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Usability of the bivalves *Dreissena polymorpha* and *Anodonta anatina* for a biosurvey of the neurotoxin BMAA in freshwater ecosystems

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ABSTRACT

The environmental neurotoxin β-methylamino-L-alanine (BMAA) may represent a risk for human health in case of chronic exposure or after short-term exposure during embryo development. BMAA accumulates in freshwater and marine organisms consumed by humans. It is produced by marine and freshwater phytoplankton species, but the range of producers remains unknown. Therefore, analysing the phytoplankton composition is not sufficient to inform about the risk of freshwater contamination by BMAA. Filter-feeders mussels have accumulation capacities and therefore appear to be relevant to monitor various pollutants in aquatic ecosystems. We investigated the suitability of the freshwater mussels *Dreissena polymorpha* and *Anodonta anatina* for monitoring BMAA in water. Both species were exposed to 1, 10, and 50 μg of dissolved BMAA/L daily for 21 days, followed by 42 days of depuration in clean water. On days 0, 1, 7, 14, and 21 of exposure and 1, 7, 14, 21 and 42 of depuration, whole *D. polymorpha* and digestive glands of *A. anatina* were sampled, and the total BMAA concentration was measured. *D. polymorpha* accumulated BMAA earlier (from day 1 at all concentrations) and at higher tissue concentrations than *A. anatina,* which accumulated BMAA from day 14 when exposed to 10 μg BMAA/L and from day 7 when exposed to 50 μg BMAA/L. As BMAA accumulation by *D. polymorpha* was time and concentration-dependent, with a significant elimination during the depuration period, this species may be able to reflect the levels and dynamics of water contamination by dissolved BMAA. The species *A. anatina* could be used for monitoring water concentrations above 10 μg BMAA/L.

1. Introduction

The environmental neurotoxin β-methylamino-L-alanine (BMAA) is of growing concern because it may be involved in the development of the amyotrophic lateral sclerosis-parkinsonism-dementia complex (ALS-PDC), a human neurodegenerative pathology (Murch et al., 2004; Nunn, 2017). Even if no etiological link has been demonstrated so far (Delcourt et al., 2017), laboratory experiments have shown that this non-proteinogenic amino acid can induce: (i) excitotoxicity mediated by glutamate receptors in the presence of bicarbonate at physiological concentrations (Weiss and Choi, 1988), (ii) dysregulation of the cellular protein homeostasis and a potent interaction with neuromelanin, which could lead to long-lasting neurotoxic activity (Frøyset et al., 2016; Karlsson et al., 2009a, 2015), and (iii) inhibition of the cysteine/glutamate antiporter, leading to potential oxidative stress (Lobner, 2009; Pierozan et al., 2018). Moreover, this toxin may also act as a developmental toxin potentially linked to neurodegeneration (Karlsson et al., 2015, 2009b; Pierozan and Karlsson, 2019). The

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onset of the ALS-PDC pathology is thought to occur following long-term chronic exposure via the consumption of BMAA contaminated food (Banack and Cox, 2003), or after short exposure during embryo development as observed with rats embryonic neural stem cells (Pierozan and Karlsson, 2019). BMAA accumulates in various seafoods (mussels, crustaceans, shark fins) (Lance et al., 2018). Marine bivalve 'molluscs have even been qualified as the most BMAA-contaminated food source for humans, and blue mussels sampled along the French coasts systematically contained BMAA (Réveillon et al., 2016b; Lance et al., 2018). However, its occurrence is not solely restricted to marine ecosystems, as it has been found in the tissues of fish from a Swedish Lake (Lage et al., 2015).

BMAA producers have not been fully characterized. Initial BMAA quantification in cyanobacteria suggested that the neurotoxin may be produced by most of the freshwater cyanobacterial genus (Cox et al., 2005). However, the wide production of BMAA by freshwater cyanobacteria was not confirmed using more selective analytical methods (e.g., LC-MS/MS). Up to now, BMAA has been demonstrated to be produced by few cyanobacterial species (*Nostoc* sp., *Leptolyngbya* sp.), and also by diatoms (*Chaetoceros* sp., *Phaeodactylum tricornutum*) and marine dinoflagellates (*Heterocapsa triquetra*, *Gymnodinium catenatum*) (Faassen et al., 2009; Monteiro et al., 2016; Réveillon et al., 2016b, 2015).

Phytoplankton proliferations in fresh waters often occur in reservoirs used for recreational activities or drinking water. BMAA may therefore represent a threat to populations using water supply or living nearby contaminated lakes. In addition to the consumption of contaminated freshwater fish, human contamination may also occur through inhalation of aerosolized toxins or accidental ingestion of lake water (Caller et al., 2009). A follow-up of BMAA in fresh waters might be useful to assess a potential risk of human exposure because phytoplankton proliferations are expected to be enhanced by increasing eutrophication and global warming (Hudnell, 2008). As very little is known about the bioavailability of BMAA in fresh waters, quantification of its dissolved form (in the medium) or intracellular form (in the phytoplankton biomass) may be required. However, quantification of dissolved BMAA in water remains a challenge because of the hydrophilic nature of this small amino acid compound and the potential co-existence of seven isomers: DAB (2,4-diaminobutyric acid), BAMA (β-amino-N-methyl-alanine), N-2(aminoethyl)glycine, DABA (2,3-diaminobutyric acid), 3,4-diaminobutyric acid, AEG (3-amino-2-(aminomethyl)-propanoic acid), and 2,3-diamino-2-methylpropanoic acid (Jiang et al., 2012; Faassen, 2017). Concerning BMAA quantification in the phytoplankton biomass, very little reliable data is currently available because highly selective analytical methods are required (Lance et al., 2018). Such data of intracellular BMAA concentrations in water range from 0.3 μg/L measured in a Canadian lake (Roy-Lachapelle et al., 2015), to 39.6 μg/L in an American lake (Al-Sammak et al., 2014), or are expressed by phytoplankton biomass and vary from 2.3 ng/g (Jonasson et al., 2010) to 42 μg/g (Faassen et al., 2009). However, BMAA analysis in the phytoplankton biomass may not be sufficient to assess the overall contamination levels of an ecosystem for the two following reasons: i) BMAA production is not steady, it varies over time and may be influenced by environmental parameters such as nitrogen availability (Scott et al., 2014), ii) phytoplankton communities are prone to display considerable short-term spatio-temporal variability (Deng et al., 2016; Qi et al., 2018; Salmaso et al., 2017). Therefore, the use of biological integrative tools such as sentinel bivalve species may be relevant to evaluate the presence of BMAA in the environment.

