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β-N-Methylamino-L-Alanine (BMAA) and Its Effects on Hatching Success, Heart Rate and Early Development Stage of *Danio Rerio* (Zebrafish)

β-N-Methylamino-L-Alanine (BMAA) dan kesannya kepada Kejayaan Penetasan, Kadar Denyutan Jantung dan Perkembangan Awal Danio Rerio (Ikan Zebra)

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Abstract

β-N-methylamino-L-alanine (BMAA) is a nonprotein amino acid found naturally in some cyanobacteria and artificial form as chemical product. BMAA is a neurotoxin and once ingested, BMAA can apparently be bound by proteins within the body, resulting in a slow release of free BMAA over years as contaminated proteins is metabolized. This study identified the effects of BMAA on Danio rerio (zebrafish) early development. Hatching success was not affected by BMAA but later developmental stages of embryos were more sensitive to BMAA than embryonic stage. Higher concentrations of BMAA (500 to 50000 μg L^{-1}) caused increases in the egg diameter, yolk sac length and width, and eye length and width of Danio rerio embryos. All investigated BMAA concentrations increased the larval heart rate during the exposure period. Abnormalities and conditions including oedema, helical body, tail deformities, bend body, flat face, eye deficiency and active performance were observed as a result of exposure to BMAA

Keywords BMAA, Danio rerio (Zebrafish), early development, heart rate, deformities, oedema

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Abstrak

β-N-methylamino-L-alanine (BMAA) adalah asid amino bukan protein dijumpai secara semulajadi dalam beberapa sianobakteria dan dalam bentuk buatan sebagai produk kimia. BMAA adalah neurotoksin dan apabila dicerna, BMAA boleh terikat dengan protein di dalam tubuh, mengakibatkan pembebasan yang perlahan BMAA bebas selama bertahun-tahun sebagai bahan cemar metabolisme protein. Kajian ini mengenalpasti kesan BMAA pada perkembangan awal *Danio rerio* (Ikan Zebra). Penetasan tidak dipengaruhi oleh BMAA tetapi tahap perkembangan embrio seterusnya adalah lebih sensitif terhadap BMAA berbanding tahap embrionik. Kepekatan BMAA yang lebih tinggi (500-50000 ng L^{-1}) menyebabkan peningkatan diameter telur, panjang dan lebar kantung yolka, dan panjang dan lebar mata embrio *Danio rerio*. Kesemua kepekatan BMAA yang dikaji meningkatkan denyutan jantung larva selama tempoh pendedahan. Kecacatan dan perubahan termasuklah edema, tubuh berbentuk heliks, kecacatan pada ekor, tubuh yang membengkuk, muka yang rata, mata yang mengecil dan aktiviti renang yang aktif dilihat sebagai kesan pendedahan kepada BMAA.

Keywords BMAA, Danio rerio (ikan zebra), perkembangan awal, denyutan jantung, kecacatan

Introduction

β-N-methylamino-L-alanine (BMAA)

β-*N*-methylamino-L-alanine (BMAA) (Fig. 1) is a non-protein amino acid (Cox *et al.*, 2003), and occurs both as a free and a protein–bound amino acid (Murch *et al.*, 2004a). It is reported to be a potent glutamate agonist at alpha-amino-3-hydroxy-5-methl-4-isoxazole propionic acid (AMPA) receptors (Weiss *et al*., 1989, Carriedo *et al*., 1996), and can be produced by cyanobacterial root symbionts of the genus *Nostoc* (Vessey *et al*., 2005).

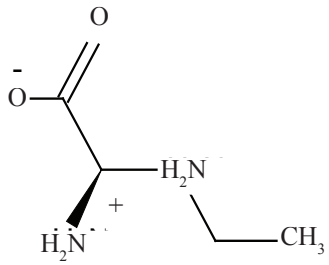

Figure 1 The Chemical Structures of BMAA

BMAA was discovered in cycad seeds (Vega and Bell, 1967) and was suggested as a potential cause of the amyotrophic lateral sclerosis/parkinsonism-dementia complex (ALS/PDC) among the native Chamorro people of Guam compared with incidence rates of ALS elsewhere (Spencer *et al*., 1986, 1987). It was discovered to biomagnify within the Guam ecosystem (Cox and Sacks, 2002, Banack and Cox, 2003, Cox *et al*., 2003, Murch *et al.*, 2004). It was found in the brain tissue of Chamorros who died of ALS/PDC, but not in patients who died

of causes unrelated to neurodegenerative disease (Cox *et al*., 2003, Murch *et al*., 2004) It was found in all known group of cyanobacteria, including cyanobacterial symbionts and free-living cyanobacteria (Cox *et al*., 2005); and caused neuro-muscular effects on *Danio rerio* (Purdie *et al.,* 2009).

