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Effects of the cyanobacterial neurotoxin β-*N*-methylamino-L-alanine on the early-life stage development of zebrafish (*Danio rerio*)

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ABSTRACT

β-*N*-Methylamino-L-alanine (BMAA), a neurotoxic amino acid, is produced by members of all known groups of cyanobacteria. In the presence of added carbonate, BMAA generates an analogue of glutamate which has been associated with motor neuron (MN) diseases via a mechanism of motor neurone specific excitotoxicity. The toxicity of BMAA has been established in various mammalian test models, but the widespread aquatic production of BMAA raises questions of BMAA toxicity to aquatic organisms. Zebrafish (*Danio rerio*) embryos were exposed to varying concentrations of BMAA (5–50,000 $\mu g\,l^{-1}$) with and without added carbonate. BMAA exposure induced a range of neuro-muscular and developmental abnormalities in *D. rerio*, which can be directly related to disruptions to glutamatergic signalling pathways. When exposed to BMAA plus added carbonate, the incidence of pericardial oedema increased by up to 21% in test subjects, correlating with a reduction in heart rate. Increased incidence of abnormal spinal axis formation was seen in all *D. rerio* larvae exposed to BMAA concentrations of $\geq$50 $\mu g\,l^{-1}$, with a further 10% increase from $\geq$500 $\mu g\,l^{-1}$ BMAA when carbonate species were present. A dose-dependent increase in clonus-like convulsions was observable in embryos exposed to $\geq$5 $\mu g\,l^{-1}$ BMAA ± added carbonate. This is the first study on the neuro-muscular and developmental effects of BMAA exposure on aquatic vertebrates. The present findings, plus the potentially widespread production of BMAA in aquatic cyanobacteria, indicate a need for information of exposure levels, duration and toxic outcomes in aquatic biota.

1. Introduction

Cyanobacteria (blue-green algae) are receiving increased scientific and public attention, due in part to an apparent increasing incidence of cyanobacterial blooms in high resource waterbodies, and the production of a diverse range of potent cyanotoxins (Codd et al., 2005). β-*N*-Methylamino-L-alanine (BMAA) is a neurotoxic amino acid, first reported over 40 years ago in extracts of the cycad tree, *Cycas micronesica* (Vega and Bell, 1967). Evidence is emerging for the widespread production of BMAA by free-living freshwater, marine, brackish water and terrestrial cyanobacterial species, as well as by symbiotic cyanobacteria (Cox et al., 2003, 2005; Murch et al., 2004; Banack et al., 2007; Metcalf et al., 2008; Esterhuizen and Downing, 2008).

A causative association between human dietary exposure to BMAA and the increased incidence of amyotrophic lateral sclerosis–parkinsonism dementia complex (ALS–PDC), a motor neuron (MN) disease among native Chamorro people on the Island of Guam in Micronesia, has long been hypothesised (Spencer et al., 1987; Cox et al., 2003; Ince and Codd, 2005). Debate on this hypothesis (Papapetropoulos, 2007) and further research with environmental and human clinical material continue. In the presence of a carbonate species, BMAA forms the product L-BMAA-β-NCO_2, an analogue of glutamate. This analogue causes motor neuron selective toxicity via hyper-activation of MN glutamate (Glu) transporters at concentrations of 30 μM and potentiates MN injury induced by other insults from 10 μM (Nunn and O'Brien, 1989; Weiss et al., 1989; Rao et al., 2006; Lobner et al., 2007). During neurodegeneration, Glu transporters load excess Glu into extracellular spaces, inducing a flood of calcium (Ca^{2+}) into motor neurons via receptor channels causing mitochondrial damage, and Glu/Ca^{2+}-mediated increases in apoptotic transcription factors. Excitotoxicity due to excess Glu occurs as part of the ischemic cascade and is associated with stroke and diseases including amyotrophic lateral sclerosis, lathyrism, and Alzheimer's disease (Van Den Bosch et al., 2006; Hara and Snyder, 2007).