Freshwater bivalves can be used as bioindicators of metals, microplastics, organochlorine contaminants and parasites (Baqar et al., 2018; Bourgeault et al., 2010; Camusso et al., 1994; Palos Ladeiro et al., 2014; Su et al., 2018; Zuykov et al., 2013). They can reveal the presence of the cyanotoxins microcystins (MC) in their medium, in the laboratory (Lauceri et al., 2017) and *in situ*, even when water analysis did not reveal it, in relation with the great spatio-temporal variability of the producers (Preece et al., 2015). There is yet no data regarding BMAA concentrations in freshwater bivalves *in situ*, but laboratory experiments have shown that freshwater mussels bioaccumulate BMAA. The toxin was found in tissues of the mussels *Anodonta cygnea* and *Dreissena polymorpha* after exposure to 100 μg of radiolabelled BMAA/L for 24 and 48 h (Downing et al., 2014). One of our previous studies showed that BMAA spread and accumulated throughout *D. polymorpha* soft tissues without a specific target organ, with similar kinetics of accumulation and elimination for all analysed fractions (total BMAA, free BMAA, BMAA associated with high- and low-molecular-weight polypeptides), when exposed to 2.5 μg BMAA/day for 21 days, followed by 21 days of depuration (Lepoutre et al., 2019). Therefore, these bivalves could be used as potential bioindicators of the presence of BMAA in fresh water by quantifying total BMAA in one organ or in the entire body. The two species *D. polymorpha* and *A. anatina* were selected for this purpose. *D. polymorpha* has long been used in biomonitoring studies and has been suggested to represent the freshwater counterpart of the marine mussel *Mytilus* in ecotoxicological studies (Binelli et al., 2015). This sessile bivalve lives mostly on hard substrates and is present in a wide range of habitats, from freshwater lakes and rivers to brackish estuaries (Van Damme, 2014). However, the use of this species as a sentinel is restricted because *D. polymorpha* is an invasive species (Van Damme, 2014). Those restrictions may not apply to *A. anatina*, a vagile bivalve living unattached on soft substrates of flowing streams and standing waters (Lopes-Lima, 2014).

The objectives of this study were to evaluate if *D. polymorpha* and *A. anatina* could reflect the levels and variations of BMAA concentrations in water and may therefore be suitable bioindicators of BMAA occurrence in aquatic environments. Due to the lack of a known microorganism that could produce BMAA steadily in laboratory conditions, *A. anatina* and *D. polymorpha* were exposed to 1, 10 and 50 μg of dissolved BMAA/L for 21 days, followed by 42 days of depuration in clean water. After 0, 7, 14, and 21 days of exposure and 1, 7, 14, 21, and 42 days of depuration, mussels were sampled to quantify total BMAA in tissues. Preliminary experiments showed that *D. polymorpha* was smaller than *A. anatina* (2 ± 0.3 cm vs. 6.5 ± 0.05 cm) with an approximately 12-times lower fresh weight (184.4 ± 9.7 mg FW vs. 2171.7 ± 107.1 mg FW). Whole *D. polymorpha* were sampled to quantify BMAA. However, concerning *A. anatina*, the quantity of tissues required to perform the analysis represented less than 1% of the whole body (dry weight), so only one of the accumulating organs (the digestive gland) was sampled to avoid large inter-individual variability. Results are discussed in terms of dynamics of accumulation and elimination of the neurotoxin, and relevance of the use of each species as a bioindicator of the presence of BMAA in fresh water.

2. Materials and methods

2.1. Mussel acclimation

2.1.1. Anodonta anatina

Mussels measuring 58.3 ± 5.6 mm were provided by Univers aquatique (Sartrouville, France), and were acclimated by groups of 50 at 14 ± 2 °C in the dark in aerated 15-L tanks containing

Cristalline® (Saint Yorre, France) spring water for two weeks. During this phase, they were fed twice a week with 3.7×10^7 cells of *Chlorella vulgaris*/individual/day (Greensea, Mèze, France). Algal density was measured with an optical microscope (Primovert, Zeiss, Oberkochen, Germany) and KOVA® slides (Kova slide, VWR, Fontenay-sous-Bois, France).

2.1.2. Dreissena polymorpha

D. polymorpha were collected at the Lac-du-Der-Chantecoq (48°36'07.7″N; 4°44'37.0″E) in June 2016. In the laboratory, they were gently cleaned, sorted according to size (25 ± 2 mm), and placed in aerated tanks containing Cristalline® (Saint Yorre, France) spring water. As they originated from the field, they were gradually acclimated from lake temperature to 14 ± 2 °C in the dark for up to seven weeks. During this phase, they were fed as described for *A. anatina*, with a ratio of 2×10^6 cells of *Chlorella vulgaris*/individual/day.

3. Experimental design

Preliminary experiments were performed to reach an equivalence in available BMAA per milligram of soft tissues (FW) of each species. As whole *D. polymorpha* fresh weight was 12 times lower than whole *A. anatina* fresh weight (184.4 ± 9.7 mg vs. 2171.7 ± 107.1 mg), the density of mussels per litre was adjusted to one *A. anatina*/L and 12 *D. polymorpha*/L. Three days before the experiment, mussels were no longer fed and randomly dispatched in six aerated 40-L tanks containing 30.5 L of spring water. Before adding BMAA in the tanks, negative controls were taken by sampling 5 digestive glands of *A. anatina* and 2 pools of 3 whole *D. polymorpha*. Exposure was conducted in duplicate (two tanks per BMAA concentration for each species). A total of 61 *A. anatina* and 192 *D. polymorpha* were used for each concentration. Both species were exposed for 21 days to 1, 10 or 50 μg of dissolved BMAA/L (L-BMAA hydrochloride B-107, Sigma–Aldrich®, Saint-Louis, MO, USA), daily added in the tanks. The mussels were not fed throughout the experiment. The water was renewed every three days. At the end of the exposure period, the mussels were transferred into new tanks containing only clear water to study depuration for 42 days. Mussels were randomly collected and sacrificed after 0, 1, 7, 14, and 21 days of exposure and after 1, 7, 14, 21, and 42 days of depuration. The water volume was reduced after each sampling to keep the same mussel:water ratio over time. Samplings consisted of 4 pools of 3 whole *D. polymorpha* and, because of their size, 5 digestive glands of *A. anatina* per concentration. Tissues were kept in Eppendorf® tubes (Eppendorf, Hamburg, Germany), frozen in liquid nitrogen and stored at −80 °C. Then they were freeze-dried and ground with a Mixer Mill MM400 (Retsch, Haan, Germany) using 4 beads, 4 min of beating at 30 Hz.

