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Miquel Lüring, Elisabeth Faassen, Justine van Eenennaam

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Effects of the cyanobacterial neurotoxin β -N-methylamino-L-alanine (BMAA) on the survival, mobility and reproduction of *Daphnia magna*

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10 *Daphnia magna*
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12 Miquel Lüring*, Elisabeth J. Faassen and Justine S. van Eenennaam
13
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16
17 Aquatic Ecology & Water Quality Management, Department of Environmental
18 Sciences, Wageningen University and Research Centre, PO Box 47, 6700 AA
19
20
21
22 Wageningen, The Netherlands
23
24
25
26
27
28
29
30
31

32 *Corresponding author
33

34 Email: miquel.lurling@wur.nl
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ABSTRACT

In short-term tests and chronic life-table assays *Daphnia magna* was exposed to the cyanobacterial neurotoxic non-protein amino acid β -*N*-methylamino-L-alanine (BMAA). BMAA was not acutely lethal to *Daphnia* ($LC_{50-48h} > 10000 \mu\text{g L}^{-1}$), but reduced mobility ($IC_{50-48h} 40 \mu\text{g L}^{-1}$) and affected life-history characteristics. Animals showed a tendency to later age at first reproduction, larger size at first reproduction, smaller clutch size and lower population growth rates with higher concentrations of BMAA. Animals that had been kept in either food-free medium or were fed the green alga *Scenedemus obliquus* with BMAA accumulated BMAA in their tissue. The highest measured bioconcentration factors were 275 in adult *D. magna* and 3821 in their neonates. This bioconcentration of the neurotoxic BMAA in *D. magna* suggests that these animals may be an important vector of BMAA in the pelagic food web.

INTRODUCTION

Pelagic cyanobacteria are ubiquitous inhabitants of freshwater systems and well-known for their ability to produce a variety of toxic secondary metabolites, cyanotoxins (Sivonen, 1996; Wiegand and Pflugmacher, 2005; Funari and Testai, 2008). Hundreds of these compounds, mostly peptides and alkaloids, have been structurally identified, but their physiological functions remain unknown (Dietrich and Hoeger, 2005).

Recently, a novel cyanotoxin, the non-protein amino acid β -*N*-methylamino-L-alanine (BMAA) was identified in cultures of the genus *Nostoc*, isolated from coralloid roots of the cycad *Cycas micronesia* (Cox *et al.*, 2003). Subsequent analysis revealed that BMAA production is not restricted to symbiotic *Nostoc*, but occurred in about 95% of free-living cyanobacterial strains from different origins and all five taxonomic sections (Cox *et al.*, 2005; Esterhuizen and Downing, 2008). Although the results of these first studies could not be reproduced by some other groups and the analytical techniques used are under discussion (Rosén and Hellenäs, 2008, Krüger *et al.*, 2010), BMAA has recently been detected in cyanobacteria by highly specific analytical techniques, albeit in lower concentrations than reported by Cox *et al.* (2005) and Esterhuizen and Downing (2008) (Faassen *et al.*, 2009; Jonasson *et al.*, 2010; Spáčil *et al.*, 2010).

Since BMAA has been linked to the human neurodegenerative disease amyotrophic lateral sclerosis–parkinsonism dementia complex (ALS/PDC), the role of BMAA in this disease has been studied over the last years (*reviewed in* Bradley and Mash, 2009). However, the effect of BMAA on organisms that are in direct contact with cyanobacteria, such as the suspension-feeding zooplankton species *Daphnia*, has not yet been studied.

1
2
3 Suspension-feeding zooplankton is often an important component of lake and
4 pond ecosystems. These key organisms form a major link between limnetic primary
5 production and higher trophic levels and therefore play an important role in the
6 trophic energy flow (McQueen *et al.*, 1986). When cyanobacteria appear however,
7 large-bodied *Daphnia* may decline and the cyanobacteria develop dense blooms
8 (Edmondson and Litt, 1982).
9

10
11 An explanation for the decrease of *Daphnia* in the presence of cyanobacteria
12 may be that cyanobacteria are in general poor food for *Daphnia* (Wilson *et al.*, 2006;
13 Tillmanns *et al.*, 2008). Several causal factors have been proposed, such as lack of
14 essential compounds, a hard-to-handle morphology and the presence of toxic
15 compounds. Traditionally, microcystins have received considerable interest, but these
16 cyanotoxins seem not to be the sole cause of cyanobacteria being a poor food, since
17 the growth of *Daphnia* is not necessarily significantly different with diets of non-
18 microcystin producing cyanobacteria compared to microcystin producing ones
19 (Wilson *et al.*, 2006; Tillmanns *et al.*, 2008). Studies in which morphology and
20 biochemical deficiencies could be eliminated support the hypothesis that yet unknown
21 cyanotoxins might be present in the cyanobacterial matrix inhibiting *Daphnia* growth
22 (Jungmann, 1992; 1995; Lürling, 2003a,b). These compounds might be peptides, but
23 might also belong to the family of non-protein amino acids. In fact, the structural
24 isomer of BMAA, 2,4-diaminobutyric acid (2,4-DAB) has been detected in a
25 cyanobacterial matrix (Rosén and Hellenäs, 2008; Faassen *et al.*, 2009). Because of
26 the many potential factors acting simultaneously on zooplankton grazers, we decided
27 not to use a cyanobacterium as transport vehicle of BMAA at this stage. Moreover,
28 upon bloom senescence, BMAA, like other cyanotoxins, may be released into the
29 surrounding environment. Although no data are available at this point on dissolved
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3 BMAA concentrations *in situ*, we assumed that BMAA could also be present in
4
5 dissolved form in surface waters, as has been found for microcystins that may be
6
7 present in dissolved form for months at concentrations between 3 – 100 $\mu\text{g L}^{-1}$
8
9 (Gurbuz *et al.*, 2009). Since *Daphnia* can take up dissolved organic carbon directly
10
11 from the water (Speas and Duffy, 1998), we expected uptake of dissolved BMAA as
12
13 well.
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17 As non-protein amino acids are classical anti-metabolites inhibiting selected
18
19 reactions in the primary metabolism of predators and herbivores (Fowden *et al.*, 1967;
20
21 Bell, 2003; Zenk and Juenger, 2007), we hypothesize that BMAA inhibits the grazer
22
23 *Daphnia*. Because one of the mechanisms of toxicity is the incorporation of non-
24
25 protein amino acids in the proteins of predators (Crine and Lemieux, 1982; Rosenthal,
26
27 2001), we also expect that *Daphnia* accumulates BMAA and thereby transports
28
29 BMAA along the pelagic food chain. To test our hypotheses, we studied the effect of
30
31 synthetic BMAA on survival, mobility, growth and reproduction of *Daphnia* in short-
32
33 term tests and life-history assays. In addition, we analyzed concentrations of BMAA
34
35 in medium and animals, using a highly selective LC-MSMS technique (Faassen *et al.*,
36
37 2009).
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46 **METHOD**

