ELSEVIER

Toxicon 45 (2005) 881–890

TOXICON

www.elsevier.com/locate/toxicon

Teratogenic effects of azaspiracid-1 identified by microinjection of Japanese medaka (*Oryzias latipes*) embryos

Jamie R. Colman[a], Michael J. Twiner[a], Philipp Hess[b], Terry McMahon[b], Masayuki Satake[c], Takeshi Yasumoto[d], Gregory J. Doucette[a], John S. Ramsdell[a,*]

[a]*Marine Biotoxins Program, Center for Coastal Environmental Health and Biomolecular Research, NOAA/National Ocean Service, 219 Fort Johnson Road, Charleston, SC 29412, USA*

[b]*Biotoxin Chemistry, Marine Environment and Food Safety Services, Marine Institute, Galway Technology Park, Parkmore West, Galway, Ireland*

[c]*Japan Food Research Laboratory, Tama Laboratory, Nagayama, Tama, Tokyo, Japan*

[d]*Graduate School of Agricultural Science, Tohoku University, Tsutsumi-dori, Amamiya, Aoba-ku, Sendai, Japan*

Received 24 November 2004; accepted 1 February 2005
Available online 2 April 2005

Abstract

Azaspiracid-1 (AZA-1) is a newly identified phycotoxin that accumulates in commercially important bivalve molluscs harvested in several European countries and causes severe human intoxications. Molluscan shellfish are known vectors for accumulation and subsequent transfer of phycotoxins such as brevetoxin and domoic acid through various trophic levels within food webs. Finfish can also accumulate phycotoxins, both directly from toxic algae or from consumption of contaminated shellfish and smaller intoxicated fish. To evaluate the teratogenic potential of AZA-1 and its relevancy to toxin accumulation in finfish, we have utilized a microinjection technique to mimic the maternal-egg toxin transfer of an AZA-1 reference standard and a shellfish extract containing azaspiracids in an embryonic Japanese medaka (*Oryzias latipes*) fish model. Microinjection of purified AZA-1 caused dose-dependent effects on heart rate, developmental rate, hatching success, and viability in medaka embryos. Within 4 days of exposure to doses $\geq$40 pg AZA-1/egg, substantial retardation in development was observed as reduced somatic growth and yolk absorption, and delayed onset of blood circulation and pigmentation. Embryos treated to $\geq$40 pg AZA-1/egg had slower heart rates (bradycardia) for the 9 days in ovo period, followed by reduced hatching success. Microinjection of a contaminated mussel (*Mytilus edulis*) extract containing AZAs (AZA-1, -2, and -3), okadaic acid, and dinophysistoxin-2 resulted in similar responses from the fish embryos at equivalent doses. These studies demonstrate that AZA-1 is a potent teratogen to finfish. This work will complement future investigations on AZA-1 accumulation in marine food webs and provide a basis for understanding its toxicity at different trophic levels.

Keywords: Phycotoxin; Azaspiracid; Medaka; Development

1. Introduction

In 1995, a novel marine phycotoxin, azaspiracid (AZA), was identified in the Netherlands following cases of shellfish intoxication from mussels cultivated in Killary Harbour, Ireland (McMahon and Silke, 1998). Azaspiracid was later associated with additional shellfish intoxications along the West coast of Europe and has been shown to accumulate in the muscular and visceral tissues of bivalve molluscs (Magdalena et al., 2003; James et al., 2002). Following consumption by humans, AZA induced gastrointestinal symptoms such as nausea, vomiting, and diarrhea. Following intraperitoneal

* Corresponding author. Tel.: +1 843 762 8510; fax: +1 843 762 8700.
E-mail address: john.ramsdell@noaa.gov (J.S. Ramsdell).

0041-0101/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.toxicon.2005.02.014

(i.p.) injection, mice showed additional neurological symptoms including respiratory difficulty, spasms, and limb paralysis, as well as pathological effects (Ito et al., 2000, 2002).

While little is known about this new phycotoxin, it is thought to be produced by the marine dinoflagellate *Protoperidinium crassipes* (James et al., 2003), though direct evidence is lacking. Accumulation of AZAs in molluscan shellfish suggests the possibility of food web transfer, as is the case with several other marine phycotoxins including brevetoxins, ciguatoxins, domoic acid that have been documented in both shellfish and finfish tissues (Bargu and Silver, 2003; Lehane and Lewis, 2000). As is the case with these toxins, AZAs can remain in tissues for extended periods of time, forcing shellfish closures for as long as 8 months (James et al., 2000; McMahon and Silke, 1998).

Lipid soluble toxins such as the brevetoxins and ciguatoxins are capable of accumulating and amplifying through the food web in concentrations dependent upon factors such as the rate of dietary uptake, absorption efficiency, and depuration (Tester et al., 2000). Typically, older, often larger predators within a given environment carry the highest toxin body burdens. While toxin storage in lipid-rich, visceral tissues such as the liver and gonads may have little or no effect on the adult, mobilization of fat stores during reproduction may provide a route of exposure from the parent to the offspring. This is particularly true in oviparous fishes, which use gonadal (fat) stores to generate the lipid-rich yolk that serves as the primary food source for developing embryos (Niimi, 1983; Smith and Cole, 1973). Lipophilic contaminants, such as DDT and PCBs have been demonstrated to accumulate in fish eggs at concentrations that are proportional to the contaminant body burden levels of the adult (Miller, 1993). Tropical ciguatoxins, which adversely affect finfish development by inducing severe spinal curvatures and death (Colman et al., 2004; Edmunds et al., 1999a), have been shown to undergo biomagnification in food webs yielding the highest toxin loads in top level reef predators. Ciguatoxins have been reported to partition into fish eggs at levels comparable to those found in the liver (Colman et al., 2004; Lehane and Lewis, 2000), even though such toxins are not readily bioavailable to the adult while stored in the gonad. However, as they are transferred to the yolk during oogenesis, the toxins become part of the direct food source to the embryo. The sensitive processes involved in embryogenesis can be disrupted, leading to downstream developmental abnormalities and ultimately to the death of the exposed fish. The maternal transfer of toxins during oogenesis could lead to overall reductions in the number of healthy offspring able to pass into subsequent age classes.