4. Total BMAA extraction and analysis by tandem mass spectrometry

Several fractions of BMAA can be analysed in tissues, requiring each a different extraction procedure (Faassen et al., 2016). BMAA can accumulate in its free form ("free BMAA") when it is extracted with polar solvents. But some BMAAs can be associated to unknown compounds that can stay in solution, suggesting a low molecular weight of the BMAA-molecule complex ("soluble bound BMAA"), while some BMAAs can be bound in the precipitate, suggesting a heavier weight ("precipitated bound BMAA"). To reach a global understanding of BMAA concentrations in tissues, we analysed the total concentrations of BMAA ("total BMAA") encompassing free, soluble bound and protein-bound BMAA.

Total BMAA was extracted as described in Faassen (2017). Briefly, 1 mg of tissues were spiked with 40 μL of D_3BMAA in 20 mmol/L HCl, an internal standard, then dried under vacuum. After adding 30 μL of HCl 6 M, tissues were hydrolysed approximately at 0.7 mbar for 20 h at 105 °C in an Eldex® hydrolysis workstation (Eldex, Napa, CA, USA). Then they were dried, resuspended twice in 500 μL of a 67:33:0.1 ACN:water:formic acid mix, and transferred into spin filter tubes and centrifuged. The underivatized extracts were analysed with a UHPLC-MS/MS system 1290 Infinity II connected with a 6490C triple quadrupole mass spectrometer (Agilent, Santa Clara, CA, USA). The method was slightly modified from the underivatized BMAA analysis described in Faassen et al. (2016): we injected 5 μL, and the compounds were separated on a HILIC column (ZIC®HILIC, 150 × 2.1 mm, 5 μm, 200 Å, Merck, Darmstadt, Germany) set at 40 °C. The mobile phase consisted of acetonitrile with 0.1% formic acid (A) and MilliQ-water with 0.1% formic acid (B). The initial conditions were 5% B for 2 min, followed by a gradient from 2 to 4 min to 35% B, from 4 to 8 min to 45% B, and up to 16 min at 45% B. Between 16 and 17 min, B was decreased to 5%, and this was held for another 5 min. The mass spectrometer was used in the positive mode with a gas flow of 12 mL/min, a source temperature of 230 °C, a nebulizer pressure of 40 psi, a sheath gas temperature of 200 °C, a sheath gas flow of 12 L/min, and a capillary voltage of 2.5 kV. The compounds were analysed in multi reaction mode (MRM) using nitrogen as the collision gas. BMAA was monitored based on the m/z transitions 119.1 > 76.2, 119.1 > 88.1 and 119.1 > 102.1 using collision energies of 9, 9 and 5 V and a fragmentor voltage of 73 V. Transitions for DAB were m/z 119.1 > 101.1 and 119.1 > 74.2 using collision energies of 5 and 13 V and a fragmentor voltage of 68 V D3BMAA was monitored based on the m/z transitions 122.1 > 76.2, 122.1 > 88.1 and 122.1 > 105.1 using collision energies of 9, 9 and 5 V and a fragmentor voltage of 75 V. Masshunter B 08.02 (Agilent, Santa Clara,CA, USA) was used for data acquisition and analysis.

A 20% relative deviation from the average ion ratios in the standards was allowed in the samples. Furthermore, the BMAA retention time was verified by D3BMAA retention time. BMAA was quantified against an external calibration curve, and the concentrations in each sample were corrected for the signal intensity of the internal standard. DAB and AEG were not quantified, but only included in the analysis to ensure that there was no co-elution with BMAA.

In addition, in our study, LOD was defined as the lowest concentration at which the quantifier and the qualifier ions all had a signal to noise (S/N) ratio > 3. LOQ was defined as the lowest concentration at which the qualifier ions had a S/N ratio >3 and the quantifier ion had a S/N > 10. As the intensity of the quantifier was clearly higher than that of both qualifiers, the criteria for LOQ were met at the same concentration as the LOD. For both sample types (*D. polymorpha* and *A. anatina*), the LOQ (and LOD) was approximately 1 μg/g. Apparent recovery (extraction recovery plus matrix effect during ionisation) was determined for each sample by adding deuterated BMAA before extraction. Average apparent recovery for *D. polymorpha* was 81% (stdev 18, n = 125). For *A. anatina*, the average apparent recovery was 47% (stdev 14, n = 184).

5. Data treatment and statistics

A cumulative percentage of BMAA accumulation (cum % acc) was calculated for each sampling time, using the total BMAA concentration in tissues (μg/g DW) converted into μg/g FW using pre-established values from preliminary experiments, the tissue weights, and the cumulative quantities of BMAA to which mussels were exposed.

$$\text{cum \% acc} = \frac{\text{Total BMAA in tissues } (\mu g/g) \text{ on day n} \times \text{tissue weight } (g)}{\text{Cumulative quantity of BMAA available per mussel in water between days 1 and n } (\mu g)} \times 100$$

Half-lives of BMAA in *A. anatina* and *D. polymorpha* were calculated for each concentration using Rutherford and Soddy (1903) formula:

$$t_{1/2} = \frac{\ln(2) \times 42}{\ln(C_{E21}|C_{D42})}$$

C_{E21} and C_{D42} were the mean BMAA concentrations in mussels exposed to BMAA after 21 days of exposure and 42 days of depuration, respectively. Polynomial regression functions were obtained using Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA).

Statistical analyses were performed with Statistica (Version 8, Statsoft, Tulsa, USA, 2007). Pearson's correlation coefficient r at a significance level of $p < 0.05$ was used to determine the correlation between variables. Normality was studied with a Shapiro-Wilk test, and the homogeneity of variances was studied with a Levene test. The comparison of multiple independent samples was done with Bonferroni-corrected Kruskal-Wallis tests. Pairwise comparisons of independent samples were done using Mann-Whitney tests.

