Toxicon
February 2016, Volume 110, Pages 35-46
http://dx.doi.org/10.1016/j.toxicon.2015.11.011
http://archimer.ifremer.fr/doc/00297/40780/

Systematic detection of BMAA (β-N-methylamino-L-alanine) and DAB (2,4-diaminobutyric acid) in mollusks collected in shellfish production areas along the French coasts

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Abstract :

The neurotoxin β-N-methylamino-L-alanine (BMAA) is naturally present in some microalgal species in the marine environment. The accumulation of BMAA has widely been observed in filter-feeding bivalves that are known to consume primary producers constituting the base of complex aquatic food webs. This study was performed to assess the occurrence of BMAA and isomers in mollusks collected from nine representative shellfish production areas located on the three French coasts (Channel, Atlantic and Mediterranean sites). The use of a highly selective and sensitive HILIC-MS/MS method, with D5DAB as internal standard, revealed the systematic detection of BMAA and DAB, in concentrations ranging from 0.20 to 6.7 µg g-1 dry weight of digestive gland tissues of mollusks. While we detected BMAA in four strains of diatoms in a previous study, here BMAA was only detected in one diatom species previously not investigated out of the 23 microalgal species examined (belonging to seven classes). The concentrations of BMAA and DAB in mussels and oysters were similar at different sampling locations and despite the high diversity of phytoplankton populations that mollusks feed on at these locations. Only small variations of BMAA and DAB levels were observed and these were not correlated to any of the phytoplankton species reported. Therefore, extensive research should be performed on both origin and metabolism of BMAA in shellfish. The levels observed in this study are similar to those found in other studies in France or elsewhere. A previous study had related such levels to a cluster of Amyotrophic Lateral Sclerosis in the South of France; hence the widespread occurrence of BMAA in shellfish from all coasts in France found in this study suggests the need for further epidemiological and toxicological studies to establish the levels that are relevant for a link between the consumption of BMAA-containing foodstuffs and neurodegenerative diseases.

Highlights

► BMAA and DAB were systematically present in bivalves sampled from the French coasts. ► Concentrations in bivalves exhibited only small geographical and temporal variations. ► No obvious link was noted between phytoplankton abundance and BMAA levels in bivalves. ► BMAA was present in 1 diatom species out of 23 cultured microalgal strains.

Keywords : BMAA, *Chaetoceros*, *Thalassiosira*, *Phaeodactylum*, bivalves, seafood

1 Introduction

BMAA (β-*N*-methylamino-L-alanine) is a non-proteinogenic amino acid whose mechanisms of toxicity have been extensively studied (reviewed by Chiu et al. (2011) and Karamyan and Speth (2008)) and associated with neurodegenerative diseases like the particular form of Amyotrophic Lateral Sclerosis (ALS) that occurred on the island of Guam in the 1950s (Whiting, 1963).

To date, BMAA and isomers have been detected in many organisms encompassing microalgae, plants, mollusks, crustaceans and fishes, hence including potential foodstuffs (Al-Sammak et al., 2014; Christensen et al., 2012; Cox et al., 2005; Lage et al., 2014; Mondo et al., 2012; Murch et al., 2004a). The bioaccumulation of BMAA, theoretically based on its misincorporation instead of L-serine during protein synthesis (Dunlop et al., 2013; Glover et al., 2014), has been suggested in geographically distinct aquatic food webs, in marine and fresh- or brackish water environments, e.g. Baltic Sea (Jonasson et al., 2010), Florida Bay (Brand et al., 2010), Gonghu Bay, China (Jiao et al., 2014) or Thau lagoon, France (Réveillon et al., 2015). Concentrations reported varied by several orders of magnitude, most likely originating from insufficient analytical performance that led to overestimation and/or misidentification of BMAA, as recently reviewed by (Faassen, 2014). Thus, the analysis of BMAA requires selective and sensitive analytical methods, relying on tandem mass spectrometry and the use of an internal standard for accurate quantification (Cohen, 2012). Considering only those studies in which highly selective methods have been employed, the widespread occurrence of BMAA in many organisms, including in primary producers like cyanobacteria, cannot be clearly verified (Faassen, 2014). However, recent reports convincingly showed the presence of BMAA in dinoflagellates and diatoms (Jiang et al., 2014a; Lage et al., 2014), expanding the number of BMAA-containing phytoplanktonic species that constitute the base of food chains.

