The role of *Azadinium spinosum* (Dinophyceae) in the production of azaspiracid shellfish poisoning in mussels

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ABSTRACT

Azaspiracids (AZAs) are a group of lipophilic polyether compounds first detected in Ireland which have been implicated in shellfish poisoning incidents around Europe. These toxins regularly effect shellfish mariculture operations including protracted closures of shellfish harvesting areas for human consumption. The armoured dinoflagellate *Azadinium spinosum* Elbrächter et Tillmann gen. et sp. nov. (Dinophyceae) has been described as the *de novo* azaspiracid toxin producer; nonetheless the link between this organism and AZA toxin accumulation in shellfish has not yet been established. In August 2009, shellfish samples of blue mussel (*Mytilus edulis*) from the Southwest of Ireland were analysed using liquid chromatography–tandem-mass spectrometry (LC–MS/MS) and were found to be above the regulatory limit (0.16 μg g−1 AZA-equiv.) for AZAs. Water samples from this area were collected and one algal isolate was identified as *A. spinosum* and was shown to produce azaspiracid toxins. This is the first strain of *A. spinosum* isolated from Irish waters. The Irish *A. spinosum* is identical with the other two available *A. spinosum* strains from Scotland (3D9) and from Denmark (UTHE2) in its sequence of the D1–D2 regions of the LSU rDNA.

A 24 h feeding trial of blue mussels (*M. edulis*) using an algal suspension of the Irish *A. spinosum* culture at different cell densities demonstrated that *A. spinosum* is filtered, consumed and digested directly by mussels. Also, LC–MS/MS analysis had shown that AZAs were accumulating in the shellfish hepatopancreas. The toxins AZA1 and -2 were detected in the shellfish together with the AZA analogues AZA3, AZA6, AZA17 and -19 suggesting that AZA1 and -2 are metabolised in the shellfish within the first 24 h after ingestion of the algae. The levels of AZA17 detected in the shellfish hepatopancreas (HP) were equivalent to the levels of AZA1 but in the remainder tissues the levels of AZA17 were four to five times higher than that of AZA1, only small quantities of AZA3 and -19 were present with negligible amounts of AZA6 detected after the 24 h period. This could have implications in the future monitoring of these toxins given that at present according to EU legislation only AZA1–AZA3 is regulated for. This is the first report of blue mussels (*M. edulis*) feeding on the azaspiracid producing alga *A. spinosum* from Irish waters.

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1. Introduction

Azaspiracids (AZAs) are a group of lipophilic polyether toxins which were first detected in shellfish in 1995 from a contaminated batch of mussels (*Mytilus edulis*) from Killary harbour, county Galway on the West coast of Ireland. This event led to a poisoning incident in the Netherlands in November of that year where eight people became ill with symptoms typical of diarrheic shellfish poisoning (DSP), however chemistry results showed that DSP toxins were only found in the shellfish at low concentrations and phytoplankton samples did not show the presence of DSP toxin producing algae in the water, yet the mousse bioassay was strongly positive (McMahon and Silke, 1996). In 1998 this novel group of compounds were isolated and chemically characterized from shellfish (Satake et al., 1998; Ofuji et al., 1999). A few years later azaspiracids were confirmed in the UK and Norway (James et al., 2002), Morocco (Taleb et al., 2006) and Portugal (Vale et al., 2008) suggesting that azaspiracid toxicity had a more extensive distribution along the west European Atlantic seaboard and as far as north Africa. There is evidence now that the distribution of azaspiracids may be worldwide with azaspiracids found in Japan (Ueoka et al., 2009), Chile (Alvarez et al., 2010; Lopez-Ribera et al., 2010) and Canada (Twiner et al., 2010).
The development of routine chemical analysis as a monitoring tool showed azaspiracid concentrations found in _M. edulis_ in Ireland between 2003 and 2010 using LC–MS/MS (Fig. 1) indicates that the presence of azaspiracids has been a recurring problem since 2005 annually. However, AZA toxins were found at concentrations below the regulatory limit in 2003 and not detected in 2004 and the first half of 2005, suggesting that there could be a cyclical element to AZA events. While the accumulation of AZA toxins has been reported in a number of different shellfish species (Table 1), levels above the regulatory limit have only been observed in blue mussels (_M. edulis_) and Pacific oysters (_Crassostrea gigas_) in Ireland.

Due to the ability to accumulate high levels of AZA toxins, mussels have become an important source of AZAs for toxin isolation (Furey et al., 2003; Perez et al., 2010). Since the first characterization of AZA1 by Satake et al. (1998) other structural variants have been isolated and characterized: AZA2 and AZA3 (Ofuji et al., 1999, 2001) and AZA6–AZA11 (James et al., 2003) with AZA12 theoretically postulated, the number of variants was increased from AZA12 to AZA32 later by Rehmann et al. (2008) with the discovery of new dihydroxy and carboxy-AZAs. Most of these AZA variants have been reported to be shellfish metabolites (Rehmann et al., 2008) rather than _de novo_ products of plankton, apart from AZA1, AZA2 and AZA3 (Furey et al., 2003). However, Krock et al. (2009) only detected AZA1 and AZA2 in the field samples from Scotland generating uncertainties over the production of AZA3 by plankton. Fux et al. (2009) did find low amounts of AZA3 using Solid Phase Adsorption Toxin Tracker Device (SPATT), a passive sampler, but concluded that a heat treatment step could have influenced the enzymatic activity of the SPATT prior to the extraction allowing the metabolism of AZA1 to AZA3.