6. Results

BMAA was found in four out of five unexposed digestive glands of *A. anatina* (Fig. 1), at a mean concentration of 2.95 ± 0.96 μg BMAA/g DW. This concentration did not significantly differ from the one quantified in *A. anatina* exposed to 1 μg BMAA/L (Mann-Whitney test, $p = 0.50$), whose concentration ranged from the

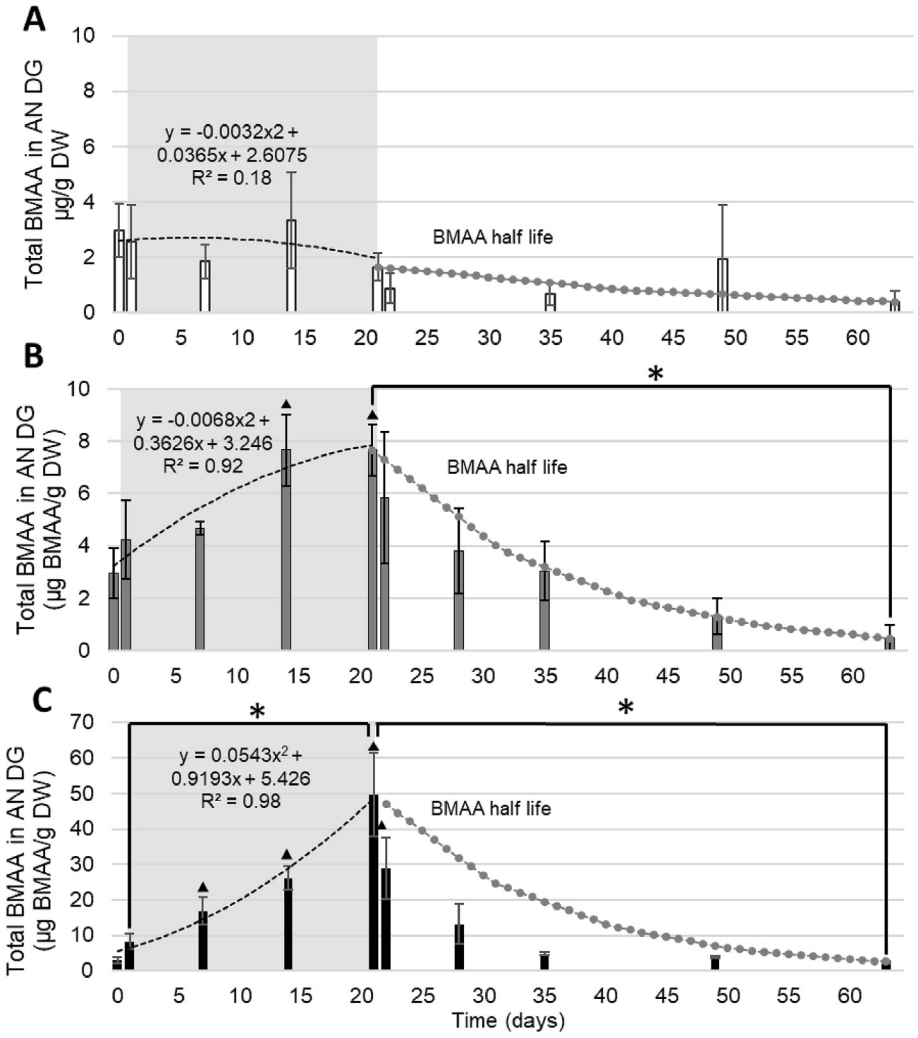

Fig. 1. Mean ± SE of total BMAA concentrations in digestive glands of *A. anatina* exposed to 1 (A), 10 (B) and 50 (C) μg/L of dissolved BMAA, and in controls (E0), during the exposure period (E, grey background) and the depuration period (D, white background), n = 5. *, significant difference in the BMAA concentrations in tissues between two dates (Mann-Whitney test, $p < 0.01$). ▲, significant difference in BMAA concentrations with the controls (Mann-Whitney test, $p < 0.05$). Polynomial regressions during the exposure period are represented by black dotted lines, and BMAA half-life curves by grey dotted lines.

detection limit (on day 7 of depuration) to 3.35 ± 1.73 μg BMAA/g DW (on day 14 of exposure). The BMAA concentration in *A. anatina* digestive glands started to differ significantly from the controls after 14 days of exposure to 10 μg BMAA/L, and after 7 days of exposure to 50 μg BMAA/L (Mann-Whitney test, $p < 0.05$). The maximum concentration of BMAA in *A. anatina* digestive glands was observed on the last day of the 3-week exposure period (7.65 ± 0.96 μg BMAA/g DW in mussels exposed to 10 μg/L, and 49.64 ± 11.74 μg BMAA/g DW in mussels exposed to 50 μg/L). Throughout the exposure period, the BMAA concentration in the digestive glands of *A. anatina* exposed to 10 μg BMAA/L was significantly higher than in the digestive glands of *A. anatina* exposed to 1 μg BMAA/L (Mann-Whitney test, $p < 0.01$). The BMAA concentration was also significantly higher in *A. anatina* exposed to 50 μg BMAA/L than in those exposed to 10 μg BMAA/L (Mann-Whitney test, $p < 0.01$) considering the entire exposure period. The BMAA concentration in *A. anatina* digestive glands was correlated with the length of exposure to the toxin only in mussels exposed to 50 μg BMAA/L (Pearson's $r = 0.74$, $p < 0.01$).

During the depuration period, BMAA elimination from *A. anatina* digestive glands was consistent, but not complete as BMAA was still detected after 42 days spent in clean water (0.39 ± 0.39 μg BMAA/g DW in mussels exposed to 1 μg/L, 0.48 ± 0.48 μg BMAA/g DW in mussels exposed to 10 μg/L, and 2.52 ± 0.27 μg BMAA/g DW in mussels exposed to 50 μg/L). Elimination was significant in *A. anatina* exposed to 10 and 50 μg BMAA/L (Mann-Whitney test, $p < 0.01$) as BMAA concentrations decreased by 94 and 95% between the last day of exposure and day 42 of depuration, respectively. The BMAA contents observed during the depuration period were similar between the treatments whatever the exposure concentration (Fig. 1). Moreover, the BMAA contents in all exposed mussels rapidly decreased during the depuration period down to levels similar to the one in the controls before the experiment, except on day 1 of depuration in the mussels exposed to 50 μg BMAA/L; the BMAA concentration in these mussels remained significantly higher than in the controls (Mann-Whitney test, $p < 0.01$).