47 ***Daphnia* culture**

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49 The cladoceran *Daphnia magna* Straus was isolated from Lake Zwemlust (The
50
51 Netherlands) in 1999 and has been cultured in the laboratory in 1 liter jars containing
52
53 800 mL artificial RT-medium with a pH of 7.6, a conductivity of 270 $\mu\text{S cm}^{-1}$ and a
54
55 total hardness of 88 mg $\text{CaCO}_3 \text{ L}^{-1}$ (Tollrian, 1993). Jars with *D. magna* were kept
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3 without agitation at 20° C in low light of about 15 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ with a 14:10
4
5 hours light-dark cycle. The animals were fed three times a week with the green alga
6
7 *Scenedesmus obliquus* (about 4 mg C L⁻¹), which was grown at 20 °C in a 1-L
8
9 chemostat on a modified WC medium (Lürling and Beekman, 2006) in continuous
10
11 light (~100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) and with a dilution rate of 1.0 d⁻¹. The *S. obliquus*
12
13 population was dominated by single cells (linear dimensions 16 x 5 μm) with some
14
15 two- and four-celled colonies.
16
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18

19
20 Prior to the experiments, a new isofemale lineage was started. One juvenile *D.*
21
22 *magna* was placed in a 125 mL glass-tube with 100 mL fresh RT-medium and ~5 mg
23
24 C L⁻¹ *S. obliquus*. Every two/three days medium and food were refreshed. The
25
26 neonates of the first brood were discarded, the neonates of the second brood were
27
28 collected and placed individually in 125 mL glass-tubes with 100 mL RT-medium and
29
30 ~5 mg C L⁻¹ *S. obliquus*. Every two/three days medium and food were refreshed.
31
32 From these second generation animals, first brood neonates were again discarded and
33
34 ten neonates of the second brood were transferred to 1 L jars with 800 mL artificial
35
36 RT-medium and ~5 mg C L⁻¹ *S. obliquus*. Again every two/three days medium and
37
38 food were refreshed. From these third generation animals, only the adults were
39
40 transferred to fresh medium, all neonates born on the same day were transferred to
41
42 separate jars to avoid crowding effects. Before each experiment, neonates (<24 h) or
43
44 juveniles (>48 h and <72 h) born on the same day were collected from the jars and
45
46 treated as described below.
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54

55 **Short-term toxicity experiment**

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57 Since no data on the effects of BMAA on *D. magna* were available, a short-term
58
59 experiment was performed where neonate *D. magna* were exposed for 48 hours to
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1
2
3 different concentrations of dissolved BMAA. Immobilization and death were chosen
4
5 as endpoints.
6

7
8 BMAA (BMAA Hydrochloride, Sigma-Aldrich) was dissolved in RT-medium at
9
10 concentrations of 0, 1, 10, 100, 1000 and 10000 $\mu\text{g L}^{-1}$. The experiment was
11
12 performed in plastic 190 mL jars with 100 mL RT-medium. Each jar received 10
13
14 neonate (< 24 h) *D. magna* and for each treatment four jars were used. The jars were
15
16 placed in a climate controlled chamber in constant light of 30 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$
17
18 and a constant temperature of 20°C. Animals were not fed during the experiment.
19
20 After 48 h the number of living, dead and immobile (not swimming, but with heart
21
22 beat) *D. magna* were recorded in each jar. The BMAA concentration at which 50% of
23
24 the *D. magna* had died (LC₅₀) or were immobilized (IC₅₀) was determined by non-
25
26 linear regression (4 parameter logistic model) in the tool pack SigmaPlot 11.0.
27
28 BMAA concentration in the medium was checked by collecting 20 mL medium from
29
30 each jar at the end of the experiment and transferring the medium to a glass-bottle.
31
32 Bottles were stored at -20°C until BMAA analysis (*see section* BMAA analysis). The
33
34 stability of BMAA in solution was checked by sampling an additional jar without *D.*
35
36 *magna*, but with a BMAA concentration of 10000 $\mu\text{g L}^{-1}$ at the start and end of the
37
38 experiment.
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49 **Short term bioconcentration experiment**

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51 The short term toxicity experiment was repeated to determine the bioconcentration of
52
53 BMAA in *D. magna* during short term exposure without food. This experiment was
54
55 conducted in the same way as the short term toxicity experiment, only older animals
56
57 (> 48 h and < 72 h) were used and exposure time was extended to 96 h. The endpoint
58
59 of this experiment was BMAA concentration per amount of wet weight (WW) and dry
60

