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Zebrafish larvae exposed to BMAA and isomers DAB and AEG: a morphological and behavioural study

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1. Introduction

BMAA (beta-methylamino L-alanine) is a neurotoxin produced mainly by cyanobacteria and the subject of several hypotheses suggesting a link with amyotrophic lateral sclerosis (ALS). It has historically been found in cycad roots, consumed in various ways by the Chamorros on the island of Guam, where the incidence of ALS is much higher than the global average. Other clusters have also been identified by the scientific communityⁱ and several studies have highlighted multiple mechanisms of toxicity of this moleculeⁱⁱ. Two isomers are also identified and studied: DAB (2,3-duaminobutyric acid) and AEG (N-(2-aminoethyl) glycine).

In France, in the Occitanie region, BMAA and/or isomers have been detected in the Thau lagoon in bivalvesⁱⁱⁱ and, depending on the time of year, in water and/or air samples (Courtier thesis, 2022). Other global studies show the possible presence of toxins in the environment at nanogram concentrations^{iv}. As highlighted by the 2017 French National Agency for Food, Environmental and Occupational Health and Safety report^v, information on the effects of environmental concentrations remains to be completed. We therefore tested BMAA, DAB and AEG on a common use toxicological model: *Danio rerio*; at the doses of 1-10-100 ng.L⁻¹ and 1-500 µg.L⁻¹.

2. Materials and Methods

Chemicals were purchased from Sigma Aldrich (Saint Louis, Missouri, USA) and Santa Cruz Biotechnologies (Dallas, Texas, États-Unis).

2.1. Zebrafish maintenance and eggs collection

Eggs were obtained from wild-type (AB strain) *Danio rerio* from the VBIC zebrafish facility. Zebrafish care and maintenance were previously described in Vergunst et al., 2010. Zebrafish were kept and handled in compliance with the guidelines of the European Union for handling laboratory animals (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm). The zebrafish facilities at VBIC are approved by the "Direction Départementale de la Protection des Populations" (DDPP) du Gard (ID 30-189-4). Toxicity studies were not classified as animal experiments according to the 2010/63/EU Directive and were terminated before 5 days post-fertilization.

2.2. FET (fish embryo acute toxicity test)

At 28-30 hpf embryos were manually dechorionated with fine forceps (Tweezers #5 Dumont) under a stereo microscope. The following experiments were inspired by the OECD 236. Zebrafish larvae were transferred in 48 well-plate. Each plate contained six E3 medium (negative controls) and two different working solutions for 20 embryos each. DCA (3,4 dichloroaniline) was used as a positive control. Tests were performed under semi-static conditions with renewal of working solutions every 24h and control of pH. Every experiment was repeated 4 times, for a total of 80 larvae per condition.

- Morphological analysis: death and multiple morphological characteristics were analyzed indicating different types of abnormalities (presence of hemorrhage, presence of edema (pericardial or yolk sac), yolk sac deformation and spine deformations (kyphosis, lordosis, scoliosis)).
- Behavioural analysis: swimming behaviour was studied using the Daniovision® observation chamber. Following a period of acclimatization, the larvae were observed for three minutes of free swimming, followed by a tap stimulus and two more minutes of free swimming.

3. Results and Discussion

3.1. Morphological analysis

Positive (DCA) and negative (E3) controls were used to validate the morphological analysis. The E3 medium did not cause any death or malformation and the DCA had an effect rate of over 80%.

Results showed that every molecule studied produced an effect (morphological abnormality or death) at each concentration studied. Considering BMAA, 1 $\mu\text{g.L}^{-1}$ seems to be the less toxic concentration from a morphological point of view. After 72 hours of exposure, the average is 2.5% of larvae with effects or death compared with 10% of larvae for 100 ng.L^{-1} or 5% of larvae for 500 $\mu\text{g.L}^{-1}$. For DAB, 1 $\mu\text{g.L}^{-1}$ appears to be the most toxic. After 72 hours of exposure the average is 7.5% of larvae with effects or death while the other concentrations tested show lower effects. For AEG, 1 - 10 ng.L^{-1} and 1 $\mu\text{g.L}^{-1}$ have around 5% of affected larvae, while concentrations of 100 ng.L^{-1} and 500 $\mu\text{g.L}^{-1}$ have an average of 10% of effects or death.