As opposed to *A. anatina* digestive glands, no BMAA was detected in unexposed *D. polymorpha* (Fig. 2). BMAA concentrations in *D. polymorpha* exposed to 1 μg BMAA/L ranged from 2.0 ± 1.2 μg BMAA/g DW on day 42 of depuration to 4.3 ± 0.2 μg BMAA/g DW on day 14 of exposure. BMAA was detected from the first day of exposure in whole *D. polymorpha* exposed to 10 (4.6 ± 0.3 μg BMAA/g DW) and 50 μg BMAA/L (19.9 ± 4.0 μg BMAA/g DW). The maximum BMAA concentrations in *D. polymorpha* exposed to 10 μg BMAA/L were observed on day 14 of exposure (22.6 ± 0.6 μg BMAA/

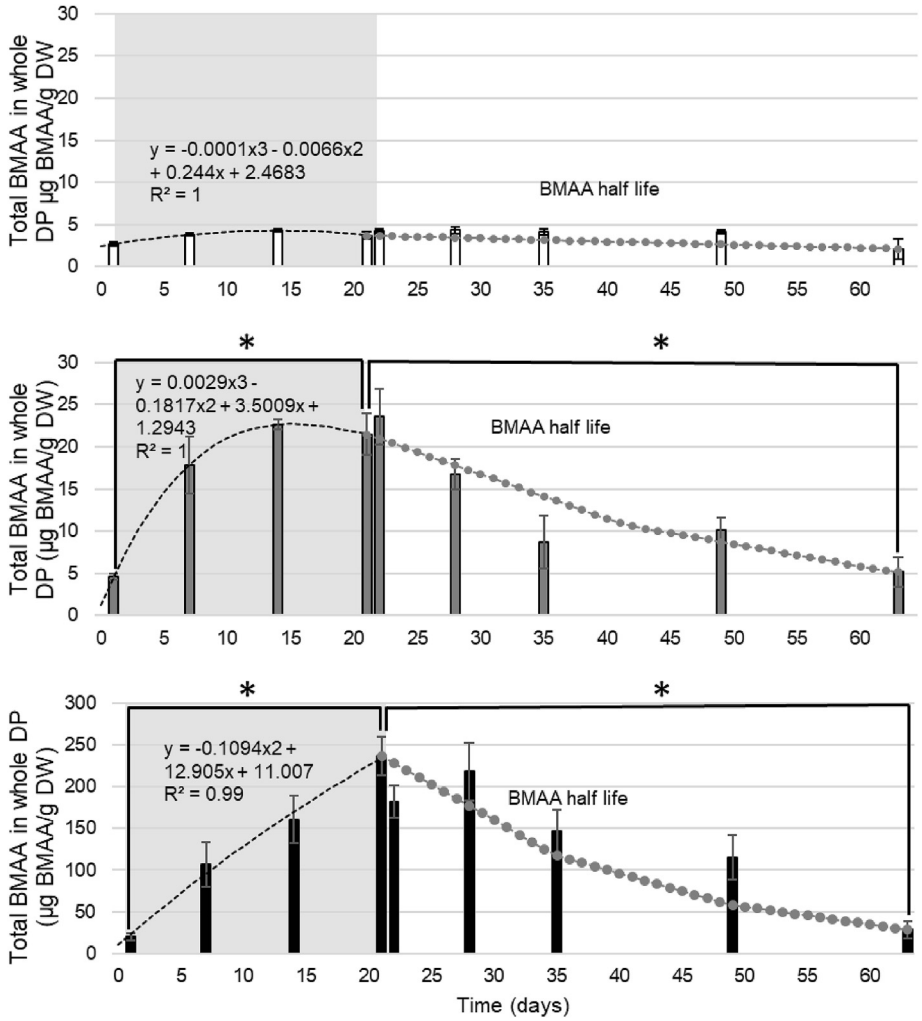

Fig. 2. Mean ± SE of total BMAA concentrations in whole *D. polymorpha* exposed to 1 (A), 10 (B) and 50 (C) μg/L of dissolved BMAA, during the exposure period (E, grey background) and the depuration period (D, white background), n = 4. *, significant difference in the BMAA concentrations in tissues between two dates (Mann-Whitney test, $p < 0.05$). Polynomial regression lines calculated during the exposure period are represented by black dotted lines, and the BMAA half-life curve by grey dotted lines.

g DW) and were the same one week later, on the last day of exposure (21.5 ± 2.4 μg BMAA/g DW). In *D. polymorpha* exposed to 50 μg BMAA/L, the maximum BMAA accumulation was observed on the last day of exposure (236.9 ± 22.9 μg BMAA/g DW). During the exposure period, BMAA concentrations in mussels exposed to 10 μg BMAA/L were significantly higher than in those exposed to 1 μg BMAA/L. Similarly, BMAA concentrations in mussels exposed to 50 μg BMAA/L were significantly higher (Mann-Whitney test, $p < 0.01$) than in those exposed to 10 μg BMAA/L. The calculation of the correlation between BMAA concentrations in whole *D. polymorpha* and the duration of exposure showed that BMAA accumulation was correlated with time in mussels exposed to 10 (Pearson's r = 0.75, $p = 0.001$) and 50 μg BMAA/L (Pearson's r = 0.89, $p = 0.001$), but not in those exposed to 1 μg BMAA/L (Pearson's r = 0.49, $p > 0.05$).

During the depuration period, BMAA was only partially eliminated as it was still found after 42 days spent in clean water in mussels previously exposed to 10 μg BMAA/L (5.2 ± 1.8 μg BMAA/g DW) and 50 μg BMAA/L (28.5 ± 10.4 μg BMAA/g DW). The elimination of BMAA was significant (Mann-Whitney test, $p < 0.05$), with decreases of 76 and 88% of the BMAA content in tissues between the last day of exposure and the 42nd day of depuration, respectively. During the depuration period, the BMAA concentrations in mussels exposed to 10 μg/L were (i) significantly higher than those in mussels exposed to 1 μg BMAA/L (Mann-Whitney test, $p < 0.01$), and (ii) significantly lower (Mann-Whitney test, $p < 0.01$) than in mussels exposed to 50 μg BMAA/L. The BMAA concentration was negatively correlated with time during the depuration period in *D. polymorpha* exposed to 10 (Pearson's r = −0.73, $p < 0.001$) and 50 μg BMAA/L (Pearson's r = −0.78, $p < 0.001$), as well as in mussels exposed to 1 μg BMAA/L (Pearson's r = −0.53, $p = 0.016$).

We compared BMAA concentrations, expressed in μg/g DW, measured in *A. anatina* digestive glands and in whole *D. polymorpha.* Whole *D. polymorpha* accumulated BMAA at significantly higher concentrations, i.e., 1.7 (during exposure to 1 μg BMAA/L) to 18.8 (during depuration after exposure to 50 μg BMAA/L) times more than *A. anatina* digestive glands, under the same exposure and depuration conditions (Table 1).