Recreational activities as well as aerosolization have been highlighted as potential routes of human exposure to BMAA (Banack et al., 2010; Cox et al., 2009; Stommel et al., 2013). However, since the detection of BMAA in the diet of the Chamorro people on the island of Guam (Cox et al., 2003), the risk arising from the consumption of BMAA-containing foodstuffs has gained attention and could constitute a more widespread route of human exposure. Indeed, recent studies reported the existence of potential BMAA-contaminated foodstuffs, for a large Swedish population (Jiang et al., 2014b), in the environment where ALS clusters have been reported (Banack et al., 2015; Masseret et al., 2013) and even in the direct diet of ALS patients (Banack et al., 2014; Field et al., 2013). The accumulation of BMAA in the human brain is still under debate (Combes et al., 2014; Murch et al.,

2004b; Pablo et al., 2009; Snyder et al., 2009), possibly due to different sample preparation techniques and analytical methods. Even though the presence of BMAA in the human central nervous system may be not specific to ALS-patients (Berntzon et al., 2015), the fact that humans are exposed to BMAA confirms that further studies are required about BMAA sources and its level of involvement in neurodegenerative disorders (Banack et al., 2015).

In France, BMAA had been detected in shellfish for the first time in Thau Lagoon, South of France (Masseret et al., 2013). Those authors also formulated the hypothesis that this contamination could be related to an ALS cluster in that area. However, many toxins produced by phytoplankton (*i.e.* phycotoxins) are accumulated in filter-feeding bivalves, including some neurotoxins currently not regulated e.g. spirolides and pinnatoxins, leading to health concerns for consumers (Amzil et al., 2007; Amzil et al., 2008; Hallegraeff, 1993; Hess et al., 2013). In the context of the French phytoplankton and phycotoxins monitoring network program (REPHY), shellfish from different locations on the French coasts are routinely screened for regulated toxins as well as fast acting phycotoxins while the phytoplankton communities are also recorded (Amzil et al., 2007; Belin and Neaud-Masson, 2012). In addition, the REPHY has established a vigilance program of shellfish for novel or emerging toxins, *i.e.* analysis by both LC-MS/MS (Liquid Chromatography coupled with tandem Mass Spectrometry) and the lipophilic mouse bioassay as a screen (Belin and Neaud-Masson, 2012).

In a previous study, we have reported the continuous presence of BMAA and isomers in mussels from Thau lagoon, French Mediterranean Sea (Réveillon et al., 2015). To assess whether BMAA was present in French ecosystems other than Thau lagoon, we screened 97 samples of mollusks (mussels and oysters) with a highly selective and sensitive HILIC-MS/MS method (*i.e.* liquid chromatography with a HILIC column). This method had previously been optimized for cyanobacterial and mollusk matrices, using D_5DAB as internal standard (Réveillon et al., 2014). The mollusks were sampled within the vigilance program, once a month during 2013, in nine locations chosen for geographical spread and to reflect important French shellfish production areas. Thanks to the phytoplankton communities (*i.e.* database of REPHY), we would be able to evaluate a possible link between phytoplankton populations and the concentration of BMAA in the mollusks, as was hypothesized between diatoms and the mussels of Thau lagoon in a recent study (Réveillon et al., 2015). In parallel, 23 microalgal species were screened for total BMAA and isomers, to assess if other species, in addition to the already analyzed diatoms and dinoflagellates, are capable of producing BMAA.

2 Material and Methods

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, France. 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.

2.1 Samples

2.1.1 Collection of shellfish as part of the vigilance surveillance program in 2013

Sampling was carried out in 2013 in nine of the ten sentinel sites defined by the vigilance surveillance program (figure 1), as part of the French phytoplankton and phycotoxins monitoring network (REPHY). The site at Ingril was omitted since previous studies had already established the presence of BMAA in Thau lagoon, an adjacent production area (Masseret et al., 2013; Réveillon et al., 2015).

