific determination method for azaspiracids by liquid chromatography mass spectrometry. Natural Toxins 7, 247–250.

Ofuji, K., Satake, M., McMahon, T., James, K.J., Naoki, H., Oshima, Y., Yasumoto, T., 2001. Structures of azaspiracid analogs, azaspiracid-4 and azaspiracid-5, causative toxins of azaspiracid poisoning in Europe. Bioscience Biotechnology and Biochemistry 65, 740–742.

Osleng, C.D., Naustvoll, L.J., Paasche, E., 2002. Grazing by the heterotrophic dinoflagellate, Protoperidinium steinii, on a Ceratium bloom. Marine Ecology Progress Series 225, 161–167.

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 Communications in Mass Spectrometry 22, 549–558.

Satake, M., Ofugi, K., James, K.J., Furey, A., Yasumoto, T., 1997. New toxic event caused by Irish mussels. In: Reguera, B., Blanco, J., Fernandez, M.L., Wyatt, T. (Eds.), Harmful Algae. Intergovernmental Oceanographic Commission, Vigo, Galicia, Spain, pp. 468–469.

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. Journal of the American Chemical Society 120, 9967–9968.

Stobo, L.A., Lacaze, J.-P.C.L., Scott, A.C., Petrie, J., Turrell, E.A., 2008. Surveillance of algal toxins in shellfish from shellfish waters. Toxicon 51, 635--648.

Suganuma, M., Fujuki, H., Suguri, H., Yoshizawa, S.H., Nakayasu, M., Ojika, M., Wakamatzu, K., Yamada, K., Sugimura, T., 1998. Okadaic acid: an additional non-phorbal 12-tetradecanoate-13-acetate type tumour promoter. Proceedings of National Academy of Science USA 85, 1768–1771.

Taleb, H., Vale, P., Amanhir, R., Benhadouch, A., Sagou, R., Chafik, A., 2006. First detection of azaspiraicds in mussels in north west Africa. J Shellfish Res 25, 1067–1070.

Tillman, U., Elbrachter, M., Krock, B., John, U., Cembella, A., 2009. Azadinium spinosum gen. et sp nov (Dinophyceae) identified as a primary producer of azaspiracid toxins. European Journal of Phycology 44, 63–79.

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.

Twiner, M.J., Hess, P., Bottein Dechraoui, M.Y., McMahon, T., Samons, M.S., Satake, M., Yasumoto, T., Ramsdell, J.S., Doucette, G.J., 2005. Cytotoxic and cytoskeletal effects of azaspiracid-1 on mammalian cell lines. Toxicon 45, 891–900.

Twiner, M.J., Rehmann, N., Hess, P., Doucette, G.J., 2008. Azaspiracid shellfish poisoning: a review on the chemistry, ecology and toxicology with an emphasis on human health impact. Marine Drugs 6, 39–72.

Ueoka, R., Ito, A., Izumikawa, M., Maeda, S., Takagi, M., Shin-ya, K., Yoshida, M., van Soest, R.W.M., Matsunaga, S., 2009. Isolation of azaspiracid-2 from a marine sponge Echinoclathria sp. as a potent cytotoxin. Toxicon 53, 680–684.

Uribe, J.C., 1993. PSP Outbreakin Chile. Sixth International Conference on Toxic Marine Phytoplancton, Nantes, France. 18–22 October, p. 210

Uribe, J.C., García, C., Rivas, M., Lagos, N., 2001. First report diarrhetic shellfish toxins in Magellanic fiords, Southern Chile. Journal of Shellfish Research 20, 69–74.

Vale, P., 2004. Is there a risk of human poisoning by azaspiracids from shellfish harvested at the Portuguese coast? Toxicon 44, 943–947.

Vale, P.J., Botelho, M.J., Rodrifues, S.M., Gomes, S.S., Sampayo, M.A, de.M., 2008. Two decades of marine biotoxin monitoring in bivalves from Portugal (1986–2006): a review of exposure assessment. Harmful Algae 7, 11–25.

Yasumoto, T., Murata, M., 1993. Marine toxins. Chemical Reviews 93, 1897–1909.

Yasumoto, T., Murata, M., Oshima, Y., Matsumoto, G.K., Clardy, J., 1984. Diarrhetic shellfish poisoning. In: Seafood Toxins. American Chemical Society, Washington, DC, pp. 207–214.

Yasumoto, T., Fukui, M., Sil, K., 1995. Determination of marine toxin in foods. Journal of AOAC International 78, 574–582.

Zhao, J., Lembeye, G., Cenci, G., Wall, B., Yasumoto, T., 1993. Determination of okadaic acid and dinophysistoxin-1 in mussels form Chile, Italy and Ireland. In: Smayda, T.J., Shimizu, Y. (Eds.), Toxic Phytoplankton Blooms in the Sea. Elsevier Science, New York, USA, pp. 587–592.