Third-degree polynomial regression was chosen to observe BMAA dynamics in tissues during exposure because it yielded better results of fitting than linear regression. Those regression lines were not fitted to BMAA dynamics in *A. anatina* exposed to 1 μg BMAA/L ($r^2 = 0.18$), but were correlated with BMAA concentrations in *A. anatina* exposed to 10 and 50 μg BMAA/L ($r^2 = 0.92$ and 0.98, respectively). However, polynomial regressions were fitted to BMAA dynamics in whole *D. polymorpha* ($r^2 > 0.9$ for all exposure concentrations). The BMAA half-life values evaluated during the 42-day depuration period were higher in whole *D. polymorpha* (13.8 and 20.4 days for mussels exposed to 10 and 50 μg BMAA/L, respectively), than in *A. anatina* digestive glands (9.8 and 10.5 days for mussels exposed to 10 and 50 μg BMAA/L, respectively). The predictive BMAA concentrations in *A. anatina* digestive glands, calculated for each day of depuration using the respective half-lives, were correlated with the observed values for each concentration (Pearson's r = 0.99 and $p < 0.01$ for 10 μg BMAA/L and r = 0.94 and $p < 0.05$ for 50 μg BMAA/L). Therefore, the mean half-life of BMAA in this matrix was used to estimate the BMAA concentration in mussels before their 2-week acclimation based on the values observed in the controls at the beginning of exposure.

Table 1
Mean difference factor between BMAA concentrations in whole *D. polymorpha* compared to *A. anatina* digestive glands during exposure to 1, 10 and 50 μg BMAA/L and the depuration period. Tabulated *p*-values for Mann-Whitney tests comparing BMAA concentrations in whole *D. polymorpha* and *A. anatina* digestive glands are given in brackets.

	Exposure	Depuration
1 μg BMAA/L	1.7 ($p = 0.009$)	4.5 ($p < 0.001$)
10 μg BMAA/L	2.7 ($p = 0.003$)	6.0 ($p < 0.001$)
50 μg BMAA/L	4.9 ($p < 0.001$)	18.8 ($p < 0.001$)

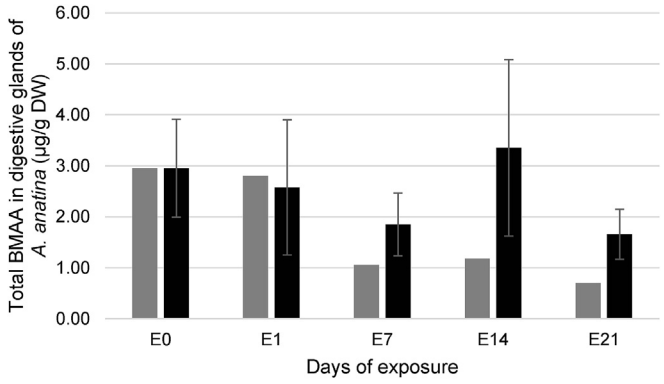

Fig. 3. Predicted BMAA elimination by *A. anatina* estimated from BMAA concentrations measured in the controls (2.95 ± 0.96 μg BMAA/g DW) over 21 days using BMAA half-life (grey bars) compared to observed BMAA concentrations in mussels exposed to 1 μg BMAA/L (black bars) in *A. anatina* digestive glands throughout the exposure period.

The hypothetical kinetics of BMAA elimination by digestive glands of *A. anatina* containing 2.95 ± 0.96 μg BMAA/g DW in their tissues (concentration observed in unexposed mussels) over three weeks were compared with the BMAA concentrations measured in mussels exposed to 1 μg BMAA/L (Fig. 3). While the BMAA concentrations in those two conditions were similar on the first day of exposure (observed concentration: 2.57 ± 1.33 μg BMAA/g DW, estimated BMAA concentration: 2.80 μg BMAA/g DW), differences seemed to appear later. After 7 days, the BMAA concentration measured in *A. anatina* exposed to 1 μg BMAA/L was twice as much as the estimated concentration with no BMAA added in the medium. The difference remained roughly the same after 21 days of exposure, when the observed concentration was 1.66 ± 0.49 μg BMAA/g DW, and the estimated concentration was 0.70 μg BMAA/g DW. This suggests BMAA accumulation by the digestive glands of *A. anatina* exposed to 1 μg BMAA/L.

The calculation of the cumulated percentage of BMAA accumulation (cum % acc, Fig. 4) in each model showed no significant variation over time (Kruskal-Wallis test, $p > 0.05$). However, the cum % acc tended to decrease between day 1 and 7 of exposure in *A. anatina* digestive glands and whole *D. polymorpha* exposed to 1 and 10 μg BMAA/L.

7. Discussion

The freshwater bivalves *A. anatina* and *D. polymorpha* were exposed to dissolved BMAA to assess their potential use as tools for biomonitoring the contamination of aquatic environments by BMAA.

Because of the difference in size (average difference factor of 2) and in soft tissue weight (average difference factor of 12) between the two bivalve species, only one organ was selected to analyse BMAA concentrations in the larger species *A. anatina*. Total BMAA was assessed from 1 mg of freeze-dried tissue, representing less than 1% of the dry mass of a whole *A. anatina,* which is unlikely to be representative of the entire organism. It is admittedly preferable to take only one organ to limit inter-sample variability and to propose a simple tool for multi-risk assessment of water contamination by cyanotoxins. Therefore, we collected the digestive gland,

