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 algae *A. spinosum* from Irish waters.

1

Highlights

• Confirmation of *Azadinium spinosum* from Ireland as the producer of azaspiracid toxins. • A 24 h feeding experiment confirms that mussels (*Mytilus edulis*) are able to ingest *A. spinosum* cells directly without the need of vector species. • AZA toxins can accumulate in mussels above the regulatory level within 24 h. • There is a rapid conversion of AZA-1 to AZA-17 and AZA-2 to AZA-19 in the mussels' tissue within 24 h. • The ratio of AZA-17 to AZA-1 is 5:1 in the mussels' remainder tissue.

Keywords: Azaspiracids ; AZP ; AZA toxins ; Biodeposits ; Dinoflagellates ; Feeding experiment ; Ireland ; LC–MS/MS ; Mussels ; Phylogeny ; Taxonomy

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 diarrhetic shellfish poisoning (DSP), however chemistry results showed that DSP toxins

51 were only found in the shellfish at low concentrations and phytoplankton samples did not

52 show the presence of DSP toxin producing algae in the water, yet the mousse bioassay was

53 strongly positive (McMahon & Silke, 1996). In 1998 this novel group of compounds were

54 isolated and chemically characterized from shellfish (Satake *et al.*, 1998, Ofuji *et al.*1999). A

55 few years later azaspiracids were confirmed in the UK and Norway (James *et al.*, 2002),

56 Morocco (Taleb *et al.*, 2006) and Portugal (Vale *et al.*, 2008) suggesting that azaspiracid

57 toxicity had a more extensive distribution along the west European Atlantic seaboard and as

58 far as north Africa . There is evidence now that the distribution of azaspiracids may be

59 worldwide with azaspiracids found in Japan (Ueoka *et al.*, 2009), Chile (Alvarez *et al.* 2010,

60 Lopez-Rivera *et al.* 2010) and Canada (Twiner *et al.*,2010) .

61 The development of routine chemical analysis as a monitoring tool showed azaspiracid

62 concentrations found in *M.edulis* in Ireland between 2003 and 2010 using LC-MS/MS (Fig.

63 1) indicates that the presence of azaspiracids has been a recurring problem since 2005

64 annually. However, AZA toxins were found at concentrations below the regulatory limit in

65 2003 and not detected in 2004 and the first half of 2005, suggesting that there could be a

66 cyclical element to AZA events. While the accumulation of AZA toxins has been reported in

67 a number of different shellfish species (Table 1), levels above the regulatory limit have only

68 been observed in blue mussels (*Mytilus edulis*) and pacific oysters (*Crasostrea gigas*) in

69 Ireland.

70 Due to the ability to accumulate high levels of AZA toxins, mussels have become an

71 important source of AZAs for toxin isolation (Furey *et al.,* 2003, Perez *et al.*, 2010). Since the

72 first characterization of AZA1 by Satake *et al.*, 1998 other structural variants have been

73 isolated and characterised: AZA2 and AZA3 (Ofuji *et al.*, 1999a: 2001) and AZA6-AZA11

74 (James *et* al., 2003a) with AZA12 theoretically postulated, the number of variants was

75   increased from AZA12-AZA 32 later by Rehmann *et al.*, 2008 with the discovery of new

76   dihydroxy and carboxy-AZAs. Most of these AZA variants have been reported to be shellfish

77   metabolites (Rehman *et al.*, 2008) rather than *de novo* products of plankton, apart from

78   AZA1, AZA2 and AZA3 (Furey *et al*, 2003). However, Krock *et al.*, 2009 only detected

79   AZA1 and AZA2 in the field samples from Scotland generating uncertainties over the

80   production of AZA3 by plankton. Fux *et al.*, 2009 did find low amounts of AZA3 using Solid

81   Phase Adsorption Toxin Tracker Device (SPATT), a passive sampler, but concluded that a

82   heat treatment step could have influenced the enzymatic activity of the SPATT prior to the

83   extraction allowing the metabolism of AZA1 to AZA3.

84   In 2008, the causative organism was discovered from the North-East coast of Scotland

85   (Krock *et al.*, 2009) and described as a genera and *species novo* (Tillman *et al.*, 2009). This

86   organism, a small armoured dinoflagellate named *A. spinosum* was shown to produce AZA

87   toxins in culture and effectively identified as *de novo* producer of azaspiracids. Later,

88   McCarron *et al*., 2009 reported that AZA17 and -19 are formed by oxidation of the 22-methyl

89   group of AZA1 and -2 respectively. In this study, heat induced decarboxylation of AZA17

90   and -19 from AZA3 and -6 was demonstrated showing the possible bioconversion pathways

91   of these toxins in shellfish.

92   The Marine Institute phytoplankton unit collected water samples between August and

93   September of 2009 following positive results in blue mussels of AZA toxins. The samples

94   yielded several isolates and cultures, one of which was shown to produce AZA1 and AZA2.

95   The Irish isolate was provisionally named SM2. Here, we report on the taxonomic identity of

96   the Irish toxin producer and the genotypic/phenotypic relationship with other *Azadinium*

97   species and its toxin profile. The link between *A.spinosum* toxicity and toxin accumulation in

98   shellfish had not yet been established. It was not known whether blue mussels were able to

99   directly ingest the small thecate dinoflagellate *A. spinosum* or if toxin accumulation in

100   mussels is mainly achieved via planktonic vector species which have potentially accumulated

101   AZA toxins by consuming the small armoured dinoflagellate. Thus, a feeding trial was

102   designed to demonstrate that blue mussels are able to feed and digest directly from an algal

103   suspension of *A.spinosum* , that AZA toxins accumulate in their digestive system and that

104   ultimately these are metabolised into other AZA analogues.

105

106   **2.   Materials and Methods**

107   *2.1 Sample collection, isolation and culture of an AZA producing dinoflagellate*

108   *2.1.1 Field collection using a submersible pump*

109   The culture of an Irish strain of *A. spinosum provisionally* designated as SM2 was established

110   from water samples collected in the South west coast of Ireland, at Gearhies pier, Bantry bay

111   (latitude: 51° 39' 4.7'' N, longitude: 9° 35' 11'' E) during September of 2009 coinciding with

112   an increase of AZA toxin in shellfish as reported by the Marine Institute biotoxin monitoring

113   programme. The water samples were collected using a pump below surface and at 3 m depth,

114   approximately100 L of seawater were pumped into a fraction sampler to separate and

115   concentrate the different size fractions (60 µm, 38 µm and 20 µm). The filtrate was also

116   collected into 25 L polyethylene drums for cell concentration down to 3 µm in size using a

117   vacuum pump at low pressure and 3 µm pore size TSTP Millipore ^{TM} membrane filters.

118   Approximately 5 L of 20 µm sample filtrate was concentrated down (3 µm) to approximately

119   50 ml volume before isolation.

120

121   *2.1.2 Isolation and culture conditions*

122   The dinoflagellates were isolated using single cell isolation by micropipette in 96 cell tissue

123   culture plates (Corning, New York, USA). The isolates were kept in F/2 without silica

124   (Guillard and Ryther 1962, Guillard 1975) made up with enriched sterile filtered seawater

125   from the site and kept at 18° C temperature, 12:12 light:dark cycle and the irradiance in the

126   incubator was 150 µmol photon $m^{-2}s^{-1}$ measured using an Iso-tech ILM350 light meter (ISO-

127   tech, Merseyside, UK). Potential AZA toxin producer isolates were discriminated from the 20

128   µm filtrate and 3 µm backwash concentrated fractions by screening for morphological

129   characteristics typical of *A. spinosum*. Four other isolates apart from SM2 suspected to be *A.*

130   *spinosum* were isolated and cultured successfully based on cell size and shape, presence of

131   thecal plates, cell movement, and presence of antapical spine, presence/absence of a

132   conspicuous pyrenoid and presence/absence of apical pore. After successful isolation, the

133   unialgal cultures were transferred to 25 x 150 mm borosilicate culture tubes (Fisherbrand™,

134   Loughborough, UK) containing 35 ml of F/2 media and kept in the incubator in the

135   conditions as outlined before.

