Marine Environmental Research
September 2015, Volume 110 Pages 8-18
http://dx.doi.org/10.1016/j.marenvres.2015.07.015
http://archimer.ifremer.fr/doc/00275/38607/

β-N-methylamino-l-alanine (BMAA) and isomers: Distribution in different food web compartments of Thau lagoon, French Mediterranean Sea

Réveillon Damien [1, *], Abadie Eric, Séchet Veronique [1], Masseret Estelle [3], Hess Philipp [1], Amzil Zouher [1]

[1] Ifremer, Laboratoire Phycotoxines, Rue de l'Ile d'Yeu, BP 21105, F-44311, Nantes, France
[2] Ifremer, Laboratoire Environnement Ressources Languedoc-Roussillon, UMR MARBEC (IRD – Ifremer – Univ. Montpellier – CNRS), Avenue Jean Monnet, CS30171 Sète, Cedex 3, France
[3] Université de Montpellier, UMR MARBEC (IRD – Ifremer – Univ. Montpellier – CNRS), cc93, Place Eugène Bataillon, 34095, Montpellier, Cedex 5, France

* Corresponding author : Damien Réveillon, email address : Damien.reveillon@ifremer.fr

Abstract :

The neurotoxin BMAA (β-*N*-methylamino-l-alanine) and its isomer DAB (2,4-diaminobutyric acid) have been detected in seafood worldwide, including in Thau lagoon (French Mediterranean Sea). A cluster of amyotrophic lateral sclerosis (ALS), a neurodegenerative disease associated with BMAA, has also been observed in this region. Mussels, periphyton (*i.e.* biofilms attached to mussels) and plankton were sampled between July 2013 and October 2014, and analyzed using HILIC-MS/MS. BMAA, DAB and AEG (N-(2-aminoethyl)glycine) were found in almost all the samples of the lagoon. BMAA and DAB were present at 0.58 and 0.83, 2.6 and 3.3, 4.0 and 7.2 μg g^{-1} dry weight in plankton collected with nets, periphyton and mussels, respectively. *Synechococcus* sp., *Ostreococcus tauri*, *Alexandrium catenella* and eight species of diatoms were cultured and screened for BMAA and analogs. While *Synechococcus* sp., *O. tauri* and *A. catenella* did not produce BMAA under our culture conditions, four diatoms species contained both BMAA and DAB. Hence, diatoms may be a source of BMAA for mussels. Unlike other toxins produced by microalgae, BMAA and DAB were detected in significant amounts in tissues other than digestive glands in mussels.

Highlights

► BMAA was detected in increasing concentrations along the food chain of Thau lagoon. ► BMAA was present in 4 out of 8 cultured diatoms species, not in picocyanobacteria. ► BMAA levels in mussels seemed to be linked to diatom abundances in Thau lagoon. ►Significant amounts of BMAA were detected in non-visceral tissues of mussels.

Keywords : BMAA, Diatoms, *Chaetoceros* sp., Seston, *Mytilus galloprovincialis*, HILIC-MS/MS

1. Introduction

BMAA and isomers (DAB, AEG and BAMA) are non proteinogenic amino acids that have been found in higher trophic levels in many ecosystems, e.g. Guam Island, the Baltic Sea and lake Taihu, China (Jiao et al., 2014, Jonasson et al., 2010 and Murch et al., 2004a). BMAA has been associated with amyotrophic lateral sclerosis (ALS)

(Spencer et al., 1987). As a polar molecule, its bioaccumulation is uncommon but has been shown in the brain of patients who died of ALS but also of other neurodegenerative diseases (Murch et al., 2004b; Pablo et al., 2009). This accumulation is possibly justified by the misincorporation of BMAA into proteins during protein synthesis (Dunlop et al., 2013; Murch et al., 2004a). Consequently, BMAA may exist in two forms, as a free amino acid and bound to proteins. Recently, BMAA has been suggested to be either incorporated into or associated with proteins, since a fraction of "bound" BMAA was released by protein denaturation (Glover et al., 2014b). Hereafter, bound BMAA refers to the BMAA amount that is not extracted as a free amino acid, irrespective of its incorporated or associated form. Many different analytical methods have been employed to detect BMAA leading to controversial reports of BMAA in the environment (Faassen, 2014). However, several sample preparation and analytical procedures have now been well characterized, including those using different internal standards for accurate identification and quantification of BMAA and isomers in biological samples (Combes et al., 2013; Jiang et al., 2013; Reveillon et al., 2014).

Several cases of ALS have been linked to a possible long-term consumption of specific foodstuffs containing BMAA, e.g. crabs or lobsters (Banack et al., 2014; Field et al., 2013). In those studies, cyanobacteria were the suggested primary source of BMAA. However, in marine ecosystems, other microalgal species (some diatoms and dinoflagellates) have recently been reported to contain bound BMAA and isomers. Similarly, marine mollusks were shown to contain BMAA and analogues in the same environments (Jiang et al., 2014a; Lage et al., 2014), thus highlighting potential bioaccumulation. Zooplankton organisms are a key component in some ecosystems, being consumers of phytoplankton and prey of higher-order consumers, thus playing an important trophic link (Gifford, 1991). Zooplankton has been suggested to be a vector of BMAA in aquatic animals in lake Taihu, China (Jiao et al., 2014), due to its ecological function and because BMAA has been reported to be bioaccumulated in zooplankton from the Baltic Sea (Jonasson et al., 2010).

In mussels and oysters from Thau lagoon, France, BMAA, DAB and AEG have independently been detected by two groups at similar levels (Masseret et al., 2013; Reveillon et al., 2014). However, the distribution of BMAA in different organs of filter-feeding bivalves has not been studied so far. Generally, cyanotoxins and phycotoxins are known to accumulate mainly in digestive glands (Ferrao and Kozlowsky-Suzuki, 2011), even more polar molecules such as saxitoxins (Bricelj and Shumway, 1998).

Bioaccumulation and depuration of aquatic toxins in mollusks have been well studied (Bricelj and Shumway, 1998; Ferrao-Filho and Kozlowsky-Suzuki, 2011; Ibelings and Chorus, 2007; Jauffrais et al., 2012) except for BMAA. Mussels are known to bioaccumulate toxins to higher concentrations than other bivalves, making them

good sentinel organisms (Bricelj and Shumway, 1998; Mafra et al., 2010). Detoxification kinetics are compound- and species-specific (Bricelj and Shumway, 1998). In the worst case, toxins can be retained for months, e.g. domoic acid in the scallop *Pecten maximus* (toxic for more than 295 days after contamination by the diatom *Pseudo-nitzschia australis* (Blanco et al., 2002)).

With the aim of improving knowledge on the distribution of BMAA in different compartments of Thau lagoon ecosystem, mussels, periphyton (biofilms attached to mussel) and plankton were collected between July 2013 and October 2014. Diatoms are dominating the micophytoplankton community and they sustain the growth of mollusks of Thau lagoon (Dupuy et al., 2000; Pernet et al., 2012). However, ciliates (including abundant tintinnid populations) and zooplankton species have also been reported in the lagoon (Dupuy et al., 2000; Lam-Hoai and Rougier, 2001) and could represent an alternative food source for the mussels. Indeed, mollusks are selective and opportunistic suspension-feeders, largely relying on phytoplankton but exhibiting different behavior depending on the season and the type of plankton communities (Lefebvre et al., 2009; Riisgard and Larsen, 2010; Trottet et al., 2007).

Samples were analyzed for both free and total BMAA, DAB and AEG to study the distribution of these molecules in different compartments of the Thau lagoon ecosystem and between digestive glands and remaining flesh of the mussels. As cyanobacteria had been considered to be the most likely source of marine-derived BMAA, and had been suggested as a source of BMAA in Thau lagoon in a previous study (Masseret et al., 2013), we screened four strains of *Synechococcus* sp., previously isolated from Thau lagoon. Furthermore, emblematic protists isolated from this lagoon and available to this study included *O. tauri*, and two strains of *A. catenella*. In parallel, as diatoms were found in large quantities in Thau lagoon, eight species of diatoms were grown and screened for total BMAA and isomers.

2 Material and Methods

2.1 Chemicals and reagents

β-*N*-methylamino-L-alanine hydrochloride (BMAA, B107) and trichloroacetic acid (TCA, 33731) were purchased from Sigma-Aldrich, France, while N-2-aminoethylglycine (AEG, A1153) and 2,4-diaminobutyric acid dihydrochloride (DAB, D0083) were obtained from TCI, Belgium. D-2,4-diaminobutyric acid-2,3,3,4,4-$^{2}D_5$ dihydrochloride (D_5DAB), used as the internal standard, was purchased from CDN isotopes (CIL, France).

Methanol (MeOH) and acetonitrile (ACN) were obtained as HPLC grade solvents from JT Baker. Water for analysis was supplied by a Milli-Q integral 3 system (Millipore, France). Solutions of formic acid (FA, 33015),

hydrochloric acid 37% (HCl, 258148) and ammonium hydroxide (NH_4OH, 221228), all reagent grade, were purchased from Sigma-Aldrich, France.

2.2 Samples

2.2.1 Samples collected in Thau lagoon in 2013-2014

The Mediterranean lagoon of Thau, France, is an important area for farming oysters (*Crassostrea gigas*) (Gangnery et al., 2003) and, to a lesser extent, mussels (*Mytilus galloprovincialis*) (Gangnery et al., 2004).

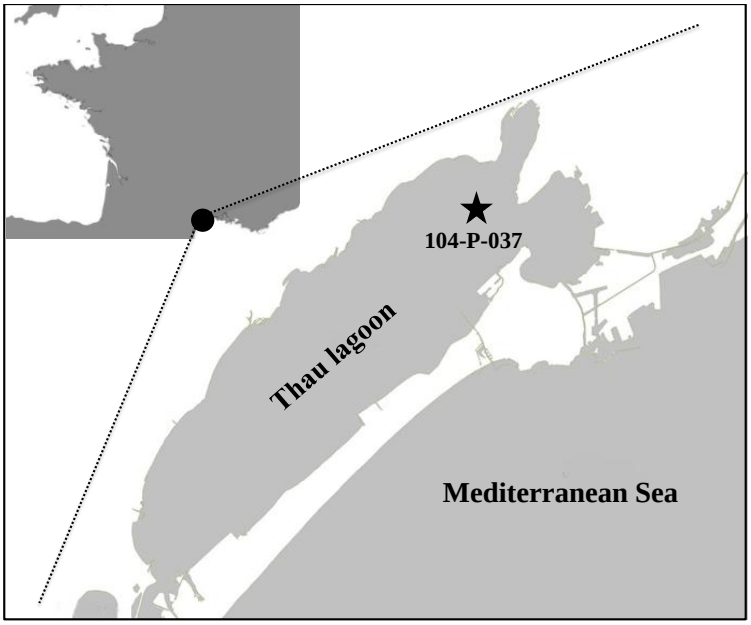


Figure 1: Map of Thau lagoon showing the sampling location (104-P-037)

Samples were collected at location 104-P-037 of the chemical contaminants monitoring network in France ROCCH (Claisse and Raffin, 2010) from July 2013 to October 2014. During this period, samples of wild mussels (17), periphyton, *i.e.* biofilms attached to mussels, (13) and of seston, *i.e.* all particles, including plankton suspended in water, (17) were collected with phytoplankton nets of 20, 40 or 63 µm mesh size. Species identification of periphyton and seston samples was not performed except for the plankton biomass collected after July 8, 2014. From this date, the procedure to collect plankton was adapted to discriminate phytoplankton from zooplankton. Thereafter, samples were pre-filtered through a 125 µm plankton net to remove any large zooplankton or algal debris and filtered through a 20 µm screen to obtain a microphytoplankton fraction (> 20 µm and < 125 µm) while the zooplankton fraction (> 63 µm) was obtained with the 63 µm mesh size net. Identification of major species of plankton was made according to the Utermöhl method (Utermöhl, 1958).