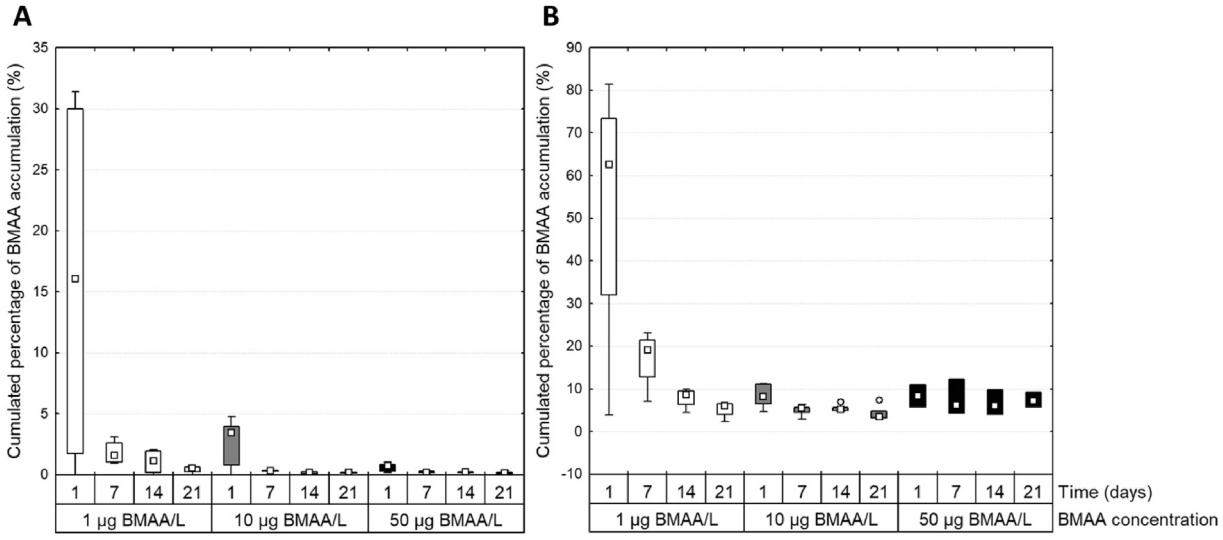

Fig. 4. Cumulated percentage of BMAA accumulation (cum % acc) over time during the exposure period in digestive glands of *A. anatina* (A) and in the whole body of *D. polymorpha* (B) exposed to 1 (white boxes), 10 (grey boxes) and 50 (black boxes) µg BMAA/L. Boxplots indicate first and third quartiles of the observations, whiskers indicate minimum and maximum values, ○ indicate outliers, and the median is indicated by a square.

which is the target organ of oligo-peptide cyanotoxins of interest such as MC and nodularins in bivalves (Chen and Xie, 2005; Kankaanpää et al., 2007). However, *D. polymorpha* is smaller, so that the sampling of its digestive gland would have required 6 to 10 individuals to pool in order to have one replicate of analyse. As this strategy would have required too many individuals for long-term monitoring in multiple sites, we analysed BMAA concentrations in whole *D. polymorpha*.

We detected BMAA at low concentrations (3.3 ± 1.6 µg BMAA/g DW) in the digestive glands of unexposed *A. anatina*. However, as no BMAA was detected in unexposed *D. polymorpha*, it is unlikely that the concentration measured in *A. anatina* digestive glands was a background noise related to matrix interference. It is also unlikely that BMAA was a constitutive molecule of the bivalve tissue or a secondary product of the extraction procedure. Indeed, no BMAA was found in the digestive glands of unexposed *A. anatina* caged *in situ*, originated from the same location as the ones used in this study (Lepoutre et al., unpublished data). Therefore, commercially available *A. anatina* might have accumulated BMAA during farming, probably through the ingestion of phytoplankton producers. Despite the two-week acclimation period, all of the initial BMAA content may not have been completely eliminated from their tissues. It is known that BMAA depuration can take long, as it was still found in *M. edulis* maintained two months in clean water (Reveillon, 2015). Moreover, BMAA was still found in all soft tissues after an exposure of *D. polymorpha* for 21 days to an equivalent of 2.5 µg BMAA/day, followed by 21 days in clean water (Lepoutre et al., 2019). We applied the calculated BMAA half-life in *A. anatina* digestive glands (obtained from the kinetics of BMAA contents observed during the depuration period) to the BMAA contents observed in the controls on day 0 of exposure. We estimated that the BMAA concentration in this tissue may have been around 8.26 µg/g DW upon their reception in the laboratory, before the two-week acclimation period. Using the estimated elimination of BMAA by the controls, we hypothesized that *A. anatina* digestive glands were potentially able to accumulate the neurotoxin when exposed to 1 µg BMAA/L. However, further analyses would be required to investigate when digestive glands of *A. anatina* could reveal the presence of 1 µg BMAA/L.

The low and quite constant BMAA levels, with no kinetics of accumulation or elimination, in both *A. anatina* and *D. polymorpha* exposed to 1 µg BMAA/L may be attributed to gradual and partial elimination of BMAA from tissues slightly after accumulation, involving detoxification mechanisms that are as yet unknown (Downing et al., 2014). Therefore, it appears that the threshold for whole *D. polymorpha* and *A. anatina* digestive glands to reflect environmental contamination of the medium may be around or slightly above 1 µg of dissolved BMAA/L. Freshwater mussels like Dreisenids can clearly take up small dissolved organic molecules (Baines et al., 2007), but they are primarily filter feeders consuming nutritious particles present in the water. Assuming that most of the BMAA present *in situ* is in particulate form (e.g., inside phytoplankton cells), the relatively high filtration rates of the mussels per gill area unit (1.2–1.3 mL min^{-1} cm^{-2} for *A. anatina* and 1.4–1.9 mL min^{-1} cm^{-2} for *D. polymorpha*; Kryger and Riisgård, 1988), suggest that the limit of detection of environmental BMAA using bivalves could be lower. To determine this, further investigations are required with either BMAA-loaded green algae as feed or with a natural stable BMAA producer.

The decrease in accumulation capacities (cum % acc) of total BMAA by the two mussels exposed to 1 and 10 µg BMAA/L between early exposure (day 1) and the remaining exposure times may be explained by three mechanisms. First, some detoxification mechanisms may have been activated between days 1 and 7 of exposure in both species and then remained constantly activated. In mussels exposed to 50 µg BMMA/L, those mechanisms were probably triggered during the first 24 h of exposure, explaining the constant cum % acc observed in both species. During the depuration period, BMAA elimination was partial but significant in whole *D. polymorpha*: 82% of the BMAA content quantified at the end of the exposure period had been eliminated after 42 days of depuration. As BMAA is a hydrophilic compound, the biotransformation of the free fraction by the enzyme glutathione-S-transferase (GST) is not expected (Downing et al., 2014). Contardo-Jara et al. (2014) exposed *A. cygnea* and *D. polymorpha* to 100 µg BMAA/L for 48 h; they measured no variation of the activity of this enzyme during these 48 h. However, BMAA was found in bivalves mainly associated with molecules that can either be soluble (soluble bound BMAA) or found in precipitates (precipitated bound BMAA) after the use of organic solvents (Réveillon et al., 2016a; Salomonsson

et al., 2015). The molecules to which BMAA may be associated are still unknown, but their precursors could be heavier than BMAA (i.e., >300 k vs 118.1 Da) (Rosén et al., 2016), and therefore could be detoxified by enzymes such as GST. The determination of the accumulation kinetics and of the activity of detoxification enzymes at a fine temporal scale remains to be investigated.