136

137   All cultured isolates were tested for the production of AZA toxins using LC-MS/MS, 1ml

138   culture aliquots of a densely growing culture were collected using a 1ml pipette and placed

139   into 0.2µm Whatmann Anopore™ spin filter, the filters were centrifuged at 14000rpm for

140   one minute using a Heraeus Multifuge 3S-R (Heraeus, Hanau, Germany) and the filter

141   extracted using methanol.

142

143   *2.2 Microscopy*

144   *2.2.1 Light microscopy (LM)*

145   Observation of live cultured isolates was carried out using an inverted microscope (Axiovert

146   200M, Zeiss, Germany) equipped with epifluorescence and differential interference contrast

147   optics. Light microscopic examination of the thecal plate was performed as per Tillmann *et*

148   *al.* 2010

149

150

151 *2.2.2 Scanning electron microscopy (SEM)*

152 The material for examination was collected from the unialgal cultures and prepared following

153 the protocol for SEM by Tillmann *et al.* 2009 with slight variations: the cell pellet -after re-

154 suspension and removal of 40% seawater -was fixed with formalin (2% final concentration)

155 instead of glutaraldehyde in cacodylate buffer and stored in the fridge for 2 h before washing

156 and dehydration steps. The filters were mounted on stubs, sputter coated (Emscope SC 500,

157 Ashford, UK) with gold-palladium and viewed under a scanning electron microscope (FEI

158 Quanta FEG 200, Eindhoven, Netherlands).

159

160 *2.3 Chemical analysis of the azaspiracids producing culture*

161 2.3.1 *Solvents and reagents*

162 Acetonitrile and methanol were purchased as pestican grade solvents from Labscan (Dublin,

163 Ireland). Formic acid, ammonium formate, ammonium hydroxide and sodium hydroxide

164 were purchased from Sigma Aldrich (Steinheim, Germany). Hydrochloric acid was purchased

165 from VWR (England). Water was obtained from a reverse-osmosis purification system

166 (Barnstead, Dublin, Ireland). AZA certified reference materials (CRM) were obtained from

167 the NRC (Halifax, Canada).

168

169 2.3.2 *Toxin extraction*

170 The culture was extracted by solid phase extraction (SPE). An Oasis HLB, 3 cc cartridge was

171 initially conditioned with 5 ml of methanol, flushed with 10 ml of a 5% methanol solution (in

172 water) and then loaded with 10 ml of culture slowly (drop wise). The cartridge was flushed

173 again with 10 ml of a 5% methanol solution followed by toxin elution with 4 ml of methanol.

174   The methanol extract was blown down to dryness, reconstituted back up in 0.5 ml of

175   methanol and transferred into a HPLC vial for analysis.

176   2.3.3 *Analysis by LC-MS/MS*

177   Analysis of AZAs was performed on a Micromass triple stage quadrupole (TSQ) Ultima

178   coupled to a Waters 2695, equipped with a Z-spray ESI source. The TSQ was operated in

179   positive ionization mode through multiple reactions monitoring (MRM). The following

180   transitions were monitored: AZA1 *m/z* 842.5>654.4 and 842.5>672.4, AZA2 856.5>654.4

181   and 856.5>672.4, AZA3 828.5>640.4 and 828.5>658.4, AZA6 842.5>640.4 and

182   842.5>658.4, AZA17 872.5>640.4 and 872.5>658.4 and AZA19 886.5>640.4 and

183   886.5>658.4 in positive ionisation mode. The cone and collision voltages were set at 60 V

184   and 40 V respectively. Cone and desolvation gas flows were set at 100 and 800 L/h,

185   respectively, while the source and desolvation temperatures were set at 150 °C and 350 °C

186   respectively.

187

188   A binary mobile phase was used, phase A (100% aqueous) and phase B (95% aqueous

189   acetonitrile), both containing 2 mM ammonium formate and 50 mM formic acid. HPLC

190   separation was achieved using a Hypersil BDS C8 column; 50 x 2.1, 3 µm; guard column, 10

191   x 2.1 mm, 3 µm (Thermo Scientific, Runcorn, UK). The flow rate was set at 0.25 ml/min and

192   the injection volume was set at 5 µl. The column and sample temperatures were set at 25 °C

193   and 6 °C respectively. A gradient elution was employed, starting with 30% B, rising to 90%

194   B over 8 mins, held for 2.5 min, then decreased to 30% B in 0.5 min and held for 4 min to

195   equilibrate the system.

196

197   *2.4 Molecular phylogenetic analysis*

198   2.4.*1 Extraction of genomic DNA*

199   A 50 ml sample of exponentially growing culture was centrifuged (Eppendorf 5810R,

200   Hamburg, Germany) at 3,220 x g for 15 min at room temperature. The cell pellets were

201   frozen at -20 °C for 20 min before being subjected to total DNA extraction with the DNeasy

202   Kit (Mini) (Qiagen, Hilden, Germany) according to manufacturer's instructions. The purity

203   and quantity of the DNA was checked by UV-spectroscopy with a NanoDrop ND-1000

204   system (Peqlab, Erlangen, Germany) and the integrity of DNA fragments of a molecular

205   weight of about 20 kb was verified on a 1 % agarose gel.

206

207   *2.4.2 PCR amplification and sequencing*

208   The total extracted DNA from the isolate was subjected to polymerase chain reaction (PCR)

209   amplification of the 28S ribosomal DNA .The forward and reverse primers for amplification

210   of 28S rDNA (D1-D2 regions) were: Dir-F (5'- ACC CGC TGA ATT TAA GCA TA - 3')

211   and Dir-2CR (5'- CCT TGG TCC GTG TTT CAA GA - 3'), respectively. For the 50 µl PCR

212   reaction, HotMasterTaq^® (Eppendorf, Hamburg, Germany) buffer 1X , 0.1 mM of dNTPs,

213   0.1 mM of each forward and reverse primer and 1.25 units of Taq polymerase were added to

214   10 ng of the extracted genomic DNA. For 28S rDNA amplifications, the reactions were

215   subjected to the following thermocycling conditions: an initial denaturation at a temperature

216   of 95 °C for 7 min was followed by 35 cycles of denaturation at 94 °C for 45 seconds,

217   annealing temperature at 54 °C for 2 min and elongation temperature at 72 °C for 1.5 min. A

218   final extension step at 72 °C was carried out for 10 min. The completed reactions were kept

219   at 10 °C until the next step. The PCR amplicons were analysed on 1% agarose by

220   electrophoresis. Sequencing was conducted with a standard cycle sequencing chemistry ABI

221   3.1 (Applied Biosystems, Darmstadt, Germany) using the PCR primer sets. Cycle sequencing

222   products were analyzed on an ABI 3130 XL capillary sequencer (Applied Biosystems,

223   Darmstadt , Germany)

224

225 *2.4.3 Sequence alignment for phylogenetic analyses*

226 Sequence alignment was done with CLUSTAL X software (Thompson *et al*., 1997) and

227 improved manually for all sequences; ambiguous alignments positions were excluded from

228 the analysis. Alignments are available upon request and sequences are available at GenBank

229 under accession number for LSU: SM2 XXXXXXXX. Maximum likelihood phylogenetic

230 tree was calculated with PhyML (Guindon & Gascuel, 2003) using a BIO-NJ (neighbour-

231 joining) tree as a starting tree, and the general time reversible (GTR) nucleotide substitution

232 model (Whelan & Goldman, 2001) with a gamma distribution parameter estimated from the

233 data, bootstrap analysis was performed with 100 replicates. The dinoflagellate *Oxyrrhis*

234 *marina* (Dujardin) was selected as the outgroup.