BMAA production by cyanobacteria suggests that this neurotoxin may be of toxicological significance to humans via aquatic exposure routes (Ince and Codd, 2005). Indeed, BMAA has been identified along with other cyanotoxins in environmental samples

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of cyanobacterial blooms from waterbodies used for abstraction for drinking water treatment, as fisheries and as recreational resources (Metcalf et al., 2008). Although the toxicity of BMAA has been investigated in various mammalian test models (Karamyan and Speth, 2008), the toxicity of BMAA to aquatic organisms has not been investigated. There is a need to develop effective models for motor neurone degeneration research, and the principles of humane experimental technique, as outlined by Russell and Burch (1959), place an emphasis on replacement, reduction, and refinement in animals used for toxicity testing, highlighting the need to further develop aquatic vertebrates as toxicity testing models. We have used the zebrafish (*Danio rerio*) as a model. This model has previously been used to identify and characterise other known cyanotoxins (Wiegand et al., 1999; Lefebvre et al., 2004; Wright et al., 2006; Berry et al., 2007).

Some of the main benefits of using zebrafish in toxicology are based on the early life-stage morphology, size and ease of husbandry of these fish. Optimal breeding and maintenance conditions are very well established and are also relatively easy to control. Their size allows standard microscopic analysis, whilst being small enough to be suitable for high-throughput screening, such as via multi-well plate analysis. The short gestational period and development of *D. rerio* make it ideal for both acute and chronic exposure analysis. Perhaps most importantly, the transparency of the embryo chorion allows straightforward identification of developmental stages and phenotype traits (Hill et al., 2005; Hinton et al., 2005; Berry et al., 2007). *D. rerio* is becoming increasingly popular as a standard vertebrate toxicity testing model and is particularly valuable for the study of MN degeneration due to conserved neurodevelopmental processes and signalling pathways. Glutamate is the key neurotransmitter in locomotor regions of the developing zebrafish, and glutamatergic synaptic drive systems in *D. rerio* show significant similarities with other vertebrates, with the universal motor pathway of synaptic drive underlying locomotion (Lein et al., 2005; Gabriel et al., 2008; Drapeau et al., 2002). *D. rerio* applications are being developed as standard Parkinson's disease and Huntington's disease models, among others (Bretaud et al., 2004; Linney et al., 2004; McKinley et al., 2005; Stehr et al., 2006; Schiffer et al., 2007).

This is to our knowledge the first investigation of the developmental effects of BMAA exposure on aquatic vertebrates. The potential for widespread BMAA production by cyanobacteria in aquatic environments means that both the toxicity to and fates of BMAA in aquatic organisms must be better understood. We report here on impacts of BMAA exposure on toxicological endpoints in *D. rerio*, including developmental, behavioural, physiological and in particular neurological and neuro-muscular responses (Fraysse et al., 2006).

2. Materials and methods

2.1. Fish

Adult zebrafish (*D. rerio*) were maintained in the University of Dundee aquarium in aerated de-chlorinated water at 26.5 ± 1.0 °C, with a light:dark cycle of 14:10 h. Spawning was induced by the onset of light and feeding. Eggs were collected from 2.25 h post-fertilisation (hpf) at the blastula (64-cell) developmental stage. Embryos were rinsed with aerated de-chlorinated water from the University of Dundee Aquarium, and placed in Petri dishes at 26.5 ± 1.0 °C where the unfertilised eggs could be distinguished and removed. Mean values for water quality in mmol l^{-1} were: Na^+ 0.19; K^+ 0.02; Ca^{2+} 0.24; Mg^{2+} 0.07; Cl^- 0.03; free CO_2 0.02; alkalinity as $CaCO_3$ 31.3 mg l^{-1}; non-bicarbonate hardness as $CaCO_3$ 10.6 mg l^{-1}; pH 8.2.