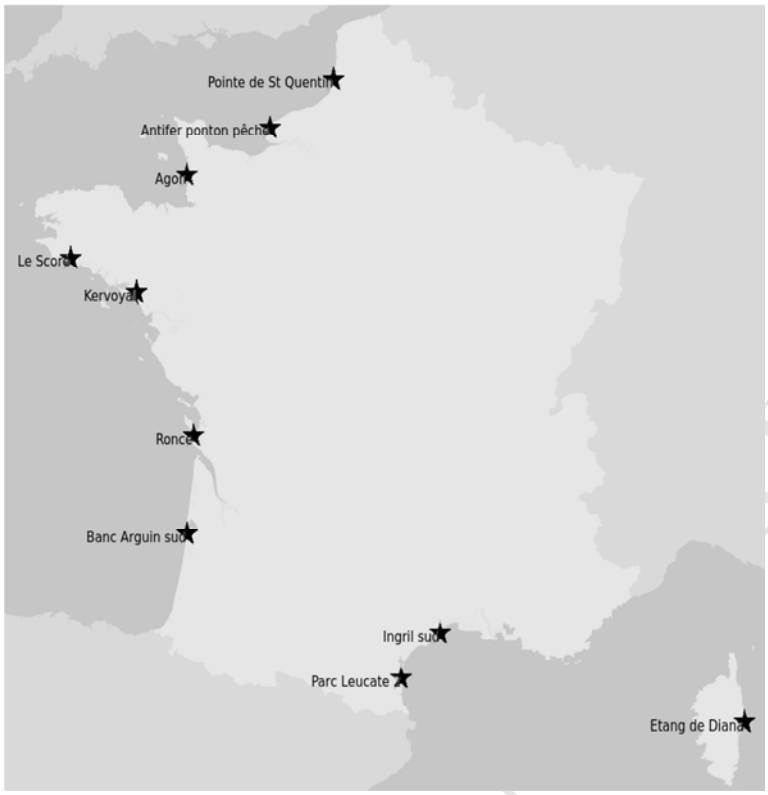


Figure 1: Map of French coastline with the ten sentinel sites selected for the vigilance surveillance program.

Digestive gland tissues (DG, ≥ 10 g) of either oysters (*Crassostrea gigas* in Ronce and Parc Leucate) or mussels (*Mytilus galloprovincialis* in Etang de Diana and *M. edulis* in all remaining sites) were collected once a month on each site, homogenized and stored at -80 °C. It should be noted that all sampling dates were available for only few sites (Le Scoré, Kervoyal, Ronce and Banc Arguin sud), while one date was missing for Pointe de St Quentin, Antifer ponton pêche, Agon and Parc Leucate and only 5 samples were available from Etang de Diana. In total, 97 freeze-dried aliquots, corresponding to nine sites, were screened. Only total BMAA and isomers were analyzed because the free form of these non-proteinogenic amino acids was rarely detected in previous studies (Al-Sammak et al., 2014; Réveillon et al., 2014), especially in digestive gland tissue of mollusks (Réveillon et al., 2015).

2.1.2 Monitoring of phytoplankton communities (REPHY)

The REPHY also monitors the spatial and temporal distribution of phytoplankton populations on French coasts. For quantitative analysis, sub-surface (0 – 1 m) water samples collected once or twice monthly were fixed with Lugol's solution and counted according to the Utermöhl method (Utermöhl, 1958). Experts identified and counted all organisms ≥ 20 µm. Smaller species were also counted if they were potentially toxic/noxious (e.g. *Chrysochromulina*, *Phaeocystis*) or had a chain structure or formed a colony. Organisms were identified to the

lowest possible taxonomic level. Taxa that were difficult to discriminate with optical microscopy were grouped (e.g. *Pseudo-nitzschia* spp. or cryptophyceae species). Further details about sampling and processing of phytoplankton are available in the literature (Belin and Neaud-Masson, 2012; Grossel, 2006; Hernandez-Farinas et al., 2014). In this study, we used the phytoplankton population data of 2013, corresponding to the closest sampling sites to those of the mollusk sampling locations.

2.1.3 Microalgal cultures

Non-axenic strains of microalgae representing possible food resources for mollusks were cultured in order to screen them for the production of BMAA, DAB and AEG (table 1).