235

236 *2.5 Blue Mussels feeding experiment*

237 *2.5.1 Experimental design*

238 A batch of approximately 10 kg of *M.edulis* were harvested from Carlingford Lough,

239 Greenore (Lat: 54.0339˚N, Long: -6.1417W) in the East coast of Ireland. The mussels were

240 cleaned of all fouling organisms and placed in 20L clear plastic carboys filled with sterile

241 filtered seawater (30 psu salinity) in the walk-in incubator at 18 ˚ Celsius. Any dead mussels

242 were removed from the carboys. The experiment consisted of a 24 h feeding experiment with

243 *M. edulis* and the azaspiracids producing dinoflagellate *A.spinosum* at three cell densities to

244 study feeding activity, toxin uptake and bioconversion of toxins in the shellfish digestive

245 system. The cultures of *A.spinosum* and the mussels were maintained under the same

246 environmental conditions for a week before the experiment was carried out.

247 Triplicate treatments were prepared in 5 L borosilicate conical flasks (Lennox, Dublin,

248 Ireland) at three cell densities; 30000cells ml^{-1}, 20000 cells ml^{-1} and 5000 cells ml^{-1} of *A.*

249   *spinosum*. The mussels used in this study were weighed, measured and labelled before the

250   start of the experiment; 100 g of mussels whole flesh was dissected from the batch of mussels

251   for LC-MS/MS analysis to test for AZAs in the mussels prior to the experiment. Also, a

252   sample of seawater (50ml) used for growing *A.spinosum* culture before inoculation of the

253   algae was extracted for AZA toxins using a SPE column as described above in 2.3.2. The

254   feeding experiment consisted of three mussels placed in each conical flask containing

255   *A.spinosum*. Two control samples were set up, one containing three mussels with no algal

256   suspension and one containing algal suspension with no mussels. The latter control was

257   prepared to account for potential decline of algae in the water column due to sedimentation. 3

258   X 10 ml suspension samples from each of the flasks were collected using an automated

259   pipette (Hirsmann Laborgerate, Heilbronn, Germany) to measure the initial cell density and

260   AZA toxin concentration. During the first two hours of the experiment, 1 ml aliquots of

261   seawater were collected from each flask every 20 min. using a 1 ml pipette (Eppendorf,

262   Cambridge, UK) and preserved with lugol's iodine (Clin-tech, Dublin, Ireland) 1% final

263   concentration to estimate cell concentrations of *A.spinosum* during the experiment. After

264   two hours samples were collected every hour and finally at 24 h. A final suspension sample

265   (10 ml) was collected at 24 h to analyse the final toxin content of remaining algal cells or

266   other particles in the water column.

267

268   Pseudo-faeces and faeces excreted by the mussels during the experiment were harvested

269   using 25ml serological pipettes (Sardstedt, Nümbrecht, Germany) and filtered after 24 h

270   using GF/C Whatman (1.2 μm, 47 mm diameter) glass microfiber filters under vacuum for

271   toxin analysis to determine the total toxin budget for the experiment. Before harvesting, the

272   water was removed from the flasks carefully avoiding the re-suspension of the faeces and

273   pseudo-faeces, while most of the water was removed, a small amount of water was left at the

274 bottom of the flasks and picked up in the pipette before filtering, which means that it is

275 possible that other material was present in the sample other than the faeces and pseudo-

276 faeces. The hypothesis here would be that since the algal suspension was depleted after 24 h,

277 no *A. spinosum* cells are believed to be present in the water at this point.

278

279 *2.5.2 Azadinium spinosum cell counts*

280 Cell counts of the 1 ml lugol's preserved aliquots were carried out using a Sedgewick-Rafter

281 cell counting chamber (Pyser-SGI, Kent, UK) for each flask at each time interval using an

282 inverted optical microscope Leica DMI 6000B (Leica, Wetzlar ,Germany).

283

284 2.5.3 *Dissection of mussels and toxin analysis of shellfish tissues*

285 After 24 h, the mussels were harvested from the flasks, weighed and dissected into

286 hepatopancreas (HP) and remainder tissues. The dissected HP and remainder were placed

287 into labeled 15 ml polypropylene centrifuge tubes (Sardstedt, Nümbrecht, Germany). A

288 volume of 1.5 ml methanol was added to each tube and the sample was homogenized using

289 an Ultra-Turrax (T25 Basic IKA®-Werke, Germany) at 11,000 rpm for 1 min. Samples were

290 then centrifuged in a Heraeus Multifuge 3S-R (Heraeus, Hanau, Germany) at 4,500 rpm for 5

291 mins. The supernatant was decanted into 5 ml volumetric flasks (Hirsmann-Techcolor,

292 Heilbronn , Germany) and this step repeated twice for each pellet. The volume was then

293 brought up to the mark using pestican grade methanol, inverted 5 times for each and filtered

294 through 0.22 µm filters(Sartorius, Surrey, UK) into HPLC vials (AGB, Dublin, Ireland) to be

295 run on the LC-MS/MS.

296

297 *2.5.4 Toxin analysis of A.spinosum samples*

298   The toxicity of *A.spinosum* cells was measured during the feeding experiment at two time

299   intervals. Triplicate 10 ml samples of *A. spinosum* were collected from each flask in 15ml

300   centrifuge tubes at initial time (T_0) and at the end of the experiment after 24 h. The samples

301   were centrifuged at 4,500 rpm for 15 min. The supernatant was decanted off and 500 µL of

302   methanol was added to each pellet. Samples were then vortexed in a V400 Multitube Vortex

303   mixer (Alpha Laboratories, Hampshire, UK) mixed for 1 min at maximum speed then

304   centrifuged again. The supernatant was collected into labeled HPLC vials. The process was

305   repeated twice to obtain a final volume of 1.5 ml for each HPLC vial. These samples were

306   blown down to dryness under nitrogen gas (BOC gases, Dublin, Ireland) and reconstituted

307   with 500 µL methanol to be analysed by LC-MS/MS.

308

309   2.5.5 *Toxin analysis of biodeposits*

310   After harvesting the mussels from the flasks, biodeposits (pseudo-faeces, faeces and any

311   detritus left in the flask and a small amount of water) were collected from the bottom of the

312   flasks and then filtrated on GF/C Whatman (1.2 µm, 47 mm diameter) glass microfiber

313   filters. Filters from each flask were placed into labeled 50 ml centrifuge tubes. A volume of

314   3 ml methanol was added to each tube and vortex mixed at maximum speed for 1 min.

315   Samples were then centrifuged at 3,500 rpm for 15 min at 4 °C. The supernatant was

316   transferred to 10 ml volumetric flasks. This was repeated twice and the volume was made up

317   to the mark using methanol. The flasks were inverted 5 times and filtered through 0.22 µm

318   filters into HPLC vials to be analysed via LC-MS/MS using the conditions described

319   previously.

320

321   **3. Results and discussion**

322   *3.1 Azadinium spinosum Irish strain*

323   The armoured dinoflagellate *A. spinosum* has been described by Tillmann *et al.* 2009 as a

324   new species and a new genus. Since then, two additional but non-toxic species have been

325   described, *A.obesum* (Tillmann *et al.* 2010) and *A.poporum* (Tillmann *et al.* 2011). The Irish

326   isolate provisionally named SM2, clearly is a new geographical strain of *A.spinosum* as it is

327   sharing all morphological details compiled by Tillmann *et al.* (2009, 2011) to describe

328   *A.spinosum* and to differentiate this species from the two other species.

329

330   Figure 2A depicts a light microscopy image of the Irish strain of *A.spinosum* showing the

331   main morphological characteristics typical of the species, a conspicuous Apical Pore

332   Complex (APC), a conspicuous pyrenoid (P) in the episome which can be used to

333   differentiate *A. spinosum* from *A.obesum*, the large spherical nucleus (N) posteriorly located

334   and the antapical spine (S).This antapical spine situated in the second antapical plate is an

335   important feature for the identification of the species as it is present in *A. spinosum* but

336   lacking in the two other described species, *A obesum* and *A. poporum* (Tillmann et al. 2010,

337   2011). For the Irish strain SM2, the spine was visible using X100 oil immersion objectives

338   under the light microscope in formalin preserved water samples, although it was difficult

339   enough to discern.