1
2
3 weight (DW). Dry weight and BMAA concentration were determined as described for
4
5 the life-history experiment (*see section* Life-history experiment), animals were pooled
6
7 per treatment for the BMAA analysis (*see section* BMAA analysis).
8
9

10 11 12 **Life-history experiment**

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14
15 Long-term effects of sub lethal BMAA concentrations on *D. magna* were tested in an
16
17 experiment where neonate *D. magna* were exposed for 15 days to different
18
19 concentrations of dissolved BMAA. Somatic growth and population growth were
20
21 chosen as endpoints, and levels of BMAA in the animals were analyzed.
22
23

24
25 Neonates (< 24 h) were selected and placed individually in 15 mL glass-tubes
26
27 containing 10 mL of RT-medium with a *S. obliquus* concentration of $\sim 5 \text{ mg C L}^{-1}$.
28

29
30 BMAA was dissolved in the RT-medium in the concentrations 0, 1, 10, 50 and $100 \mu\text{g}$
31
32 L^{-1} ; each treatment was replicated ten times. The glass-tubes were incubated in a
33
34 temperature-controlled incubator at 22°C in darkness. The animals were transferred
35
36 daily to new tubes with fresh medium containing $\sim 5 \text{ mg C L}^{-1}$ *S. obliquus* and the
37
38 assigned BMAA concentration. Before the animals were transferred into these new
39
40 tubes their body length was measured under a stereo-binocular microscope. Body
41
42 length is defined as the distance from the most posterior point on the eye to the base
43
44 of the junction of the tail spine with the carapace. The number of survivors, time of
45
46 reproduction, and number of neonates were also recorded daily. The neonates were
47
48 counted and then discarded, except for the last brood. The experiment ran until the
49
50 control animals had reached their third broods. The mother animals as well as the
51
52 neonates were then rinsed with RT-medium and placed separated from each other for
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54 3-4 hours in clean tubes with fresh RT-medium. After wet weight determination,
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60 animals were transferred into pre-weighed aluminum boats in groups and were dried

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3 for 24 h at 55°C. After drying, dry weight was determined on a Sartorius MC5
4
5
6 microbalance ($\pm 1 \mu\text{g}$). After weighing, dried animals were pooled per treatment, but
7
8 juveniles and adults were kept separate from each other. These groups of animals
9
10 were stored at -20°C until BMAA-analysis (*see section BMAA-analysis*).

11
12 To assess the weight and length of the animals at the start of the experiment, twenty
13
14 neonates that would not be used in the experiment were collected at the same time as
15
16 the neonates that were used in the experiment. Body-length was measured for each of
17
18 these twenty animals and animals were pooled in five groups of four animals for the
19
20 dry weight determination.
21
22

23
24 At the end of the experiment, the increase in body-length over time (somatic growth)
25
26 was statistically evaluated and population growth rates were calculated. Somatic
27
28 growth was evaluated statistically by a repeated-measures ANOVA, which was
29
30 followed by a Tukey's post-hoc comparison in the program PASW Statistics 17
31
32 (SPSS Inc.).
33

34
35 Population growth rates, or the instantaneous rates of population increase (r) were
36
37 estimated from the abbreviated life-tables including the first two broods using the
38
39

40
41 equation: $r \cong \frac{\ln \sum_{x=0}^{\infty} l_x m_x}{T}$, where r = rate of population increase (d^{-1}), x = age class
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46
47 ($0 \dots N$), l_x = probability of surviving to age x , m_x = fecundity at age x , and T the
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49 generation time (d). The life-history characteristics age at first reproduction (AFR, d),
50
51 size at first reproduction (SFR, d) and total number of offspring per female, as well as
52
53 the dry-weight (DW, μg) of the experimental animals at the end of the experiment
54
55 were analyzed by one-way ANOVAs (PASW Statistics 17). Means that were
56
57 significantly different were distinguished by a Tukey post-hoc comparison test ($p <$
58
59 0.05).
60

Growth experiment with *S. obliquus*

S. obliquus served as food in the life history experiment and was therefore also exposed to different concentrations of BMAA. Because it was unknown whether BMAA has a negative effect on the survival and growth of *S. obliquus* and therefore on the food availability of *D. magna*, we exposed *S. obliquus* to different concentrations of BMAA and measured different growth parameters.

S. obliquus was inoculated to a concentration of 5 mg C L⁻¹ in 300 mL Erlenmeyer flasks that contained 150 mL sterile WC-medium. BMAA was added to the flasks at concentrations of 0, 1, 10, 100, 1000 and 10000 µg L⁻¹. The flasks were closed with a cellulose plug and the experiment was run in triplicate for 72 hours in a climate-controlled incubator at 22°C, in continuous light of 150 µmol photons m⁻² s⁻¹ and continuous orbital shaking at 80 rpm. Every 24 hours cell densities (# mL⁻¹) and biovolumes (µm³ mL⁻¹) were measured with a cell-counter system (Innovatis Casy® model TT), while chlorophyll-*a* concentrations (µg L⁻¹) and Photosystem II efficiencies were determined using a PHYTO-PAM phytoplankton analyzer (Walz). Growth rates (µ, d⁻¹) were estimated using linear regression over natural logarithm transformed particle, biovolume and chlorophyll-*a* concentrations against time. Growth rates were statistically compared in a one-way ANOVA (PASW Statistics 17).