In 2008, the causative organism was discovered from the North-East coast of Scotland (Krock et al., 2009) and described as a _genus_ and _species novo_ (Tillmann et al., 2009). This organism, a small armoured dinoflagellate named _Azadinium spinosum_ was shown to produce AZA toxins in culture and effectively identified as _de novo_ producer of azaspiracids. Later, McCarron et al. (2009) reported that AZA17 and -19 are formed by oxidation of the 22-methyl group of AZA1 and -2, respectively. In this study, heat induced decarboxylation of AZA17 and -19 from AZA3 and -6 was demonstrated showing the possible bioconversion pathways of these toxins in shellfish.

Table 1

<table>
<thead>
<tr>
<th>Shellfish species</th>
<th>Scientific name</th>
<th>Concentration (mg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common Cockle</td>
<td>Cerastoderma edule</td>
<td>0.08</td>
</tr>
<tr>
<td>Pacific oyster</td>
<td>Crassostrea gigas</td>
<td>0.31</td>
</tr>
<tr>
<td>Razor clam</td>
<td>Ensis arcaucus</td>
<td>0.05</td>
</tr>
<tr>
<td>Razor clam</td>
<td>Ensis siliqua</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Dog cockle</td>
<td>Glycemeris glycermis</td>
<td>0.01</td>
</tr>
<tr>
<td>Abalone</td>
<td>Halotis discus hannah</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>Blue mussel</td>
<td>Mytilus edulis</td>
<td>8.97</td>
</tr>
<tr>
<td>Native oyster</td>
<td>Ostrea edulis</td>
<td>0.07</td>
</tr>
<tr>
<td>Common limpet</td>
<td>Patella vulgata</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>Surf Clam</td>
<td>Spisula solida</td>
<td>0.15</td>
</tr>
<tr>
<td>Manila Clam</td>
<td>Tapes philippinarum</td>
<td>0.10</td>
</tr>
<tr>
<td>Clam</td>
<td>Tapes semidesusscatus</td>
<td>0.01</td>
</tr>
<tr>
<td>Pullet carpet shell</td>
<td>Venerapis senegalensis</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>Venus Clam</td>
<td>Venus verrucosa</td>
<td>&lt;LOQ</td>
</tr>
</tbody>
</table>

The Marine Institute phytoplankton unit collected water samples between August and September of 2009 following positive results in blue mussels of AZA toxins. The samples yielded several isolates and cultures, one of which was shown to produce AZA1 and AZA2. The Irish isolate was provisionally named SM2. Here, we report on the taxonomic identity of the Irish toxin producer and the genotypic/phenotypic relationship with other _Azadinium_ species and its toxic profile. The link between _A. spinosum_ toxicity and toxin accumulation in shellfish had not yet been established. It was not known whether blue mussels were able to directly ingest the small thecate dinoflagellate _A. spinosum_ or if toxin accumulation in mussels is mainly achieved via planktonic vector species which have potentially accumulated AZA toxins by consuming the small armoured dinoflagellate. Thus, a feeding trial was designed to demonstrate that blue mussels are able to feed and digest directly from an algal suspension of _A. spinosum_, that AZA toxins accumulate in their digestive system and that ultimately these are metabolised into other AZA analogues.

![Fig. 1. AZA equivalents concentration (mg kg⁻¹) found in blue mussels (Mytilus edulis) in Ireland between 2003 and 2010. Please note that two data points are not shown in this table as the AZA equivalent concentrations were too high compared to the rest of the results and it would have caused the graph to collapse at the lower concentations. The following data points are not shown: (7.37 mg kg⁻¹ AZA-equiv. (22/08/08) and 8.97 mg kg⁻¹ AZA-equiv. (01/10/2005)).](image)
2. Materials and methods

2.1. Sample collection, isolation and culture of an AZA producing dinoflagellate

2.1.1. Field collection using a submersible pump

The culture of an Irish strain of _A. spinosum_ provisionally designated as SM2 was established from water samples collected in the Southwest coast of Ireland, at Garehies pier, Bantry bay (latitude: 51°39’4.7”N, longitude: 9°35’11”E) during September of 2009 coinciding with an increase of AZA toxin in shellfish as reported by the Marine Institute biotoxin monitoring programme. The water samples were collected using a pump below surface and at 3 m depth, approximately 100 L of seawater was pumped into a fraction sampler to separate and concentrate the different size fractions (60 µm, 38 µm and 20 µm). The filtrate was also collected into 25 L polyethylene drums for cell concentration down to 3 µm in size using a vacuum pump at low pressure and 3 µm pore size TSTP Millipore® membrane filters. Approximately 5 L of 20 µm filtrate was concentrated down (3 µm) to approximately 50 ml volume before isolation.