340

341   Anyhow, the best way to visualise the spine and distinguish the delicate thecal plates in this

342   dinoflagellate is to use Scanning Electron Microscopy (SEM) which allowed us to identify

343   the plate tabulation to be Po, cp, X, 4', 3a, 6'', 6C, 5?S, 6''', 2'''' which is the same as

344   reported in Tillmann *et al.* 2009. Figure 2B & C show complete cells of *A.spinosum*. In

345   ventral view (Fig.2B) the ventral pore (vp) is clearly visible in the left suture of the first

346   apical plate, the thecal plates cleaned of the outer membrane appear smooth and some

347   scattered pores can be seen on the plates. Figure 3B shows the three intercalary plates on the

348   episome of the cell; the second intercalary plate (2a) is smaller than the other two plates and

349   is located above the third pre-cingular plate.

350

351   Figure 3C shows the complete epithecal plates of *A. spinosum*, the detail view of the APC

352   (Fig 3D) shows an identical configuration of the pore described for *A. spinosum* and

353   *A.obesum (*Tillmann *et al.* 2010). The apical pore located centrally in the pore plate (Po)

354   which is topped by the cover plate (cp). The X plate is situated between the first apical and

355   the pore plate which protrudes and extends to touch the cover plate.

356

357   The sulcal plates (Fig. 4A & B) were determined by SEM and we can distinguish 5 plates

358   from the outer side of the theca. The anterior sulcal (Sa) plate is large and roughly the same

359   height as the width of the cingulum, the right sulcal (Sd) and median sulcal (Sm) plates form

360   an intricate cavity around the emerging point of the flagella with the left sulcal (Ss) plate

361   extending from the cingulum where it touches the first cingular (C1) and Sa plates then

362   follows the contour of the Sm and Sd on the left side and finally touches the Sulcal posterior

363   (Sp) in the hypotheca. The cingulum is wide and composed of 6 cingular plates (Fig. 4A &

364   C).The hypothecal plates can be seen from the antapical view in figure 4D, 6 post-cingular

365   and 2 antapical plates with the spine positioned in the second antapical plate.

366

367   *3.2 Azaspiracid composition and content in culture*

368   The cultured Irish strain SM2 of *A.spinosum* produces the azaspiracid analogues AZA1 and -

369   2. AZA1 is the major toxin component in the sample and AZA2 is found at lower

370   concentrations. The cell quota from parallel cultures kept in the same conditions as the

371   culture used for the feeding experiment ranged from ~15 to 25fg /cell for AZA1 and ~1 to

372   5fg/cell for AZA2 (data not included). This toxin profile correlates with that found by Krock

373 *et al.,* 2009 in the 3D9 isolate from the Scottish coast. Krock, also reported a potential new

374 analogue (AZAx) which was later found to be an extraction (with methanol) artefact,

375 personal comms.

376 *3.3 Molecular genetic analysis*

377 We amplified and sequenced the D1-D2 regions of the nuclear ribosomal RNA gene from

378 strain SM2. The sequenced region encompassed 436 base pairs, and was exactly identical to

379 the homologous sequences from two previously characterized *A. spinosum* strains (strains

380 3D9 and UTHE2), and differed in 10 and 8 substitutions from sequences available from *A.*

381 *poporum* and *A. obesum*, respectively (Figure 5).

382

383 3.4 *Feeding experiment*

384 *3.4.1 Mussels feeding activity*

385 All mussels started feeding after a few minutes of being introduced into the flasks containing

386 the algae; this continued for the 24 h that the experiment lasted. Figure 6 illustrates the

387 decreasing concentration of algae in the different treatments over 24 h. The control line

388 demonstrates that sedimentation of the algal suspension in the control treatment during the

389 experiment was negligible. The data suggest that most of the algae have been consumed

390 within three hours of commencement of the experiment, low baseline algal cell

391 concentrations below ~3000 cells ml^{-1} were found after 5 h in the 30000 cells ml^{-1} treatment,

392 ~850 cells ml^{-1} in the 20000 cells ml^{-1} treatment and ~67 cells ml^{-1} in the 5000 cells ml^{-1}

393 treatment and after 24 h the estimates were ~73 cells ml^{-1}, ~50 cells ml^{-1} and ~4 cells ml^{-1}

394 respectively.

395

396 During feeding, all individual mussels were observed to produce pseudo-faeces in all

397 treatments. The amount of pseudo-faeces produced appeared to slow down after

398   approximately one hour, while some faeces were also produced after approximately two

399   hours. One mussel in one of the 30000 cells ml^{-1} treatment replicates started spawning after

400   two hours copiously but continued filtering afterwards, which could suggest some level of

401   stress however this appeared to be an isolated episode.

402   *3.4.2 Shellfish azaspiracid toxin analysis*

403   The mussels and the media used for this experiment were analysed by LC-MS/MS for AZAs

404   to demonstrate that there weren't any toxins initially in the seawater used to grow the algae or

405   in the shellfish tissue prior to carrying out the feeding experiment. Both controls were below

406   the limit of quantification (LOQ) for AZAs. Also, a control using mussels without algal

407   suspension throughout the 24 h experiment was analysed using LC-MS/MS and found to be

408   negative for AZAs (data not included).

409

410   After 24 h the mussels were harvested, dissected and analysed via LC-MS/MS. Figure 7

411   shows the concentration of AZA toxins detected in the mussels hepatopancreas for each

412   treatment. In all treatments, significant amounts of AZA1and AZA2 were found in the

413   mussels with considerably higher concentrations for the two highest cell concentrations of

414   *A.spinosum* compared to the lower concentration. This demonstrates that mussels do ingest

415   *A.spinosum* directly and accumulate AZA toxins in their digestive system with toxicity being

416   related to the density of the algae in the water.

417

418   The amount of AZA1 in both the 30000 cells ml^{-1} treatment and the 20000 cells ml^{-1}

419   treatment was already above the regulatory limit for AZA equivalent toxins suggesting that

420   mussels can become intoxicated with AZAs at *A.spinosum* cell concentrations of 20000 cells

421   ml^{-1} over a 24 h period. The concentration of AZA1 and -2 toxins is higher in the HP tissue

422   compared to the toxin concentration found in the remainder with negligible amounts between

423 the limit of detection (0.01 $\mu g\ g^{-1}$) and the limit of quantification for the instrument (0.02 μg

424 g^{-1}) with the highest amount detected ~0.015 $\mu g\ g^{-1}$ of AZA1 in the 20000 cells ml^{-1}

425 treatment. The concentration of the toxin analogue AZA3 in the mussel HP was below the

426 limit of quantification suggesting that the decarboxylation of AZA17 to AZA3 probably

427 occurs over a longer period of time than 24 h.

428

429

430 3.4. 3 *Azaspiracid toxin analogues results*

431 The amount of toxins found varied significantly between mussels, replicates and treatments;

432 this is possibly due to normal physiological differences like size, weight, age and condition of

433 the mussels. We found that AZA1 and -2 toxins were already bioconverting into their

434 carboxylated analogues AZA17 and -19 within the first 24 h .This is evidence that toxin

435 bioconversion takes place in the shellfish digestive tract quite rapidly. The ratio of AZA17 to

436 AZA1 toxins were found in all treatments (n=9) on average to be 1:1 in the mussel

437 hepatopancreas.

438

439 High levels of AZA17 compared to AZA1were also found in the remainder tissue supporting

440 the study performed by O'Driscoll *et al.* 2010 (unpublished) which shows that the oxidation

441 process of AZA toxins occurs primarily in the gills. The ratio of AZA17 to AZA1 toxins in

442 the remainder tissues was approximately 5:1 in all treatments.

443

444 The screening of AZA17 in shellfish samples is not a monitoring requirement in the current

445 legislation as AZA17 is converted naturally to AZA3 in the shellfish over time, and it is not

446 thought to be a large component of the total AZA toxin content, however as this feeding

447 experiment indicates, it is possible that if mussels have been contaminated recently with

448   AZAs, the amount of AZA17can equal that of AZA1 or even exceed that of AZA1 resulting

449   potentially in an underestimation of the total amount of AZA toxins in the samples. As

450   shellfish samples are analysed raw, AZA17 won't convert readily to AZA3 unless mussels

451   are cooked or the method incorporates a heating step in the extraction process.