2.2. In vivo exposure of D. rerio embryos

After washing, embryos (n = 30) were placed in 25 well 12 mm cell macro-culture plates (Falcon 3503, Becton Dickinson and Co.) with 2 ml of Hank's egg hatching medium, with a composition of NaCl 80 g l^{-1}; KCl 4 g l^{-1}; Na_2HPO_4 3.58 g l^{-1}; KH_2PO_4 6 g l^{-1}; $CaCl_2$ 14.4 g l^{-1}; $MgSO_4{\cdot}7H_2O$ 24.6 g l^{-1}; $NaHCO_3$ 35 g l^{-1}. At 24 h post-fertilisation (hpf) embryos exposed to 0, 5, 50, 500, 5000 or 50,000 μg l^{-1} BMAA in 2 ml aerated de-chlorinated water from the University of Dundee Aquarium (composition outlined in section 2.1). Some embryos were exposed to a combination of BMAA and 0.1 mM Na_2CO_3. These were placed in 2 ml of aerated de-chlorinated water with 10.6 μg l^{-1} (0.1 mM) Na_2CO_3, ±0, ±5, ±500, ±5000 or ±50,000 μg l^{-1} BMAA. All treatments were performed in triplicate. Embryos were incubated at 26.5 ± 1.0 °C. The embryos were exposed to treatments for 5 days post-fertilisation (dpf). After hatching, the chorions were removed. After the 5 dpf exposure period, the larvae were placed in fresh aerated de-chlorinated water, referred to as the "recovery state" and observed until point of mortality (8–14 dpf).

2.3. Observations and data collection

Data for the morphological study and heart rate measurements were collected every 24 h. Physiological aspects monitored included heart rate, embryo area and yolk sac area, pericardial area, head and eye length and width, larval spinal axis angle, fin and tail length and width, and occurrence of clonus-like convulsions. Every individual (n = 30) from each treatment was observed. Any observable abnormalities or deformities were noted and photographed. At least 5 apparently normal individuals per group were randomly selected, along with all affected individuals, for photography every 24 h using phase contrast microscopy and a graticule. Morphological features were measured using Image Tool software (UTHSCSA ImageTool Version 3.0). Every day, the numbers of hatched and damaged embryos, abnormal and dead larvae were recorded. All damaged embryos and dead larvae were removed from the wells.

Heart rate (beats per minute, bpm) was observed via light microscopy, due to the transparency of the embryo/larvae. Heart rate monitored until the development of pigmentation occludes observation (96–120 hpf). Heart rate calculated by time taken for 20 heart beats, converted to bpm. Clonus-convulsions were observed as extended periods of rapidly alternating muscular contraction and relaxation, apparently affecting the entire body (dorsal and caudal regions observed). Each well monitored for 3 min and the number of convulsing individuals within that period recorded. Distinguished from reflex tail flicks as must involve full body, and only recorded when convulsion duration over 3 s.

2.4. Chemicals

Synthetic L-BMAA hydrochloride was purchased from Sigma–Aldrich. A stock solution of 10 mg ml^{-1} in deionised water was diluted to 5, 50, 500, 5000 and 50,000 μg l^{-1} of BMAA with de-chlorinated water for fish-exposure studies. All other chemicals, including anhydrous sodium carbonate, were of analytical grade quality and were purchased from BDH Laboratory Supplies (Poole, UK).