Materials and Methods

BMAA preparation

BMAA was purchased from Sigma and a stock solution of 10 mg/ml was prepared in deionised water. The stock solution was diluted to 5, 50, 500, 5000 and 50000 mg L^{-1} of BMAA with local supply of Dundee de-chlorinated tap water as final concentrations to be used in the experiments. The treatment and control (Dundee de-chlorinated tap water) were prepared in triplicate.

Danio rerio (Zebrafish)

Selected *Danio rerio* embryos were placed in experimental solutions by putting 20 embryos per 5 ml solution in six wells of macroplates designed for cell culture. Three replicates per treatment were used and each experiment was repeated at least twice. All experimental work carried out on the embryos were done at 26.5 ± 1°C. Embryos at the blastula stage were used for 48- and 96- hour exposure to experimental solutions and larvae at the long pec stage (48 hour) to early larvae (96 hour) were also used for the experiments. Larvae were used to the point when feeding would have commenced, usually about 5 day post fertilization (dpf). No experiments on larvae were performed after this point.

The hatching success of embryos exposed to different treatments was recorded to determine the 50% hatching for each treatment. The larval mortality was recorded and from the information the 50% mortality was established.

The activity state of embryos or larvae in each treatment was recorded. Live embryos and larvae were photographed for measuring morphology as shown in Figure 2 and Figure 3 accordingly. *In situ* measuring was difficult to conduct because embryos and larvae were small and larvae were quite active at 3 dpf. The dead embryos and larvae were removed and survival was determined.

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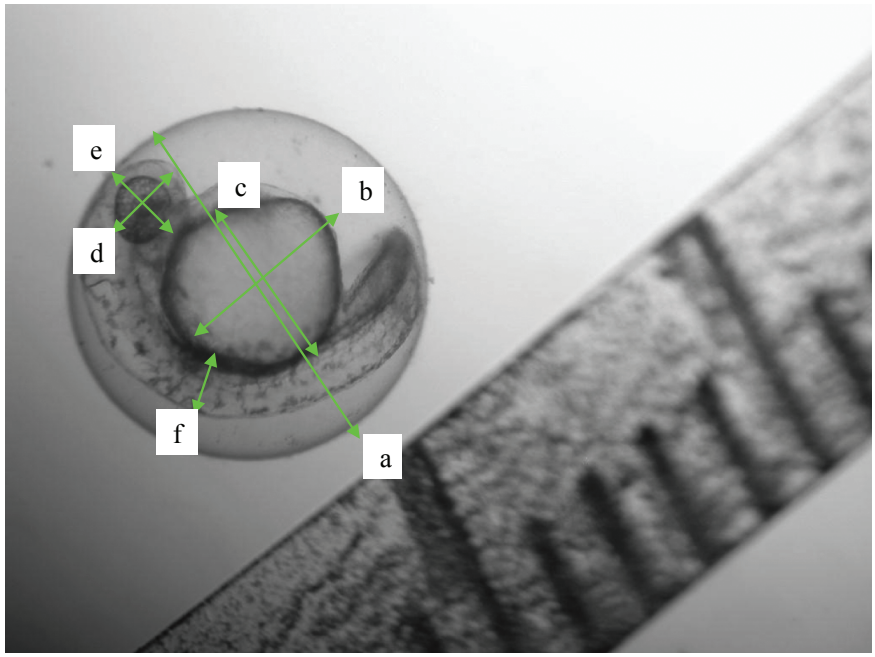

Figure 2 Photograph of a control *Danio rerio* embryo at 1 day post-fertilization (24 hour post-fertilization). Magnification 40X. a = egg diameter, b = yolk sac length, c = yolk sac width, d = eye length, e = eye width and f = body height.