While AZAs have not been identified in fish tissues, *Protoperidinium* spp. are a known prey species for a variety of copepods and ciliates (Jeong, 1999; Jeong et al., 2002), which constitute a fundamental trophic level in marine food webs. It can therefore be inferred that planktivores such as fish may accumulate AZAs in their tissues. In this study, we characterized the effects of the most common azaspiracid congener, AZA-1, as well as extracts from mussels (*Mytilus edulis*) naturally contaminated with AZA-1, -2, -3, okadaic acid (OA), and dinophysistoxin-2 (DTX-2) on embryonic finfish development using microinjection to mimic maternal toxin transfer.

2. Materials and methods

2.1. Medaka

Breeding sets (6 females:4 males) of wild type Medaka (*Oryzias latipes*) were obtained from Carolina Biological Supply (Burlington, NC). Fish were housed in 8-l aquaria in a balanced salt solution (17 mM NaCl, 0.4 mM KCl, 0.2 mM $CaCl_2$, 0.3 mM $MgSO_4$, 0.24 mM $NaHCO_3$) under a 16:8 h light:dark cycle. Water temperature was maintained at 25–28 °C during the light phase and declined approximately 3 °C during the dark phase. These conditions were optimal for day-length and temperature induced reproduction of breeders. Medaka were fed twice daily with Wardley's Spirulina Plus flake food or live *Artemia*. Eggs were collected from the female fish each morning and inspected for fertilization. Healthy fertilized eggs were selected for microinjection.

2.2. Sample preparation

Azaspiracid (AZA-1) was extracted from 2 kg mussels (*Mytilus edulis*) that were collected in 1996 from Killary Harbour, on the west coast of Ireland, and Bantry Bay in 1999 on the southwest coast of Ireland. Toxins were extracted in 2001, as described by Satake et al. (1998) and Ofuji et al. (1999) with slight modifications. Stock AZA-1 (2.4 mg) was determined to be $>93\%$ pure by NMR and showed $<1\%$ impurity of other AZA subtypes/congeners by liquid chromatography–mass spectrometry (LC–MS). Subsequent LC–MS analysis comparing this lot of AZA-1 to a previously prepared lot found no statistically significant differences.

Crude extracts were prepared from both non-toxic and AZA-contaminated mussels (*Mytilus edulis*) originating from Bantry Bay, Ireland in August 2001. Extracts were prepared by tissue homogenization with water, extraction with 2 mL of 80% methanol per gram of homogenate, vortexing and sonication, followed by centrifugation (1000*g* for 10 min at 4 °C), and 0.22 μm filtration of the supernatant. To prepare the samples for injection, extracts were dried down under nitrogen gas and resuspended in acetone. Using extraction methods employed by the Irish Marine Institute, the contaminated mussel extract was previously shown by LC–MS to contain 0.17 μg/g AZA-1, 0.03 μg/g AZA-2, 0.04 μg/g AZA-3 (0.24 μg/g AZA_{total}) in addition to 0.19 μg/g okadaic acid (OA) and 1.15 μg/g dinophysistoxin-2 (DTX-2).

2.3. Egg collection and microinjection

Six to 8 h post fertilization (gastrula), healthy eggs were embedded in 2% agarose dissolved in 12.5% Hank's solution for stabilization during microinjection. After injection, each egg was removed from the agarose, rinsed for approximately 1 min in 12.5% Hanks solution, then transferred to one well in a sterile 24-well plate (Corning Life Sciences, Acton, MA) containing Yamamoto's solution (133 mM NaCl, 2.7 mM KCl, 2.1 mM $CaCl_2$, 0.2 mM $NaHCO_3$, pH 7.3). Egg plates were maintained at 25 °C under the same light regimen as the adult fish. For the injections, aluminosilicate micropipettes (O.D. 1 mm; Sutter Instrument Co., Novato, CA) were pulled using a P-87 Sutter micropipette puller and bevelled with a BV-10 Sutter micropipette beveller. Each pipette was coated in Sigmacote (Sigma, St Louis, MO) to increase durability and reduce clogging. Micropipettes were set in a three-dimensional manipulator (Narashige Group, MO-150, Long Island, NY) and front loaded with triolein oil (Sigma) vehicle by means of a nitrogen gas pico-injector (Harvard Apparatus, PL1-100, Holliston, MA). All injections were carried out and visualized with the aid of a stereomicroscope (Leica MZ 12) with an ocular micrometer, as was development of the fish embryos. Digital images were captured using a RGB autoimagecam (MicroImage Video Systems Co. A209, Boyertown, PA) mounted onto the microscope. Images were enhanced using Flashpoint 128 video frame grabbing software (Integral Technologies Inc., Indianapolis, IN).