Alexandrium minutum AM99PZ, *Heterocapsa triquetra* HT99PZ and *Scrippsiella trochoïdea* ST97PZ were isolated from Bay of Morlaix, English Channel Sea, France. *Prorocentrum micans* PM85BV and the unidentified cryptophyceae were isolated from Bay of Vilaine while *Pseudo-nitzschia delicatissima* was isolated from Môle Saint-Anne, all from Atlantic Ocean, France. The other strains were coming from culture collections. The culture media were prepared with filter-sterilized seawater (0.2 µm) at a salinity of 35. Microalgae were grown in batch cultures (except *H. triquetra* in a 100 L bioreactor) and were harvested via centrifugation at 4000 *g* for 30 min at 4 °C. Supernatant was carefully discarded and the resulting pellet was freeze-dried, homogenized and stored at room temperature until extraction of total BMAA, DAB and AEG.

Table 1: Origin and conditions of culture of the microalgae species that were screened for total BMAA, DAB and AEG. The culture media were prepared accordingly to Guillard (1975) for f/2 medium, Guillard and Hargraves (1993) for L1 medium, Walne (1970) for Conway medium, Tompkins et al. (1995) for PE medium and Provasoli (1968) for ESP medium. For L1 modified medium, 190 µM of $NaHCO_3$ were added.

Strain	Origin	Culture media / days of growth (d)	Irradiance (µmol/m²/s) / Light:dark cycle (h) / Temperature (°C)
Bacillariophyceae			
Halamphora coffeaeformis	CCAP 1001/2	f/2 / 18	200 / 12:12 / 20
Asterionellopsis glacialis	CCMP 139	f/2 / 15	200 / 12:12 / 20
Odontella aurita	AC 815	f/2 / 22	200 / 12:12 / 20
Pseudo-nitzschia delicatissima	France (P5C1)	L1 / 10	100 / 12:12 / 16
Dinophyceae			
Alexandrium minutum	France (AMP99PZ)	L1 (27‰) - Si / 15	90 / 16:8 / 17
Heterocapsa triquetra	France (HT99PZ)	L1 - Si / (*)	250 / 16:8 / 20
Prorocentrum micans	France (PM85BV)	ESP / 19	100 / 12:12 / 16
Pyrocystis noctulica	CCMP 732	f/2 - Si / 34	200 / 12:12 / 20
Scrippsiella trochoïdea	France (ST97PZ)	f/2 - Si / 19	150 / 12:12 / 16
Symbiodinium microadriaticum	CCMP 828	f/2 - Si / 18	200 / 12:12 / 20
Cryptophyceae			
Hemiselmis sp.	RCC 659	L1 – Si (modified) / 12	100 / 16:8 / 17
Proteomonas sp.	RCC 3072	L1 – Si (modified) / 12	100 / 16:8 / 17
Rhinomonas sp.	RCC 821	L1 – Si (modified) / 12	100 / 16:8 / 17
Rhodomonas salina	RCC 1506	L1 – Si (modified) / 12	100 / 16:8 / 17
Rhodomonas sp.	RCC 1978	L1 – Si (modified) / 12	100 / 16:8 / 17
Unidentified cryptophyceae	France	L1 – Si (modified) / 12	100 / 16:8 / 17
Microalgae of other classes			
Chlamydomonas reginae	CCAP 11/78	Conway - Si / 20	200 / 12:12 / 20
Chlorella vulgaris	CCAP 211/25	Conway - Si / 20	200 / 12:12 / 20
Dunaliella salina	CCAP 19/18	f/2 - Si / 20	200 / 12:12 / 20
Emiliana huxleyi	CCMP 371	f/2 - Si / 27	200 / 12:12 / 20
Eutreptiella gymnastica	CCMP 1594	f/2 - Si / 20	200 / 12:12 / 20
Porphyridium purpureum	CCAP 1380/5	PE / 27	200 / 12:12 / 20
Tisochrysis lutea	CCAP 927/14	ESP / 27	50 / 12:12 / 16

(*) culture in a 100 L bioreactor; 27‰, diluted natural seawater; - Si, without silicate