2.1.2. Isolation and culture conditions

The dinoflagellates were isolated using single cell isolation by micropipette in 96 cell tissue culture plates (Corning, New York, USA). The isolates were kept in F2 without silica (Guillard and Ryther, 1962; Guillard, 1975) made up with enriched sterile filtered seawater from the site and kept at 18 °C temperature, 12:12 light:dark cycle and the irradiance in the incubator was 150 µmol photon m⁻² s⁻¹ measured using an Iso-tech ILM350 light meter (ISO-tech, Merseyside, UK). Potential AZA toxin producer isolates were discriminated from the 20 µm filtrate and 3 µm backwash concentrated fractions by screening for morphological characteristics typical of _A. spinosum_. Four other isolates apart from SM2 suspected to be _A. spinosum_ were isolated and cultured successfully based on cell size and shape, presence of thecal plates, cell movement, and presence of antapical spine, presence/absence of a conspicuous pyrenoid and presence/absence of apical pore. After successful isolation, the unialgal cultures were transferred to 25 mm x 150 mm borosilicate culture tubes (Fisherbrand™, Loughborough, UK) containing 35 ml of F2 media and kept in the incubator in the conditions as outlined before.

All cultured isolates were tested for the production of AZA toxins using LC–MS/MS, 1 ml culture aliquots of a densely growing culture were collected using a 1 ml pipette and placed into 0.2 µm Whatman Anopore™ spin filter, the filters were centrifuged at 14,000 rpm for 1 min using a Heraeus Multifuge 35-R (Heraeus, Hanau, Germany) and the filter extracted using methanol.

2.2. Microscopy

2.2.1. Light microscopy (LM)

Observation of live cultured isolates was carried out using an inverted microscope (Axiovert 200M, Zeiss, Germany) equipped with epifluorescence and differential interference contrast optics. Light microscopic examination of the thecal plate was performed as per Tillmann et al. (2010).

2.2.2. Scanning electron microscopy (SEM)

The material for examination was collected from the unialgal cultures and prepared following the protocol for SEM by Tillmann et al. (2009) with slight variations: the cell pellet – after resuspension and removal of 40% seawater – was fixed with formalin (2% final concentration) instead of glutaraldehyde in cacodylate buffer and stored in the fridge for 2 h before washing and dehydration steps. The filters were mounted on stubs, sputter coated (Emscope SC 500, Ashford, UK) with gold–palladium and viewed under a scanning electron microscope (FEI Quanta FEG 200, Eindhoven, Netherlands).

2.3. Chemical analysis of the azaspiracids producing culture

2.3.1. Solvents and reagents

Acetonitrile and methanol were purchased as pesticide grade solvents from Labscan (Dublin, Ireland). Formic acid, ammonium formate, ammonium hydroxide and sodium hydroxide were purchased from Sigma Aldrich (Steinheim, Germany). Hydrochloric acid was purchased from VWR (England). Water was obtained from a reverse-osmosis purification system (Barnstead, Dublin, Ireland). AZA certified reference materials (CRM) were obtained from the NRC (Halifax, Canada).

2.3.2. Toxin extraction

The culture was extracted by solid phase extraction (SPE). An Oasis HLB, 3 cm³ cartridge was initially conditioned with 5 ml of methanol, flushed with 10 ml of a 5% methanol solution (in water) and then loaded with 10 ml of culture slowly (drop wise). The cartridge was flushed again with 10 ml of a 5% methanol solution followed by toxin elution with 4 ml of methanol. The methanol extract was blown down to dryness, reconstituted back up in 0.5 ml of methanol and transferred into a HPLC vial for analysis.

2.3.3. Analysis by LC–MS/MS

Analysis of AZAs was performed on a Micromass triple stage quadrupole (TSQ) Ultima coupled to a Waters 2695, equipped with a Z-spray ESI source. The TSQ was operated in positive ionization mode through multiple reactions monitoring (MRM). The following transitions were monitored: AZA1 m/z 842.5 > 654.4 and 842.5 > 672.4, AZA2 856.5 > 654.4 and 856.5 > 672.4, AZA3 828.5 > 640.4 and 828.5 > 658.4, AZA6 842.5 > 640.4 and 842.5 > 658.4, AZA17 872.5 > 640.4 and 872.5 > 658.4 and AZA19 886.5 > 640.4 and 886.5 > 658.4 in positive ionization mode. The cone and collision voltages were set at 60 V and 40 V, respectively. Cone and desolvation gas flows were set at 100 and 800 L/h, respectively, while the source and desolvation temperatures were set at 150 °C and 350 °C, respectively.

A binary mobile phase was used, phase A (100% aqueous) and phase B (95% aqueous acetonitrile), both containing 2 mM ammonium formate and 50 mM formic acid. HPLC separation was achieved using a Hypersil BDS C8 column; 50 mm x 2.1 mm, 3 µm; guard column, 10 mm x 2.1 mm, 3 µm (Thermo Scientific, Runcorn, UK). The flow rate was set at 0.25 ml/min and the injection volume was set at 5 µl. The column and sample temperatures were set at 25 °C and 6 °C, respectively. A gradient elution was employed, starting with 30% B, rising to 90% B over 8 min, held for 2.5 min, then decreased to 30% B in 0.5 min and held for 4 min to equilibrate the system.