Plankton biomass was harvested via centrifugation at 4000 *g* for 30 min at 4 °C. All samples were freeze-dried, homogenized and stored at room temperature. Both free (when material was available) and total BMAA, DAB and AEG were analyzed. The concentration of bound BMAA and isomers was obtained as the difference between total and free concentrations. For mussels, aliquots of freeze-dried digestive glands (DG) and remaining flesh (RF) tissues ($n \geq 12$) were separately quantified.

2.2.2 Microalgal cultures

The non-axenic strains of microalgae that were screened for the presence of BMAA, DAB and AEG and their conditions of culture are summarized in table 1.

Table 1: Origin and culture conditions of microalgal strains screened for total BMAA, DAB and AEG. Culture media were prepared accordingly to Walne, 1970 for Conway medium, Provasoli, 1968 for ESP medium, Guillard and Hargraves, 1993 for L1 medium, Keller et al., 1987 for K medium and Harrison et al., 1980 for ESAW medium. Here, the ESAW medium was modified by adding ammonium chloride (final concentration of 563 µM).

Strain	Origin	Culture media / days of growth (d)	Irradiance (µmol/m²/s) / Light:dark cycle (h) / Temperature (°C)
Diatoms			
Chaetoceros calcitrans	CCMP 1315	Conway / 11	60 / 16:8 / 22
Chaetoceros sp.	Argenton (France)	Conway / 11	60 / 16:8 / 22
Chaetoceros pumilum	Argenton (France)	Conway / 11	60 / 16:8 / 22
Phaeodactylum tricornutum	CCAP 1055/1	Conway / 11	60 / 16:8 / 22
Skeletonema marinoi	CCMP 1332	L1 / 11	60 / 16:8 / 22
Skeletonema pseudocostatum	Bouin (France)	ESP / 28	50 / 12:12 / 16
Thalassiosira pseudonana	CCMP 1015	Conway / 11	60 / 16:8 / 22
Thalassiosira weiss flogii	CCMP 1336	Conway / 11	60 / 16:8 / 22
Dinoflagellates			
Alexandrium catenella	Thau lagoon (C2-4)	L1 - Si / 10	90/ 16:8 / 17
Alexandrium catenella	Thau lagoon (C11-4)	L1 - Si / 10	90/ 16:8 / 17
Cyanobacteria			
Synechococcus sp.	Thau lagoon (TES 206V)	Modified ESAW / 11	20 / 16:8 / 22
Synechococcus sp.	Thau lagoon (TES 206R)	Modified ESAW / 11	20 / 16:8 / 22
Synechococcus sp.	Thau lagoon (TES 206H6)	Modified ESAW / 11	20 / 16:8 / 22
Synechococcus sp.	Thau lagoon (TES 206D8)	Modified ESAW / 11	20 / 16:8 / 22
Prasinophyceae			
Ostreococcus tauri	Thau lagoon (H95)	K - Si / 41	200 / 16:8 / 23

- Si, without silicate

Most of the diatom strains originated from culture collections while some were isolated from different coastal locations in France. *Synechococcus* sp., *Alexandrium catenella* (C2-4 and C11-4) and *Ostreococcus tauri* were

all isolated from Thau lagoon. *Synechococcus* sp. TES 206V (chlorophyll-rich), TES 206R (phycoerythrin-rich) are polyclonal while TES 206H6 and 206D8 are assumed to be monoclonal strains isolated from TES 206R. *Skeletonema pseudocostatum* was isolated from Bouin, Atlantic coast, France. *Chaetoceros* sp. and *Chaetoceros pumilum* were isolated from Argenton, English Channel, France.

The culture media were prepared with natural filter-sterilized seawater (0.2 µm) at a salinity of 35. All microalgae were grown in batch culture, and were harvested via centrifugation at 4000 *g* for 30 min at 4 °C. Supernatant was carefully removed using Pasteur pipetting and the resulting pellet was freeze-dried, homogenized and stored at room temperature until extraction of total BMAA, DAB and AEG.

2.3 Sample preparation

The procedures for extraction of BMAA, DAB and AEG (both in free and total form), and for analysis were optimized in a previous study (Reveillon et al., 2014). Briefly, deuterated DAB (D_5DAB at a final concentration of 50 ng.mL^{-1}), used as internal standard (750 µL solution, in 0.1 M TCA), was added to freeze-dried shellfish and periphyton (15 mg) or wild and cultured phytoplankton (10 mg). The mixture was ground with glass beads (0.5 g) in a mixer mill (Retsch MM400, Germany) for 30 min. For the extraction of free analytes, supernatants were filtered after grinding and directly cleaned up by SPE. For the extraction of total BMAA and isomers, supernatants were collected and evaporated to dryness under a stream of nitrogen at 40 °C. The residue was dissolved in 600 µL HCl (6 M) and hydrolyzed at 99 °C for 24 h. HCl was dried, and the residue was dissolved in 1 mL of TCA (0.1 M) before SPE clean-up. SPE was performed on Bond Elut® Plexa PCX cartridges (Agilent Technologies, VWR, France).

2.4 HILIC-MS/MS analysis

The LC-MS/MS system consisted of an Ultra-Fast Liquid Chromatograhy (UFLC) (Shimadzu, France) coupled with a triple-quadrupole mass spectrometer (5500 QTRAP, AB Sciex, France). BMAA, DAB and AEG were separated on a ZIC®-HILIC column (150 × 2.1 mm, 5 µm, Merck Sequant®, France) with a TSK gel amide 80 guard column (2 × 10 mm, 5 µm). The linear gradient elution program of the binary mobile phase (solvent A: water with 0.1% formic acid; solvent B: acetonitrile with 0.1% formic acid) was as follows: 0 min, 37% A; 18 min, 55% A, 20 min, 55% A; 23 min, 37% A and 38 min, 37% A. The flow rate was 0.2 mL.min^{-1}, the column temperature 30 °C while samples were kept at 4°C and the injection volume was 5 µL.

The MS/MS analysis was performed in positive ion mode with multiple reaction monitoring (MRM) detection. BMAA, DAB and AEG were unambiguously distinguished thanks to chromatographic resolution, specific mass

spectral transitions and ion ratios (the peak area ratio between the mass spectral transitions *m/z* 119>76 and 119>88 was also considered for the identification of BMAA). Transition *m/z* 119 > 102 (common to all three analogues) was used to quantify BMAA, DAB and AEG, while transition *m/z* 124 > 47 was used to quantify D_5DAB.

BMAA and isomers were quantified using a five-point calibration curve of pure standards prepared by dilution series (1–100 ng.mL^{-1}) of stock solutions in a mixture of ACN and water (63:37, v/v, both containing 0.1% FA), i.e. the starting condition of the chromatographic gradient. A corrective factor derived from D_5DAB recovery was applied to more accurately quantify BMAA, DAB and AEG. All other instrument parameters were the same as mentioned previously (Reveillon et al., 2014). The software Analyst 1.5.1 was used to reprocess the acquired raw data.

2.5 Statistical Analysis

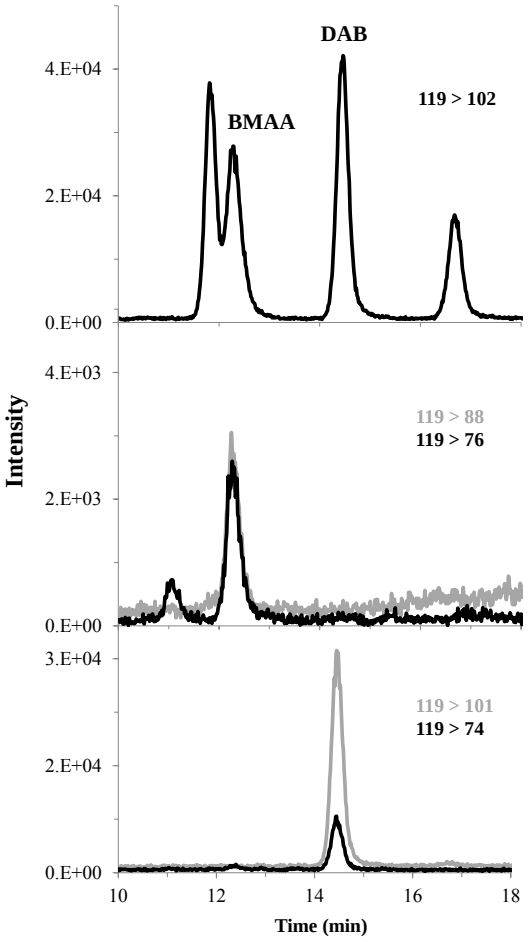
SigmaPlot 11 (Systat Software Inc., Chicago, IL, USA) was used to perform t-tests.

3 Results and Discussion

3.1 Sample preparation and analysis

We used a selective and sensitive HILIC-MS/MS method that had previously been optimized (Reveillon et al., 2014). This method allowed us to accurately identify and quantify BMAA, DAB and AEG in biological matrices, using D_5DAB as internal standard. In this study, the total recovery of D_5DAB was consistent with the results that we have reported in (Reveillon et al., 2014) but also with (Jiang et al., 2013) who observed a total recovery of BMAA of 63.3% for spirulina tablets extracted with a similar sample preparation procedure. Indeed, average recoveries ranged from 53.9 to 60.4% for microalgae and plankton collected with nets and from 56 to 66% for mollusk matrices, extracted for both free and total BMAA and isomers. The relative standard deviation was between 8.9 and 18.8% indicating adequate reproducibility of the sample preparation procedures. However, the recoveries in periphyton matrix were significantly lower (from 28.1 to 29.4%). A group working with freshwater biofilms has also reported lower recoveries of BMAA and DAB in this matrix, in comparison to cyanobacteria and water samples (Combes et al., 2013).

A compound eluting just before BMAA was observed in hydrolyzed environmental samples of periphyton (figure 1) and plankton, as well as in bivalves as previously reported for hydrolyzed mussels and oysters (Reveillon et al., 2014).



184

185 Figure 2: Extracted ion chromatograms of periphyton of March 2014

186 *3.2 Temporal and spatial distribution of BMAA and isomers in environmental samples*

187 The presence of BMAA, DAB and AEG has been reported in sporadically collected mollusks from Thau lagoon
188 (Masseret et al., 2013; Reveillon et al., 2014). For this study, monthly sampling was carried out for over a year
189 (July 2013 to October 2014). In addition to mussels, periphyton (*i.e.* biofilms attached to mussel) and seston
190 were also collected with phytoplankton nets in order to verify the presence and the distribution of BMAA and
191 isomers in different compartments of the Thau lagoon ecosystem and in different mussel tissues.