452

453   *3.4.4 Toxin budget feeding experiment*

454   The total toxin budget for the feeding experiment included the initial and final toxin content

455   of the algal suspension, the toxin found in the mussel tissues (HP and remainder) and the

456   toxin found in the biodeposits (faeces/pseudo-faeces). This budget doesn't include any

457   extracellular dissolved toxin fraction. The budget measures the amount of the principal toxins

458   AZA1 and -2, the oxidation analogues AZA17 and -19 and the decarboxylated analogues

459   AZA3 and-6. The final toxin budget illustrated by the 30000 cells ml^{-1} treatment (Table 2)

460   shows a high % recovery of toxins from the experiment accounting on average for ~87% of

461   AZA1 and AZA2 of the initial toxin content in the algal suspension, ~ 36% of AZA1 and

462   34% of AZA2 were recovered in the mussel tissues and ~16% of AZA1 and ~21 % of AZA2

463   were found in the biodeposits which indicates some level of toxin excretion , however, after

464   24 h ~35% of AZA1and ~32% of AZA2 toxins were still found in the final water samples

465   which at this point should only contain baseline cell densities of *A.*spinosum suggesting that

466   some toxins were possibly re-suspended from the biodeposits due to aeration, so it is possible

467   that the amount of toxins in the biodeposits could be larger than the reported budgets. The

468   remaining ~13% of AZA1and AZA2 were not accounted for.

469

470   The toxin budget of the 20000 cells ml^{-1} and 5000 cells ml^{-1} treatments (supplementary

471   material) returned a recovery above 100%, ~113% AZA1 and ~120% AZA2 in the 20000

472   cells ml^{-1} budget and ~150% AZA1 and 175% AZA2 in the 5000 cells ml^{-1} budget. The high

473   recovery in the 5000 cells ml^{-1} treatment could be explained by the lower toxin content and

474   the uncertainty in the measurement of the AZA peaks at these concentrations. The recovery

475   in the 20000 cells ml^{-1} while above the 100%, demonstrates overall that there must be very

476   little loss of toxins into the dissolved phase in any case. This latter point is only hypothetical

477   as we have not calculated the dissolved fraction for this experiment.

478

479   **Conclusions**

480   Based upon the morphological characteristics described and the Kofoidean tabulation of the

481   recently isolated AZA toxin producer Irish strain SM2 we can conclude that the Irish isolate

482   fits perfectly with the description of *A. spinosum*. The sequence of the variable D1-D2

483   regions of the nuclear LSU rDNA of the Irish strain SM2 is also identical to those available

484   from the Scottish 3D9 and Danish UTHE2 strains, further supporting their conspecificity.

485   This is the third strain of *A.spinosum* reported to produce AZAs from a different geographical

486   location in the North Atlantic after the discovery of the 3D9 isolate from the Scottish coast

487   and the UTH E2 isolate from the Danish coast (Tillmann *et al*., 2009, 2011). So far no other

488   species of the genus *Azadinium* that have been tested to date have produced AZAs other than

489   the type species *A.spinosum*. From a monitoring perspective this organism is very small and

490   difficult to identify to species level in preserved water samples, yet using a good research

491   compound microscope fitted with oil immersion lenses, it is possible to view several

492   important morphological features that describes this species, in particular the antapical spine,

493   which is typical of *A.spinosum* .While this feature provides reliable identification of the

494   species together with other morphological characteristics typical of the genus, it will be

495   ultimately the development of gene probes that will prove a useful tool in the future of

496   monitoring, quantification and identification of the genus. Molecular methods however may

497   yet take some time to become available for real time monitoring as this genus is still only

498   recently discovered and the continued discovery of new species of *Azadinium* and strains of

499   *A.spinosum* and other species in this size range, means that gene probes will have to be

500   exhaustedly tested for cross reactivity between the genus and within the different strains

501   before they can be relied upon.

502

503   Here we have for the first time proof of a direct toxin transfer of AZA toxins from

504   *A.spinosum* by feeding *M. edulis* without the need for vector species. Mussels will actively

505   filter, ingest, accumulate and bioconvert azaspiracid toxins quite readily into other AZA

506   analogues. AZA1 and -2 were found to be concentrated mainly in the HP tissue whereas

507   AZA17 and -19 were distributed throughout the whole flesh. The results show that the ratio

508   of AZA17 in the remainder tissue can be up to five or six times the amount of AZA1. AZA17

509   is known to convert readily to the decarboxylated analogue AZA3 upon cooking, however

510   monitoring samples are analysed raw and the analogue AZA17 is not monitored as the

511   present legislation only sets limits for the forms AZA1, AZA2 and AZA3. This means that

512   the total AZA toxin content in shellfish samples could be underestimated. The toxin budget

513   indicated that most of the AZA toxins detected in the plankton can be accounted for in the

514   shellfish tissues, the biodeposits and the particulates in the water suggesting that the dissolved

515   fraction of AZAs in the water should be quite small. These results illustrate the need for

516   further experiments in the kinetics of the principal plankton AZA toxins in shellfish tissues.

517

529

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Tables

Table 1. Maximum concentration of AZA equivalents (mg kg_1) found in shellfish species cultured in Ireland between 2003 and 2010 analysed using LC–MS/MS. LOQ, limit of quantification.

Shellfish species		Concentration
Common name	Scientific name	AZA-equiv. $(mg\,kg^{-1})$
Common Cockle	*Cerastoderma edule*	0.08
Pacific oyster	*Crassostrea gigas*	0.31
Razor clam	*Ensis arcuatus*	0.05
Razor clam	*Ensis siliqua*	<0.01
Dog cockle	*Glycymeris glycymeris*	0.01
Abalone	*Haliotis discus hannai*	<LOQ
Blue mussel	*Mytilus edulis*	8.97
Native oyster	*Ostrea edulis*	0.07
Common limpet	*Patella vulgata*	<LOQ
Surf Clam	*Spisula solida*	0.15
Manila Clam	*Tapes philippinarum*	0.10
Clam	*Tapes semidescussatus*	0.01
Pullet carpet shell	*Venerupis senegalensis*	<LOQ
Venus Clam	*Venus verrucosa*	<LOQ

Table 2. Azaspiracid toxins budget in the 30,000 treatment.

Flasks	Initial toxin culture (ng)	Final toxin culture		Tissue after 24 h (ng)			Biodeposits (ng)			Total (ng)	%
Replicates	AZA1	AZA1	AZA17	AZA1	AZA17	AZA3	AZA1	AZA17	AZA3	Total recovered	
30,000 F1	2291.67	708.33	5.61	414.18	371.10	32.45	454.00	6.32	3.36	1995.34	87.1
30,000 F2	2258.33	808.33	172.23	136.61	370.84	25.32	285.00	9.17	1.83	1809.34	80.1
30,000 F3	2500.00	625.00	68.44	292.07	480.13	32.76	391.00	7.91	2.26	1899.57	76.0
Mean	2350.00	713.89	82.09	280.95	407.36	30.18	376.67	7.80	2.48	1901.41	81.1
% toxins in mussel tissue											30.6
% toxins in suspended particulate matter											33.9
% toxins in biodeposits											16.5
Replicates	AZA2	AZA2	AZA6	AZA2	AZA19	AZA6	AZA2	AZA19	AZA6	Total recovered	
30,000 F1	312.62	121.17	0.00	86.20	29.95	0.00	58.56	0.85	0.00	296.74	94.9
30,000 F2	263.84	103.14	0.00	35.06	29.31	0.00	50.95	1.61	0.00	220.07	83.4
30,000 F3	348.87	71.39	0.00	69.32	43.88	0.00	79.10	1.58	0.00	265.28	76.0
Mean	308.44	96.57	0.00	63.53	34.38	0.00	62.87	1.35	0.00	260.69	84.8
% toxins in mussel tissue											31.7
% toxins in suspended particulate matter											32.0
% toxins in biodeposits											20.8