2.4. Molecular phylogenetic analysis

2.4.1. Extraction of genomic DNA

A 50 ml sample of exponentially growing culture was centrifuged (Eppendorf 5810R, Hamburg, Germany) at 3220 x g for 15 min at room temperature. The cell pellets were frozen at −20 °C for 20 min before being subjected to total DNA extraction with the DNeasy Kit (Mini) (Qiagen, Hilden, Germany) according to manufacturer’s instructions. The purity and quantity of the DNA were checked by UV-spectroscopy with a NanoDrop ND-1000 system (Peglab, Erlangen, Germany) and the integrity of DNA fragments of a molecular weight of about 20 kb was verified on a 1% agarose gel.
2.4.2. PCR amplification and sequencing

The total extracted DNA from the isolate was subjected to polymerase chain reaction (PCR) amplification of the 28S ribosomal DNA. The forward and reverse primers for amplification of 28S rDNA (D1–D2 regions) were: Dir-F (5'-ACC CGG TGA ATT TAA GCA TA-3') and Dir-2CR (5'-CTT TGG GCC GTG TTT CAA GA3'), respectively. For the 50 µL PCR, HotMasterTaq™ (Eppendorf, Hamburg, Germany) buffer 1×, 0.1 mM of dNTPs, 0.1 mM of each forward and reverse primer and 1.25 units of Taq polymerase were added to 10 ng of the extracted genomic DNA. For 28S rDNA amplifications, the reactions were subjected to the following thermocycling conditions: an initial denaturation at a temperature of 95 °C for 7 min was followed by 35 cycles of denaturation at 94 °C for 45 s, annealing temperature at 54 °C for 2 min and elongation temperature at 72 °C for 1.5 min. A final extension step at 72 °C was carried out for 10 min. The completed reactions were kept at 10 °C until the next step. The PCR amplicons were analysed on 1% agarose by electrophoresis. Sequencing was conducted with a standard cycle sequencing chemistry ABI 3.1 (Applied Biosystems, Darmstadt, Germany) using the PCR primer sets. Cycle sequencing products were analysed on an ABI 3130 XL capillary sequencer (Applied Biosystems, Darmstadt, Germany).

2.4.3. Sequence alignment for phylogenetic analyses

Sequence alignment was done with CLUSTAL X software (Thompson et al., 1997) and improved manually for all sequences; ambiguous alignment positions were excluded from the analysis. Alignments are available upon request and sequences are available at GenBank under accession number for LSU: SM2 JN165101. Maximum likelihood phylogenetic tree was calculated with PhyML (Guindon and Gascuel, 2003) using a BLO-NJ (neighbour-joining) tree as a starting tree, and the general time reversible (GTR) nucleotide substitution model (Whelan and Goldman, 2001) with a gamma distribution parameter estimated from the data, bootstrap analysis was performed with 100 replicates. The dinoflagellate Oxyrrhis marina (Dujardin) was selected as the outgroup.

2.5. Blue mussels feeding experiment

2.5.1. Experimental design

A batch of approximately 10 kg of M. edulis was harvested from Carlingford Lough, Greenore (latitude: 54.0339 N, longitude: −6.1417 W) in the East coast of Ireland. The mussels were cleaned of all fouling organisms and placed in 20 L clear plastic carboys filled with sterile filtered seawater (30 psu salinity) in the walk-in incubator at 18 °C. Any dead mussels were removed from the carboys. The experiment consisted of a 24 h feeding experiment with M. edulis and the azaspiracids producing dinoflagellate A. spinosum at three cell densities to study feeding activity, toxin uptake and bioconversion of toxins in the shellfish digestive system. The cultures of A. spinosum and the mussels were maintained under the same environmental conditions for a week before the experiment was carried out.

Triplicate treatments were prepared in 5 L borosilicate conical flasks (Lennox, Dublin, Ireland) at three cell densities; 30,000 cells ml⁻¹, 20,000 cells ml⁻¹ and 5000 cells ml⁻¹ of A. spinosum. The mussels used in this study were weighed, measured and labeled before the start of the experiment; 100 g of mussels’ whole flesh was dissected from the batch of mussels for LC–MS/MS analysis to test for AZAs in the mussels prior to the experiment. Also, a sample of seawater (50 ml) used for growing A. spinosum culture before inoculation of the algae was extracted for AZA toxins using a SPE column as described above in Section 2.3.2. The feeding experiment consisted of three mussels placed in each conical flask containing A. spinosum. Two control samples were set up, one containing three mussels with no algal suspension and one containing algal suspension with no mussels. The latter control was prepared to account for potential decline of algae in the water column due to sedimentation. 3 × 10 ml suspension samples from each of the flasks were collected using an automated pipette (Hirsmann Laborgerate, Heilbronn, Germany) to measure the initial cell density and AZA toxin concentration. During the first 2 h of the experiment, 1 ml aliquots of seawater was collected from each flask every 20 min using a 1 ml pipette (Eppendorf, Cambridge, UK) and preserved with lugol’s iodine (Clin-tech, Dublin, Ireland) 1% final concentration to estimate cell concentrations of A. spinosum during the experiment. After 2 h samples were collected every hour and finally at 24 h. A final suspension sample (10 ml) was collected at 24 h to analyse the final toxin content of remaining algal cells or other particles in the water column.