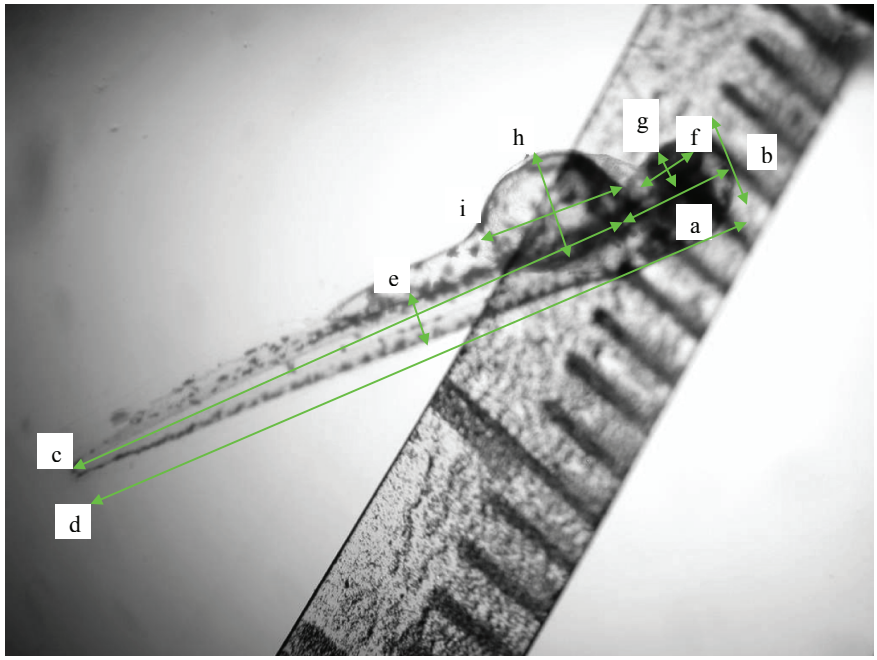

Figure 3 Photograph of a control Danio rerio larva at 2 day post-fertilization (48 hour post-fertilization). Magnification 40X. a = head length, b = head width, c = body length, d = total length, e = body width, f = eye length, g = eye width, h = yolk sac length and i = yolk sac width.

The data collected for heart rate was conducted *in situ* and was calculated by using the formula below:

$60/y \times 20 = w$ *beats/min*

60 = 60 second

y = the reading in seconds

20 = 20 beats

Data analysis

All data was tabulated into Excel 2000 and statistical analyses were performed by using SigmaStat (Systat Software, Inc). Two-way ANOVA analysis was carried out to compare all the treatment groups for three variables (e.g. treatments, time and measurements). If there was a significant difference ($p<0.05$) in the Two-way ANOVA test, the Holm-Sidak method were performed to compare differences between any two groups. Kruskal-Wallis Analysis of Variance on Ranks was conducted when there were non-normal populations or when the data did not have equal variances. One-way ANOVA analysis was carried out to compare all the treatment groups for two variables e.g. treatments and measurements. The Student t-test, Dunn's test and Tukey test were also performed to compare any two groups. Values represented are means and ± represent the standard error of the means.

Results and Discussions

Hatching Success

Exposure to BMAA concentrations at 24 hour post-fertilization (hpf) have delayed hatching and early mortality (Table 1). Harvey and Chamberlain (1982) and Adams *et al*. (2005) reported higher membrane permeability in *Danio rerio* embryos at later stages of development (early high blastula and one-half epiboly). In the present study, the hatching process was delayed by one day in *Danio rerio* embryos exposed to BMAA concentrations at 24 hpf (50 and 500 µg L^{-1}) compared to the control and 5 hpf groups. This showed that the later exposure (24 hpf) of *Danio rerio* embryos to BMAA was sensitive in influencing the time of hatching which agreed to Harvey and Chamberlain (1982) and Adams *et al*. (2005). This finding suggested that the BMAA can easily enter the chorion.

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Table 1 Time to 50% hatch and mortality for controls; 5, 50, 500, 5000 and 50000 μg L^{-1} of BMAA

Treatments	5 hpf		24 hpf	
	Time to 50% hatch (dpf)	Time to 50% mortality (dpf)	Time to 50% hatch (dpf)	Time to 50% mortality (dpf)
Control	2	12 - 13	2 - 3	12
5	2	12	2 - 3	11 - 12
50	2	13	3	12
500	2	12	3	12
5000	2	12	2 - 3	11 - 12
50000	2	12	2 - 3	12

hpf = hour post-fertilization dpf = day post-fertilization

Development

Over the measurement period, the eye length measured for the control group for the 24 hpf series were 0.044 ± 0.002 cm at 1 dpf and 0.043 ± 0.001 cm at 2 dpf (Figure 4). All treated groups showed a significant increase in eye length at 2 dpf compared to the control group.