AZA-1 in methanol, or contaminated mussel (*Mytilus edulis*) extracts in acetone were added to triolein oil, which served as a vehicle for toxin delivery. The toxins and oil were mixed by sonication and the excess solvent was removed by evaporation under a nitrogen gas stream. The stock concentration of the AZA-1 in oil was 40 ng/μL, while the stock concentration of the mussel extract was an estimated 24 ng/μL AZA_{total}. Following injection, the diameter and resultant volume of each droplet was measured using an ocular micrometer. Given the concentration of the toxin in oil, the amount injected into each individual egg could be accurately determined. A minimum of 8 eggs were injected for each dose of the AZA-1 studies and doses were grouped by range. Up to 4 eggs were injected with mussel extract for each dose range. Ten eggs were also injected with toxin-free oil or non-toxic extract as controls. Previous research has indicated that injection of the vehicle oil at volumes equivalent to those used to administer the toxins does not adversely affect development (Colman and Ramsdell, 2003). Finally, eggs were reared without injection as a control for the vehicle-injected eggs.

2.4. Fish development monitoring and data analysis

Injected embryos were allowed to grow for 13 days before being removed from the study. The optimal times for observing developmental effects in embryos were previously determined to occur following the initiation of a heart beat (approximately post-fertilization day 3) and prior to hatching (approximately post-fertilization day 9) (Colman and Ramsdell, 2003). The developing fish were monitored for physical abnormalities, bradycardia, tachycardia, circulation, hyperkinetic twitches, and hatching success. Heart rate (beats/min) and any other observable parameters were measured and recorded for each embryo on days 3, 5, and 7 following injection. On day 13, the final status of each fish was recorded as hatched, not hatched, or dead. Larval survivability was not examined due to complications in rearing the Medaka fry. One way analysis of variance, followed by a Dunnett's multiple comparisons test (JMP™ statistical software, SAS Institute Inc., Cary, NC or GraphPad Prism™) was used to compare the responses of embryos in each dose range to the responses of the embryos exposed only to the oil vehicle, or non-toxic extract, and the non-injected embryos. Heart rate data was compared within each observational day for each AZA treatment to the oil-vehicle controls on days 3, 5, 7 and 9.

3. Results

3.1. Effect of AZA-1 on developing cardiac function and circulation in medaka embryos

Microinjections exceeding 20 pg AZA-1/egg elicited several observable adverse effects in the developing Japanese medaka embryos. In healthy embryos, a heart beat is visible by the end of day 3 post-fertilization (PF). Prior to the normal hatching time (9–10 days PF) embryos dosed with 1–40 pg AZA-1/egg were not different from control embryos ($p=0.06$), however, microinjections of $\geq$40 pg AZA-1/egg showed significantly ($p<0.01$) lower heart rates (bradycardia) on days 3, 5, 7 and 9 as compared with control embryos (Fig. 1). As doses increased, heart rates were depressed wherein embryos exposed to the highest doses never obtained heart rates greater than 75 beats/min during the first 9 days. This AZA-1-induced bradycardic effect was observed during the normal in ovo time frame; however, greater than normal heart rates occurred at the time of hatching. This spike resulted in heart rates comparable to those seen in the control embryos (i.e. normal) for the treatment groups $>$80 pg AZA-1/egg. Concurrent with the cardiac effects induced by AZA-1, circulatory abnormalities, expressed as a percentage of eggs in each treatment group ($n=8$–15), also occurred in a dose-dependent fashion (Table 1). With increasing doses of AZA-1, the onset of circulation and blood pigmentation, which are normally evident 4 and 5 days post-fertilization, respectively, were delayed. Most individuals injected with the highest doses (80–120 pg/egg) failed to exhibit circulating blood and none contained pigmented blood. In addition, in some fish exposed to AZA-1, a beating heart had developed;

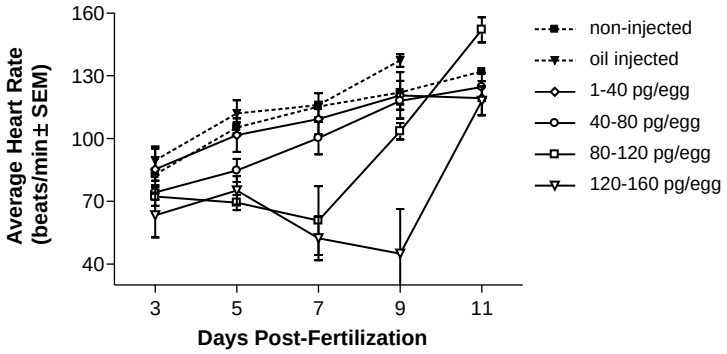

Fig. 1. Average heart rate (beats/min ± SEM) measured in medaka embryos on days 3, 5, 7, 9, and 11 in six different treatment groups ($n = 7$–15). Embryos were microinjected with a range of doses of AZA-1 (1–40, 40–80, 80–120, 120–160 pg) dissolved in triolein oil. Non-injected and triolein oil controls are also shown. Microinjections occurred on day 0, 6–8 h following fertilization.

however, during the latter half of the in ovo period, there was an apparent recession of the circulatory system wherein fish maintained a beating heart but the normally visible arterial network over the periphery of the yolk was no longer evident (Table 1).

3.2. Effect of AZA-1 on morphology of the developing embryos

Microinjection of AZA-1 into medaka eggs resulted in acute developmental retardation wherein the embryos failed to develop at the same rate as the controls. Beginning approximately 4 days post-fertilization, embryos exposed to ≥40 pg AZA-1/egg demonstrated dose-dependent retardation of all observable morphological development and, in turn, were smaller, contained less pigmentation, and absorbed substantially less yolk mass than control embryos of the same age (Fig. 2A–D). These AZA-1-induced abnormalities were also evident throughout embryogenesis, as demonstrated in eggs exposed to a single dose of AZA-1 (89 pg/egg) monitored over the entire in ovo duration (Fig. 3A–F). The embryos were smaller, contained less pigmentation and utilized less yolk mass relative to the controls. Yolk absorption in the treated eggs did not occur in a uniform manner, but rather appeared to be taken up primarily around the head of the fish, resulting in a flattened surface beneath the head and a semicircular shaped yolk (Fig. 4).