BMAA analysis

BMAA concentrations were analyzed as described in Faassen *et al.* (2009) with slight adaptations. BMAA was analysed by LC-MSMS using an Agilent 1200 LC and an Agilent G6410A QQQ. Chromatography was performed on a 2.1 × 150 mm, 5 µm diameter ZIC®-HILIC column (SeQuant, Sweden) with a Direct-Connect™ Filter

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2
3 (Grace Alltech). The mobile phase consisted of acetonitrile with 0.1 % formic acid
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5 (v:v, eluent A) and nanopure water with 0.1% formic acid (v/v, eluent B). The elution
6
7 program was 0 to 2 min: 95% A, 4 min: 65% A, 8 to 17 min 55% A, 17 to 23 min
8
9 95% A. HPLC flow was 0.4 mL min⁻¹, injection volume 5 µL and column temperature
10
11 40 °C. The LC-MSMS was operated in positive mode with an ESI source. Nitrogen
12
13 was used as the drying and the collision gas. Both quadrupoles were operated in unit
14
15 mode, fragmentor voltage was 50 V. In MRM mode, quadrupole 1 selected ions with
16
17 a mass-to-charge ratio (*m/z*) of 119 and quadrupole 2 recorded the abundance of the
18
19 following product ions: *m/z* 102 at 4 V collision energy and *m/z* 76 and 88 at 8 V.
20
21 Calibration standards of BMAA were prepared on the day of analysis.
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23 Samples of RT-medium were analyzed without pretreatment. For these measurements,
24
25 calibration standards of BMAA were dissolved in RT-medium.
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32 *D. magna* samples were hydrolysed before analysis in an Eldex
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34 Hydrolysis/Derivatization WorkStation. Hydrolysis was performed in 6 N HCl vapour
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36 at 105 °C for 20 h in the absence of oxygen. After hydrolysis, samples were dried
37
38 down and reconstituted in 300 µl acetonitrile / nanopure water / formic acid (v/v
39
40 65:35:0.1) and were measured on the LC-MSMS. Calibration standards of BMAA for
41
42 these measurements were also dissolved in acetonitrile / nanopure water / formic acid
43
44 in the same ratio. The limit of BMAA detection in hydrolyzed *Daphnia* samples is 5
45
46 µg L⁻¹; the limit of quantification (LOQ) is 15 µg L⁻¹. Recovery of this method,
47
48 determined by spiking unexposed *D. magna* prior to hydrolysis, was 40%.
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52
53 The bioconcentration factor (BCF) of BMAA in the animals was calculated
54
55 according to Walker *et al.* (2001): $BCF = \frac{[organism]}{[medium]}$
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3 in which values higher than 1 indicate that the compound is bioconcentrated in the
4
5 organism. BCF was expressed per unit WW and DW ($\mu\text{g g}^{-1}$).
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10 RESULTS

11 Short-term toxicity experiment

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17 The short-term exposure (48 h) to BMAA had a significant effect on the activity of *D.*
18
19 *magna*. The number of swimming animals in the 0 and 1 $\mu\text{g BMAA L}^{-1}$ treatments
20
21 was significantly higher than in the treatments with 10 – 10000 $\mu\text{g BMAA L}^{-1}$ (Fig. 1;
22
23 Tukey test following one-way ANOVA; $F_{5,18} = 23.1$; $p < 0.001$).
24
25

26
27 The BMAA concentration at which the number of swimming animals had been
28
29 reduced to 50% was estimated at 40 $\mu\text{g BMAA L}^{-1}$ ($r^2_{\text{adj}} = 0.798$). In parallel, the
30
31 number of immobile animals increased significantly ($\chi^2 = 17.4$; $p = 0.004$) with
32
33 increasing concentrations of BMAA (Fig. 1). Mortality also increased with higher
34
35 concentrations of BMAA reaching a maximum of 45% (SD 17%) at the highest
36
37 BMAA concentration (Fig. 1). Hence, the $\text{LC}_{50-48\text{h}}$ is higher than 10000 $\mu\text{g BMAA L}^{-1}$.
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42 BMAA remained stable in RT-medium during the experiment; the levels in the
43
44 control jar did not decrease. BMAA levels in the exposure media with 0 and 10 μg
45
46 BMAA L^{-1} were below level of quantification ($\sim 15 \mu\text{g L}^{-1}$), while the mean
47
48 concentration in the 100 $\mu\text{g BMAA L}^{-1}$ treatments was 77 (SD 2) $\mu\text{g L}^{-1}$, 804 (SD 6)
49
50 $\mu\text{g L}^{-1}$ in the 1000 $\mu\text{g BMAA L}^{-1}$ treatments and 9932 (SD 85) $\mu\text{g L}^{-1}$ in the 10000 μg
51
52 BMAA L^{-1} .
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59 Short-term bioconcentration experiment

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2
3 During the short-term bioconcentration experiment dose related immobilization and
4 death were observed (data not shown). BMAA was not detected in control animals. In
5
6 contrast, BMAA was detected in *D. magna* that had been exposed to BMAA in
7
8 exposures of 1 to 1000 $\mu\text{g L}^{-1}$, albeit below the level of quantification (sample
9
10 concentration 4.3 – 7.1 $\mu\text{g L}^{-1}$; LOQ = 15 $\mu\text{g L}^{-1}$). BMAA could be quantified in
11
12 animals exposed to 10000 $\mu\text{g BMAA L}^{-1}$ (Table 1). Based on their dry-weights,
13
14 animals exposed to 10000 $\mu\text{g L}^{-1}$ had an averaged 125 times higher concentration of
15
16 BMAA in their tissue than in the surrounding medium (Table 1). When wet-weights
17
18 were used the BCFs was 44 for animals from the 10000 $\mu\text{g L}^{-1}$ treatment (Table 1).
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28 **Life-history experiment**