192 3.2.1 Monitoring of BMAA and isomers in mussels from Thau lagoon

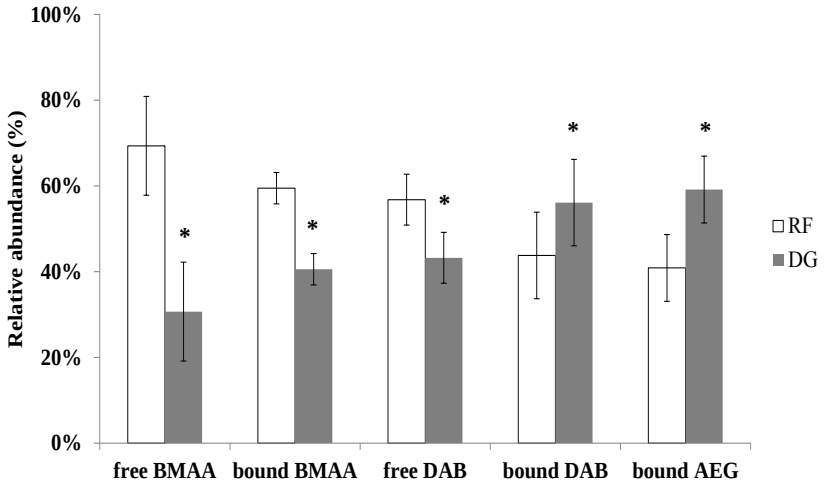
193 BMAA was always detected but quantified in its free form in only 16 out of 34 mussel samples (LOQ was 0.15
194 $\mu g.g^{-1}$ DW), mostly during the summer but in significantly lower concentrations compared to bound BMAA
195 (Appendix A). AEG was found in 31 of 34 samples and only 5 samples, collected during summer 2014, showed
196 quantifiable amounts of AEG in the free form. On the opposite, DAB was detected both in free and bound forms,
197 in all samples. For all isomers, higher amounts were detected as bound form. Indeed, free concentrations of

198 BMAA, DAB and AEG ranged from below the limit of detection to 1.2, 6.2 and 0.31 $\mu g.g^{-1}$ DW, respectively,
199 while maximal concentrations of their total form were 9.7, 10.7 and 1.2 $\mu g.g^{-1}$ DW, respectively. In a previous
200 study (Reveillon et al., 2014), mollusks from Thau lagoon had been screened as a cluster of ALS had recently
201 been reported in this important bivalve farming area, concurrently with the presence of BMAA and DAB in
202 reared mollusks (Masseret et al., 2013). In that study, oysters collected in 2009 and mussels collected at different
203 seasons between 1995 and 2008 contained similar amounts of BMAA and DAB (0.6-6 $\mu g.g^{-1}$ DW), suggesting
204 that these two isomers were repeatedly present in mollusks over several decades. Here, we report the presence of
205 BMAA, DAB and AEG in all samples of Thau lagoon mussels (*Mytilus galloprovincialis*) collected monthly
206 between July 2013 and October 2014.
207 The presence of BMAA in seafood (as well freshwater foodstuffs) was reported worldwide. Indeed, blue crabs
208 from Chesapeake Bay, USA (< 115.2 $\mu g.g^{-1}$ DW) (Field et al., 2013), lobsters collected in Florida Bay
209 (< 27 $\mu g.g^{-1}$ DW) (Banack et al., 2014), fishes from Nebraska reservoirs (< 2.6 $\mu g.g^{-1}$ DW) (Al-Sammak et al.,
210 2014), dietary supplements from shark cartilage (< 352 $\mu g.g^{-1}$ DW) (Mondo et al., 2014), cockles from
211 Portuguese coasts (< 0.43 $\mu g.g^{-1}$ DW) (Lage et al., 2014), mussels, oysters and different fish species from the
212 Baltic Sea (< 1.3 $\mu g.g^{-1}$ DW and < 1.6 $\mu g.g^{-1}$ wet weight) (Jonasson et al., 2010; Salomonsson et al., 2013), even
213 seafood purchased in Swedish markets (< 0.9 $\mu g.g^{-1}$ wet weight) (Jiang et al., 2014b) and fishes and
214 invertebrates from Gonghu Bay, China (< 36 $\mu g.g^{-1}$ DW) (Jiao et al., 2014) were shown to contain various
215 amounts of BMAA, depending on the matrix and the analytical method used. DAB and/or AEG were only
216 analyzed for in few of these studies but DAB concentrations were generally similar to those of BMAA.
217 The survey from July 2013 to October 2014 provided insight into the distribution of BMAA and isomers in
218 mussels from Thau lagoon, including seasonal variations. Concentrations of DAB were relatively constant
219 throughout the sampling period, irrespective of the free or total form and the organ considered (Appendix A).
220 The overall mean concentration of total DAB in remaining flesh (RF) and digestive gland (DG) tissues was 7.2
221 $\mu g.g^{-1}$ DW, with a relative standard deviation of only 22%. On the opposite, the concentration of total BMAA
222 varied and showed two time-dependent increases in summer/autumn seasons in 2013 and 2014. High levels of
223 total BMAA (from 3.8 to 14.4 $\mu g.g^{-1}$ DW) has also been observed throughout the summer of 2009 in mussels
224 from Thau in a previous study (Reveillon et al., 2014). Similarly, Masseret et al., 2013 also reported higher
225 concentrations of BMAA in mussels collected at the same location during summer. The pattern of BMAA
226 presence in mussels of the lagoon could be due to cycles of accumulation/depuration, as a result of feeding, in
227 analogy to the hypothesis formulated for contaminated cockles in Portugal, feeding at least partially on the

228 dinoflagellate *Gymnodinium catenatum* that was shown to contain bound BMAA (Lage et al., 2014). The higher
229 seasonal variability in BMAA concentrations compared to DAB may also be due to the generally much wider
230 presence of DAB in all phytoplankton and cyanobacterial species analyzed.

231 3.2.2 Distribution of BMAA and isomers in mussels digestive glands (DG) and remaining flesh (RF) tissues

232 We discriminated concentrations of BMAA and its isomers between digestive glands (DG) and remaining flesh
233 (RF), so these tissues were separately analyzed (figure 2).



234

235 Figure 3: Relative abundance (mean ± SD) of free and bound BMAA and isomers between digestive gland (DG)
236 and remaining flesh (RF) tissues of all mussel samples collected during the sampling period. Free AEG
237 concentrations were rarely detected so only relative abundance of bound AEG are represented. * $P < 0.05$ in
238 comparison to the relative abundance in the remaining flesh tissue (t-test).

239 Relative abundance of BMAA, DAB and AEG all differed significantly between digestive glands and remaining
240 flesh of mussels ($P < 0.05$). Indeed, free and bound BMAA were more present in RF tissues and accounted
241 respectively for 69.3% ± 17% and 59.5% ± 6% (mean ± RSD) of the BMAA concentrations in mussels. On the
242 opposite, concentration of bound AEG was significantly higher in DGs. Concerning DAB, while free
243 concentration was significantly higher in RFs, the tendency was reversed for bound DAB which was
244 significantly more present in DGs. This differential distributionis somewhat surprising, considering the small
245 differences at molecular level of these amino acids. However, differences between analogs had already been
246 observed in an *in vitro* model for protein incorporation (Glover et al., 2014a).
247 The distribution of BMAA and DAB within mussel tissues was unusual in comparison to other cyanotoxins and
248 phycotoxins Toxins accumulating in filter-feeding bivalves as a function of consumption of microalgae were
249 mainly found in digestive glands or viscera, independently of the polarity of the toxins, with proportions ranging

from 70 to 95% in the digestive gland for both freshwater and marine mollusks (Blanco et al., 2007; Bricelj et al., 1990; Jauffrais et al., 2012; Lassus et al., 2007; Mafra et al., 2010; Medhioub et al., 2010; Negri and Jones, 1995; Strogyloudi et al., 2006; Vasconcelos, 1995).. As far as we know, few data concerning the distribution of BMAA in mollusks are available in the bibliography. Only one study focused on the bioaccumulation of dissolved labeled $^{5+}$BMAA but no distinction was made between organs of the freshwater mussels (Downing et al., 2014). However, our results confirmed the report of (Andrys et al., 2015) about the presence of BMAA both in the visceral (gut) and non-visceral (mantle) tissues of *M. edulis*, raising a question about the bio-incorporation and the metabolization of BMAA in blue mussel.

3.2.3 BMAA and DAB, as non proteinogenic amino acids, have recently been suggested to be incorporated into proteins during protein synthesis or associated with proteins through non-specific binding (Dunlop et al., 2013; Glover et al., 2014b; Xie et al., 2013). The amount of incorporated BMAA was even higher in organs with a high rate of protein synthesis, like the liver for rat (Karlsson et al., 2014). Even if it remains to be demonstrated in controlled conditions, BMAA and DAB could be accumulated in mussels via the incorporation into proteins, as it was suggested to explain the biomagnification of BMAA in the Guam ecosystem (Murch et al., 2004a).

3.2.3 BMAA and isomers in periphyton and seston collected in the Thau lagoon

To further increase our knowledge about the distribution of BMAA in different compartments of the Thau lagoon ecosystem, we collected different samples of plankton. Both periphyton (*i.e.* biofilms attached to the shell of mussels) and seston were analyzed for free and total BMAA and isomers.

Periphyton consists of biofilms that are assemblages of aggregated microorganisms and particles that are attached to a substrate (*i.e.* on the shell of mussels in this study). Unfortunately, species identification of periphyton samples from Thau lagoon was not performed, however, in a previous unpublished study diatom species had been observed in periphyton from Thau lagoon (E. Masseret, personal communication). Similar concentrations of total BMAA and DAB were observed, ranging from 1.3 to 4.3 and 1.3 to 4.8 µg g^{-1} DW while concentrations of total AEG were lower (figure 3). Only DAB was detected in its free form, in 11 out of the 13 samples and in low concentrations (mean of 0.49 µg g^{-1} DW).

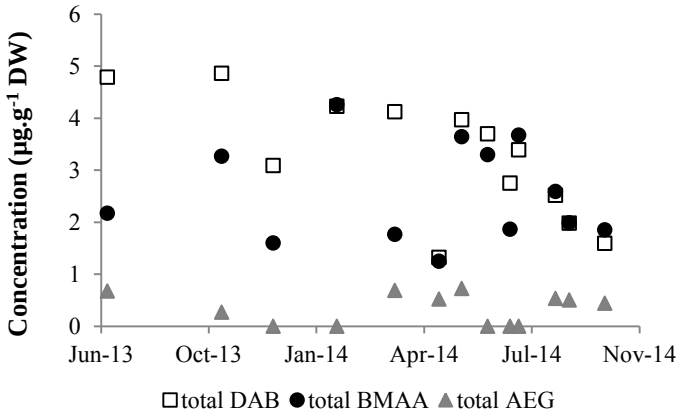


Figure 4: Concentrations of total DAB (empty squares), BMAA (black circles) and AEG (grey triangles) found in periphyton samples.

Temporal variability was observed in our study for both BMAA and DAB in periphyton. Periphyton is a dynamic structure whose communities respond very rapidly to changes in environmental conditions, for both natural and anthropogenic origins, making them useful tools and indicator of biotic integrity and water quality (Dahl and Blanck, 1996; Gaiser et al., 2011; Hill et al., 2000; Nayar et al., 2003). Therefore, seasonal variability is expected to occur. It is not proven that mussels directly feed on periphyton but they may possibly feed on periphyton grazers (Hillebrand et al., 2002), or on diatoms and other organisms constitutive to periphyton in Thau lagoon.