2.5. Statistical analysis

For endpoints described by 'normal' law or Gaussian distribution, statistical analysis was performed in the form of single ANOVA, assuming equal variances between exposure groups ($p^* < 0.05$; $p^{**} < 0.01$; $p^{***} < 0.001$). For all data expressed the statistical dif-

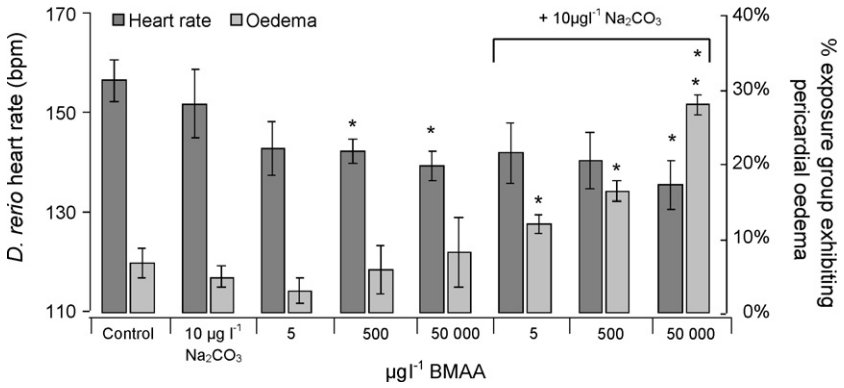

Fig. 1. Effects of exposure of BMAA and 10.6 µg l^{-1} sodium carbonate on heart rate and presence of pericardial oedema in *Danio rerio* larvae at 72 hpf. Reduced heart rate directly correlated with presence of pericardial oedema in *D. rerio* due to BMAA exposure ± sodium carbonate (Significant Pearson correlation $R = -0.59402$; $^{***}p < 0.01$; $n = 3$). Statistical analysis by single ANOVA assuming equal variances ($^{*}p < 0.05$; $^{**}p < 0.01$; $n = 3$).

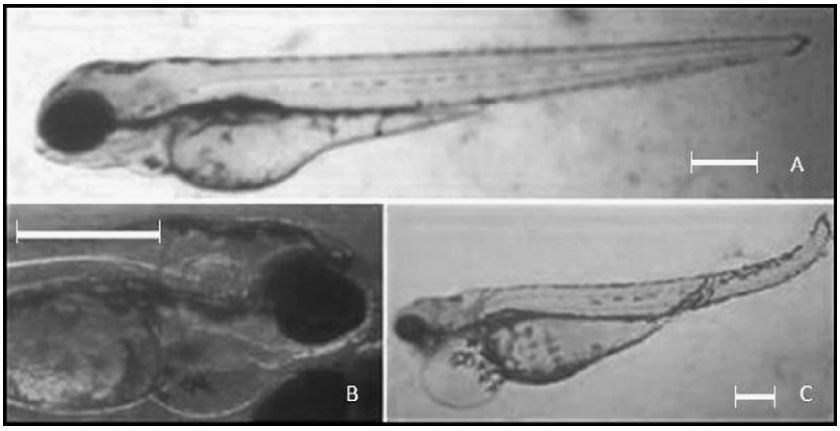

Fig. 2. Phase contrast microscopy of *D. rerio* larvae. (A) *D. rerio* at 120 hpf, control; (B) pericardial oedema of *D. rerio* exposed to 5 µg l^{-1} BMAA + 10.6 µg l^{-1} sodium carbonate for 96 hpf; (C) *D. rerio* at 120 hpf, exposed to 500 µg l^{-1} BMAA + 10.6 µg l^{-1} sodium carbonate showing pericardial oedema with lordosis of the spine. Horizontal bar: 0.25 mm.

ferences refer to difference between controls and treatments within specific experiments. Data are expressed as mean percentage from triplicate groups each containing 30 individuals. For determination of the correlation between heart rate and pericardial oedema, Pearson's coefficient correlation method was used.