2.2 Sample extraction

BMAA, DAB and AEG (total form) were extracted and analyzed as previously described (Réveillon et al., 2014). Briefly, 750 µL of TCA 0.1 M containing the internal standard D_5DAB (50 ng mL^{-1}) were added to 10 mg (for algae) or 15 mg (for mollusk) of freeze-dried material before grinding with glass beads in a mixer mill (Retsch MM400, Germany) for 30 min. The supernatant was subsequently collected, evaporated to dryness, the residue dissolved in 600 µL HCl 6 M and hydrolyzed at 99 °C for 24 h. HCl was dried, and the residue dissolved in 1 mL of TCA 0.1 M before SPE clean-up on Bond Elut® Plexa PCX cartridges (Agilent Technologies, VWR, France).

2.3 Instrumentation and analytical method

Liquid chromatography was performed on a ZIC®-HILIC column (150 × 2.1 mm, 5 µm, Merck Sequant®) with a TSK gel amide 80 guard column (2 × 10 mm, 5 µm) using a Nexera Ultra-Fast Liquid Chromatography system

(Shimadzu, France). Separation was achieved using a linear gradient elution at 0.2 mL min^{-1} with a column temperature set at 30 °C while samples were kept at 4 °C and the injection volume was 5 µL. The elution program (solvent A: water and solvent B: acetonitrile, both 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 MS/MS analysis was performed with an API 5500 QTRAP triple-quadrupole mass spectrometer (AB Sciex, France) 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 qualitative to quantitative ion ratios. The peak area ratio between the specific product ions *m/z* 76 and 88 was also verified for identification of BMAA. The common transition *m/z* 119 > 102 was used to quantify BMAA, DAB and AEG, while the transition *m/z* 124 > 47 was used to quantify D_5DAB. To avoid overestimation of BMAA in digestive gland tissues, BMAA was quantified with the specific transition *m/z* 119 > 76, for all mollusk samples (see paragraph 3.1).

Quantitation was performed relatively to pure standards of BMAA, DAB and AEG. The limits of detection (LOD) and quantification (LOQ) were the same as reported previously (Réveillon et al., 2014). For all isomers, LOQ was 0.23 µg g^{-1} and 0.15 µg g^{-1} DW for microalgae and mollusk matrices, respectively. A corrective factor derived from D_5DAB recovery was applied to compensate for losses during samples preparation and matrix effects. All other instrument parameters were the same as previously mentioned (Réveillon et al., 2014). The software Analyst 1.5.1 was used to analyze acquired raw data.

3 Results

3.1 HILIC-MS/MS performance

A compound eluting just before BMAA was detected in all digestive gland tissue of mollusks, especially for oysters extracts (Appendix). In this study, the mean values (± RSD) of 88/102 and 76/102 ion ratios used for correct identification of BMAA were 13.2% (± 4.4%) and 12.1% (± 4.1%), respectively. These ratios were inconsistent between oyster digestive gland samples and the BMAA standard. These discrepancies were attributed to the interfering compound that only generated a product ion at *m/z* 119 > 102. Therefore, the ratio of specific product ions (76/88) was assessed to further confirm BMAA identity. The 76/88 ion ratio of BMAA standard was 92.6 ± 2.2%. For all mollusk extracts, there was a difference ≤ 10% of this ratio between samples and the BMAA standard (with 93% of samples showing a difference ≤ 5%). Thus, the 76/88 ion ratio was included as a criterion used to confirm the identity of BMAA, in all biological matrices. The interfering