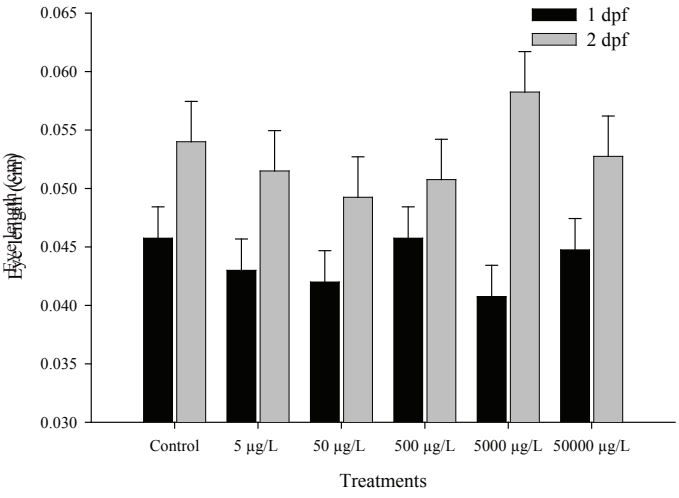
Eye length and eye width of the embryos treated to all BMAA concentrations experienced a significant increase at 2 dpf, compared to the control group. It is probable that the embryos in this present study showed retinal lesions as reported by Zeevalk and Nicklas (1989) in chicks. No further explanation is possible until investigations have been conducted.

It can be concluded that higher concentrations of BMAA caused the increases in egg diameter, yolk sac length and width, and eye length and width of *Danio rerio* embryos. Later developmental stages of embryos were more sensitive to BMAA. It would be interesting to investigate how this neurotoxin can affect the embryos' developmental processes if exposure started a few hours before hatching.

Heart Rate

The heart rate response to BMAA was measured in *Danio rerio* embryos (at 1 dpf) and larvae (from 2 to 7dpf). All treated group were compared to the control group. The 500, 5000 and 50000 μg L^{-1} BMAA treated groups showed a significant increase at 1 dpf and the 50 μg L^{-1} BMAA group showed a significant increase at 2 dpf. All treatment groups showed a significant increase at 3, 4 and 5 dpf but the 5 and 50 μg L^{-1} BMAA groups showed a significant decrease at 6 dpf. Only the 5000 μg L^{-1} BMAA group showed a significant increase at 7 dpf. Over the measurement period, all treatments showed a significant increase in heart rate compared to the controls (Figure 5).

Graph A



Graph B

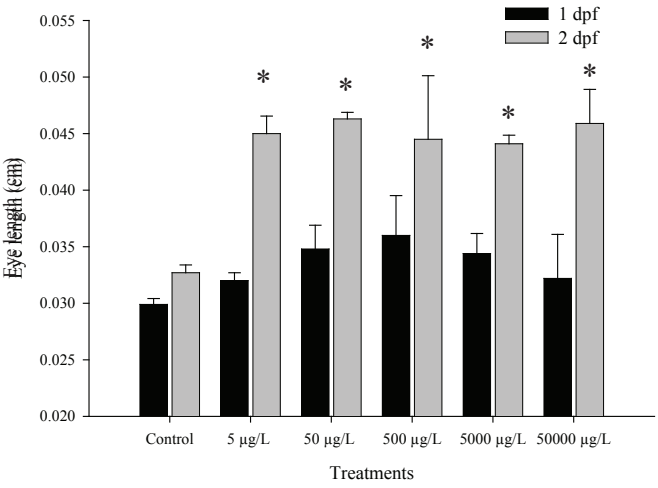


Figure 4 Eye length (cm) results of *Danio rerio* (zebrafish) embryos for controls and BMAA concentrations: 5, 50, 500, 5000 and 50000 µg L^{-1}. Embryos were exposed to the above concentrations at 5 hpf (Graph A) and 24 hpf (Graph B) until hatching. After hatching the exposures were continued. Measurements were taken at 0 dpf (8 hpf), 1 dpf (24 hpf) and 2 dpf (48 hpf) in Graph A, and 0 dpf (8 hpf), 1 dpf (28 hpf) and 2 dpf (48 hpf) in Graph B. Values significantly different from the control group at 2 dpf are indicated by (*) for each measurement day at $p<0.05$ (Two-way ANOVA, Holm–Sidak method).