3. Results

3.1. Reduced heart rate and increased pericardial oedema

D. rerio embryos exposed to BMAA ± Na_2CO_3, exhibited a significant correlation between reduced heart rate and pericardial oedema (Pearson correlation coefficient; $r = -0.59$, $p^{***} < 0.001$; $n = 3$). Embryos exposed to ≥5 µg l^{-1} BMAA showed a reduction in heart rate, with a significant reduction of up to 11% (139.5 bpm ± 2.9 bpm) from ≥500 µg l^{-1} BMAA, compared to controls (156.7 bpm ± 4.2 bpm), and signs of increased oedema compared to controls, although the difference was not statistically significant. However, when *D. rerio* was exposed to BMAA supplemented with 10.6 µg l^{-1} sodium carbonate (Na_2CO_3) a significant dose-dependent increase in the occurrence of pericardial oedema up to a maximum of 21% of test group (±1.3%; $p^{**} < 0.001$; $n = 3$), as well as a statistically significant reduction in heart rate, by up to 13% at highest BMAA concentration (135.7 bpm ± 4.95 bpm; $p^{*} < 0.05$; $n = 3$). Exposure of embryos to sodium carbonate-only controls resulted in no significant difference to either heart rate or the incidence of pericardial oedema among test groups (Figs. 1 and 2).

3.2. Abnormal spinal axis formation

The incidence of abnormal spinal, dorsal and caudal axis formation increased significantly upon exposure to BMAA concentrations of ≥50 µg l^{-1}, with an increase from 3.3% (±0.8%) in control groups to 21% (±0.9%; $p^{**} < 0.01$; $n = 3$) at 5000 µg l^{-1} BMAA. The addition of 10.6 µg l^{-1} Na_2CO_3 alone, had no significant impact on the rate of abnormal axis formation, and had no impact on the toxicity of BMAA at ≤50 µg l^{-1}, but resulted in a significant increase in BMAA toxicity at ≥500 µg l^{-1} (Fig. 3). Statistical significance refers to differences

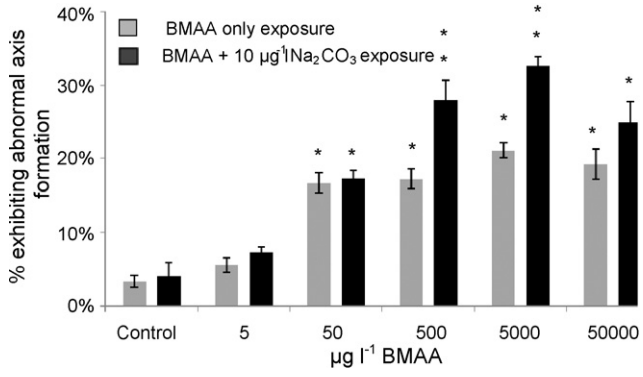

Fig. 3. Increasing occurrence of abnormal axis formation in *D. rerio* due to exposure to BMAA ± 10.6 µg l^{-1} sodium carbonate. Deformity observed from 72 hpf onwards, once larvae hatched. Statistical analysis by single ANOVA assuming equal variances ($^{*}p < 0.05$; $^{**}p < 0.01$; $n = 3$).

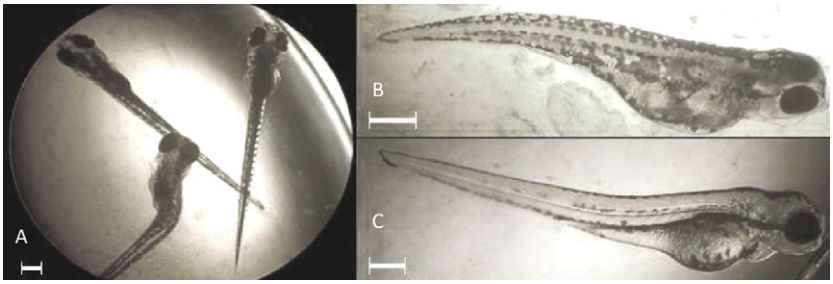

Fig. 4. Phase contrast microscopy of *D. rerio* larvae. (A) *D. rerio* larvae at 168 hpf exposed to 5000 μg l^{-1} BMAA showing two unaffected larvae and one larva with helical spinal curvature; (B) *D. rerio* larvae at 168 hpf exposed to 500 μg l^{-1} showing cyphosis of the spinal axis (downwards curvature); (C) *D. rerio* larvae at 96 hpf exposed to 5 μg l^{-1} BMAA showing lordosis of the spinal axis (upwards curvature). Horizontal bar: 0.25 mm.

between treatments and controls within specific experiments. Several forms of abnormal axis formation were observed from 80 hpf onwards, including lordosis, cyphosis and helical spine (Fig. 4).