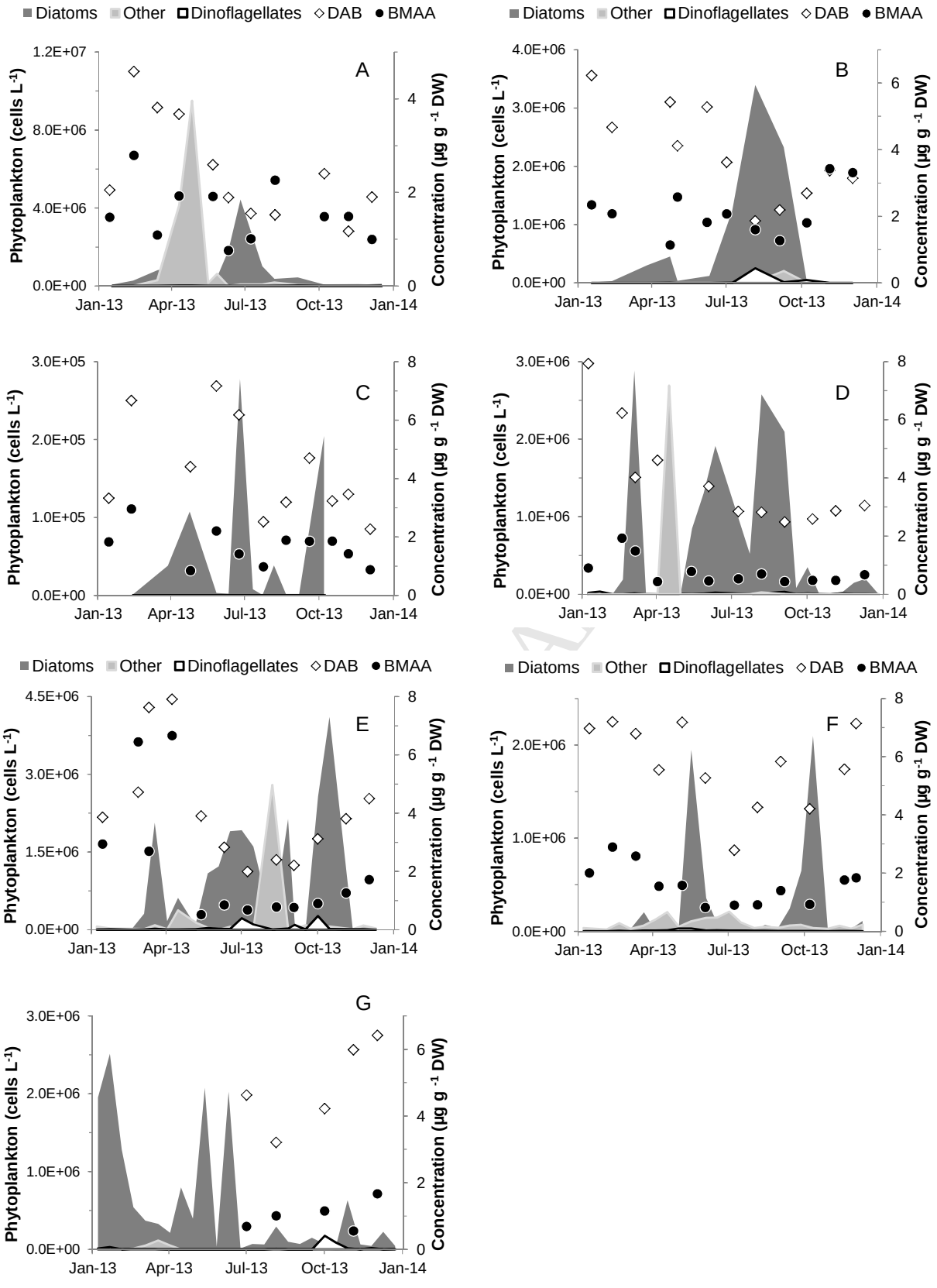
compound could lead to an overestimation of BMAA since the mass spectral transition *m/z* 119 > 102 was used to quantify the samples. As an alternative, the quantification with either the product ion *m/z* 88 or 76 was tested and no difference was observed with these two ions for BMAA-calculated concentrations. Therefore, all digestive gland extracts of mollusks were quantified with the mass spectral transition *m/z* 119 > 76, for consistent comparison between oysters and mussels. As a function of this amendment, LOQs for BMAA in shellfish were somewhat higher than in the originally reported method (Réveillon et al., 2014), *i.e.* (0.45 µg g^{-1} DW). Nevertheless, the signal-to-noise (S/N) ratio for the transition *m/z* 119 > 88 was ≥ 10 for all mollusks extracts, except for two oysters samples (S/N ≥ 6).

In this study, the mean total recovery (± RSD) of the internal standard D_5DAB was 62.6 (± 16%, n= 23) in microalgal samples, while recoveries of 64.9 (± 15%, n=23) and 69.1% (± 12.7%, n=74) were obtained for digestive gland tissues of oysters and mussels, respectively.