Figures

Figure 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 compare to the rest of the results and it would have caused the graph to collapse at the lower concentrations. The following data points are not shown (7.37 mg kg^{-1} AZA-equiv. (22/08/05) and 8.97 mg kg^{-1} AZA-equiv. (01/10/2005)).
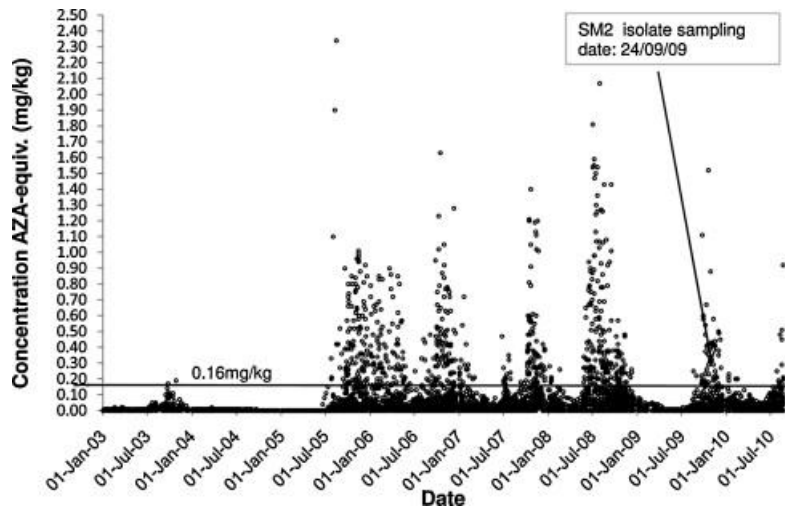