3.2. Behavioural analysis

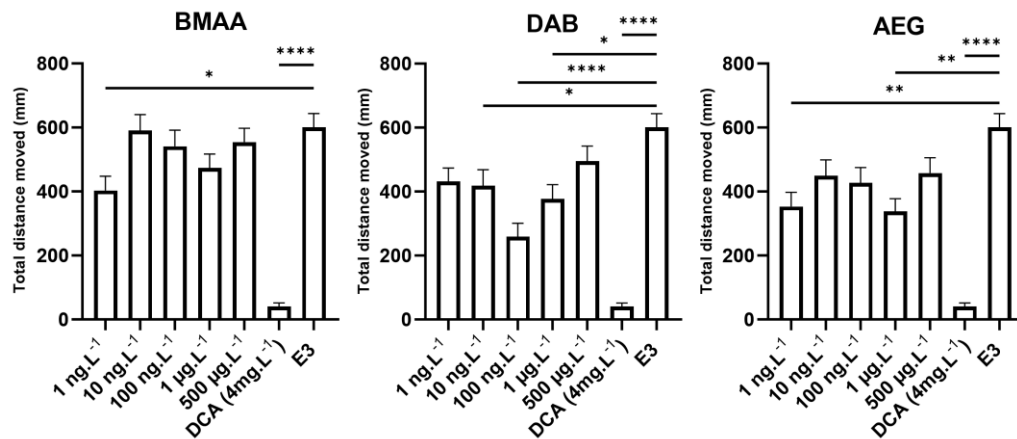


Figure 1 Effect of different concentrations of BMAA, DAB and AEG on *Danio rerio* behaviour. Swimming behaviour was analysed for a total of 5 min following 72h of exposure and 10 min of adaptation. A “tap” stimulus was applied by Daniovision © after 3 minutes of recording (n=80). Mean \pm SEM (P<0,05 Kruskal-Wallis test and the post hoc Dunn's multiple comparisons test)

Figure 1 shows the total distance covered by larvae exposed to different molecules at different concentrations. Larvae exposed to BMAA and AEG, at a concentration of 1 ng.L^{-1} , have a significantly lower mean than the negative control (E3). Larvae exposed to DAB show 3 concentrations (10 – 100 ng.L^{-1} and 1 $\mu\text{g.L}^{-1}$) significantly lower than the negative control. Larvae exposed to DCA, which is known to be toxic, travel a significantly shorter distance than larvae not exposed to toxic substances (E3). The total distance covered indicates the potential toxicity of the molecules, as the larvae show effects similar to those obtained with the positive control. Also, the capacity of exposed larvae to respond to the tap stimulus was affected by the three toxins at nanogram concentrations (analysis of 1 second and 5 seconds time frame before and after the tap stimulus; data not shown).

Concluding, whether for morphological or behavioural analysis, the highest concentrations tested do not appear to be the most toxic.

4. Conclusions

From these results, DAB and AEG seem to have a higher toxicity if compared to BMAA as their impact is stronger and highly statistically significant. To note, these data suggest also a nonlinear dose-response relationship, supporting the value of studies at environmental concentrations. To our knowledge, this is the first study to focus on such low doses. Overall, the morphological and behavioural analysis show complementary results and indicate the importance of pursuing research.

5. References

- ⁱ Spencer, Lagrange, et Camu, « ALS and Environment ».
- ⁱⁱ Courtier, Potheret, et Giannoni, « Environmental Bacteria as Triggers to Brain Disease ».
- ⁱⁱⁱ Réveillon et al., « β -N-Methylamino-L-Alanine (BMAA) and Isomers ».
- ^{iv} Abbes et al., « Occurrence of BMAA Isomers in Bloom-Impacted Lakes and Reservoirs of Brazil, Canada, France, Mexico, and the United Kingdom ».
- ^v ANSES, « Données relatives à la toxicité aiguë et chronique de la bêta-méthylamino-L-alanine (BMAA) ».