3.2 BMAA and isomers in mollusks collected within the vigilance surveillance program

In total, 97 aliquots of digestive gland tissues of mollusks collected in 2013 were screened for BMAA and isomers. Surprisingly, BMAA and DAB were detected in all samples (both mussels and oysters), at all sampling stations and dates (figures 2 and 3). Mean BMAA concentrations (in µg g^{-1} DW, with the lowest and highest concentrations noted in brackets) were as follows: Kervoyal, 2.2 (0.52 – 6.7) < Antifer ponton pêche, 2.1 (1.1 – 3.4) < Agon, 1.7 (0.85 – 3.0) < Banc Arguin sud, 1.6 (0.82 – 2.9) < Pointe de St Quentin, 1.6 (0.76 – 2.8) < Diana, 1.0 (0.55 – 1.7) < Le Scoré, 0.78 (0.44 – 1.9) < Ronce, 0.76 (0.25 – 2.4) < Leucate, 0.61 (0.19 – 1.0). For DAB, the decreasing order was: Leucate, 7.5 (4.3 – 9.2) < Banc Arguin sud, 5.7 (2.8 – 7.2) < Diana, 4.9 (3.2 – 6.4) < Ronce, 4.8 (2.8 – 6.6) < Agon, 4.3 (2.3 – 7.2) < Kervoyal, 4.1 (2.0 – 7.9) < Le Scoré, 3.9 (2.5 – 7.9) < Antifer, 3.9 (1.9 – 6.2) < Pointe de St Quentin, 2.5 (1.2 – 4.6).

BMAA concentrations were generally higher in mussels, while for DAB, higher concentrations were observed in oysters (means of 1.6 *versus* 0.68 for BMAA and 4.2 *versus* 6.2 µg g^{-1} DW for DAB).



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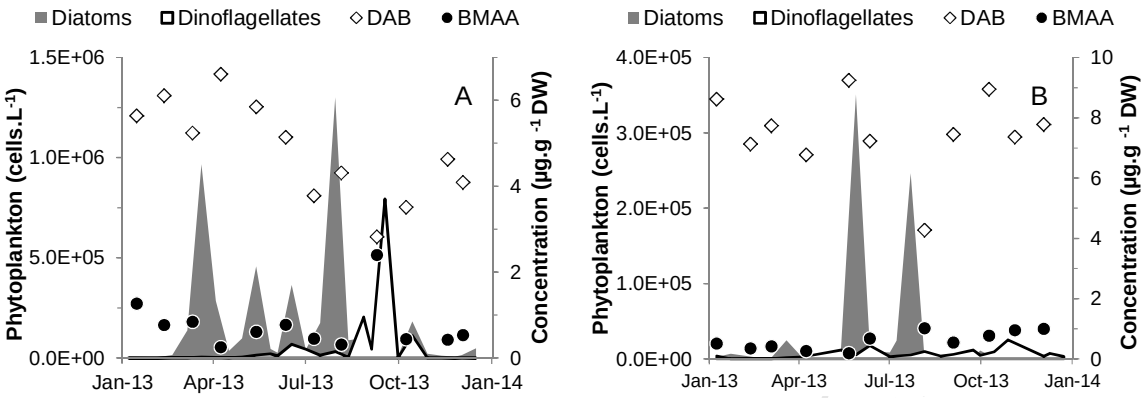
208 Figure 2: Abundances of diatoms, dinoflagellates and other classes of phytoplankton reported by REPHY in
209 2013 and BMAA and DAB concentrations obtained in the digestive glands of mussels sampled at (A) Pointe de
210 St Quentin, (B) Antifer ponton pêche, (C) Agon, (D) Le Scoré, (E) Kervoyal, (F) Banc Arguin sud and (G) Etang

211 de Diana, within the vigilance surveillance program. Only partial counting was available for Agon
212 phytoplankton populations.



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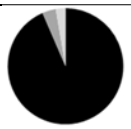
214 Figure 3: Abundances of diatoms and dinoflagellates reported by REPHY in 2013 and BMAA and DAB
215 concentrations obtained in the digestive glands of oysters sampled at (A) Ronce and (B) Parc Leucate, within the
216 vigilance surveillance program.