While we do not know if mussels typically feed on periphyton in Thau lagoon, they are known to feed on phytoplankton or zooplankton, the major components of seston. We detected BMAA and DAB, almost exclusively in their bound form, in seston collected with phytoplankton nets (figure 4). Unlike for periphyton matrix, no AEG was detected in these samples. We detected BMAA and DAB in seston dominated by either microalgae or zooplankton, and even when species of both organisms were found in similar proportion (e.g. September 3 and October 6, 2014). The mean concentrations (± SD) of BMAA were 0.49 (± 0.30) and 0.63 (± 0.30) µg.g^{-1} DW in the microalgae and zooplankton-dominated fractions, respectively. Concerning DAB, the concentrations in the two fractions were 0.69 (± 0.39) and 0.92 (± 0.44) µg.g^{-1} DW.

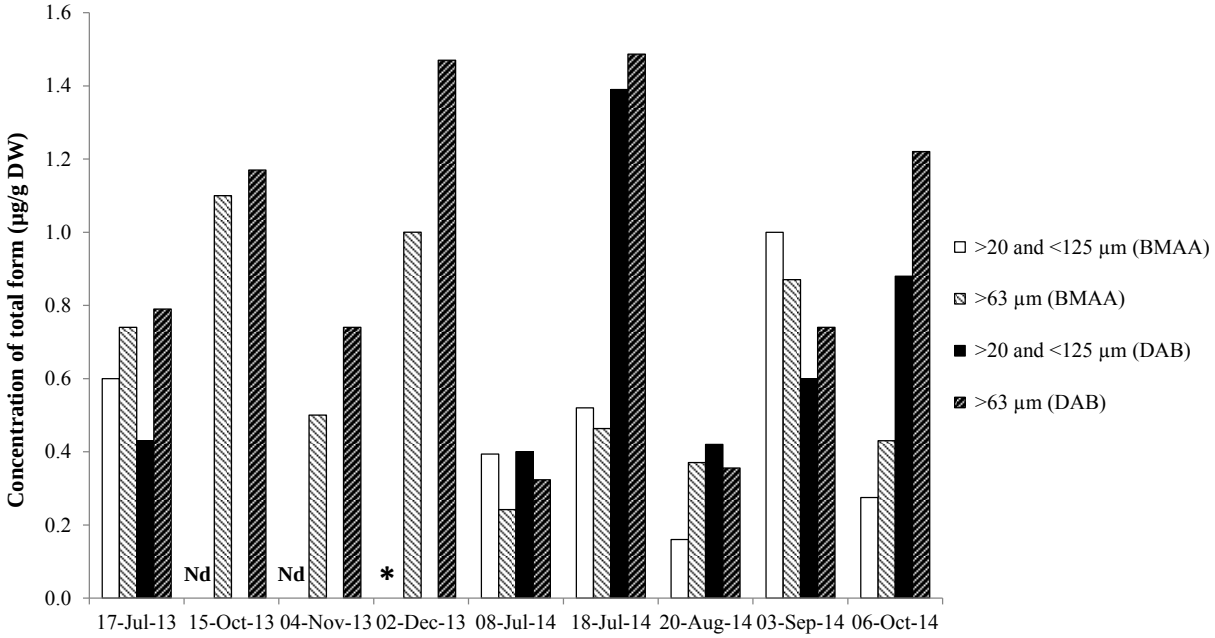


Figure 5: Concentrations of total BMAA and DAB in seston collected with phytoplankton nets of 20 (plain colors) and 63 µm (hatched), depending on the date of sampling. Note: * only biomass collected with a 63 µm mesh size net was sampled on December 2, 2013 while Nd: BMAA and DAB were not detected in the seston collected with a 20 µm screen on October 15 and November 4, 2013.

The composition of plankton collected from July 8, 2014 was reported in Appendix B. Unfortunately sampling procedure did not lead to size fractions that clearly contained only phyto- or only zooplankton. As expected, phytoplankton (mostly diatoms) were more abundant in seston > 20 µm while zooplankton (mainly copepods) dominated the biomass in seston > 63 µm. The screening of seston samples did not allow us to identify specific organisms containing BMAA in Thau lagoon. However, the presence of BMAA and DAB in these samples suggested that some plankton species, on which mussels can feed, did contain these two non proteinogenic amino acids. However, the plankton community in the seston collected occasionally did not reflect the overall plankton community between July 2013 and October 2014. Therefore, the database of the French national monitoring program on phytoplankton and phycotoxins (REPHY) was interrogated for species specific information during our sampling period.

3.3 Phytoplankton community and culture of organisms as possible sources of BMAA in Thau lagoon

3.3.1 Phytoplankton species identification from the national monitoring program REPHY

In this program, phytoplankton experts identify and count all phytoplankton organisms >20 µm, as well smaller organisms if these are chain- or colony-forming, according to the Utermöhl method (Utermöhl, 1958). From July

2013 to October 2014, diatom abundances accounted for 89% of the microphytoplankton, with high proportions of *Chaetoceros* sp., *Pseudo-nitzschia* sp., *Ceratoneis closterium* and *Skeletonema costatum,* depending on the season (figure 6). Diatoms were always present but bloomed mainly in summer. *Chaetoceros* sp. was largely dominating the biomass except in winter, when a peak of *Skeletonema costatum* occurred. Previous studies have shown that *Chaetoceros* sp. was the main diatom species in the microplankton on several occasions separated by several years, indicating that this species is regularly abundant in Thau lagoon (Pernet et al., 2012; Vaquer et al., 1996). Data from this study, as well from previous studies, thus confirmed that diatoms, in particular *Chaetoceros* sp., were highly abundant during the sampling period and could have been an important food source for zooplankton and mussels. As previously mentioned, the presence of diatoms is noteworthy since diatoms have recently been suggested to contain bound BMAA (Jiang et al., 2014a) and they sustain the growth of mollusks in Thau lagoon (Dupuy et al., 2000; Pernet et al., 2014; Pernet et al., 2012). However, the production of BMAA by microalgae from Thau lagoon has not been studied so far and the assertion of Jiang et al., 2014a saying that "BMAA production might be common among diatoms" should be confirmed. Therefore, we have undertaken culture of several phytoplankton organisms that have been identified in Thau lagoon, either during previous studies or as part of the REPHY program.

3.3.2 BMAA and its isomers in cultivated cyanobacteria and other microalgae

Cyanobacteria have been the first organisms suspected to be BMAA-producers (Cox et al., 2005). The dominance of diatoms and dinoflagellates in some marine ecosystems, concurrently with the occurrence of BMAA in mollusks, has led some groups to investigate the potential production of BMAA by these abundant microalgal species (Jiang et al., 2014a; Jiang and Ilag, 2014; Lage et al., 2014), increasing the number of potential BMAA-producing microalgae. We screened fours strains of the cyanobacteria *Synechococcus*, previously isolated from Thau lagoon as well as several other, emblematic protists isolated from this lagoon: *O. tauri*, and two strains of *A. catenella.* As diatoms were abundant in Thau lagoon, including several reports of *Chaetoceros* sp., we also screened eight species of diatoms, including three *Chaetoceros* species. BMAA was detected in four out of the eight species of diatoms (table 2) and was not detected in any other class. Concerning the two isomers of BMAA, DAB was found in all microalgae while AEG was only detected in four of the eukaryotic strains.

342 Table 2: Concentrations of total BMAA, DAB and AEG in the 15 lab-cultured microalgae.

Strain	Origin	Concentration of total form (µg.g^{-1} DW)		
Diatoms		BMAA	DAB	AEG
Chaetoceros calcitrans	CCMP 1315	0.32	3.9	< LD
Chaetoceros sp.	Argenton (France)	0.58	29	< LD
Chaetoceros pumilum	Argenton (France)	< LD	5.5	< LD
Phaeodactylum tricornutum	CCAP 1055/1	0.51	1.3	< LD
Skeletonema marinoi	CCMP 1332	< LD	1.1	< LD
Skeletonema pseudocostatum	Bouin (France)	< LD	1.6	0.28
Thalassiosira pseudonana	CCMP 1015	0.75	18	< LD
Thalassiosira weiss flogii	CCMP 1336	< LD	1.1	< LD
Dinoflagellates				
Alexandrium catenella	Thau lagoon (C2-4)	< LD	1.3	0.53
Alexandrium catenella	Thau lagoon (C11-4)	< LD	3.7	0.61
Cyanobacteria				
Synechococcus sp.	Thau lagoon (TES 206V)	< LD	1.3	< LD
Synechococcus sp.	Thau lagoon (TES 206R)	< LD	2.0	< LD
Synechococcus sp.	Thau lagoon (TES 206H6)	< LD	1.9	< LD
Synechococcus sp.	Thau lagoon (TES 206D8)	< LD	4.7	< LD
Prasinophyceae				
Ostreococcus tauri	Thau lagoon (H95)	< LD	8.7	4.7

343 < LD: no peak or a peak inferior to the limit of detection (0.23 µg.g^{-1} DW) was observed at a retention time
344 corresponding to the standard
345

346 The concentrations of BMAA in the four diatom species ranged from 0.32 to 0.75 µg g^{-1}. BMAA has recently
347 been detected in all diatom strains analyzed by a Swedish group (Jiang et al., 2014a), including one
348 *Thalassiosira* sp. (CCAP 1085/15) and two *Skeletonema marinoi* isolates. In contrast to our results, this group
349 reported concentrations < 0.003 µg.g^{-1} DW, i.e. ca. 100-fold lower than concentrations in the present study. That
350 group focused on the concentration of bound BMAA while we analyzed the total form. As sample preparation in
351 case of the Swedish study included a protein precipitation step (not used in our study), the difference in results
352 may derive from this difference. The Swedish study introduced this step due to column effects observed when
353 injecting hydrolyzed crude extracts. We did not observe such effects in our method and did also not observe any
354 interference for BMAA in diatom extracts (Figure 5). Ion ratios for the different transitions in diatom extracts
355 were equivalent to those of standards for all samples in which BMAA was detected.
356