Pseudo-faeces and faeces excreted by the mussels during the experiment were harvested using 25 ml serological pipettes (Sardstedt, Nürnberg, Germany) and filtered after 24 h using GF/C Whatman (1.2 µm, 47 mm diameter) glass microfiber filters under vacuum for toxin analysis to determine the total toxin budget for the experiment. Before harvesting, the water was removed from the flasks carefully avoiding the re-suspension of the faeces and pseudo-faeces, while most of the water was removed, a small amount of water was left at the bottom of the flasks and picked up in the pipette before filtering. This means that it is possible that other material was present in the sample other than the faeces and pseudo-faeces. The hypothesis here would be that since the algal suspension was depleted after 24 h, no A. spinosum cells are believed to be present in the water at this point.

2.5.2. A. spinosum cell counts

Cell counts of the 1 ml lugol’s preserved aliquots were carried out using a Sedgewick-Rafter cell counting chamber (Pyser-SGI, Kent, UK) for each flask at each time interval using an inverted optical microscope Leica DMi 6000B (Leica, Wetzlar, Germany).

2.5.3. Dissection of mussels and toxin analysis of shellfish tissues

After 24 h, the mussels were harvested from the flasks, weighed and dissected into hepatopancreas (HP) and remainder tissues. The dissected HP and remainder were placed into labeled 15 ml polypropylene centrifuge tubes (Sardstedt, Nürnberg, Germany). A volume of 1.5 ml methanol was added to each tube and the sample was homogenized using an Ultra-Turrax (T25 Basic IKA®-Werke, Germany) at 11,000 rpm for 1 min. Samples were then centrifuged in a Heraeus Multifuge 35-R (Heraeus, Hanau, Germany) at 4500 rpm for 5 min. The supernatant was decanted into 5 ml volumetric flasks (Hirschmann-Techcolor, Heilbronn, Germany) and this step repeated twice for each pellet. The volume was then brought up to the mark with pestican grade methanol, inverted 5 times for each and filtered through 0.22 µm filters (Sartorius, Surrey, UK) into HPLC vials (AGB, Dublin, Ireland) to be run on the LC–MS/MS.

2.5.4. Toxin analysis of A. spinosum samples

The toxicity of A. spinosum cells was measured during the feeding experiment at two time intervals. Triplicate 10 ml samples of A. spinosum were collected from each flask in 15 ml centrifuge tubes at initial time (t0) and at the end of the experiment after 24 h. The samples were centrifuged at 4500 rpm for 15 min. The supernatant was decanted off and 500 µl of methanol was added to each pellet. Samples were then vortexed in a V400 Multitube Vortex mixer (Alpha Laboratories, Hampshire, UK) mixed for 1 min at maximum speed then centrifuged again. The supernatant was collected into labeled HPLC vials. The process was repeated twice to obtain a final volume of 1.5 ml for each HPLC vial. These samples were blown down to dryness under nitrogen gas (BOC gases, Dublin, Ireland) and reconstituted with 500 µl methanol to be analysed by LC–MS/MS.
2.5.5. Toxin analysis of biodeposits

After harvesting the mussels from the flasks, biodeposits (pseudo-faeces, faeces and any detritus left in the flask and a small amount of water) were collected from the bottom of the flasks and then filtrated on GF/C Whatman (1.2 μm, 47 mm diameter) glass microfiber filters. Filters from each flask were placed into labeled 50 ml centrifuge tubes. A volume of 3 ml methanol was added to each tube and vortex mixed at maximum speed for 1 min. Samples were then centrifuged at 3500 rpm for 15 min at 4 °C. The supernatant was transferred to 10 ml volumetric flasks. This was repeated twice and the volume was made up to the mark using methanol. The flasks were inverted 5 times and filtered through 0.22 μm filters into HPLC vials to be analysed via LC–MS/MS using the conditions described previously.

3. Results and discussion

3.1. A. spinosum Irish strain

The armoured dinoflagellate A. spinosum has been described by Tillmann et al. (2009) as a new species and a new genus. Since then, two additional but non-toxic species have been described, A. obesum (Tillmann et al., 2010) and A. poporum (Tillmann et al., 2011). The Irish isolate provisionally named SM2, clearly is a new
geographical strain of *A. spinosum* as it is sharing all morphological details compiled by Tillmann et al. (2009) to describe *A. spinosum* and to differentiate this species from the two other species.