- *in Graph A, 0 dpf =5 hpf*
- *in Graph B, exposure started at 1 dpf*

There are several reasons for these results. The BMAA might have dilated the blood vessels and increased the blood pressure and the heart rate. After placing the larvae in the de-chlorinated water, vasoconstriction might have taken place and resulted in decreased blood pressure and heart rate to the normal level as in the controls. To confirm this, further work should be carried out to investigate the effect of BMAA on the blood vessels.

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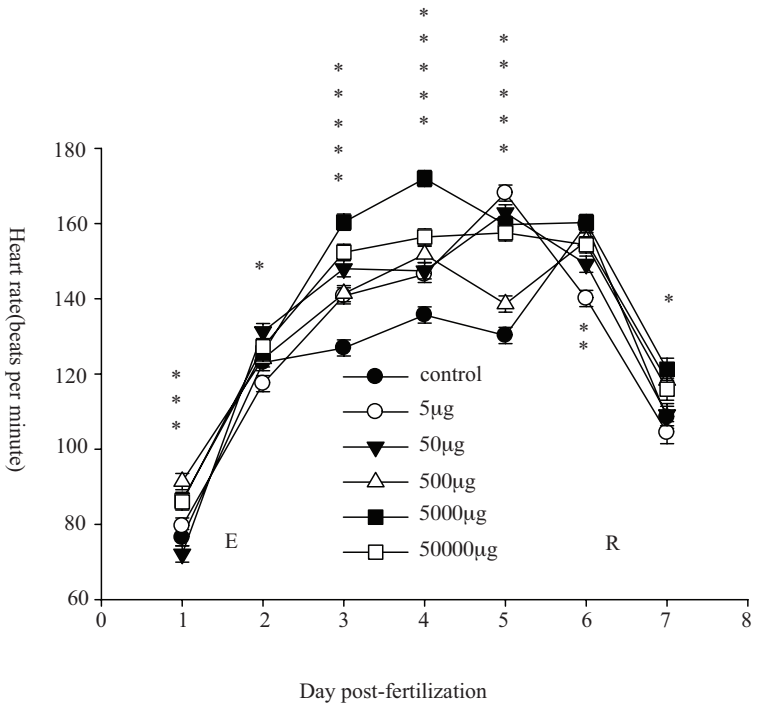

Figure 5 Heart rate (beats per min) in *Danio rerio* (zebrafish) embryos and larvae in response to different concentrations of BMAA exposure at 5 hpf to 96 hpf. The embryos and larvae were exposed to the concentrations immediately until 4 dpf. After that stage the exposures were discontinued and the larvae were placed in de-chlorinated water. Measurements were taken at 1 (embryo), 2, 3, 4, 5, 6 and 7 dpf (n=13 for every measurement taken each time); (T= 26.5 ± 1.0ºC). Values represented are means, vertical bars represent the standard error of the means (S. E. M). Values significantly different from the control group are indicated by (*) for each measurement day at $p<0.05$ (Two-way ANOVA, Holm-Sidak method).

E= exposure period; R=recovery state
Significant different (*) at 1 dpf = 500, 5000 and 50000 µg L^{-1} BMAA
Significant different (*) at 2 dpf = 50 µg L^{-1} BMAA
Significant different (*) at 3, 4, 5 dpf = 5, 50, 500, 5000 and 50000 µg L^{-1} BMAA
Significant different (*) at 6 dpf = 5 and 50 µg L^{-1} BMAA
Significant different (*) at 7 dpf = 5000 µg L^{-1} BMAA

Abnormalities

All BMAA concentrations tested showed effects on *Danio rerio* early development. Table 2 represents the abnormalities in response to the BMAA exposure. Abnormalities were observed as early as 1 dpf.