3.3. Clonus-like convulsions

Pre- and post-hatch *D. rerio* larvae exposed to BMAA at all concentrations, ±10.6 μg l^{-1} Na_2CO_3, presented with extended periods of rapidly alternating muscular contraction and relaxation, apparently affecting the entire body (dorsal and caudal regions observed). No clonus-like convulsions were observed in the control groups and low levels of convulsions were present in sodium carbonate control groups, although this was not significant overall. Exposure at ≥500 μg l^{-1} BMAA ± Na_2CO_3, caused significant dose-dependent increases in the occurrence of the clonus-like convulsions ($p^* < 0.05$; $p^{**} < 0.01$; $n = 3$) (Fig. 5).

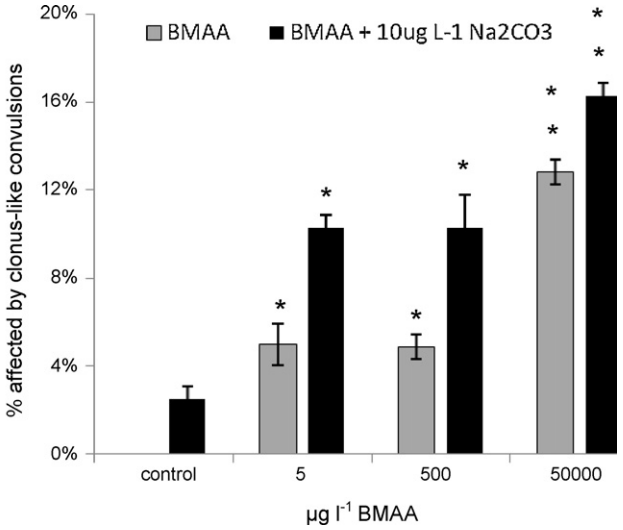

Fig. 5. Incidence of clonus-like convulsions in *D. rerio* larvae at 96 hpf, exposed to BMAA alone or with 10.6 μg l^{-1} sodium carbonate. Effect exacerbated by presence of 10.6 μg l^{-1} Na_2CO_3. Data expressed in mean percentage from triplicate groups containing 30 individuals. Statistical analysis in form of single ANOVA assuming equal variances between exposure groups ($^*p < 0.05$; $^{**}p < 0.01$; $n = 3$).

3.4. Hatching

BMAA addition alone induced a slight delay in the rate of hatching although this effect was not significant overall (Fig. 6). The addition of 10.6 μg l^{-1} Na_2CO_3, with ≥5 μg l^{-1} BMAA resulted in a significant premature hatch at 48 hpf, to a maximum of 29.5% of larvae premature hatched (±2.7%) at 50,000 μg l^{-1} BMAA, compared to 8.5% (±0.7%) in control groups ($p^{**} < 0.01$; $n = 3$). The sodium carbonate-only control groups showed no significant difference from standard control groups, all showing less than 10% of group hatched by 48 hpf (Fig. 6).