Fig. 2A depicts a light microscopy image of the Irish strain of *A. spinosum* showing the main morphological characteristics typical of the species, a conspicuous apical pore complex (APC), a conspicuous pyrenoid (P) in the episeeme which can be used to differentiate *A. spinosum* from *A. obesum*, the large spherical nucleus (N) posteriorly located and the antapical spine (S). This antapical spine situated in the second antapical plate is an important feature for the identification of the species as it is present in *A. spinosum* but lacking in the two other described species, *A. obesum* and *A. poporum* (Tillmann et al., 2010, 2011). For the Irish strain SM2, the spine was visible using 100× oil immersion objectives under the light microscope in formalin preserved water samples, although it was difficult enough to discern.

Anyhow, the best way to visualise the spine and distinguish the delicate thecal plates in this dinoflagellate is to use scanning electron microscopy (SEM) which allowed us to identify the plate tabulation to be Po, cp, X, 4’, 3a, 6’, 6C, 57S, 6m”, 2*a* which is the same as reported in Tillmann et al. (2009). Fig. 2B and C shows complete cells of *A. spinosum*. In ventral view (Fig. 2B) the ventral pore (vp) is clearly visible in the left suture of the first apical plate, the thecal plates cleaned of the outer membrane appear smooth and some scattered pores can be seen on the plates. Fig. 3B shows the three intercalary plates on the episme of the cell; the second intercalary plate (2a) is smaller than the other two plates and is located above the third pre-circular plate.

Fig. 3C shows the complete epithecal plates of *A. spinosum*, the detail view of the APC (Fig. 3D) shows an identical configuration of the pore described for *A. spinosum* and *A. obesum* (Tillmann et al., 2010). The apical pore located centrally in the pore plate (Po) which is topped by the cover plate (cp). The X plate is situated between the first apical and the pore plate which protrudes and extends to touch the cover plate.

The sulcal plates (Fig. 4A and B) were determined by SEM and we can distinguish 5 plates from the outer side of the theca. The anterior sulcal (Sa) plate is large and roughly the same height as the width of the cingulum, the right sulcal (Sm) and median sulcal (Sd) plates form an intricate cavity around the emerging point of the flagella with the left sulcal (Ss) plate extending from the cingulum where it touches the first cingular (C1) and Sa plates then follows the contour of the Sm and Sd on the left side and finally touches the Sulcal posterior (Sp) in the hypotheca. The cingulum is wide and composed of 6 cingular plates (Fig. 4A and C). The hypothecal plates can be seen from the antapical view in Fig. 4B. 6 post-cingular and 2 anterior plates with the spine positioned in the second antapical plate.

3.2. Azaspiracid composition and content in culture

The cultured Irish strain SM2 of *A. spinosum* produces the azaspiracid analogues AZA1 and -2. AZA1 is the major toxin component in the sample and AZA2 is found at lower concentrations. The cell quota from parallel cultures kept in the same conditions as the culture used for the feeding experiment ranged

**Fig. 4. Azadinium spinosum** (SM2 isolate). SEM micrographs of hypothecal plates from different cells showing details of hypotheca, cingulum and sulcus. (A) Hypotheca and cingulum in dorsal view; (B) detailed view of the sulcal region. Sa, anterior sulcal plate; Sm, median sulcal plate; Sd, right sulcal plate; Ss, left sulcal plate; Sp, posterior sulcal plate. (C) Dorsal view showing antapical spine. (D) Antapical view. Scale bars: 5 μm (Fig. 5A, C and D), 1 μm (Fig. 5B).
from ~15 to 25 fg/cell for AZA1 and ~1 to 5 fg/cell for AZA2 (data not included). This toxin profile correlates with that found by Krock et al. (2009) in the 3D9 isolate from the Scottish coast. Krock also reported a potential new analogue (AZAx) which was later found to be an extraction (with methanol) artefact, personal comms.

3.3. Molecular genetic analysis

We amplified and sequenced the D1–D2 regions of the nuclear ribosomal RNA gene from strain SM2. The sequenced region encompassed 436 base pairs, and was exactly identical to the
homologous sequences from two previously characterized A. spinosum strains (strains 3D9 and UTHE2), and differed in 10 and 8 substitutions from sequences available from A. poporum and A. obesum, respectively (Fig. 5).

3.4. Feeding experiment

3.4.1. Mussel feeding activity

All mussels started feeding after a few minutes of being introduced into the flasks containing the algae; this continued for the 24 h that the experiment lasted. Fig. 6 illustrates the decreasing concentration of algae in the different treatments over 24 h. The control line demonstrates that sedimentation of the algal suspension in the control treatment during the experiment was negligible. The data suggest that most of the algae have been consumed within 3 h of commencement of the experiment, low baseline algal cell concentrations below ~3000 cells ml\(^{-1}\) were found after 5 h in the 30,000 cells ml\(^{-1}\) treatment, ~850 cells ml\(^{-1}\) in the 20,000 cells ml\(^{-1}\) treatment and ~67 cells ml\(^{-1}\) in the 5000 cells ml\(^{-1}\) treatment and after 24 h the estimates were ~73 cells ml\(^{-1}\), ~50 cells ml\(^{-1}\) and ~4 cells ml\(^{-1}\), respectively.