3.3. Effect of AZA-1 on hatching success of medaka fish

Similar to the cardiovascular and developmental effects, hatching success was significantly ($p < 0.05$) reduced in fish exposed to ≥80 pg AZA-1 (Fig. 5). Increasing doses resulted in reduced hatching success in a dose-dependent manner. While some embryos survived the duration of our observational period (up to 13 days), they failed to hatch from the egg. Doses of 1–40 pg AZA-1/egg ($n = 15$) and 40–80 pg AZA-1/egg ($n = 10$) prohibited successful hatching of nearly 50% of the embryos. Less than 10% of the eggs exposed to 80–120 pg AZA-1 ($n = 12$) hatched successfully, and none of the embryos exposed to >120 pg AZA-1 ($n = 7$) hatched. In the non-injected controls, 9 of 11 embryos (82%) hatched successfully. Although the remaining two did not die, they did not hatch during the observational time course. Failing to hatch within the normal embryonic incubation time does happen naturally and, as a result, hatch rates for control animals are not

Table 1
Number of medaka embryos (as a percentage) expressing circulating blood and pigmented blood at days 3, 5, and 7 post-fertilization following microinjection of triolein oil (Oil) or AZA-1

	Day	NI ($n = 11$)	Oil ($n = 10$)	AZA-1 dose (pg/egg)			
				1–40 ($n = 15$)	40–80 ($n = 8$)	80–120 ($n = 11$)	120–160 ($n = 11$)
Circulation	3	100	100	100	69	13	0
	5	100	100	100	100	56	14
	7	100	100	100	100	86	3
Pigmentation	3	46	40	33	0	0	0
	5	100	100	100	75	0	0
	7	100	100	100	38	10	0

Non-injected (NI) controls are also shown. Note: in some treatment groups the embryos died. Percentage data are calculated for the live embryos only.

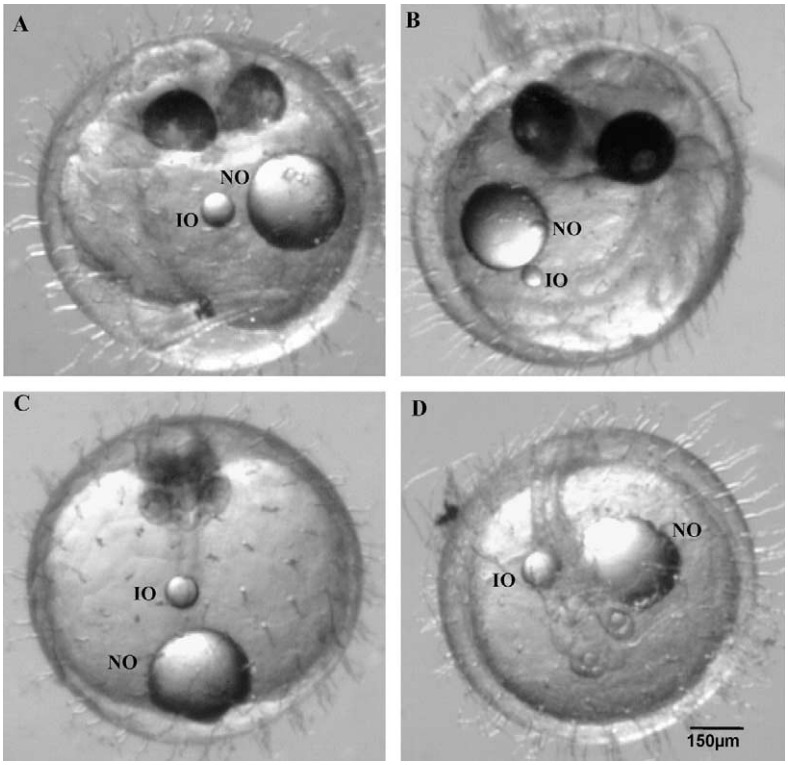

Fig. 2. Photomicrographs (80× magnification) of 5-day old medaka embryos injected with (A) triolein oil vehicle; 89 pg oil equivalents, (B) 26 pg AZA-1, (C) 89 pg AZA-1, and (D) 144 pg AZA-1. Microinjections occurred on day 0, 6–8 h following fertilization. Natural oil (NO) and injected oil (IO) droplets are marked on the figures.

always 100%, as was observed in our vehicle-treated embryos.

3.4. Effects of AZA extracts from contaminated mussels on developing medaka embryos

Crude extracts from the tissues of naturally contaminated mussels (*Mytilus edulis*) were also tested to confirm the activity of multiple AZAs (AZA-1, -2, and -3) in a naturally incurred matrix. While exposure to multiple doses of the non-toxic control extract at was comparable in dose to the toxic extract, the non-toxic control did not affect embryonic growth or survival. However, the toxic extract elicited similar responses in the fish embryos at the same doses as the purified AZA-1 (Fig. 6A–D). Development was retarded in fish injected with doses exceeding 40 pg AZA_{total}/egg. Heart rates were depressed, the onset of circulation and pigmentation, expressed as a percentage within each treatment group, were delayed (Table 2), and yolk absorption was irregular (Fig. 6C). Additionally, hyperactivity, which was not observed in the purified AZA-1 treated embryos, was observed in embryos treated with the mussel extract during the first 3–5 days of development, possibly as a result of exposure to the other AZA subtypes (AZA-2 or -3), the DTX-2, or OA. Nevertheless, this hyperactive response dissipated quickly and did not occur in a dose-dependent manner.