Figure 2. *Azadinium spinosum* (SM2 isolate). Light microscopy view (A) of live cell at 100× objective, SEM (scanning electron microscopy) micrographs of thecae (B) ventral view; (C) dorsal view. Scale bars: 5 µm. APC, apical pore complex; P, pyrenoid; N, nucleus; S, spine.
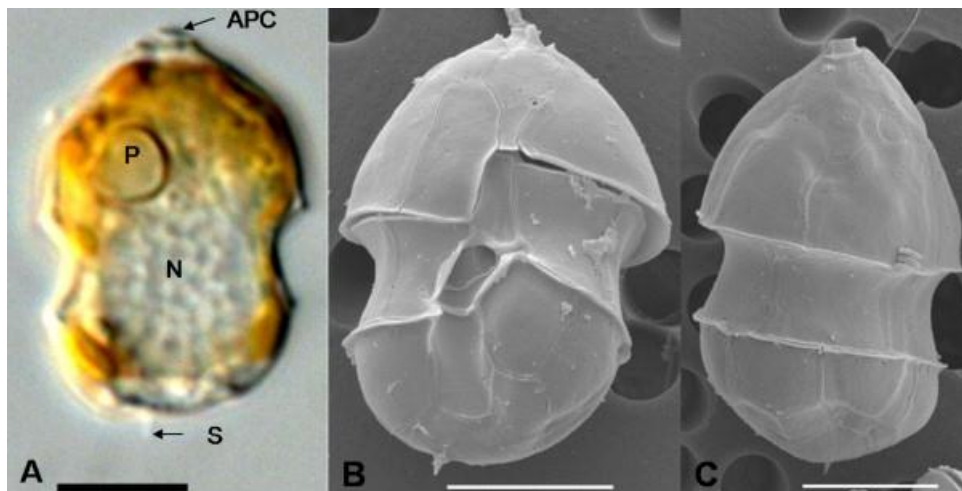
Figure 3. *Azadinium spinosum* (SM2 isolate). SEM micrographs of epithecal plates from different cells in (A) ventral view; (B) mid-dorsal view; (C) full epitheca from an apical view; (D) apical pore complex detail (APC). Scale bars: 2 µm (Fig. 4A and C), 5 µm (Fig. 4B) and 0.25 µm (Fig. 4D).
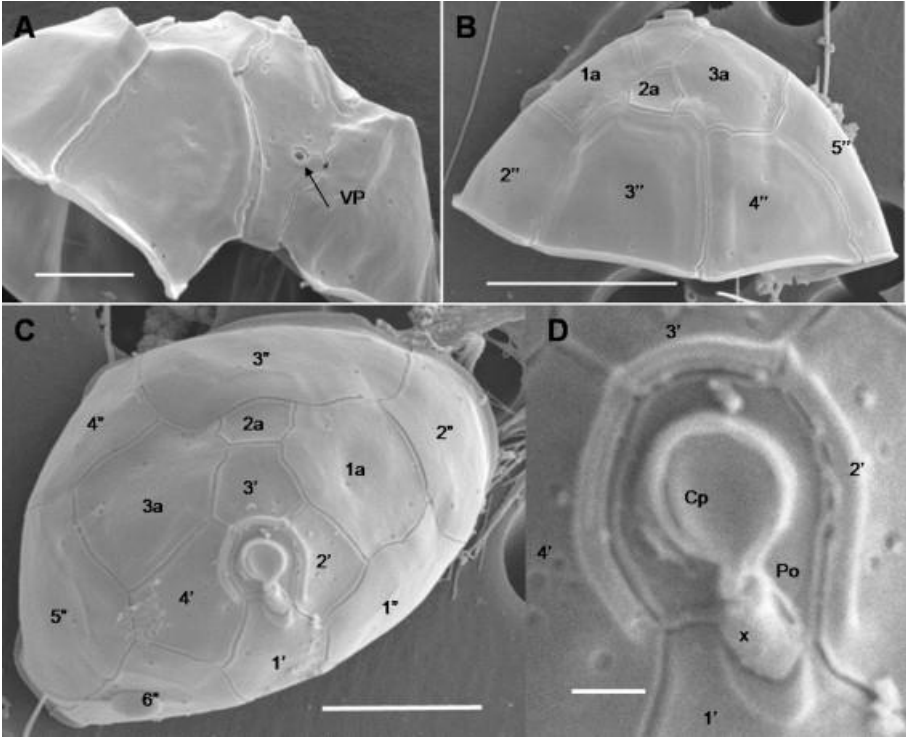
Figure 4. *Azadinium spinosum* (SM2 isolate). SEM micrographs of hypothecal plates from different cells showing details of hypotheca, cingulum and sulcus. (A) Hypotheca and cingulum in dorsal view; (B) detailed view of the sulcal region. Sa, anterior sulcal plate; Sm, median sulcal plate; Sd, right sulcal 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).
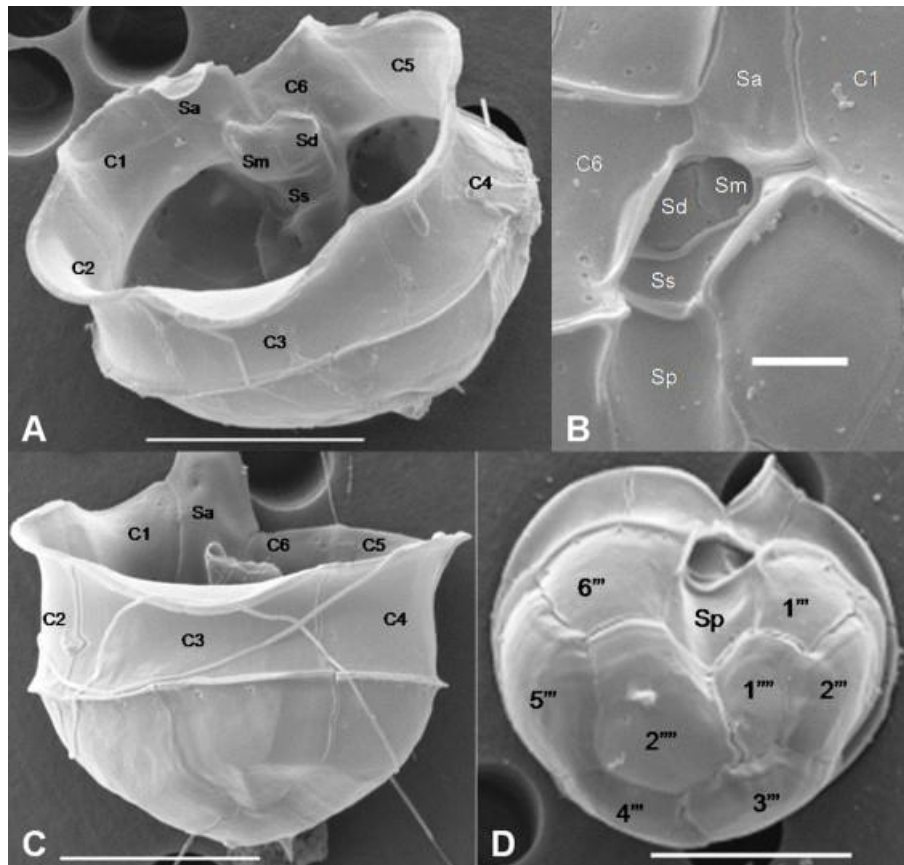
Figure 5. Maximum likelihood (ML) phylogenetic tree of the dinoflagellates inferred from the D1–D2 region of the 28S/large subunit (LSU) from the rDNA operon. The branch of the dinoflagellate *Oxyrrhis marina*, which was used as outgroup, is not shown proportionally. Bootstrap analysis was done with 100 replicates.
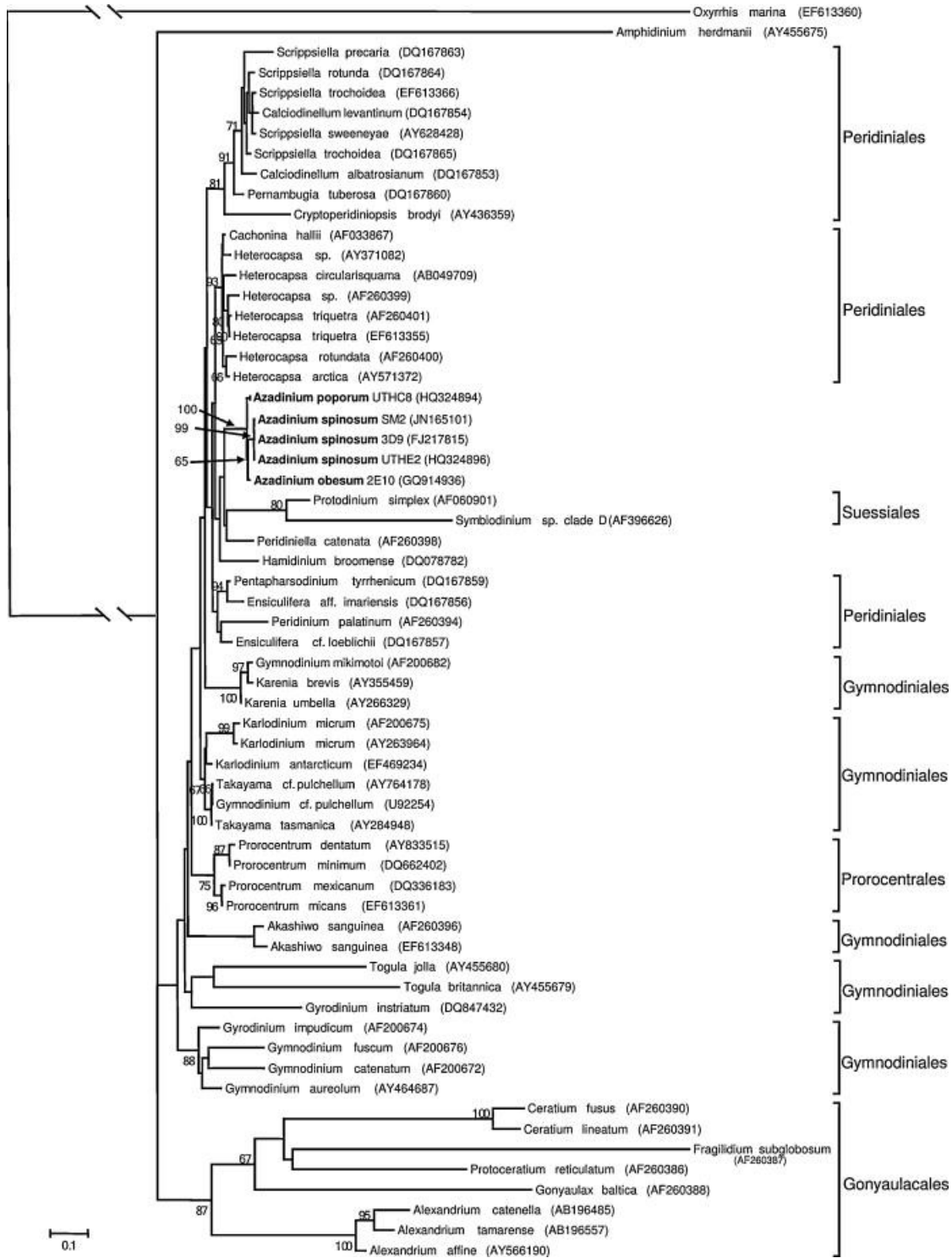
Figure 6. Cell densities of *A. spinosum* over 24 h in three treatments (30,000, 20,000, 5000) and control.
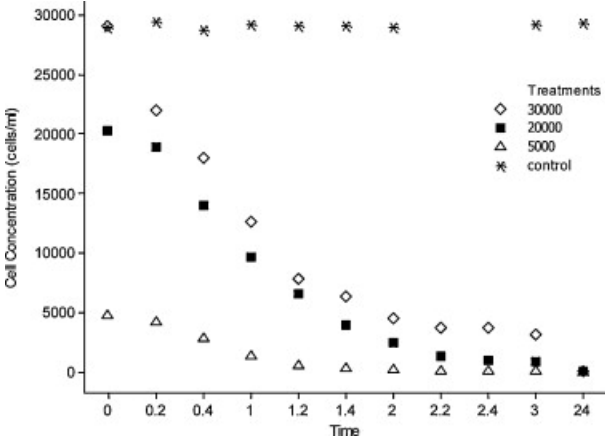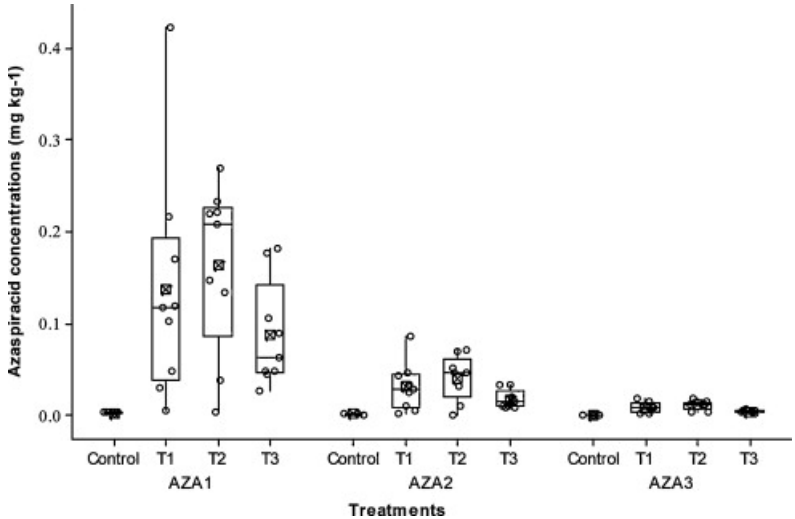

Figure 7. Azaspiracid concentrations (mg kg^{-1}) found in mussels hepatopancreas ($n = 9$ per treatment). T1 = 30,000 cells ml^{-1}, T2 = 20,000 cells ml^{-1}, T3 = 5000 cells ml^{-1} and control.