29
30 Survival in the life-history experiment varied between 70 and 90% (Table 2), the
31
32 majority of deaths were caused by handling the animals. All living animals were
33
34 swimming, no immobility was observed.
35

36
37 *D. magna* showed somatic growth in all treatments. Body-length increased from 0.91
38
39 (± 0.02) mm at the beginning of the experiment to on average 2.75 (± 0.03) mm at the
40
41 end (Fig. 2). Despite the fact that differences in size were minor and between -1% to
42
43 12% in treatments compared to control animals, BMAA had a significant effect on
44
45 body-size (repeated-measure ANOVA $F_{4,35} = 3.83$; $p = 0.011$), where animals
46
47 exposed to 10 and 50 $\mu\text{g BMAA L}^{-1}$ were significantly (Tukey test; $p < 0.05$) larger
48
49 than the controls (Fig. 2). At the end of the experiment the DW of animals from these
50
51 treatments was also higher (Table 2). Animals in the 50 $\mu\text{g BMAA L}^{-1}$ were
52
53 significantly larger than those in the controls (t -test; $t = 5.71$; $p = 0.029$), and the 1 μg
54
55 BMAA L^{-1} treatments ($t = 7.33$; $p = 0.018$), but they were not different from the 10
56
57 and 100 $\mu\text{g BMAA L}^{-1}$ treatments ($p \geq 0.251$).
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1
2
3 In line with the somatic growth over time, animals exposed to BMAA were
4
5 significantly larger at first reproduction than control animals (one-way ANOVA $F_{4,36}$
6
7 $= 3.41$; $p = 0.018$; Table 2), but only control animals and those at $10 \mu\text{g BMAA L}^{-1}$
8
9 were significantly different from each other (Tukey test). Neonates that were
10
11 produced at the end of the experiment were equally heavy and had an average dry-
12
13 weight of 6.7 (SD 0.5) μg per individual
14
15
16

17
18 The mean number of offspring produced per female decreased slightly from
19
20 11.3 to 8.8 with higher concentrations of BMAA, but was not significantly different
21
22 among treatments ($F_{4,36} = 1.08$; $p = 0.382$; Table 2). Age at first reproduction also
23
24 showed a tendency to increase with higher BMAA concentration, but this was not a
25
26 significant increase (one-way ANOVA on natural-log transformed data $F_{4,36} = 1.39$; p
27
28 $= 0.259$; Table 2). Nonetheless, population growth rates decreased gradually and
29
30 linearly from 0.174 d^{-1} to 0.159 d^{-1} with increasing BMAA concentrations (Table 2): r
31
32 $= 0.173 - 1.36 \cdot 10^{-4} \times [\text{BMAA}]$ ($r^2_{\text{adj}} = 0.964$, $F = 109.6$; $p = 0.002$).
33
34
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37
38 BMAA was detected in adults exposed to 50 and $100 \mu\text{g BMAA L}^{-1}$ and their
39
40 neonates, no BMAA was detected in adults and their neonates exposed to 0 , 1 and 10
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42 $\mu\text{g BMAA L}^{-1}$ (Table 3). On a wet weight basis, for adults the BCF was 31 and 51 ,
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44 while it was 1574 and 3327 for their neonates (Table 3). On a dry-weight basis BCFs
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46 were higher: 196 and 275 for adults, and 1808 and 3821 for their neonates. The
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48 neonates, that had been in the medium for less than 24 hours, had a 31 to 108 times
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50 higher BMAA concentration than their parents based on wet weight; based on dry
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52 weight their concentration was 7 to 20 times higher (Table 3).
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59 **Growth experiment with *S. obliquus***

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3 The green alga *S. obliquus* showed good growth in controls and the different BMAA
4 treatments. Chlorophyll-*a* based growth rates were significantly different among
5
6 treatments ($F_{5,12} = 4.75$; $p = 0.013$). Mean growth rates ($\pm 1SD$) at 10000 $\mu\text{g BMAA}$
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8 L^{-1} were 0.92 (0.01) d^{-1} and significantly lower than those in controls, the 100 and
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10 1000 $\mu\text{g BMAA L}^{-1}$ treatments ($0.98 \pm 0.03 \text{ d}^{-1}$). However, differences were small and
11
12 only significant because of low within group variability. Particle-based growth rates
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14 ($0.86 \pm 0.02 \text{ d}^{-1}$) and volume-based growth rates ($0.97 \pm 0.03 \text{ d}^{-1}$) were similar among
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16 controls and treatments ($F_{5,12} = 2.55$; $p = 0.086$ and $F_{5,12} = 1.52$; $p = 0.256$,
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18 respectively). Photosystem II efficiencies were also similar between treatments (0.70
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20 ± 0.01).