4. Discussion

Studies have shown BMAA to be produced by cyanobacteria from waterbodies on a worldwide basis, at concentrations ranging from 3 to 6478 μg g^{-1} (Cox et al., 2005; Metcalf et al., 2008). The widespread aquatic production of BMAA by cyanobacteria is potentially a cause for concern, as the neurotoxic effects observed in this study may have consequences at both individual and community level among aquatic biota, as well as reinforcing the hypothesis that exposure to BMAA poses a significant risk to human health. BMAA exposure induced a range of neuro-muscular and developmental abnormalities in several of the early life-stage endpoints monitored

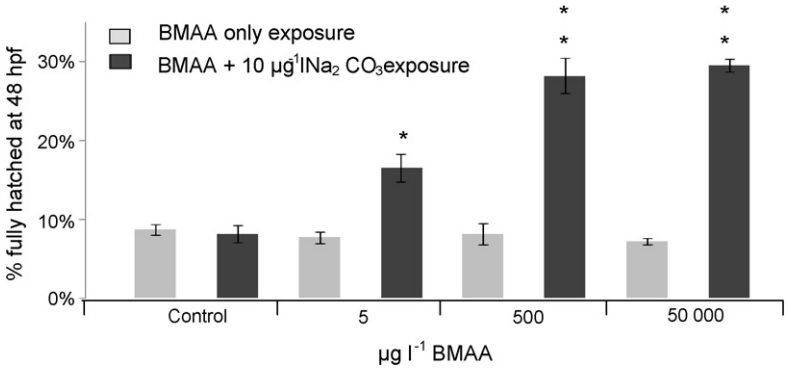

Fig. 6. Percentage of *D. rerio* fully hatched by 48 hpf. BMAA exposed embryos exhibit a reduction in % hatched at 48 hpf (not significant). BMAA and 10.6 μg l^{-1} sodium carbonate exposed embryos showed a premature hatch response as compared to controls. Statistical analysis by single ANOVA assuming equal variances ($^*p < 0.05$; $^{**}p < 0.01$; $n = 3$).

in *D. rerio* and the addition of sodium carbonate significantly exacerbated the observed toxicity of BMAA in all endpoints investigated (Figs. 1 and 6).

Calcium signalling is essential for heart formation and muscle contraction (Webb and Miller, 2000) and is directly affected by glutamatergic motor neuron activation (Gabriel et al., 2008). Abnormal intracellular calcium activation in cardiac myocytes causes reduced muscle contraction, resulting in reduced cardiac muscle size and reduced blood pressure, inducing pericardial oedema (Billiard et al., 1999; Hu et al., 2000). *D. rerio* embryos exposed to BMAA plus and minus added sodium carbonate presented reduced heart rate and pericardial oedema, with a significant correlation between these two abnormalities. When exposed to BMAA $\pm$ 10.6 $\mu g\,l^{-1}$ Na_2CO_3, the incidence of pericardial oedema significantly increased among test organisms, coinciding with a significant reduction in heart rate as compared to controls (Fig. 1).

It is not possible to identify either factor as being causative, and whilst correlation does not necessarily imply causation, there is a clearly defined relationship between blood pressure and pericardial oedema (Yelon et al., 2002). No visible sign of altered heart looping or heart muscle deformity was apparent in BMAA-exposed embryos to directly explain the observed reduced heart rate. It is notable that the *D. rerio* mutant strain 'island beat' (*isl*) has a defect in L-type calcium channels which are essential for myocardial contraction. Studies have found that disruption to calcium channels directly alters heart contraction, impairing the cardiovascular system. A reduced heart rate and reduction in heart muscle size can directly result in low blood tail circulation, with this reduced blood circulation causing pericardial oedema as well as disrupting hatching and movement (Yelon et al., 2002).

Abnormal axis formation was one of the most prominent adverse effects of exposure to BMAA. Exposure of *D. rerio* embryos to BMAA resulted in significant increase in abnormal spinal axis formation as evidenced by the occurrence of helical spine, lordosis and cyphosis (Fig. 4). Glutamatergic-associated calcium signalling controls the major phases of pattern formation in the developing zebrafish, including major body axis determination and spine formation (Webb and Miller, 2000). These neuro-muscular defects are likely to hinder *D. rerio*'s ability to survive by potentially disrupting hatching; reducing 'cruising' swim speed and the ability to maintain position in water column. These impairments can lead to an increase in susceptibility to predation and a severe reduction in food-sourcing (Von Westernhagen, 1988a,b).