Appendix A. Supplementary data

Table S1. Azaspiracid toxins budget in the 20,000 treatment.

| Flasks	Initial toxin culture (ng)	Final toxin culture (ng)		Tissue after 24 hrs (ng)			Biodeposits (ng)			Total (ng)	%
Replicates	AZA1	AZA1	AZA17	AZA1	AZA17	AZA3	AZA1	AZA17	AZA3	Total recovered	
20000F1	1116.67	250.00	0.00	309.02	375.30	28.31	188.00	0.00	1.75	1152.38	103.2
20000F2	1066.67	325.00	0.00	316.49	354.13	28.42	176.00	0.00	1.63	1201.67	112.7
20000F3	1116.67	358.33	0.00	222.33	306.88	20.55	167.00	0.00	1.34	1076.44	96.4
Mean	1100.00	311.11	0.00	282.61	345.44	25.76	177.00	0.00	1.57	1143.49	104.1
									% toxins in mussel tissue		59.4
								% toxins in suspended particulate matter			28.3
									% toxins in biodeposits		16.2
Replicates	AZA2	AZA2	AZA6	AZA2	AZA19	AZA6	AZA2	AZA19	AZA6	Total recovered	
20000F1	158.08	29.75	0.00	76.55	37.27	0.00	31.06	0.00	0.00	174.63	110.5
20000F2	139.46	38.96	0.00	70.21	31.40	0.00	28.59	0.00	0.00	169.16	121.3
20000F3	129.01	37.25	0.00	56.95	21.94	0.00	33.46	0.00	0.00	149.59	116.0
Mean	142.18	35.32	0.00	67.90	30.20	0.00	31.04	0.00	0.00	164.46	115.9
									% toxins in mussel tissue		69.0
								% toxins in suspended particulate matter			24.8
									% toxins in biodeposits		21.8

Table S2. Azaspiracid toxins budget in the 5000 treatment.

| Flasks	Initial toxin culture (ng)	Final toxin culture (ng)		Tissue after 24 hrs (ng)			Biodeposits (ng)			Total (ng)	%
Replicates	AZA1	AZA1	AZA17	AZA1	AZA17	AZA3	AZA1	AZA17	AZA3	Total recovered	
5000F1	225.00	41.67	0.00	146.10	135.26	7.02	24.00	0.00	0.00	354.04	157.4
5000F2	216.67	33.33	0.00	97.05	109.07	6.88	24.00	0.00	0.00	270.33	124.8
5000F3	241.67	33.33	0.00	114.75	106.27	6.61	30.00	0.00	0.00	290.96	120.4
Mean	227.78	36.11	0.00	119.30	116.87	6.84	26.00	0.00	0.00	305.11	134.2
									% toxins in mussel tissue		106.7
								% toxins in suspended particulate matter			15.9
									% toxins in biodeposits		11.4
Replicates	AZA2	AZA2	AZA6	AZA2	AZA19	AZA6	AZA2	AZA19	AZA6	Total recovered	
5000F1	25.21	9.77	0.00	28.37	12.52	0.00	5.60	0.00	0.00	56.25	223.1
5000F2	33.99	0.00	0.00	20.70	10.74	0.00	5.61	0.00	0.00	37.05	109.0
5000F3	25.20	0.00	0.00	23.42	11.90	0.00	6.56	0.00	0.00	41.88	166.2
Mean	28.13	3.26	0.00	24.16	11.72	0.00	5.92	0.00	0.00	45.06	166.1
									% toxins in mussel tissue		127.6
								% toxins in suspended particulate matter			11.6
									% toxins in biodeposits		21.0

Supplementary Figure S1. LC–MS/MS chromatogram (multi reaction monitoring (MRM) mode) of *Azadinium spinosum* SM2 isolate showing ion traces for AZA1, -2 and -3.
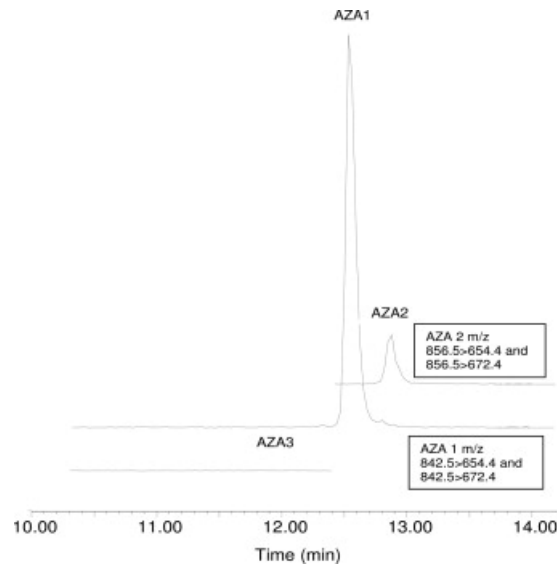

Supplementary Figure S2. : Azaspiracid concentration (mg kg^{-1}) found in the mussel remainder tissue ($n = 9$ per treatment). T1 = 30,000 cells ml^{-1}, T2 = 20,000 cells ml^{-1}, T3 = 5000 cells ml^{-1} and control. Please note that most AZA concentrations found in the reminder tissue were below the formal limit of quantification for the instrument.
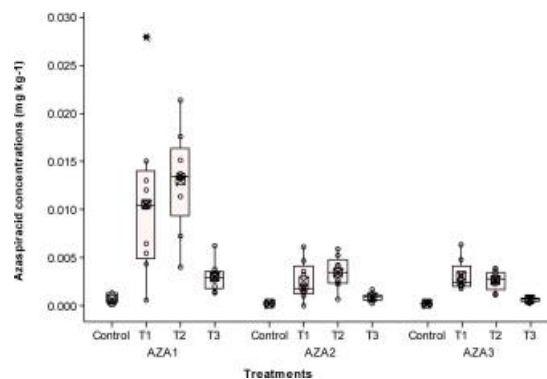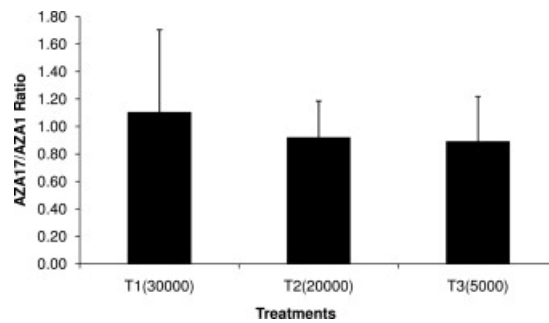

Supplementary Figure S3. AZA analogues found in mussel HP in treatment 30,000 cells ml^{-1} after 24 h.
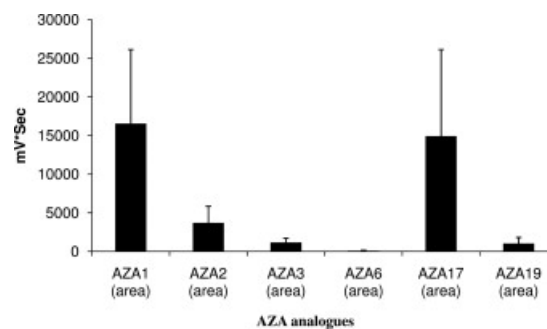
Supplementary Figure S4. AZA analogues found in mussel remainder tissues in treatment 30,000 cells ml^{-1} after 24 h (n = 9).
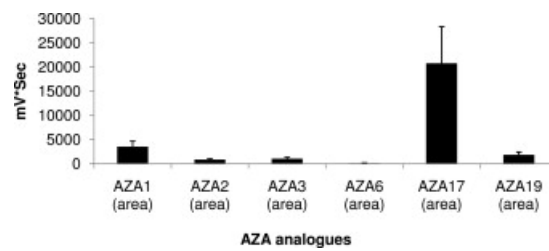
Supplementary Figure S5. Ratio of AZA 17/AZA 1 per treatment in mussel hepatopancreas after 24 h.

Supplementary Figure S6. Ratio of AZA 17/AZA 1 per treatment in mussel remainder tissues after 24 h.