4. Discussion

Lipophilic marine phycotoxins such as brevetoxins and ciguatoxins have been shown to undergo trophic transfer and accumulate in the tissues of marine invertebrates and in fishes (Lewis and Jones, 1997; Tester et al., 2000; Colman et al., 2004). Toxins that accumulate in ovarian tissues may then be mobilized during oogenesis and become incorporated into the yolk material that nourishes developing fish embryos. While the toxins may not be particularly deleterious to the parents while compartmentalized within the gonadal tissues, their bioavailability changes when transferred to the yolk sack that serves as the primary embryonic food source.

Microinjection studies have been used to mimic the maternal transfer of both marine phycotoxins (e.g. brevetoxins, ciguatoxins, microcystins) and anthropogenic contaminants (e.g. DDT, DDE), and to determine the effects of these toxins on developmental processes (Kimm-Brinson and Ramsdell, 2001; Colman and Ramsdell, 2003; Colman et al., 2004; Jacquet et al., 2004; Edmunds et al., 1999b;

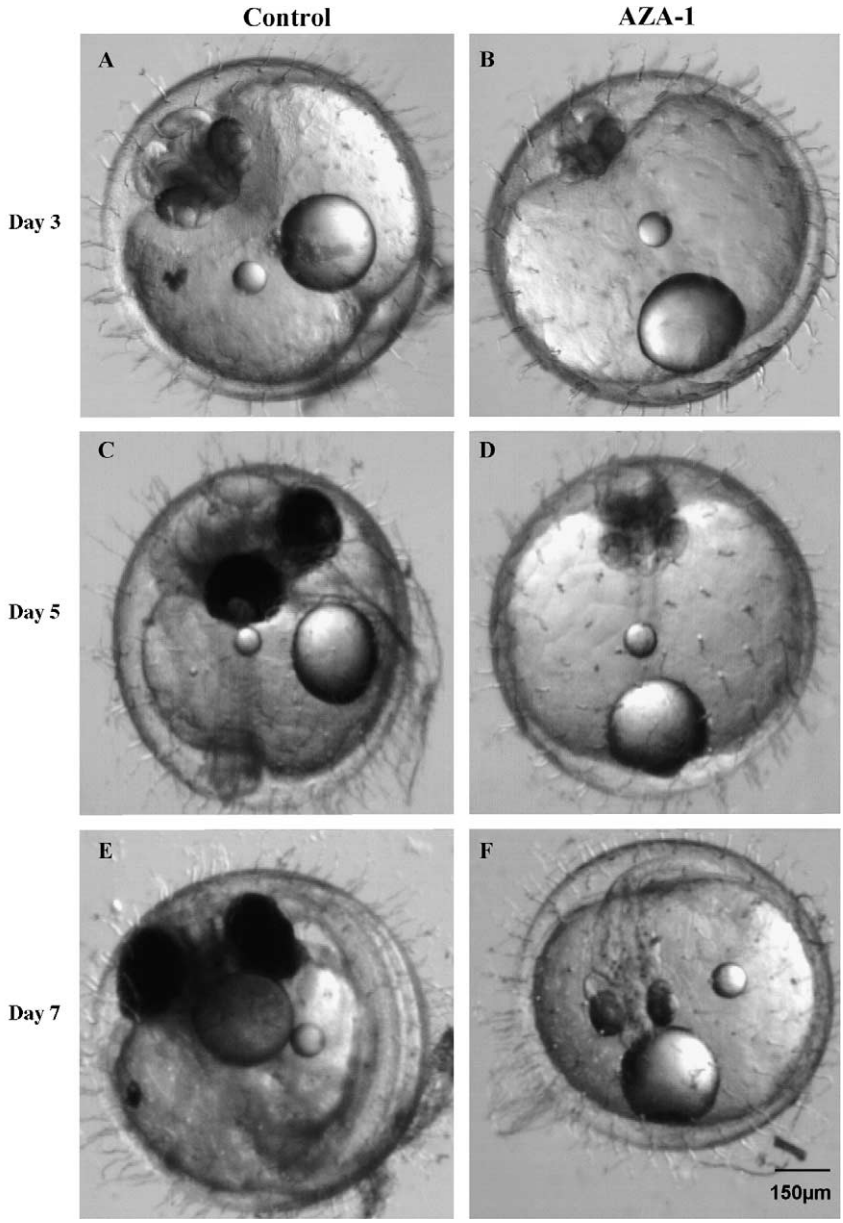

Fig. 3. Photomicrographs (80× magnification) of developing medaka embryos injected with AZA-1. Embryos were injected with 89 pg equivalents of triolein oil (A, C, E) or 89 pg AZA-1 (B, D, F) and photomicrographs were taken on day 3 (A, B), day 5 (C, D), and day 7 (E,F). Microinjections occurred on day 0, 6–8 h following fertilization. In all frames the smaller droplet is the injected oil and the larger droplet is the natural oil.

Papoulias et al., 2003). These studies have enabled the determination of the effects of maternally transferred toxins on embryonic development with speculative impacts on the fitness of natural populations.