During feeding, all individual mussels were observed to produce pseudo-faeces in all treatments. The amount of pseudo-faeces produced appeared to slow down after approximately 1 h, while some faeces were also produced after approximately 2 h. One mussel in one of the 30,000 cells ml\(^{-1}\) treatment replicates started spawning after 2 h copiously but continued filtering afterwards, which could suggest some level of stress however this appeared to be an isolated episode.

3.4.2. Shellfish azaspiracid toxin analysis

The mussels and the media used for this experiment were analysed by LC–MS/MS for AZAs to demonstrate that there were not any toxins initially in the seawater used to grow the algae or in the shellfish tissue prior to carrying out the feeding experiment. Both controls were below the limit of quantification (LOQ) for AZAs. Also, a control using mussels without algal suspension throughout the 24 h experiment was analysed using LC–MS/MS and found to be negative for AZAs (data not included).

After 24 h the mussels were harvested, dissected and analysed via LC–MS/MS. Fig. 7 shows the concentration of AZA toxins detected in the mussels’ hepatopancreas for each treatment. In all treatments, significant amounts of AZA1 and AZA2 were found in the mussels with considerably higher concentrations for the two highest cell concentrations of A. spinosum compared to the lower concentration. This demonstrates that mussels do ingest A. spinosum directly and accumulate AZA toxins in their digestive system with toxicity being related to the density of the algae in the water.

The amount of AZA1 in both the 30,000 cells ml\(^{-1}\) treatment and the 20,000 cells ml\(^{-1}\) treatment was already above the regulatory limit for AZA equivalent toxins suggesting that mussels can become intoxicated with AZAs at A. spinosum cell concentrations of 20,000 cells ml\(^{-1}\) over a 24 h period. The concentration of AZA1 and -2 toxins is higher in the HP tissue compared to the toxin concentration found in the remainder with negligible amounts between the limit of detection (0.01 µg g\(^{-1}\)) and the limit of quantification for the instrument (0.02 µg g\(^{-1}\)) with the highest amount detected ~0.015 µg g\(^{-1}\) of AZA1 in the 20,000 cells ml\(^{-1}\) treatment. The concentration of the toxin analogue AZA3 in the mussel HP was below the limit of quantification suggesting that the decarboxylation of AZA17 to AZA3 probably occurs over a longer period of time than 24 h.

![Fig 6. Cell densities of A. spinosum over 24 h in three treatments (30,000, 20,000, 5000) and control.](image)

![Fig 7. Azaspiracid concentrations (mg kg\(^{-1}\)) found in mussels hepatopancreas (n = 9 per treatment). T1 = 30,000 cells ml\(^{-1}\), T2 = 20,000 cells ml\(^{-1}\), T3 = 5000 cells ml\(^{-1}\) and control.](image)
Table 2
Azaspiracid toxins budget in the 30,000 treatment.

<table>
<thead>
<tr>
<th>Flasks</th>
<th>Initial toxin culture (ng)</th>
<th>Final toxin culture</th>
<th>Tissue after 24 h (ng)</th>
<th>Biodeposits (ng)</th>
<th>Total (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AZA1</td>
<td>AZA1</td>
<td>AZA1</td>
<td>AZA1</td>
<td>AZA17</td>
</tr>
<tr>
<td>Replicates</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30,000 F1</td>
<td>2291.67</td>
<td>708.33</td>
<td>414.18</td>
<td>371.10</td>
<td>32.45</td>
</tr>
<tr>
<td>30,000 F2</td>
<td>2258.33</td>
<td>808.33</td>
<td>136.61</td>
<td>370.84</td>
<td>25.32</td>
</tr>
<tr>
<td>30,000 F3</td>
<td>2500.00</td>
<td>625.00</td>
<td>292.07</td>
<td>480.13</td>
<td>32.76</td>
</tr>
<tr>
<td>Mean</td>
<td>2350.00</td>
<td>713.89</td>
<td>280.95</td>
<td>407.36</td>
<td>30.18</td>
</tr>
</tbody>
</table>

% toxins in mussel tissue 30.6
% toxins in suspended particulate matter 33.9
% toxins in biodeposits 16.5

3.4.3. Azaspiracid toxin analogue results

The amount of toxins found varied significantly between mussels, replicates and treatments; this is possibly due to normal physiological differences like size, weight, age and condition of the mussels. We found that AZA1 and -2 toxins were already bioconverting into their carboxybetaldehydes AZA17 and -19 within the first 24 h. This is evidence that toxin bioconversion takes place in the shellfish digestive tract quite rapidly. The ratio of AZA17 to AZA1 toxins was found in all treatments (n = 9) on average to be 1:1 in the mussel hepatopancreas.

High levels of AZA17 compared to AZA1 were also found in the remainder tissue supporting the study performed by O’Driscoll et al. (2010) (unpublished) which shows that the oxidation process of AZA toxins occurs primarily in the gills. The ratio of AZA17 to AZA1 toxins in the remainder tissues was approximately 5:1 in all treatments.