The rate of axis curvature increased significantly with exposure to BMAA concentrations $\geq$50 $\mu g\,l^{-1}$ BMAA. Embryos exposed to $\geq$500 $\mu g\,l^{-1}$ BMAA with sodium carbonate, presented a further 10% increase in the incidence of abnormal axis formation, consistent with earlier studies with vertebrates and neuronal cell lines (Nunn and O'Brien, 1989; Weiss et al., 1989; Rao et al., 2006). Potential mechanisms for abnormal axis formation also include musculature contraction disturbance, as abnormal axis formation may be a result of uncontrolled contractions of axis musculature and/or deformation of vertebral elements, also directly attributable to glutamate-associated Ca^{2+} signalling (Adams et al., 2005). Alternatively, abnormal axis formation may be due to neuronal vacuolation and death in the spinal cord cells due to BMAA exposure (Nunn et al., 1987).

Reflex activity patterns in embryonic spinal cords can be directly related to glutamate-induced motor circuit development (Gabriel et al., 2008) and previous studies have related clonus-like convulsions in *D. rerio*, induced by domoic acid (an excitotoxin with similar mechanism of toxicity to BMAA), to a neurological pattern in glutamate receptors (Baraban et al., 2005). The embryos exhibited clonus-like convulsions upon exposure to BMAA with or without the addition of carbonate, almost instantly from point of BMAA exposure from 24 hpf, continuing as an observable response in larvae throughout the full time-course of the experiments.

The *D. rerio* hatching process is a combination of biochemical and behavioural activity, with zinc-metallo-proteases synthesised to digest the chorion, followed by manual destruction by twisting movements of the embryo (Adams et al., 2005). Delayed hatching in some of the individuals (not statistically significant) were probably attributable to the neuro-muscular defects induced by BMAA exposure. However, when exposed to $\geq$5 $\mu g\,l^{-1}$ BMAA and sodium carbonate, the point of hatch of *D. rerio* embryos was significantly premature (Fig. 6). This response may be partly linked to the increased occurrence and force of clonus-like convulsions from around 24 hpf in the embryos exposed to BMAA and sodium carbonate. These apparently violent movements may contribute to the destruction of the integrity of the chorion, accelerating the hatch process. Comparison of toxin exposure at varying developmental stages (6 and 24 hpf) was also performed (data not shown). Although toxicity in all endpoints measured was most pronounced following 24 hpf exposure to BMAA $\pm$ sodium carbonate, significant toxic effects were also observed at 6hpf exposure for all endpoints measured (data not shown). The thinning of the embryo chorion by 24 hpf, in preparation for hatch is likely to be a contributing factor to this (Adams et al., 2005).

4.1. Conclusions

Overall, these data reinforce the evidence that BMAA forms a potent glutamate analogue, which may contribute to motor neuron degeneration. BMAA alone and with the addition of carbonate induces a wide range of developmental and neuro-muscular abnormalities, including reduced heart rate and increased pericardial oedema, abnormal spinal axis formation, and clonus-like convulsions. All endpoints monitored are associated with glutamate signalling, such as glutamatergic motor circuit development responsible for spinal activity patterns, and glutamatergic Ca^{2+} signalling involved in heart formation, muscle contraction and major body axis formation (Webb and Miller, 2000).

Indeed, this study suggests that further research is required, and that *D. rerio* is an ideal model for this purpose. Whilst investigations at the organ, cellular and molecular levels are required to fully explain the observed effects, this study has shown *D. rerio* to be a valuable model for investigations into vertebrate neurodevelopment and motor neuron excitotoxicity in response to BMAA. Whilst the debate continues as to whether BMAA is indeed a causative factor in human motor neurone disease, its widespread production by aquatic cyanobacteria may be of ecotoxicological significance for aquatic biota, as well as a potential risk to human health.

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