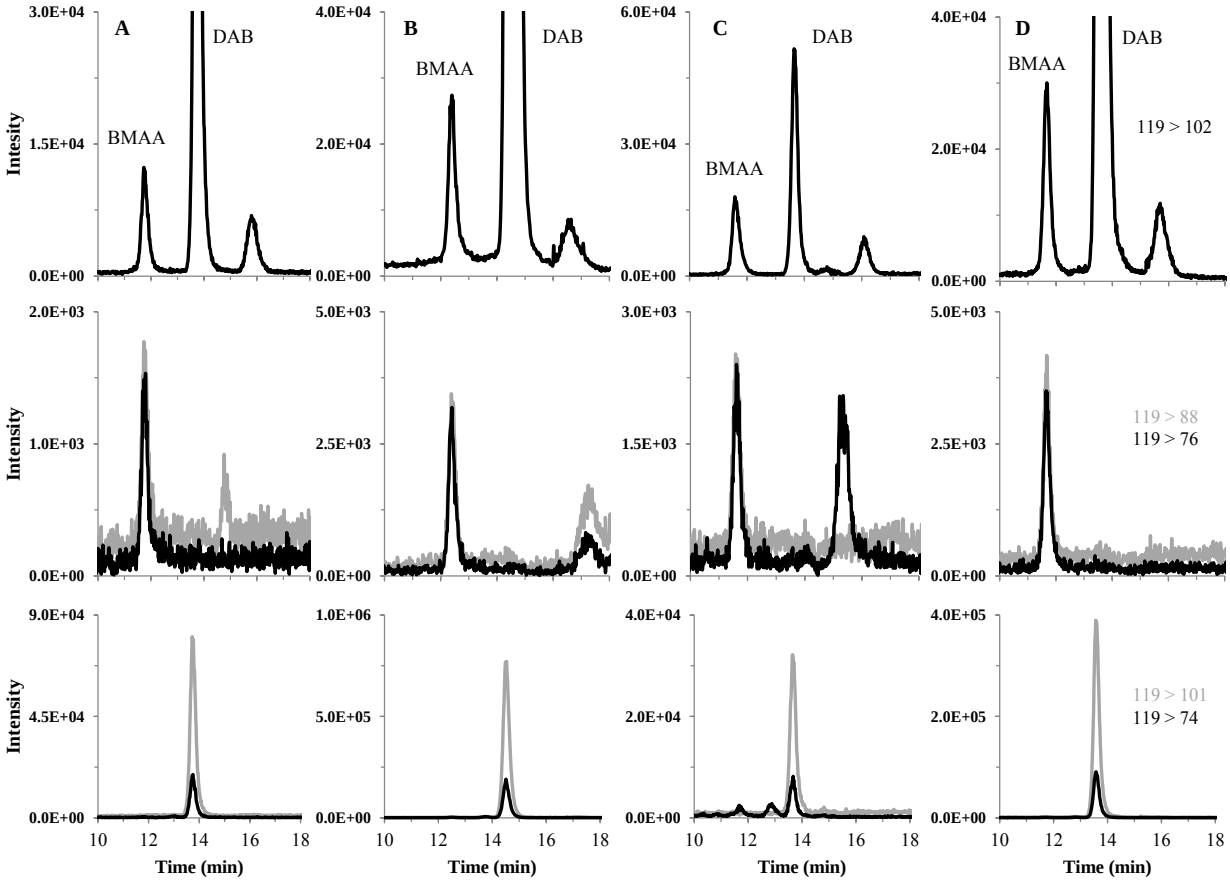


Figure 6: Extracted ion chromatograms of (A) *Chaetoceros calcitrans* CCMP 1315, (B) *Chaetoceros* sp. from Argenton, (C) *Phaeodactylum tricornutum* CCAP 1055/1 and (D) *Thalassiosira pseudonana* CCMP 1015.

The confirmation that diatoms contained BMAA is of particular importance since they contribute significantly to the diet of mussels and oysters in Thau lagoon (Dupuy et al., 2000; Pernet et al., 2012). Specifically, BMAA was found in two out of the three *Chaetoceros* strains (including one isolated from the French coast) and *Chaetoceros* sp. was one of the most abundant microalgae in Thau lagoon between July 2013 and October 2014 (figure 6). DAB and AEG were observed in previous studies focusing on the production of BMAA by dinoflagellates and diatoms, but no concentrations have been reported to date (Jiang et al., 2014a; Lage et al., 2014). DAB concentrations in our cultures showed variability and ranged from 1.1 to 29 $\mu g.g^{-1}$ DW, which is in the same order of magnitude (between 0.5 and 370 $\mu g.g^{-1}$ DW) and variability as concentrations reported for cyanobacteria (McCarron et al., 2014; Reveillon et al., 2014; Rosen and Hellenas, 2008).

The toxic dinoflagellate *Alexandrium catenella* was screened since some dinoflagellates have been suggested to produce BMAA (Lage et al., 2014). *Ostreococcus tauri*, the smallest known eukaryote, is also very abundant in Thau lagoon (Dupuy et al., 2000; Vaquer et al., 1996), and was therefore also tested as a potential source organism. Interestingly, these two species did not produce BMAA under our culture conditions, yet they did

produce DAB and AEG, and could thus be a primary source for these isomers in the zooplankton and the mussels of the lagoon. However, *O. tauri* was reported to be poorly retained by oysters of Thau lagoon (Dupuy et al., 2000), suggesting that this picoeukaryote could not be a valuable direct trophic resource for mussels.

According to Masseret et al., 2013, Thau lagoon experienced a bloom of picocyanobacteria (mostly *Synechococcus* sp.) during the summer of 2009. Neither free nor bound BMAA were found in the four strains of *Synechococcus* sp. isolated from Thau lagoon in 2006. However, further work should be done on a nitrogen stress because the BMAA content has been suggested to be modulated by nitrogen availability in cultured and environmental samples of cyanobacteria (Downing et al., 2011; Scott et al., 2014). While picocyanobacteria could have been a source of BMAA, bioaccumulation in filter-feeding bivalves would require an additional indirect vector (Masseret et al., 2013).

Finally, another hypothesis would be based on the presumption that BMAA production in diatoms could be derived from bacterial symbionts. Interactions between bacteria and diatoms are known to occur and may affect growth, aggregation or toxin production (Doucette, 1995; Gardes et al., 2011; Grossart et al., 2005). For example, bacteria played an important but nonessential role in domoic acid production by *Pseudo-nitzschia multiseries* (Bates et al., 1995). Even though it was reported for DAB [see (Banack et al., 2010) for more precisions], it is not known if bacteria can directly produce BMAA. We did not use axenic cultures of diatoms, and generally, mussels are able to accumulate and assimilate bacteria from the surrounding environment (Cavallo et al., 2009), and more efficiently when bound in marine aggregates (Kach and Ward, 2008). At this stage, we can therefore not infer whether or not bacterial symbionts play a role in bacterial synthesis of BMAA in association with diatoms. There are also no reports on the biosynthetic pathway of BMAA in cyanobacteria and thus, the presence of BMAA in *Phaeodactylum tricornutum* is particularly interesting as this diatom species has been sequenced (Bowler et al., 2008), which would be helpful in studying the biosynthesis and metabolism of BMAA. Overall, and particularly following recent methodological developments (Faassen, 2014), biosynthesis of BMAA should be revisited in a number of species to gain a clearer picture on the distribution throughout all classes of microorganisms.

3.4 Hypothesized bioaccumulation pathway in different compartments of the Thau lagoon ecosystem

Based on a previous study (Cox et al., 2005), picocyanobacteria of the genus *Synechococcus* were suggested to contain BMAA, being grazed upon by zooplankton which would be the carrier of BMAA to mollusks of Thau lagoon (Masseret et al., 2013). Nevertheless, diatoms, now reported as BMAA-producers, are highly abundant in

the phytoplankton community and are suggested to sustain the growth of mollusks in the important bivalve farming area of Thau lagoon (Pernet et al., 2012). This trend was also confirmed in our study (Figure7). The presence of BMAA in several diatom cultures (both from culture collections and from French coastal regions) and in environmental samples (from Thau lagoon) and the absence of BMAA in the four *Synechococcus* strains from Thau lagoon all suggest that the bioaccumulation pathway of BMAA in this ecosystem should be revised. However, our results cannot exclude the earlier hypothesis of cyanobacterial BMAA production in this lagoon as the picoplanktonic fraction was not sampled in this study.

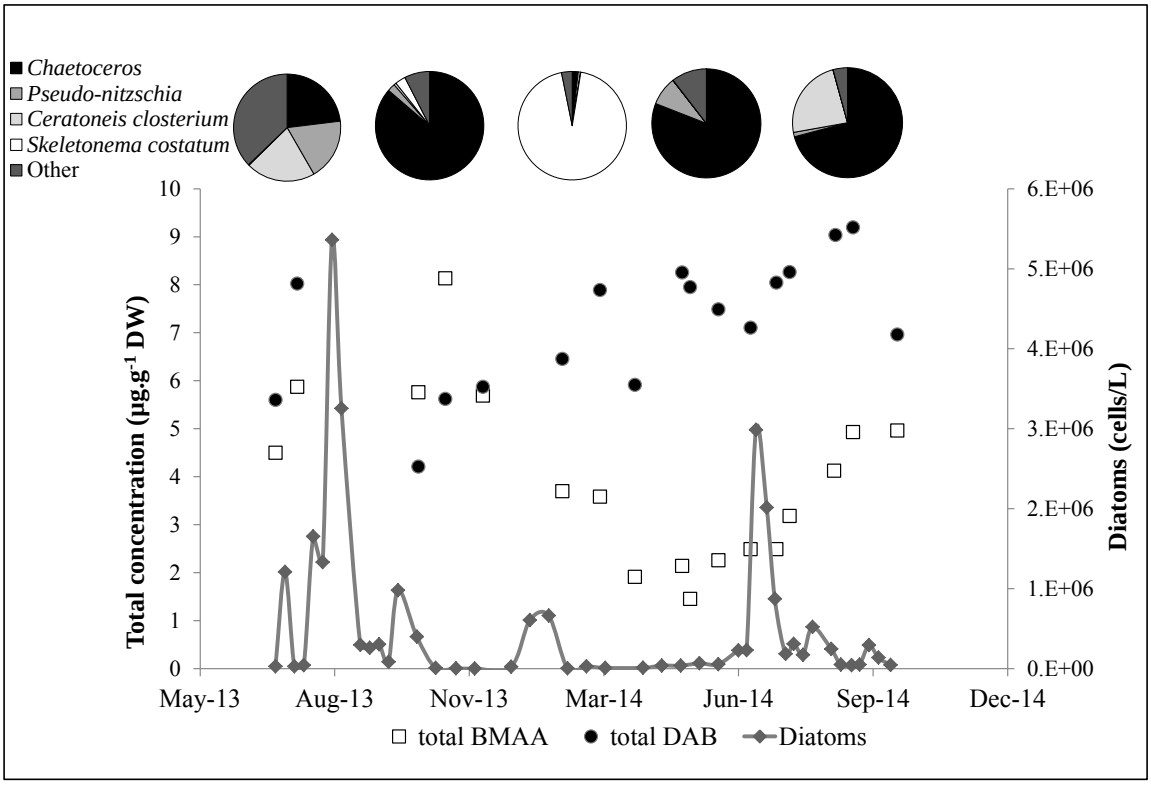


Figure 7: Total BMAA and DAB (µg.g^{-1} dry weight, expressed as the mean of concentrations in digestive glands and remaining flesh) in mussels collected in Thau lagoon, and diatom abundance (cells.L^{-1}) determined by the REPHY during the same period at the same sampling point. The pie charts on the top of the graph represent proportions of major species of diatoms as a function of the season (sum over three month periods).