The screening of AZA17 in shellfish samples is not a monitoring requirement in the current legislation as AZA17 is converted naturally to AZA3 in the shellfish over time, and it is not thought to be a large component of the total AZA toxin content, however as this feeding experiment indicates, it is possible that if mussels have been contaminated recently with AZAs, the amount of AZA17 can equal that of AZA1 or even exceed that of AZA1 resulting potentially in an underestimation of the total amount of AZA toxins in the samples. As shellfish samples are analysed raw, AZA17 will not convert readily to AZA3 unless mussels are cooked or the method incorporates a heating step in the extraction process.

3.4.4. Toxic budget feeding experiment

The total toxin budget for the feeding experiment included the initial and final toxin content of the algal suspension, the toxin found in the mussel tissues (HP and remainder) and the toxin found in the biodeposits (faeces/pseudo-faeces). This budget does not include any extracellular dissolved toxin fraction. The budget measures the amount of the principal toxins AZA1 and -2, the oxidation analogues AZA17 and -19 and the decarboxylated analogues AZA3 and -6. The final toxin budget illustrated by the 30,000 cells ml⁻¹ treatment (Table 2) shows a high % recovery of toxins from the experiment accounting on average for ~87% of AZA1 and AZA2 of the initial toxin content in the algal suspension, ~36% of AZA1 and 34% of AZA2 were recovered in the mussel tissues and ~16% of AZA1 and ~21% of AZA2 were found in the biodeposits which indicates some level of toxin excretion, however, after 24 h ~35% of AZA1and ~32% of AZA2 toxins were still found in the final water samples which at this point should only contain baseline cell densities of A. spinosum suggesting that some toxins were possibly re-suspended from the biodeposits due to aeration, so it is possible that the amount of toxins in the biodeposits could be larger than the reported budgets. The remaining ~13% of AZA1 and AZA2 were not accounted for.

The toxin budget of the 20,000 cells ml⁻¹ and 5000 cells ml⁻¹ treatments (supplementary material) returned a recovery above 100%, ~113% AZA1 and ~120% AZA2 in the 20,000 cells ml⁻¹ budget and ~150% AZA1 and 175% AZA2 in the 5000 cells ml⁻¹ budget. The high recovery in the 5000 cells ml⁻¹ treatment could be explained by the lower toxin content and the uncertainty in the measurement of the AZA peaks at these concentrations. The recovery in the 20,000 cells ml⁻¹ while above the 100%, demonstrates overall that there must be very little loss of toxins into the dissolved phase in any case. This latter point is only hypothetical as we have not calculated the dissolved fraction for this experiment.

4. Conclusions

Based upon the morphological characteristics described and the Kofoidean tabulation of the recently isolated AZA toxin producer Irish strain SM2 we can conclude that the Irish isolate fits perfectly with the description of A. spinosum. The sequence of the variable D1–D2 regions of the nuclear LSU rDNA of the Irish strain SM2 is also identical to those available from the Scottish 3D9 and Danish UTHE2 strains, further supporting their conspecificity. This is the third strain of A. spinosum reported to produce AZAs from a different geographical location in the North Atlantic after the discovery of the 3D9 isolate from the Scottish coast and the UT62 isolate from the Danish coast (Tillmann et al., 2009, 2011). So far no other species of the genus Azadinium that have been tested to date have produced AZAs other than the type species A. spinosum. From a monitoring perspective this organism is very small and difficult to identify to species level in preserved water samples, yet using a good research compound microscope fitted with oil immersion lenses, it is possible to view several important morphological features that describes this species, in particular the antapical spine, which is typical of A. spinosum. While this feature provides reliable identification of the species together with other morphological characteristics typical of the genus, it will be ultimately the development of gene probes that will prove a useful tool in the future of monitoring, quantification and identification of the genus. Molecular methods however may yet take some time to become available for real time monitoring as this genus is still only recently
discovered and the continued discovery of new species of *Azadinium* and strains of *A. spinosum* and other species in this size range, means that gene probes will have to be exhaustedly tested for cross reactivity between the genus and within the different strains before they can be relied upon.

Here we have for the first time proof of a direct toxin transfer of AZA toxins from *A. spinosum* by feeding *M. edulis* without the need for vector species. Mussel will actively filter, ingest, accumulate and bioconvert azaspiracid toxins quite readily into other AZA analogues. AZA1 and -2 were found to be concentrated mainly in the HP tissue whereas AZA17 and -19 were distributed throughout the whole flesh. The results show that the ratio of AZA17 in the remainder tissue can be up to five or six times the amount of AZA1. AZA17 is known to convert readily to the decarboxylated analogue AZA3 upon cooking, however monitoring samples are analysed raw and the analogue AZA17 is not monitored as the present legislation only sets limits for the forms AZA1, AZA2 and AZA3. This means that the total AZA toxin content in shellfish samples could be underestimated. The toxin budget indicated that most of the AZA toxins detected in the plankton can be accounted for in the shellfish tissues, the biodeposits and the particulates in the water suggesting that the dissolved fraction of AZAs in the water should be quite small. These results illustrate the need for further experiments in the kinetics of the principal plankton AZA toxins in shellfish tissues.

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhal.2011.06.010.

**References**