Table 2 Abnormalities observed in embryos and larvae exposed to BMAA concentrations

Treatment (BMAA)	Oedema	Helical Body	Tail deformities	Bend body	Flat face	Eye deficiency	SP
Control							
5 µg L^{-1}	√(3)	√(2)					
50 µg L^{-1}	√(5)	√(5)	√(3 curl)	√(3)			Active
500 µg L^{-1}	√(7)	√(6)	√(2)	√(4)		√(3)	Very active (4)
5000 µg L^{-1}	√(8)	√(7)	√(2)	√(4)	√(3)	√(4)	Very active (6)
50000 µg L^{-1}	√(6)			√(2)			

SP= swimming performance
Numbers in brackets represent numbers of observation

Oedema was observed in all treatment groups. This might be caused by the failure of circulatory function especially the heart, cardiovascular malfunction (Guiney *et al*. 1990); due to accumulation of blood and body fluid. Such deformities as helical body, tail deformities and bent body might be the end result of neuronal vacuolation and death in spinal cord cells as investigated by Nunn *et al.* (1987). Neuronal degeneration might have taken place as reported earlier in other systems (Ross and Spencer, 1987, Ross *et al.,* 1987, Spencer *et al.,* 1987, Weiss and Choi, 1988, Weiss *et al.* 1989, Zeevalk and Nicklas, 1989). The larvae might have suffered immobility which would agree with Dawson *et al.* (1998) who reported permanent developmental damage to motor function and spinal cord neurochemistry in rats. Larvae with these abnormalities might have abnormal movements which is in agreement with Seawright *et al.* (1990). The flat head feature might have been caused by neuronal cell death in the cerebellum as reported by Seawright *et al.* (1990) who investigated the effect of BMAA in young rats.

There are few previous studies on the effects of BMAA on animals where the available information is not enough to relate to the findings in the present study. Purdie *et al.* (2009) reported that BMAA may contribute to motor neuron degeneration. There might be a synergistic effect of BMAA with the addition of carbonate which induces a wide range of developmental and neuro-muscular abnormalities. Finally, it can be concluded that the BMAA shows various adverse effects on *Danio rerio* embryos and larvae developmental process and heart rate. More investigations on organ, cell and molecular level should be performed to explain the above effects.

Conclusion

This was a preliminary study on the effect of purified BMAA on fish, and in particular on the *Danio rerio*. A range of BMAA concentrations from 5 to 50000m ng L^{-1} were tested on *Danio rerio* embryos and larvae. BMAA did not influence the hatching process but higher concentrations of BMAA increased growth development and cause morphological aberrations in *Danio rerio* embryos and larvae. The heart rate has increased in all BMAA concentrations during the exposure and took about 48 hour to recover. BMAA treatments produced abnormalities in *Danio rerio* larvae and the 5000m ng L^{-1} treatment produced all seven listed aberrations.

References

Adams, S.L, Zhang, T. and Rawson, D. M. (2005). The effect of external medium composition on membrane water permeability of Zebrafish (*Danio rerio*) embryos. *Theriogenology,* 64(7), 1591-1602.

Banack, S.A. and Cox, P.A. (2003). Biomagnification of cycad neurotoxins in flying foxes - Implications for ALS-PDC in Guam. *Neurology,* 61(3), 387-389.

Carriedo, S.G., Yin, A. and Weiss, J.H. (1996). Motor neurons are selectively vulnerable to AMPA/ Kainate receptor-mediated injury in vitro. *J. Neuroscience,* 16,4069-4079.

Cox, P.A. and Sacks, O.W. (2002). Cycad neurotoxins, consumption of flying foxes, and ALS-PDC disease in Guam. *Neurology,* 58(6), 956-959.

Cox, P.A., Banack, S.A. and Murch, S.J. (2003). Biomagnification of cyanobacterial neurotoxins and neurodegenerative disease among the Chamorro people of Guam. *Proc. Nat. Ac. Sc. U.S.A.,*100(23), 13380-13383.

Cox, P.A., Banack, S.A. Murch, S.J. Rasmussen, U., Tien, G., Bidigare, R.R., Metcalfe, J.S., Morrison, L.F., Codd, G.A. and Bergmann, B. (2005). Diverse taxa of cyanobacteria produce β-*N*-methylamino-L-alanine, a neurotoxic amino acid. *Proc. Nat. Ac. Sc. U.S.A.,* 102, 5074-5078.