The concentration of BMAA in mussels seemed to be linked to the abundance of diatoms during the sampling period (figure 7). Indeed, the peaks of diatom abundance, in summer 2013 and 2014, were followed by accumulation of BMAA in mussels. On the opposite, BMAA concentration decreased slowly during winter and spring, when diatom abundances dropped, until a new bloom of diatoms appeared the following spring. DAB concentrations did not depend on diatoms abundances, which is coherent with the presence of this analogue in all

421 species analyzed. BMAA concentrations in the remaining flesh of mussels never decreased to less than 2 $\mu g.g^{-1}$
422 DW between July 2013 and October 2014. The long accumulation and retention of BMAA in mussels may be
423 coherent with the strong association with (and potentially incorporation into) proteins. Furthermore, the increase
424 of BMAA concentrations was relatively slow and lasted for a few months after the peak of diatom blooms,
425 which suggests that additional uptake routes via zooplankton might play a significant role in the BMAA food
426 chain of Thau lagoon
427 . As suspension-feeding aquatic animals, bivalve mollusks have specialized in grazing on particles of a wide
428 range of size, ranging from bacteria (< 2µm) to zooplankton (> 200µm) (Riisgard and Larsen, 2010). Thus,
429 mussels may accumulate BMAA from diatoms as well as from zooplankton. This hypothesis is supported by the
430 occurrence of BMAA in fractions of plankton dominated by either diatoms or zooplankton. BMAA has already
431 been reported in zooplankton, in 6-fold higher concentrations than in cyanobacterial samples from the Baltic Sea,
432 suggesting a bioaccumulation through feeding processes (Jonasson et al., 2010). This bioaccumulation may have
433 also occurred in Thau lagoon as zooplankton is assumed to feed on microalgae and some dinoflagellate and
434 diatom species present in this ecosystem are reported to contain BMAA.
435 In view of our results confirming the presence of BMAA in cultured diatoms, periphyton and seston sampled in
436 Thau lagoon, we propose a theoretical bioaccumulation pathway within Thau lagoon based on accumulation
437 from these three compartments (figure 7). Hitherto, only one study focused on bioaccumulation and depuration
438 of BMAA in four freshwater mussel species (Downing et al., 2014). This study used isotopically labeled BMAA,
439 yet could not provide any evidence of either degradation of BMAA nor incorporation into or association of
440 BMAA with proteins. Thus, further work is required to better understand accumulation and metabolism of
441 BMAA in mussels and to confirm the validity of the hypothesized BMAA bioaccumulation pathway in Thau
442 lagoon.

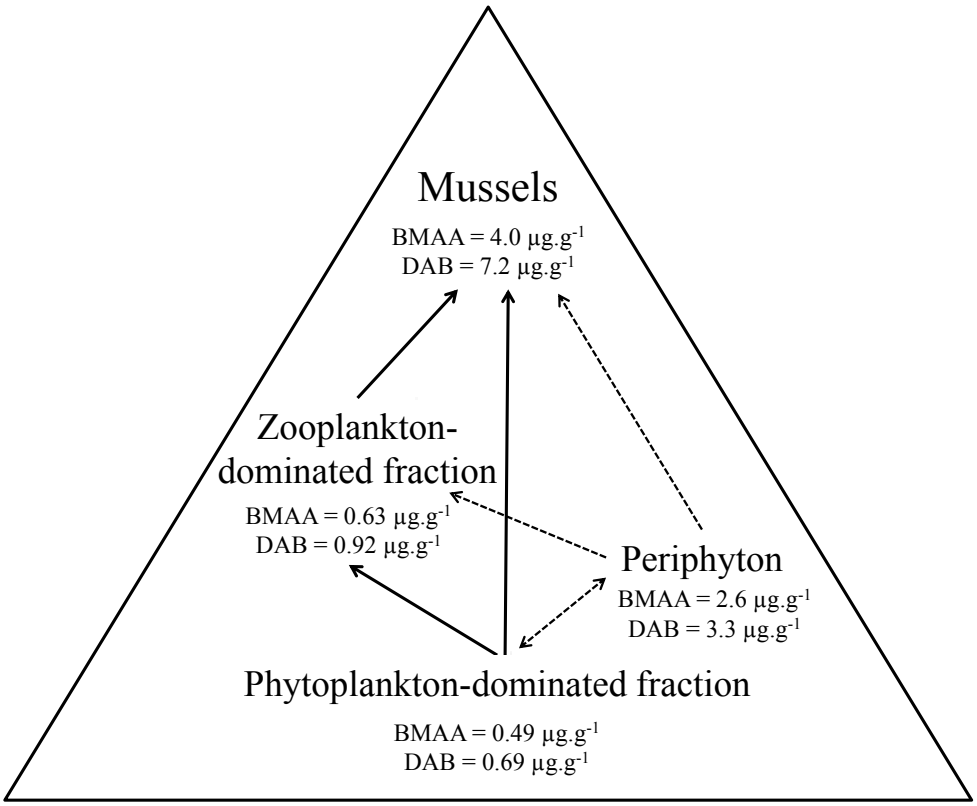


Figure 8: Hypothesized bioaccumulation pathway within the Thau lagoon ecosystem. Mean concentrations of total BMAA and DAB are expressed as $\mu g.g^{-1}$ dry weight. The plain arrows represent the hypothesized trophic links while dotted arrows represent non-confirmed links.

4 Conclusions and perspectives

We performed a survey of different trophic compartments of the Thau lagoon ecosystem, including seston (phytoplankton and zooplankton), periphyton and mussels, between July 2013 and October 2014. BMAA, DAB and AEG were present in mussels at any time point through the year. Interestingly, bound forms of both BMAA and DAB were also detected in phytoplankton-dominated fractions, zooplankton-dominated fractions and periphyton, all representing possible food sources for mussels. The increase in BMAA and DAB between lower and higher trophic levels led us to hypothesize that bioaccumulation of BMAA and DAB occurs as a result of feeding processes in mussels of Thau lagoon. The phytoplankton community of this ecosystem is dominated by diatoms, as confirmed by both the observation of the phytoplankton-dominated fractions and the REPHY surveillance program. This is of particular importance since diatoms have recently been suggested to produce BMAA and they sustain the growth of mollusks in Thau lagoon. Indeed, we confirmed through controlled cultures of diatoms in the laboratory that they may constitute a source of BMAA. In particular, we found BMAA in two out of the three *Chaetoceros* species that were screened and this genus is frequently present in Thau

lagoon at high concentrations. These results strengthened the hypothesis that diatoms are a major source of BMAA and that they are a likely source of BMAA in Thau lagoon. However, the sampling procedure of seston should be improved to collect specifically the different fractions of plankton (*i.e.* picophyto-, microphyto- and zooplankton) and to revise the bioaccumulation pathway of BMAA in the Thau lagoon ecosystem. In future, the direct accumulation of BMAA in mussels as a result of feeding on diatoms should still be confirmed in controlled contamination experiments.

AKNOWLEDGMENTS

This study was carried out under the RISALTOX project (Ifremer) and co-funded by the Regional Council of the "Pays de la Loire". The authors would like to thank all of the members of the laboratory Phycotoxins at the Atlantic Centre of Ifremer for their help and advice during this study, Michèle BARDOUIL, Virginie RAIMBAULT for the culture of some microalgal species, and Véronique SAVAR and Fabienne HERVE for their technical assistance. The authors would also like to thank Aurélie CHARRIER of the PBA laboratory, Ifremer, for providing some diatoms cultures.

REFERENCES

Al-Sammak, M.A., Hoagland, K.D., Cassada, D., Snow, D.D., 2014. Co-occurrence of the cyanotoxins BMAA, DABA and anatoxin-a in Nebraska reservoirs, fish, and aquatic plants. Toxins 6, 488-508.

Andrys, R., Zurita, J., Zguna, N., Verschueren, K., De Borggraeve, W.M., Ilag, L.L., 2015. Improved detection of beta-N-methylamino-L-alanine using N-hydroxysuccinimide ester of N-butylnicotinic acid for the localization of BMAA in blue mussels (Mytilus edulis). Anal Bioanal Chem.

Banack, S.A., Downing, T.G., Spácil, Z., Purdie, E.L., Metcalf, J.S., Downing, S., Esterhuizen, M., Codd, G.A., Cox, P.A., 2010. Distinguishing the cyanobacterial neurotoxin β-N-methylamino-l-alanine (BMAA) from its structural isomer 2,4-diaminobutyric acid (2,4-DAB). Toxicon 56, 868-879.

Banack, S.A., Metcalf, J.S., Bradley, W.G., Cox, P.A., 2014. Detection of cyanobacterial neurotoxin β-N-methylamino-l-alanine within shellfish in the diet of an ALS patient in Florida. Toxicon 90, 167-173.

Bates, S.S., Douglas, D.J., Doucette, G.J., Leger, C., 1995. Enhancement of domoic acid production by reintroducing bacteria to axenic cultures of the diatom Pseudo-nitzschia multiseries. Nat Toxins 3, 428-435.

Blanco, J., Acosta, C.P., Bermúdez de la Puente, M., Salgado, C., 2002. Depuration and anatomical distribution of the amnesic shellfish poisoning (ASP) toxin domoic acid in the king scallop Pecten maximus. Aquatic Toxicology 60, 111-121.

Blanco, J., Mariño, C., Martín, H., Acosta, C.P., 2007. Anatomical distribution of diarrhetic shellfish poisoning (DSP) toxins in the mussel Mytilus galloprovincialis. Toxicon 50, 1011-1018.

Bowler, C., Allen, A.E., Badger, J.H., Grimwood, J., Jabbari, K., Kuo, A., Maheswari, U., Martens, C., Maumus, F., Otillar, R.P., Rayko, E., Salamov, A., Vandepoele, K., Beszteri, B., Gruber, A., Heijde, M., Katinka, M., Mock, T., Valentin, K., Verret, F., Berges, J.A., Brownlee, C., Cadoret, J.-P., Chiovitti, A., Choi, C.J., Coesel, S., De Martino, A., Detter, J.C., Durkin, C., Falciatore, A., Fournet, J., Haruta, M., Huysman, M.J.J., Jenkins, B.D., Jiroutova, K., Jorgensen, R.E., Joubert, Y., Kaplan, A., Kroger, N., Kroth, P.G., La Roche, J., Lindquist, E., Lommer, M., Martin-Jezequel, V., Lopez, P.J., Lucas, S., Mangogna, M., McGinnis, K., Medlin, L.K., Montsant, A., Secq, M.-P.O.-L., Napoli, C., Obornik, M., Parker, M.S., Petit, J.-L., Porcel, B.M., Poulsen, N., Robison, M., Rychlewski, L., Rynearson, T.A., Schmutz, J., Shapiro, H., Siaut, M., Stanley, M., Sussman, M.R., Taylor, A.R., Vardi, A., von Dassow, P., Vyverman, W., Willis, A., Wyrwicz, L.S., Rokhsar, D.S., Weissenbach, J., Armbrust, E.V., Green,