21 22 23 24 25 26 27 28 29 30 **DISCUSSION**

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32 Our results show that the neurotoxic non-protein amino acid BMAA is not acutely
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34 lethal to *D. magna* when dissolved in medium. The acute LC_{50} found in this study for
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36 neonate *D. magna* after 48 hours of exposure is higher than 10000 $\mu\text{g BMAA L}^{-1}$. *D.*
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38 *magna* therefore seems to be less sensitive to BMAA than some other aquatic
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40 organisms: fish larvae *Danio rerio* ($\text{LC}_{50} 10 \mu\text{g BMAA L}^{-1}$), brine shrimps *Artemia*
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42 *salina* ($\text{LC}_{50} 1500 - 5000 \mu\text{g BMAA L}^{-1}$) and the ciliate *Nassula sorex* ($\text{LC}_{50} 5000 \mu\text{g}$
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44 BMAA L^{-1}) all showed mortality at lower BMAA concentrations (Purdie *et al.*, 2009).
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47 The fifteen day exposure to lower concentrations in the medium (maximum 100 μg
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49 BMAA L^{-1}) also did not cause death in *D. magna*. Although *D. magna* with a history
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51 of regular exposure to cyanobacteria could be more tolerant to cyanobacterial toxins
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53 than naïve specimens (Gustafson & Hansson, 2004; Sarnelle *et al.*, 2010), our clone
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55 has been in the laboratory for more than 10 years without any exposure to
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57 cyanobacteria making increased resistance unlikely. Nevertheless, it would be
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3 worthwhile quantifying the between and within-species variation in tolerance of
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6 *Daphnia* to BMAA.
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8 While BMAA was not acutely lethal to *D. magna*, it reduced mobility when
9
10 the animals were exposed without food. Behavioral changes have also been reported
11
12 for the fruit-fly *Drosophila* where exposure to BMAA reduced the climbing
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14 performance (Zhou *et al.*, 2009) and for the brine shrimp *A. salina* where
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16 concentrations $\geq 50 \mu\text{g BMAA L}^{-1}$ caused a loss of the group swarm response and
17
18 loss of phototaxis (Purdie *et al.*, 2009). However, in the life-history experiment, in the
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20 presence of a good quality green algal food, no immobility of the *Daphnia* was
21
22 observed. The presence of good quality food therefore seemed to reduce the toxic
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24 effects of BMAA, which could be through uptake and inactivation of BMAA by the
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26 algae and/or a stronger health of fed animals. Interestingly, Zhou *et al.* (2009) found a
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28 similar pattern in fruit-flies: females that were given a diet enriched with BMAA and
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30 leucine did not show a reduced climbing performance, whereas females that were only
31
32 given a BMAA enriched diet did. The authors suggested that leucine blocked the
33
34 same amino acid transporter system used by BMAA, thereby reducing toxicity.
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36 However, in our study it remains unknown whether the presence of algae decreased
37
38 the toxicity of BMAA as has been found for pesticides (Barry *et al.*, 1995), or that
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40 *Daphnia* are less capable of dealing with the adverse effects of toxicants in the
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42 absence of food.
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51 BMAA did not cause mortality or immobilization during the 15 day exposure
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53 period in the life-history experiment, but it had an effect on reproduction. Exposed
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55 animals showed a tendency to a delayed age at first reproduction and produced a
56
57 lower number of offspring. Consequently, population growth rates declined with
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59 increasing concentrations of BMAA in the medium. Interestingly, the animals that
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3 showed delayed reproduction and smaller clutch sizes were larger than the non-
4 exposed controls. In general, at a high resource level *Daphnia* has a larger size at
5 maturity, and these larger *Daphnia* have larger eggs and carry more eggs than smaller
6 individuals (Lampert, 1993). However, in our study, the tax *Daphnia* seemed to pay
7 for increased body-size was a delay in maturation, thus at the expense of early
8 reproduction (Cole, 1954). The reaction of *D. magna* in the life-history experiment
9 shows some resemblance with the inducible defense reactions of certain *Daphnia*
10 species to the chemical cues from their invertebrate natural enemy, the midge larva
11 *Chaoborus* (Tollrian and Dodson, 1999). When these *Daphnia* sense the presence of
12 *Chaoborus* larvae, they allocate a higher portion of energy to maternal growth (larger
13 size at first reproduction, later reproduction, lower relative reproductive investment)
14 to avoid size-selective predation on the smaller juvenile instars (Tollrian and Dodson,
15 1999).

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34 The population growth rate r has often been used as a measure of *Daphnia*
35 fitness (e.g. McCauley *et al.*, 1990; Lüring, 2003a,b) and represents a relevant
36 measure of ecological consequences of stressors (Forbes and Calow, 1999). Although
37 a good estimate of r requires life-table experiments with at least the first three broods
38 included (Stearns, 1992), a close proxy for r can also be obtained by assays including
39 only the period until release of the first clutch (Guilhermino *et al.*, 1999; Trubetskova
40 and Lampert, 2002). Hence, the chronic assay employed in this study might be
41 sufficient to predict population level consequences of BMAA to *Daphnia*; r was
42 about 9% lower when animals were exposed chronically to 100 $\mu\text{g L}^{-1}$ BMAA.
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3 $\mu\text{g l}^{-1}$ during the entire study period May – October (Gurbuz *et al.* 2009). However,
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6 even though no data on dissolved BMAA concentrations *in situ* are available, the
7
8
9 concentrations required to observe negative effects were exceedingly high (10-100 μg
10
11 L^{-1}). In a recent study, a BMAA concentration of 1.9 $\mu\text{g L}^{-1}$ was found in a
12
13 cyanobacteria bloom from the Baltic Sea (Spáčil *et al.*, 2010), but more data on
14
15
16 particulate and dissolved BMAA concentrations are required.