Using microinjection, the current study has shown that in a dose-dependent manner AZA-1 and mussel extracts containing multiple AZA congeners adversely affect the health and development of finfish embryos. As a result, there was a considerable reduction in the number of embryos that survive development which, in nature, may impact the abundance and distribution of future populations. Our results demonstrate that observable effects of AZA-1 on medaka embryos occur following microinjection of $\geq$40 pg/egg and become morphologically and physiologically apparent approximately 4 days following fertilization. Developmental impediments were visible in the form of

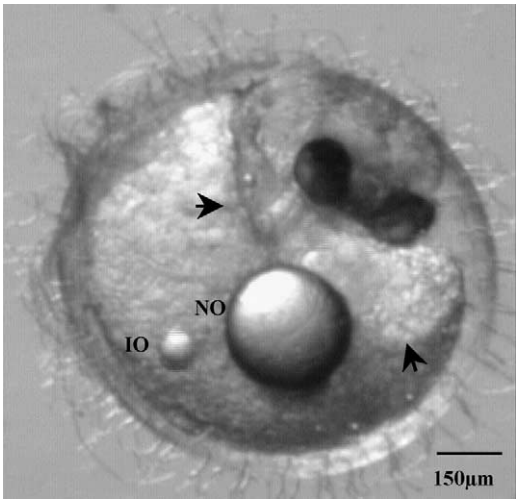

Fig. 4. Photomicrograph (80× magnification) of a 7-day old medaka embryo injected with 63 pg AZA-1. Microinjection occurred on day 0, 6–8 h following fertilization. The arrows emphasize the heterogeneous absorption of the yolk sac. The natural oil (NO) and injected oil (IO) droplets are marked on the figure.

depressed developmental rate, reduction in heart rate, and delayed onset of circulation and blood pigmentation. Hatching success and ultimately survival were negatively impacted by AZA-1, where fish that failed to develop in the same time frame as the controls either failed to hatch or died before the hatching stage.

Previous microinjection studies modeling maternally transferred marine phycotoxins have revealed a myriad of cardiac effects that were dependent upon the modes of action of the toxins (Kimm-Brinson and Ramsdell, 2001; Colman et al., 2004; Colman and Ramsdell, 2003; Edmunds et al., 1999a). While AZA-1 induced what appeared to be a bradycardic response in the developing medaka, embryonic development was delayed. This observation suggests that heart rates in these animals may not have been necessarily bradycardic, but rather representative of the physiological rates for control embryos at the same developmental stage. This cardiac effect was more severe in embryos exposed to higher doses of the toxin. Although heart rates of fish exposed to the higher doses of AZA-1 ($\geq$80 pg/egg) were significantly depressed during the first 7 days of development, they spiked during the normal hatching period (days 9–11) to normal physiological levels for that age. While these fish ultimately failed to exit the chorion, their increased heart rates during the hatching phase may indicate that the temporal regulation of hatching was not affected directly by AZA-1 or AZA-1-induced developmental retardation. Hatching is a complex process involving the release of multiple signaling factors such as a specific hatching enzyme (from the hatching enzyme gland) (Yamagami, 1997). Nonetheless, it is unlikely that AZA-1-affected embryos, which were substantially reduced in size and delayed in development, would be able to force themselves from the eggs under such circumstances. As such, the effects of the toxin on a fish's ability to hatch were strikingly evident in that only half the fish exposed to less than 80 pg/egg succeeded in breaking free of the chorion.

Prior to substantial growth of healthy medaka embryos during the first 4 days of development, embryos display a rhythmic pulsating movement throughout the yolk. This amoeboid movement becomes increasingly difficult to observe during the remainder of the in ovo period. The yolks of medaka treated with more than 80 pg of AZA-1 or AZA_{total} rarely exhibited this behavior. Rather, based on microscopic observation, the yolk remained a stationary structure during development. Furthermore, we did not observe the development of the circulatory system over the periphery of the yolk in these eggs. While the heart beat was visible, the overlaying circulatory structures failed to develop. Finally, many of these embryos were unable to uniformly consume the yolk, possibly as a secondary result of this circulatory effect, generating the flattened yolk surfaces. This overall lack of egg yolk motility, blood circulation, and nutrient uptake suggests a possible link to the effects of AZAs on the cytoskeleton, as originally proposed by Roman et al. (2002) and subsequently investigated by Twiner et al. (2005). Embryonic development is a highly dynamic process wherein cellular migration may occur across the entire diameter of the egg (Kimmel et al., 1995). Cell generation, differentiation, and proliferation require the guidance of cytoskeletal elements and are often initiated and/or maintained by calcium signals or waves, which may be visualized in vivo via fluorescent dyes (Ashworth et al., 2001; Webb and Miller, 2000). Cell migration during early developmental stages, and later in

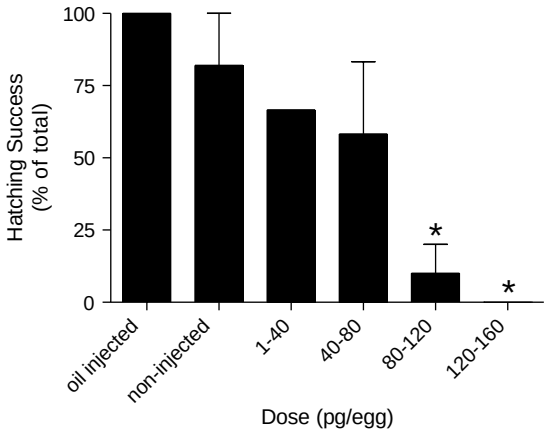

Fig. 5. Effect of AZA-1 on percent hatching success (mean ± SEM) of developing medaka embryos. Embryos (n = 7–15) were microinjected with a range of doses of AZA-1 (1–40, 40–80, 80–120, 120–160 pg) dissolved in triolein oil. Non-injected and triolein oil only controls are also shown. Microinjections occurred on day 0, 6–8 h following fertilization. Significant differences ($p < 0.05$) are denoted by an asterisk (*).