504 B.R., Van de Peer, Y., Grigoriev, I.V., 2008. The Phaeodactylum genome reveals the evolutionary
505 history of diatom genomes. Nature 456, 239-244.
506 Bricelj, V.M., Lee, J.H., Cembella, A.D., Anderson, D.M., 1990. Uptake kinetics of Paralytic Shellfish Toxins
507 from the dinoflagellate *Alexandrium fudyense* in the mussel *Mytilus edulis*. Mar Ecol Prog Ser 63, 177-
508 188.
509 Bricelj, V.M., Shumway, S.E., 1998. Paralytic Shellfish Toxins in Bivalve Molluscs: Occurrence, Transfer
510 Kinetics, and Biotransformation. Reviews in Fisheries Science 6, 315-383.
511 Cavallo, R.A., Acquaviva, M.I., Stabili, L., 2009. Culturable heterotrophic bacteria in seawater and Mytilus
512 galloprovincialis from a Mediterranean area (Northern Ionian Sea – Italy). Environmental monitoring
513 and assessment 149, 465-475.
514 Claisse, D., Raffin, B., 2010. Surveillance chimique ROCCH - Inventaire cartographique des points de
515 prélèvements ROCCH échantillonnés au titre de la surveillance sanitaire, p. 55.
516 Combes, A., El Abdellaoui, S., Sarazin, C., Vial, J., Mejean, A., Ploux, O., Pichon, V., group, B., 2013.
517 Validation of the analytical procedure for the determination of the neurotoxin beta-N-methylamino-l-
518 alanine in complex environmental samples. Anal Chim Acta 771, 42-49.
519 Cox, P.A., Banack, S.A., Murch, S.J., Rasmussen, U., Tien, G., Bidigare, R.R., Metcalf, J.S., Morrison, L.F.,
520 Codd, G.A., Bergman, B., 2005. Diverse taxa of cyanobacteria produce β-N-methylamino-l-alanine, a
521 neurotoxic amino acid. Proceedings of the National Academy of Sciences of the United States of
522 America 102, 5074-5078.
523 Dahl, B., Blanck, H., 1996. Toxic effects of the antifouling agent irgarol 1051 on periphyton communities in
524 coastal water microcosms. Mar Pollut Bull 32, 342-350.
525 Doucette, G.J., 1995. Interactions between bacteria and harmful algae: a review. Nat Toxins 3, 65-74.
526 Downing, S., Banack, S.A., Metcalf, J.S., Cox, P.A., Downing, T.G., 2011. Nitrogen starvation of cyanobacteria
527 results in the production of β-N-methylamino-L-alanine. Toxicon 58, 187-194.
528 Downing, S., Contardo-Jara, V., Pflugmacher, S., Downing, T.G., 2014. The fate of the cyanobacterial toxin
529 beta-N-methylamino-L-alanine in freshwater mussels. Ecotoxicology and Environmental Safety 101,
530 51-58.
531 Dunlop, R.A., Cox, P.A., Banack, S.A., Rodgers, K.J., 2013. The Non-Protein Amino Acid BMAA Is
532 Misincorporated into Human Proteins in Place of L-Serine Causing Protein Misfolding and
533 Aggregation. Plos One 8.
534 Dupuy, C., Vaquer, A., Lam-Hoai, T., Rougier, C., Mazouni, N., Lautier, J., Collos, Y., Le Gall, S., 2000.
535 Feeding rate of the oyster Crassostrea gigas in a natural planktonic community of the Mediterranean
536 Thau Lagoon. Mar Ecol Prog Ser 205, 171-184.
537 Faassen, E.J., 2014. Presence of the neurotoxin BMAA in aquatic ecosystems: what do we really know? Toxins
538 6, 1109-1138.
539 Ferrao-Filho, A.d.S., Kozlowsky-Suzuki, B., 2011. Cyanotoxins: Bioaccumulation and Effects on Aquatic
540 Animals. Marine Drugs 9, 2729-2772.
541 Ferrao, A.D., Kozlowsky-Suzuki, B., 2011. Cyanotoxins: Bioaccumulation and Effects on Aquatic Animals.
542 Marine Drugs 9, 2729-2772.
543 Field, N.C., Metcalf, J.S., Caller, T.A., Banack, S.A., Cox, P.A., Stommel, E.W., 2013. Linking beta-
544 methylamino-L-alanine exposure to sporadic amyotrophic lateral sclerosis in Annapolis, MD. Toxicon
545 70, 179-183.
546 Gaiser, E.E., McCormick, P.V., Hagerthey, S.E., Gottlieb, A.D., 2011. Landscape Patterns of Periphyton in the
547 Florida Everglades. Crit Rev Env Sci Tec 41, 92-120.
548 Gangnery, A., Bacher, C., Buestel, D., 2004. Application of a population dynamics model to the Mediterranean
549 mussel, Mytilus galloprovincialis, reared in Thau Lagoon (France). Aquaculture 229, 289-313.
550 Gangnery, A., Chabirand, J.-M., Lagarde, F., Le Gall, P., Oheix, J., Bacher, C., Buestel, D., 2003. Growth model
551 of the Pacific oyster, Crassostrea gigas, cultured in Thau Lagoon (Méditerranée, France). Aquaculture
552 215, 267-290.
553 Gardes, A., Iversen, M.H., Grossart, H.P., Passow, U., Ullrich, M.S., 2011. Diatom-associated bacteria are
554 required for aggregation of Thalassiosira weissflogii. Isme J 5, 436-445.
555 Gifford, D.J., 1991. The Protozon-Metazoan trophic link in pelagic ecosystems. Journal of Protozoology 38, 81-
556 86.
557 Glover, W.B., Mash, D., Murch, S., 2014a. The natural non-protein amino acid N-β-methylamino-l-alanine
558 (BMAA) is incorporated into protein during synthesis. Amino Acids 46, 2553-2559.
559 Glover, W.B., Mash, D.C., Murch, S.J., 2014b. The natural non-protein amino acid N-beta-methylamino-L-
560 alanine (BMAA) is incorporated into protein during synthesis. Amino Acids 46, 2553-2559.
561 Grossart, H.P., Levold, F., Allgaier, M., Simon, M., Brinkhoff, T., 2005. Marine diatom species harbour distinct
562 bacterial communities. Environmental Microbiology 7, 860-873.

563 Guillard, R.R.L., Hargraves, P.E., 1993. *Stichochrysis immobilis* is a diatom, not a chrysophyte. Phycologia 32,
564 234-236.
565 Harrison, P.J., Waters, R.E., Taylor, F.J.R., 1980. A broad-spectrum artificial seawater medium for coastal and
566 open ocean phytoplankton. Journal of Phycology 16, 28-35.
567 Hill, B.H., Herlihy, A.T., Kaufmann, P.R., Stevenson, R.J., McCormick, F.H., Johnson, C.B., 2000. Use of
568 periphyton assemblage data as an index of biotic integrity. Journal of the North American Benthological
569 Society 19, 50-67.
570 Hillebrand, H., Kahlert, M., Haglund, A.L., Berninger, U.G., Nagel, S., Wickham, S., 2002. Control of
571 microbenthic communities by grazing and nutrient supply. Ecology 83, 2205-2219.
572 Ibelings, B.W., Chorus, I., 2007. Accumulation of cyanobacterial toxins in freshwater "seafood" and its
573 consequences for public health: A review. Environ. Pollut. 150, 177-192.
574 Jauffrais, T., Marcaillou, C., Herrenknecht, C., Truquet, P., Séchet, V., Nicolau, E., Tillmann, U., Hess, P., 2012.
575 Azaspiracid accumulation, detoxification and biotransformation in blue mussels (Mytilus edulis)
576 experimentally fed Azadinium spinosum. Toxicon 60, 582-595.
577 Jiang, L., Eriksson, J., Lage, S., Jonasson, S., Shams, S., Mehine, M., Ilag, L.L., Rasmussen, U., 2014a. Diatoms:
578 A Novel Source for the Neurotoxin BMAA in Aquatic Environments. PLoS One 9, e84578.
579 Jiang, L., Ilag, L.L., 2014. Detection of endogenous BMAA in dinoflagellate (Heterocapsa triquetra) hints at
580 evolutionary conservation and environmental concern. PubRaw Science.
581 Jiang, L., Johnston, E., Åberg, K.M., Nilsson, U., Ilag, L., 2013. Strategy for quantifying trace levels of BMAA
582 in cyanobacteria by LC/MS/MS. Anal Bioanal Chem 405, 1283-1292.
583 Jiang, L., Kiselova, N., Rosen, J., Ilag, L.L., 2014b. Quantification of neurotoxin BMAA ([bgr]-N-methylamino-
584 L-alanine) in seafood from Swedish markets. Sci. Rep. 4.
585 Jiao, Y., Chen, Q., Chen, X., Wang, X., Liao, X., Jiang, L., Wu, J., Yang, L., 2014. Occurrence and transfer of a
586 cyanobacterial neurotoxin beta-methylamino-l-alanine within the aquatic food webs of Gonghu Bay
587 (Lake Taihu, China) to evaluate the potential human health risk. The Science of the total environment
588 468, 457-463.
589 Jonasson, S., Eriksson, J., Berntzon, L., Spáčil, Z., Ilag, L.L., Ronnevi, L.-O., Rasmussen, U., Bergman, B.,
590 2010. Transfer of a cyanobacterial neurotoxin within a temperate aquatic ecosystem suggests pathways
591 for human exposure. Proceedings of the National Academy of Sciences 107, 9252-9257.
592 Kach, D., Ward, J.E., 2008. The role of marine aggregates in the ingestion of picoplankton-size particles by
593 suspension-feeding molluscs. Mar Biol 153, 797-805.
594 Karlsson, O., Jiang, L., Andersson, M., Ilag, L.L., Brittebo, E.B., 2014. Protein association of the neurotoxin and
595 non-protein amino acid BMAA (β-N-methylamino-l-alanine) in the liver and brain following neonatal
596 administration in rats. Toxicology letters 226, 1-5.
597 Keller, M.D., Selvin, R.C., Claus, W., Guillard, R.R.L., 1987. Media for the Culture of Oceanic
598 Ultraphytoplankton. Journal of Phycology 23, 633-638.
599 Lage, S., Costa, P.R., Moita, T., Eriksson, J., Rasmussen, U., Rydberg, S.J., 2014. BMAA in shellfish from two
600 Portuguese transitional water bodies suggests the marine dinoflagellate Gymnodinium catenatum as a
601 potential BMAA source. Aquatic Toxicology 152, 131-138.
602 Lam-Hoai, T., Rougier, C., 2001. Zooplankton assemblages and biomass during a 4-period survey in a northern
603 Mediterranean coastal lagoon. Water Research 35, 271-283.
604 Lassus, P., Amzil, Z., Baron, R., Séchet, V., Barillé, L., Abadie, E., Bardouil, M., Sibat, M., Truquet, P., Bérard,
605 J.-B., Gueguen, M., 2007. Modelling the accumulation of PSP toxins in Thau Lagoon oysters (
606 Crassostrea gigas) from trials using mixed cultures of Alexandrium catenella and Thalassiosira
607 weissflogii. Aquatic Living Resources 20, 59-67.
608 Lefebvre, S., Marín Leal, J.C., Dubois, S., Orvain, F., Blin, J.-L., Bataillé, M.-P., Ourry, A., Galois, R., 2009.
609 Seasonal dynamics of trophic relationships among co-occurring suspension-feeders in two shellfish
610 culture dominated ecosystems. Estuarine, Coastal and Shelf Science 82, 415-425.
611 Mafra, L.L., Bricelj, V.M., Fennel, K., 2010. Domoic acid uptake and elimination kinetics in oysters and mussels
612 in relation to body size and anatomical distribution of toxin. Aquatic Toxicology 100, 17-29.
613 Masseret, E., Banack, S., Boumédiène, F., Abadie, E., Brient, L., Pernet, F., Juntas-Morales, R., Pageot, N.,
614 Metcalf, J., Cox, P., Camu, W., the French Network on, A.L.S.C.D., Investigation, 2013. Dietary
615 BMAA Exposure in an Amyotrophic Lateral Sclerosis Cluster from Southern France. PLoS ONE 8,
616 e83406.
617 McCarron, P., Logan, A., Giddings, S., Quilliam, M., 2014. Analysis of beta-N-methylamino-L-alanine (BMAA)
618 in spirulina-containing supplements by liquid chromatography-tandem mass spectrometry. Aquatic
619 Biosystems 10, 5.
620 Medhioub, W., Gueguen, M., Lassus, P., Bardouil, M., Truquet, P., Sibat, M., Medhioub, N., Soudant, P.,
621 Kraiem, M., Amzil, Z., 2010. Detoxification enhancement in the gymnodimine-contaminated grooved
622 carpet shell, Ruditapes decussatus (Linne). Harmful Algae 9, 200-207.