Dawson, R., Marschall, E.G., Chan, K.C., Millard, W.J., Eppler, B. and Patterson, T.A. (1998). Neurochemical and neurobehavioral effects of neonatal administration of beta-N-methylamino-L-alanine and 3,3 '-iminodipropionitrile. *Neurotoxicology And Teratology,* 20(2), 181-192.

Guiney, P.D., Walker, M.K. and Peterson, R.E. (1990). The edema in TCDD-exposed lake trout sac fry is an ultrafiltrate of blood. Abstracts. Society of Environmental Toxicology and Chemistry Meeting, Arlington, VA, USA.

Harvey, B., and Chamberlain, B. (1982). Water permeability in the developing embryo of the Danio rerio, *Brachydanio rerio*. *Can. J. Zool*, 60, 268-270.

Murch, S.J., Cox P.A. and Banack, S.A. (2004a). A mechanism for slow release of biomagnified cyanobacterial neurotoxins and neurodegenerative disease in Guam. *Proc. Nat. Ac. Sci. U.S.A.* 101: 12228-12231.

Murch, S.J., Cox P.A., Banack, S.A, Steele, J.C. and Sacks, O.W. (2004b). Occurrence of β-N-methylamino-L-alanine (BMAA) in ALS/PDC patients from Guam. *Acta Neurologica Scandinavica* 110: 267-269.

Nunn, P.B., Seelig, M., Zagoren J.C. and Spencer, P.S. (1987). Stereospecific acute neuronotoxicity of uncommon plant amino-acids linked to human motor-system diseases. *Brain Research* 410(2): 375-379.

Purdie, E.L, Samsudin, S., Eddy, F.B. and G.A. Codd. (2009). Effects of the cyanobacterial neurotoxin β-*N*-methylamino-L-alanine, on the early-life stage development of Zebrafish (*Danio rerio*). *Aquatic Toxicology*, doi:10.1016/j.aquatox.2009.02.009.

Ross, S.M. and Spencer, P.S. (1987). Specific antagonism of behavioural action of uncommon amino-acids linked to motor-system diseases. *Synapse,* 1(3), 248-253.

Ross, S.M, Seelig, M. and Spencer, P.S. (1987). Specific antagonism of excitotoxic action of uncommon amino-acids assayed in organotypic mouse cortical cultures. *Brain Research,* 425(1), 120-127.

Seawright, A.A, Brown, A.W, Nolan, C.C. and Cavanagh, J.B. (1990). Selective degeneration of cerebellar cortical-neurons caused by cycad neurotoxin, L-beta-methylaminoalanine (L-BMAA), in rats. *Neuropathol. Appl. Neurobiol*, 16(2), 153-169.

Spencer, P.S., Nunn, P., Hugon, J. Ludolph, A.C. and Roy, D.N. (1986). Motor-neuron disease on Guam - Possible role of a food neurotoxin. *Lancet,* 1(8487), 965-965.

Spencer, P.S., Nunn, P., Hugon, J. Ludolph, A.C., Ross, S.M., Roy, D.N. and Robertson, R.C. (1987). Guam amyotrophic lateral sclerosis-parkinsonism-dementia linked to a plant excitant neurotoxin. *Science,* 237, 517-522.

Vega, A. and Bell, E.A. (1967). α-Amino-β-methyl-aminopropionic acid, a new amino acid from seeds of *Cycas circinalis*. *Phytochemistry,* 6, 759-762.

Vessey, J.K., Pawlowski, K. and Bergman, B. (2005). Root-based N-2-fixing symbioses: Legumes, actinorhizal plants, *Parasponia* sp and cycads *Plant and Soil,* 266, 205-230.

Weiss, J.H. and Choi, D.W. (1988). Beta-N-methylamino-l-alanine neurotoxicity - requirement for bicarbonate as a cofactor. *Science,* 241(4868), 973-975.

Weiss, J.H, Koh, J.and Choi, D.W. (1989). Neurotoxicity of β-*N*-methylamino-L-alanine (BMAA) and β-*N*-oxalylamino-L-alanine (BOAA) on cultured cortical neurons. *Brain Research,* 497, 64-71.

Zeevalk, G.D. and Nicklas, W.J. (1989). Acute excitotoxicity in chick retina caused by the unusual amino-acids BOAA and BMAA - effects of MK-801 and kynurenate. Neuroscience Letters 102(2-3), 284-290.