17
18 In this study, when BMAA was added to the medium without food, BMAA
19
20 accumulation in the animals was observed, which is in line with the findings that
21
22 *Daphnia* can take up dissolved organic carbon directly from the water (Speas and
23
24 Duffy, 1998). The presence of protease activity in the gut of *D. magna* (Von Elert *et*
25
26 *al.*, 2004) supports the ability of the animals to take up amino acids actively from
27
28 ingested water. *Daphnia* can, however, be viewed as a highly efficient filter-feeder
29
30 that concentrates food items from the water before ingestion (Lampert, 1987). Hence,
31
32 intake of a toxin dissolved in water is presumably less than the uptake of toxin
33
34 containing particles collected from the water, which suggests that the mode of toxin
35
36 delivery (dissolved or in food particles) might be very important as a determinant for
37
38 the actual dose. For example, intact cells of the cyanobacterium *Microcystis*
39
40 *aeruginosa* containing the toxin microcystin were lethal at a concentration of four
41
42 orders of magnitude lower than dissolved microcystin (DeMott *et al.*, 1991). Although
43
44 presence of other toxic compounds in the cyanobacterial matrix can not be excluded
45
46 in that study, the huge difference suggests a strong influence of the mode of delivery.
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49 In analogy, when BMAA is offered as particles, such as cyanobacterial cells, higher
50
51 BMAA uptake might be possible. We decided not to use a cyanobacterium as
52
53 transport vehicle of BMAA, since the presence of other metabolites, a deficiency in
54
55 essential compounds, grazing resistance and/or feeding deterrents could all influence
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3 the outcomes of a life-table experiment (Lürling, 2003a,b). We did not detect any
4 effect of BMAA on *S. obliquus*, our algal food source, and since BMAA might be
5 incorporated into proteins (Murch *et al.*, 2004; Rodgers and Shiozawa, 2008) further
6 studies will focus on the green alga *S. obliquus* as possible transport vehicle for
7 BMAA and selected toxins to zooplankton grazers. Moreover, synergistic effects of
8 BMAA with other cyanobacterial neurotoxins on zooplankton grazers should also be
9 studied, since low concentrations of BMAA can enhance the effects of other
10 neurotoxins at the cellular level (Lobner *et al.*, 2007) and BMAA co-occurrence with
11 other cyanotoxins has already been observed (Metcalf *et al.*, 2008; Faassen *et al.*,
12 2009).

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Although we measured a direct effect of BMAA on population growth rates, we did not include possible transgenerational effects in our experimental design. Because toxicants can significantly reduce neonate fitness, transgenerational effects cannot be excluded *a priori* (Hammers-Wirtz and Ratte, 2000). The high BMAA concentrations in neonates (up to 3821 times as high as the medium and up to 108 times as high as their mothers) that were exposed for a maximum of 24 hours to BMAA containing medium indicate that the effect of BMAA exposure might also be transgenerational, cumulative and/or delayed. Significantly higher bioconcentration in *D. magna* neonates than in adults has also been observed for other compounds, such as nonylphenol (Preuss *et al.*, 2008). A higher concentration in neonates could be the result of the dependency of bioconcentration kinetics on the surface-to-volume ratio as bioconcentration seems to be inversely related to body-size (Preuss *et al.*, 2008). Another possible mechanism is an active maternal transfer to neonates, as observed for mercury (Tsui and Wang, 2004).

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4 In the tropical Guam ecosystem, cyanobacterially produced BMAA is
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6 biomagnified in cycads, pigs, deer and flying foxes, until it reaches the top trophic
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8 level in the Guam ecosystem: the indigenous Chamorro people (Murch *et al.*, 2004).
9
10 BMAA concentrations in zooplankton from the Baltic Sea were 6-fold higher than in
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12 cyanobacteria and up to 200-fold higher in certain fish species (Jonasson *et al.*, 2010),
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14 indicating biomagnification scenarios of BMAA may also be present in temperate
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16 regions (Jonasson *et al.*, 2008). Our study provides independent evidence that BMAA
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18 may be biomagnified in aquatic systems as BMAA was clearly bioconcentrated in
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20 *Daphnia*.
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28
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32
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TABLE LEGENDS

Table 1. BMAA concentrations (corrected for sample recovery) and the calculated bioconcentration factors (BCF) in juvenile *Daphnia magna* based on wet- and dry-weight ($\mu\text{g g}^{-1}$) after 96 h exposure to different concentrations of BMAA in the medium ($\mu\text{g L}^{-1}$) in the absence of food. *n.d.* means not detected in sample (LOD = 5 $\mu\text{g L}^{-1}$); *n.q.* means detected, but not quantified (LOQ = 15 $\mu\text{g L}^{-1}$).

Table 2. Survival (%) until the end of the experiment (15 d), age at first reproduction (AFR, d), size at first reproduction (SFR, mm), dry-weight after 15 d (DW, μg), mean number of offspring per female and the population growth rates (r , d^{-1}) for *Daphnia magna* grown for 15 days at different concentration of BMAA and a fixed food concentration of 5 mg C L^{-1} green algae *Scenedesmus obliquus*. Similar letters A,B in column SFR indicate homogeneous groups that are not significantly different at $p = 0.05$ (Tukey test).

Table 3. BMAA concentrations (corrected for sample recovery) and the calculated bioconcentration factors (BCF) in tissue of adult *Daphnia magna* based on wet- and dry-weight ($\mu\text{g g}^{-1}$) and their neonates (< 24 h) after 15 days of exposure to different concentrations of BMAA ($\mu\text{g L}^{-1}$) with the green alga *Scenedesmus obliquus* as food. *n.d.* means not detected in sample (LOD = 5 $\mu\text{g L}^{-1}$).

FIGURE LEGENDS

Fig. 1. Percentage of swimming (black bars), immobile (white symbols) and dead (grey bars) *Daphnia magna* after 48 h exposure to different concentrations of BMAA. Error bars indicate 1 standard deviation ($n = 4$). Each jar started with 10 juvenile *D. magna* (<24 h) in 100 mL artificial RT-medium.

Fig. 2. Course of the body-length (mm) of *Daphnia magna* exposed to different concentrations of BMAA. Error bars indicate 1 standard deviation ($n = 10$).

Table 1.