Second, in *A. anatina* digestive gland, some of the BMAAs measured during the first day of exposure may have been part of the pre-contamination observed in the controls. This could potentially explain the higher cum % acc observed in the early days of exposure than in the rest of the exposure period. However, cum % acc values were also higher during the first day of exposure than on other sampling days in whole *D. polymorpha* although no BMAA was detected in unexposed *D. polymorpha*. Moreover, as BMAA was measured only in *A. anatina* digestive gland, it is possible that after a first accumulation in the digestive gland BMAA was further distributed to other tissues through the haemolymph during the intoxication process. Pollutants can be mobile once taken up by bivalves as they may bind to haemocytes or to dissolved haemoproteins (Beyer et al., 2017; Marigómez et al., 2002). We previously demonstrated that BMAA was present in the haemolymph of *D. polymorpha*, and further in all organs, and associated to negative cytotoxic and genotoxic effects on haemocytes (Lepoutre et al., 2018; Lepoutre et al., 2019).

Finally, the health status of both species might have been impaired by BMAA. The neurotoxin is known to form an analogue of glutamate when bicarbonate is present at physiological concentrations (Weiss and Choi, 1988). Glutamate is a potential neurotransmitter in bivalves (Bone and Howarth, 1980; Vitellaro-Zuccarello et al., 1990). Therefore, BMAA may have altered their physiology, but not enough to impair their survival as no mortality was observed for either species throughout the experiment.

The higher BMAA concentration in whole *D. polymorpha* than in *A. anatina* digestive glands (up to 223.5 ± 48.5 vs. 38.3 ± 8.0 μg BMAA/g DW when exposed to 50 μg BMAA/L) was expected. A study of BMAA distribution in *D. polymorpha* organs showed that BMAA diffused through all tissues, and that the digestive gland could represent 4.2 ± 1.2% of the total BMAA in the whole body when considering the haemolymph, and 12.3 ± 3.5% when considering only soft tissues (Lepoutre et al., 2019). This could also explain why the cumulated percentages of accumulation measured in *D. polymorpha* were higher than in *A. anatina* (overall means of 6.4 ± 0.6 and 0.6 ± 0.3% when exposed to 10 and 50 μg BMAA/L, respectively). However, BMAA concentrations in *A. anatina* digestive glands were overall 7.3 times higher than 4% of the measured BMAA concentration in whole *D. polymorpha*, and 2.4 times higher than 12% of the measured BMAA concentration in whole *D. polymorpha*. This suggests greater accumulation capacities of BMAA by *A. anatina* than *D. polymorpha*. For instance, microcystin concentrations in whole *A. anatina* were higher than in whole *D. polymorpha* sampled in northern Latvian lakes the difference was attributed to *D. polymorpha* selective feeding and strong microcystin biotransformation (Barda et al., 2015).

8. Relevance of the use of the two bivalves as bioindicators of the presence of BMAA in fresh water

The bivalves *A. anatina* and *D. polymorpha* were exposed to environmental concentrations of BMAA in fresh water, i.e., from 0.3 to 39.6 μg/L (Al-Sammak et al., 2014; Roy-Lachapelle et al., 2015). The results suggest that BMAA accumulation may be time- and concentration-dependent in whole *D. polymopha*. Therefore, whole *D. polymorpha* could be suitable to potentially reflect BMAA contamination levels in water. As total BMAA was detected from day 1 of exposure in whole *D. polymorpha* exposed from 1 to 50 μg BMAA/L, it analysis in this mussel could be used as an early warning of the onset of a freshwater contamination. Regarding *A. anatina* digestive gland, BMAA accumulation was concentration-dependent during exposure and correlated with time during the exposure to 50 μg BMAA/L. However, using *A. anatina* digestive gland may be less relevant to reveal low contamination levels of water by BMAA because i) very little BMAA present in the medium was accumulated in tissues (0.6 ± 0.3%), and ii) accumulation was only significant compared to the controls from day 14 at 10 μg/L.

Because of the relatively slow BMAA elimination by digestive glands of *A. anatina* and whole *D. polymorpha*, the emphasis should be laid on the monitoring strategy. To analyse the presence of BMAA within a specific time-frame, active monitoring using cages with unexposed mussels seems to be more appropriate than passive monitoring, based on one-time grab samples. This technique also makes it possible to use unexposed individuals, and thus to control the length of exposure and also to use sized animals with the same filtering capacities (Phillips and Segar, 1986; Salazar and Salazar, 2006, 1996; Sylvester et al., 2005). Active monitoring also allows for a better characterization of BMAA accumulation by freshwater mussels, and therefore a comparison of the contamination levels of different sites. Similarly, to analyse the dynamics of BMAA in water within a small time-frame, it appears more relevant to cage mussels for a short time. This will avoid overestimating the environmental contamination if a proliferation of BMAA-producing organisms occurs while some of the BMAA accumulated during a previous contamination event is still present in sentinel species.

Modelling tools may be considered to establish a link between the BMAA content in those potential bioindicator species caged *in situ* and environmental contamination. The development of an accumulation model would require additional exposure periods to characterise the kinetics of BMAA in *A. anatina* and *D. polymorpha* by integrating the potential influence of environmental factors (e.g., resource availability, temperature) on their filtration rate and their accumulation and detoxification capacities (Englund and Heino, 1996; Reeders and Bij de Vaate, 1990; Sylvester et al., 2005). Such additional investigations are required to use bivalves as sentinel species because the kinetics of accumulation has an impact on how to extrapolate environmental contamination. Additionally, data from active biomonitoring of BMAA in areas with contrasting levels of contamination may implement this approach.

9. Conclusion

Whole *D. polymorpha* would be suitable as a bioindicator of the presence of BMAA in fresh water because they can reveal the presence of 10 μg/L BMAA within one day and because BMAA concentrations in their tissues are correlated with water BMAA concentrations. However, further investigations are required at finer temporal and concentration scales. The significant but incomplete elimination of BMAA from *D. polymorpha* tissues and from *A. anatina* digestive glands after 42 days of depuration shows that both models may not be suitable to rapidly reveal the end of a water contamination event. Therefore, active caging of regularly renewed uncontaminated mussels would be more suitable to characterise the current BMAA risk in water, whereas passive samplings may overestimate it in case of a previous contamination event. Moreover, as we do not know whether BMAA is dissolved in water or remains inside producers, information regarding BMAA occurrence and bioavailability in fresh water is needed.

Author contributions

Conceptualization: E.L. and A.L.; experiment and sample extraction: A.L.and J.H.; sample analysis: E.J.F. and A.J.Z.; data

curation: A.L.; writing—original draft preparation: A.L.; writing—review and editing: A.L., E.J.F, A.J.Z, M.L., A.G. and E.L.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2019.113885.

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