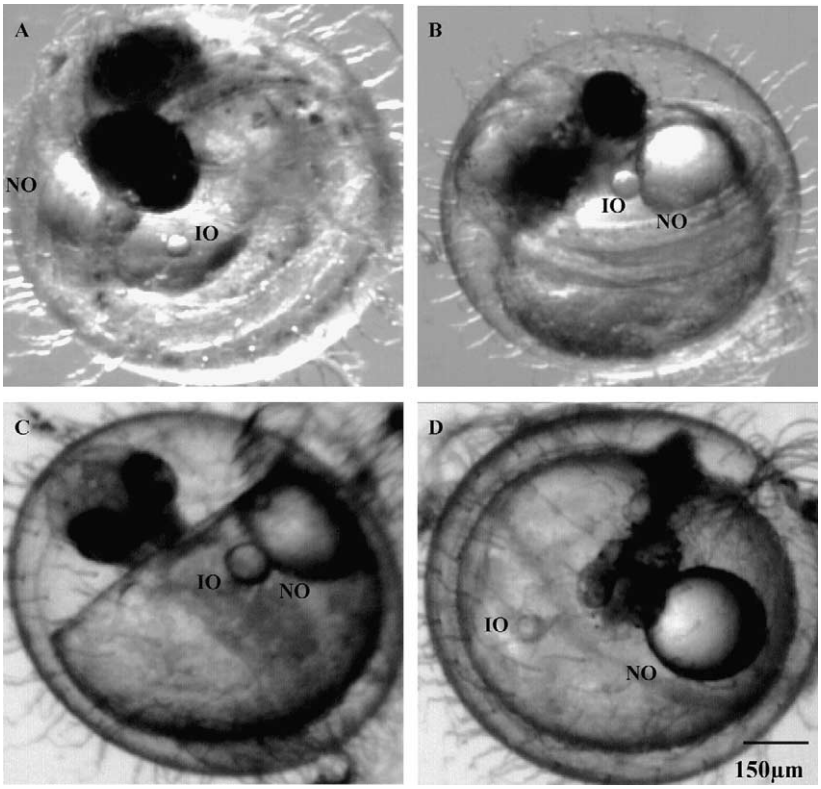

Fig. 6. Photomicrographs (80× magnification) of 5-day old medaka embryos injected with (A) non-toxic control mussel extract; 12 pg oil equivalents, (B) 25 pg AZA_{total}, (C) 84 pg AZA_{total}, and (D) 111 pg AZA_{total}. Microinjections occurred on day 0, 6–8 h following fertilization. Natural oil (NO) and injected oil (IO) droplets are marked on the figures.

rudimentary organ formation, require a high degree of fidelity to be successful (Webb and Miller, 2003). Putative disruption of these processes by AZA-1 could adversely affect developmental processes, or even prevent the processes from occurring altogether by inhibiting cell motility. In vitro studies using AZA-1 (Twiner et al., 2005) have demonstrated that AZA-1 induces cytotoxicity and affects F-actin arrangement in a human T lymphocyte cells in a concentration- and time-dependent fashion. Pure AZA-1, as well as the AZA-contaminated mussel extracts used in this study, were shown by Twiner et al. (2005) to cause a reduction and subsequent elimination of pseudopodia in these cells. Pseudopodia are actin-containing appendages necessary for chemotaxis, mobility, and cell-to-cell communication (Wehrle-Haller and Imhof, 2003). Evidence for cytotoxicity subsequent to alterations in cellular signaling molecules such as cAMP and Ca^{2+} (Roman et al., 2002) may lend insight into the effects of

Table 2
Number of medaka embryos (as a percentage) expressing circulating blood and pigmented blood at days 3, 5, and 7 post-fertilization following microinjection of non-contaminated mussel extract (Control EXT) or crude mussel extracts containing multiple AZAs (AZA_{total})

	Day	NI ($n=9$)	Control EXT ($n=10$)	AZA_{total} dose (pg/egg)		
				1–40 ($n=4$)	40–80 ($n=2$)	80–120 ($n=2$)
Circulation	3	100	100	25	0	0
	5	100	100	100	0	0
	7	100	100	100	0	0
Pigmentation	3	67	50	25	0	0
	5	100	100	100	0	0
	7	100	100	100	0	0

Non-injected (NI) controls are also shown. Note: in some treatment groups the embryos died. Percentage data are calculated for the live fish only.

AZA-1 on developing medaka, as embryonic calcium currents are essential to proper development (Ashworth et al., 2001).

The field of AZA research is still developing with much work to be done, particularly concerning its origins, mode of action, toxicity and carcinogenicity, and its potential effects on organisms at higher trophic levels. Due principally to a lack of sampling and analytical efforts AZAs have not yet been identified in fish tissues. Nevertheless, their teratogenic potential in embryonic fish is clear from this work. There is currently a lack of information concerning the trophic transfer of AZAs; however, there is sufficient evidence of chemically similar phycotoxins such as brevetoxins and ciguatoxins bioconcentrating in food webs. Subsequently, these toxins can be maternally transferred to the eggs during oogenesis in oviparous species (Colman et al., 2004). We have demonstrated the adverse effects of both purified AZA-1 and an AZA-contaminated shellfish extract in embryonic finfish. Our findings suggest that should AZA accumulation and transfer occur in marine fishes, that these toxins could have teratogenic effects and in turn detrimentally affect fecundity and population fitness of species exposed to AZAs.

Acknowledgements

This work was funded by the National Oceanic and Atmospheric Administration/National Ocean Service (NOAA/NOS and supported (in part) by the National Science Foundation under Grant No. 0212921). Funding for the Marine Institute, Ireland, and collaborative efforts was also obtained from the Irish National Development Plan (NDP) under the marine research strategic project ST-02-02, Azaspiracids Standards and Toxicology (ASTOX). MJT was supported by a National Research Council Associateship Award through NOAA/NOS/NCCOS/CCEHBR. NOS does not approve, recommend, or endorse any proprietary product or material mentioned in this publication. No reference shall be made to NOS, or to this publication furnished by NOS in any advertising or sales promotion which would indicate or imply that NOS approves, recommends, or endorses any proprietary product or proprietary material mentioned herein or which has as its purpose any intent to cause directly or indirectly the advertised product to be used or purchased because of NOS publication.