BMAA in medium ($\mu\text{g L}^{-1}$)	Wet-weight		Dry-weight	
	in tissue ($\mu\text{g g}^{-1}$)	BCF	in tissue ($\mu\text{g g}^{-1}$)	BCF
0	<i>n.d.</i>	--	<i>n.d.</i>	--
10	<i>n.q.</i>	--	<i>n.q.</i>	--
100	<i>n.q.</i>	--	<i>n.q.</i>	--
1000	<i>n.q.</i>	--	<i>n.q.</i>	--
10000	435.4	44	1246	125

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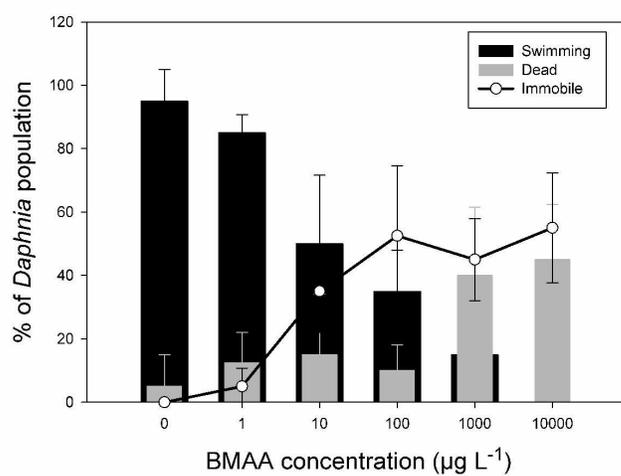
Table 2.

BMAA ($\mu\text{g L}^{-1}$)	Survival (%)	AFR (d)	SFR (mm)	DW (μg)	Offspring per female	<i>r</i> (d^{-1})
0	80	8.9 (0.6)	2.47 (0.14) ^A	164 (5)	11.3 (3.0)	0.174
1	80	9.1 (0.4)	2.59 (0.10) ^{AB}	164 (2)	11.4 (2.1)	0.172
10	70	9.5 (0.8)	2.66 (0.07) ^B	197 (24)	10.0 (3.0)	0.170
50	80	9.5 (0.9)	2.61 (0.11) ^{AB}	191 (5)	9.9 (3.0)	0.166
100	90	9.7 (1.1)	2.61 (0.10) ^{AB}	166 (16)	8.8 (3.6)	0.159

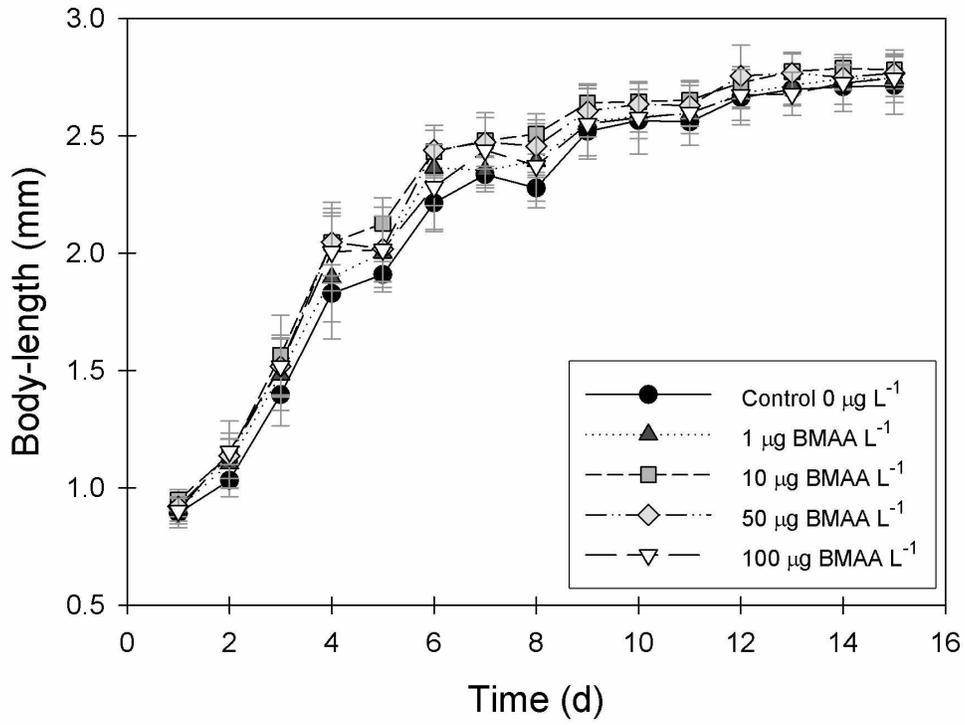
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Table 3.

BMAA		Wet-weight		
in medium ($\mu\text{g L}^{-1}$)	in adults ($\mu\text{g g}^{-1}$)	in neonates ($\mu\text{g g}^{-1}$)	BCF adults	BCF neonates
0	<i>n.d.</i>	<i>n.d.</i>	--	--
1	<i>n.d.</i>	<i>n.d.</i>	--	--
10	<i>n.d.</i>	<i>n.d.</i>	--	--
50	2.5	78.7	51	1574
100	3.1	332.7	31	3327
BMAA		Dry-weight		
in medium ($\mu\text{g L}^{-1}$)	in adults ($\mu\text{g g}^{-1}$)	in neonates ($\mu\text{g g}^{-1}$)	BCF adults	BCF neonates
0	<i>n.d.</i>	<i>n.d.</i>	--	--
1	<i>n.d.</i>	<i>n.d.</i>	--	--
10	<i>n.d.</i>	<i>n.d.</i>	--	--
50	13.7	90.4	275	1808
100	19.6	382.1	196	3821



216x278mm (600 x 600 DPI)



152x115mm (300 x 300 DPI)

Review