623 Mondo, K., Broc Glover, W., Murch, S.J., Liu, G., Cai, Y., Davis, D.A., Mash, D.C., 2014. Environmental
624 neurotoxins beta-N-methylamino-l-alanine (BMAA) and mercury in shark cartilage dietary
625 supplements. Food and chemical toxicology : an international journal published for the British
626 Industrial Biological Research Association 70, 26-32.
627 Murch, S.J., Cox, P.A., Banack, S.A., 2004a. A mechanism for slow release of biomagnified cyanobacterial
628 neurotoxins and neurodegenerative disease in Guam. Proceedings of the National Academy of Sciences
629 of the United States of America 101, 12228-12231.
630 Murch, S.J., Cox, P.A., Banack, S.A., Steele, J.C., Sacks, O.W., 2004b. Occurrence of beta-methylamino-L-
631 alanine (BMAA) in ALS/PDC patients from Guam. Acta Neurologica Scandinavica 110, 267-269.
632 Nayar, S., Goh, B.P.L., Chou, L.M., Reddy, S., 2003. In situ microcosms to study the impact of heavy metals
633 resuspended by dredging on periphyton in a tropical estuary. Aquatic Toxicology 64, 293-306.
634 Negri, A.P., Jones, G.J., 1995. Bioaccumulation of paralytic shellfish poisoning (PSP) toxins from the
635 cyanobacterium Anabaena circinalis by the freshwater mussel Alathyria condola. Toxicon 33, 667-678.
636 Pablo, J., Banack, S.A., Cox, P.A., Johnson, T.E., Papapetropoulos, S., Bradley, W.G., Buck, A., Mash, D.C.,
637 2009. Cyanobacterial neurotoxin BMAA in ALS and Alzheimer's disease. Acta Neurologica
638 Scandinavica 120, 216-225.
639 Pernet, F., Lagarde, F., Jeannee, N., Daigle, G., Barret, J., Le Gall, P., Quere, C., D'orbcastel, E.R., 2014. Spatial
640 and Temporal Dynamics of Mass Mortalities in Oysters Is Influenced by Energetic Reserves and Food
641 Quality. Plos One 9.
642 Pernet, F., Malet, N., Pastoreaud, A., Vaquer, A., Quéré, C., Dubroca, L., 2012. Marine diatoms sustain growth
643 of bivalves in a Mediterranean lagoon. Journal of Sea Research 68, 20-32.
644 Provasoli, L., 1968. Media and prospects for the cultivation of marine algae, in: Watanabe, A., Hattori, A. (Eds.),
645 Cultures and Collections of Algae. Japan Soc. Plant Physiol, Tokyo, Japan, pp. 63-75.
646 Reveillon, D., Abadie, E., Sechet, V., Brient, L., Savar, V., Bardouil, M., Hess, P., Amzil, Z., 2014. Beta-N-
647 Methylamino-L-Alanine: LC-MS/MS Optimization, Screening of Cyanobacterial Strains and
648 Occurrence in Shellfish from Thau, a French Mediterranean Lagoon. Marine Drugs 12, 5441-5467.
649 Riisgard, H.U., Larsen, P.S., 2010. Particle capture mechanisms in suspension-feeding invertebrates. Mar Ecol
650 Prog Ser 418, 255-293.
651 Rosen, J., Hellenas, K.E., 2008. Determination of the neurotoxin BMAA (beta-N-methylamino-L-alanine) in
652 cycad seed and cyanobacteria by LC-MS/MS (liquid chromatography tandem mass spectrometry).
653 Analyst 133, 1785-1789.
654 Salomonsson, M.L., Hansson, A., Bondesson, U., 2013. Development and in-house validation of a method for
655 quantification of BMAA in mussels using dansyl chloride derivatization and ultra performance liquid
656 chromatography tandem mass spectrometry. Anal Methods-Uk 5, 4865-4874.
657 Scott, L.L., Downing, S., Phelan, R.R., Downing, T.G., 2014. Environmental modulation of microcystin and
658 beta-N-methylamino-l-alanine as a function of nitrogen availability. Toxicon : official journal of the
659 International Society on Toxinology 87, 1-5.
660 Spáčil, Z., Erkisson, J., Jonasson, S., Rasmussen, U., Ilag, L.L., Bergman, B., 2010. Analytical protocol for
661 identification of BMAA and DAB in biological samples. Analyst 135, 127-132.
662 Spencer, P.S., Nunn, P.B., Hugon, J., Ludolph, A.C., Ross, S.M., Roy, D.N., Robertson, R.C., 1987. Guam
663 amyotrophic lateral sclerosis-parkinsonism-dementia linked to a plant excitant neurotoxin. Science 237,
664 517-522.
665 Strogyloudi, E., Giannakourou, A., Legrand, C., Ruehl, A., Granéli, E., 2006. Estimating the accumulation and
666 transfer of Nodularia spumigena toxins by the blue mussel Mytilus edulis: An appraisal from culture
667 and mesocosm experiments. Toxicon 48, 359-372.
668 Trottet, A., Roy, S., Tamigneaux, E., Lovejoy, C., 2007. Importance of heterotrophic planktonic communities in
669 a mussel culture environment: the Grande Entrée lagoon, Magdalen Islands (Québec, Canada). Mar
670 Biol 151, 377-392.
671 Utermöhl, H., 1958. Zur Vervollkomnung der quantitativen Phytoplankton-Methodik. Mitt. int. Ver. ther. angew.
672 Limnol 9, 1-38.
673 Vaquer, A., Troussellier, M., Courties, C., Bibent, B., 1996. Standing stock and dynamics of picophytoplankton
674 in the Thau Lagoon (northwest Mediterranean coast). Limnol Oceanogr 41, 1821-1828.
675 Vasconcelos, V.M., 1995. Uptake and depuration of the heptapeptide toxin microcystin-LR in Mytilus
676 galloprovincialis. Aquatic Toxicology 32, 227-237.
677 Walne, P.R., 1970. Studies on the food value of nineteen genera of algae to juvenile bivalves of the genera
678 Ostrea, Crassostrea, Mercenaria and Mytilus. H.M.S.O., London,.
679 Xie, X.B., Basile, M., Mash, D.C., 2013. Cerebral uptake and protein incorporation of cyanobacterial toxin beta-
680 N-methylamino-L-alanine. Neuroreport 24, 779-784.
681
682

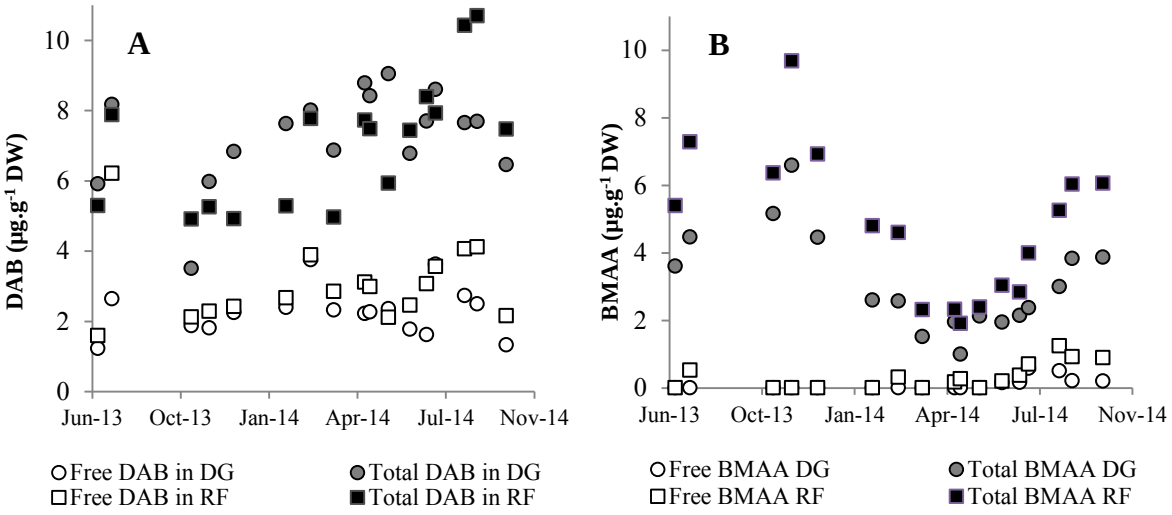
683 **Appendices**

684 A: Free (empty symbols) and total (dark symbols) concentration in µg.g^{-1} DW for both digestive glands (DG,
685 circle) and remaining flesh (RF, square) of (A) DAB and (B) BMAA in mussels collected in Thau lagoon
686 between July 2013 and October 2014.

687 B: Composition of the plankton community in seston collected with phytoplankton nets. Major species of
688 diatoms, dinoflagellates and zooplankton were mentioned in brackets.

689

690



691

692 Appendix A: Free (empty symbols) and total (dark symbols) concentration in µg.g^{-1} DW for both digestive
693 glands (DG, circle) and remaining flesh (RF, square) of (A) DAB and (B) BMAA in mussels collected in Thau
694 lagoon between July 2013 and October 2014.

695

Appendix B: Composition of the plankton community in seston collected with phytoplankton nets. Major species of diatoms, dinoflagellates and zooplankton were mentioned in brackets. Overall abundance was evaluated between low concentration (+) and bloom (++++) as an indication of the density of plankton organisms.

Date	Plankton > 20 µm and < 125 µm	Abundance	Plankton > 63 µm	Abundance
08-Jul-14	- 80 % diatoms (70% *Chaetoceros* sp., *Nitzschia* sp., *Navicula* sp.) - 15% dinoflagellates (*Prorocentrum micans, Peridinium* sp.) - 5% zooplankton	+++	- 80% zooplankton (copepods) - 20% diatoms	++
18-Jul-14	- 90% zooplankton (tintinnids) - 5% dinoflagellates (*Peridinium* sp.) - 5% diatoms	++++	- 90% zooplankton (copepods) - 10% diatoms	++++
20-Aug-14	- 90% diatoms (45% *Chaetoceros* sp.) - 10% zooplankton (copepods)	+++	- 60% zooplankton (copepods) - 40% diatoms (*Chaetoceros* sp., *Navicula, Nitzschia*)	++
03-Sep-14	- 60% dinoflagellates (*Peridinium* sp. and *Prorocentrum* sp.) - 40% zooplankton (tintinnids)	++	- 99% zooplankton (copepods) - 1% diatoms	++++
06-Oct-14	- 85% diatoms (*Nitzschia* sp. and *Pseudo-nitzschia* sp.) - 10% zooplankton - 5% dinoflagellates (*Prorocentrum* sp.)	+	- 60% zooplankton (copepods and tintinnids) - 40% diatoms (*Nitzschia* sp. and *Pseudo-nitzschia* sp.)	++