References

Ashworth, R., Zimrich, F., Bolsover, S.R., 2001. Buffering intracellular calcium disrupts motorneuron development in intact zebrafish embryos. Developmental Brain Research 129, 169–179.

Bargu, S., Silver, M.W., 2003. Field evidence of krill grazing on the toxic diatom genus *Pseudo-nitzschia* in Monterey Bay, California. Bulletin of Marine Science 72, 629–638.

Colman, J.R., Ramsdell, J.S., 2003. The type B brevetoxin (PbTx-3) adversely affects development, cardiovascular function, and survival in Medaka (*Oryzias latipes*) embryos. Environmental Health Perspectives 111, 1920–1925.

Colman, J.R., Dechraoui, M.Y.B., Dickey, R.W., Ramsdell, J.S., 2004. Characterization of the developmental toxicity of Caribbean ciguatoxins in finfish embryos. Toxicon 44, 59–66.

Edmunds, J.S.G., McCarthy, R.A., Ramsdell, J.S., 1999a. Ciguatoxin reduces larval survivability in finfish. Toxicon 37, 1827–1832.

Edmunds, J.S.G., McCarthy, R.A., Ramsdell, J.S., 1999b. Permanent and functional male-to-female sex reversal in d-rR strain medaka (*Oryzias latipes*) following egg microinjection of o,p′-DDT. Environmental Health Perspectives 108, 219–224.

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

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

Jacquet, C., Thermes, V., de Luze, A., Puiseux-Dao, S., Bernard, C., Joly, J.-S., Bourrat, F., Edery, M., 2004. Effects of microcystin-LR on development of medaka fish embryos (*Oryzias latipes*). Toxicon 43, 141–147.

James, K.J., Furey, A., Satake, M., Yasumoto, T., 2000. Azaspiracid poisoning (AZP): a new shellfish toxic syndrome in Europe. Harmful Algal Blooms pp. 250–253.

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

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

Jeong, H.J., 1999. The ecological roles of heterotrophic dinoflagellates in marine planktonic community. Journal of Eukaryotic Microbiology 46, 390–396.

Jeong, H.J., Yoon, J.Y., Kim, J.S., Yoo, Y.D., Seong, K.A., 2002. Growth and grazing rates of the prostomatid ciliate *Tiarina fusus* on red-tide and toxic algae. Aquatic Microbial Ecology 28, 289–297.

Kimm-Brinson, K.L., Ramsdell, J.S., 2001. The red tide toxin, brevetoxin, induces embryo toxicity and developmental abnormalities. Environmental Health Perspectives 109 (4), 377–381.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullman, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Developmental Dynamics 203, 253–310.

Lehane, L., Lewis, R.J., 2000. Ciguatera: recent advances but the risk remains. International Journal of Food Microbiology 61, 91–125.

Lewis, R.J., Jones, A., 1997. Characterization of ciguatoxins and ciguatoxin congeners present in ciguateric fish by gradient reverse-phase high-performance liquid chromatography/mass spectrometry. Toxicon 35, 159–168.

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

McMahon, T., Silke, J., 1998. Re-occurrence of winter toxicity. In Harmful Algae News p. 12.

Miller, M.A., 1993. Maternal transfer of lipophilic contaminants in salmonines to their eggs. Canadian Journal of Fisheries and Aquatic Sciences 49, 1405–1413.

Niimi, A.J., 1983. Biological and toxicological effects of environmental contaminants in fish and their eggs. Canadian Journal of Fisheries and Aquatic Sciences 40, 306–312.

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

Papoulias, D.M., Villalobos, S.A., Meadows, J., Noltie, D.B., Giesy, J.P., Tillitt, D.E., 2003. In ovo exposure to o,p-DDE affects sexual development but not sexual differentiation in Japanese medaka (*Oryzias latipes*). Environmental Health Perspectives 111, 29–32.

Roman, Y., Alfonso, A., Louzao, M.C., de la Rosa, L.A., Leira, F., Vieites, J.M., Vieytes, M.R., Ofuji, K., Satake, M., Yasumoto, T., Botana, L.M., 2002. Azaspiracid-1, a potent, nonapoptotic new phycotoxin with several cell targets. Cellular Signaling 14, 703–716.

Satake, M., Ofuji, K., Naoki, H., James, K.J., Furey, A., McMahon, T., Silke, J., Yasumoto, T., 1998. Azaspiracid, a new marine toxin having unique spiro ring assemblies from Irish mussels, *Mytilus edulis*. Journal of the American Chemical Society 120, 9967–9968.

Smith, R.M., Cole, C.F., 1973. Effects of egg concentrations of DDT and dieldren on development in winter flounder (*Pseudopleuronectes americanus*). Journal of Fisheries Research Board of Canada 30, 1894–1898.

Tester, P.A., Turner, P.T., Shea, D., 2000. Vectorial transport of toxins from the dinoflagellate *Gymnodinium breve* through copepods to fish. Journal of Plankton Research 22, 47–61.

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

Webb, S.E., Miller, A.L., 2000. Calcium signaling during zebrafish embryonic development. Bioessays: News and Reviews in Molecular, Cellular and Developmental Biology 22, 113–123.

Webb, S.E., Miller, A.L., 2003. Calcium signaling during embryonic development. Nature Reviews Molecular Cell Biology 4, 539–551.

Wehrle-Haller, B., Imhof, B.A., 2003. Actin, microtubules and focal adhesion dynamics during cell migration. The International Journal of Biochemistry and Cell Biology 35, 39–50.

Yamagami, K., 1997. A short history of the hatching enzyme studies in medaka. Fish Biology Journal, Medaka 9, 5–15.