217 *3.3 Phytoplankton populations database of REPHY*

218 The phytoplanktonic populations at the sampling sites of REPHY varied both qualitatively and quantitatively
219 during 2013 (table 2). Depending on the location, the number of identified taxa or groups of taxa ranged from 43
220 at Parc Leucate (Mediterranean lagoon) to 126 at Banc Arguin sud (entrance of the semi-enclosed Arcachon
221 Bay). Diatoms and dinoflagellates represented the majority of observed taxa and diatom species were generally
222 more diverse. The total abundance of phytoplankton cells was chosen as a proxy to estimate the quantity of food
223 available for bivalve mollusks. Thus, phytoplankton relative abundance was low at Parc Leucate (<1 million
224 cells.L^{-1}), intermediate at Ronce, Antifer ponton pêche and Banc Arguin sud (5.6 – 8.5 million cells.L^{-1}) and high
225 at the other sentinel sites (15 – 27 million cells.L^{-1}). Diatom abundances were significantly higher than
226 dinoflagellate cell concentrations or other classes; except at Pointe de St Quentin where dense blooms of
227 *Phaeocystis sp.* (prymnesiophyceae) were observed.

228

229 Table 2: Phytoplankton data (2013) of REPHY corresponding to the closest sentinel sites of the vigilance
230 surveillance program. The number of identified taxa/groups, the proportion (qualitative and quantitative) of
231 major classes, the total abundance and the most abundant species were reported. Only partial phytoplankton
232 counting was available for Agon (adjacent to Antifer) thus the results were not included.

Sentinel site (distance between phytoplankton and mollusk sampling sites)	Number of identified taxa (% of diatoms/ dinoflagellates /other classes)	Total abundance of phytoplankton in 2013 (cells.L^{-1})	Proportion of major classes (% of total abundance) ■= diatoms; ■= dinoflagellates; ■= other classes	Most abundant species (% of total abundance)
Pointe de St Quentin (8 km)	87 (65/25/10)	27 million		*Phaeocystis* (51%) *Leptocylindrus danicus* (25%)
Antifer ponton pêche (2.5 km)	63 (62/27/11)	8.5 million		*Chaetoceros* spp. (64%) *Leptocylindrus* spp. (14%)
Le Scoré (6 km)	110 (51/42/7)	16 million		*Chaetoceros* spp. (19%) Cryptophyceae (17%) *Pseudo-nitzschia delicatissima* complex (15%) *Skeletonema costatum* (15%)
Kervoyal (8 km)	105 (53/36/11)	25 million		*Leptocylindrus* spp. (41%) Cryptophyceae (15%) *Chaetoceros* spp. (13%)
Ronce (2 km)	93 (67/28/5)	5.9 million		*Skeletonema* spp. (25%) *Leptocylindrus* spp. (24%) Gymnodiniaceae (20%)
Banc Arguin Sud (5 km)	126 (44/44/12)	8.2 million		*Pseudo-nitzschia delicatissima* complex (41%) Cryptophyceae (21%) *Leptocylindrus* spp. (20%)
Parc Leucate (0 km)	43 (47/47/7)	0.87 million		*Chaetoceros* spp. (68%) *Prorocentrum* spp. (7%)
Etang de Diana (0 km)	76 (41/46/13)	15 million		*Nitzschia longissima* (45%) *Leptocylindrus minimus* (34%)

233

234 In 5 out of the 8 sites, the sum of abundances of *Chaetoceros* spp., *Leptocylindrus* spp., *Skeletonema* spp. and
235 species of both *Pseudo-nitzschia delicatissima* complex and cryptophyceae accounted for 69 to 84% of the total
236 abundance of phytoplankton. At the other sites, *Phaeocystis*, *Nitzschia longissima* (diatom) and species of

Gymnodiniaceae (dinoflagellates) were more abundant. It should be noted that the water sampling sites for phytoplankton monitoring were sometimes distant from the sampling sites of mollusks for the vigilance surveillance program. However, they were chosen to be representative of phytoplankton communities in the surrounding area (including the bivalve production areas).

3.4 BMAA and isomers in lab-cultured microalgae

Some diatom and dinoflagellate species have recently been shown to contain bound BMAA in the marine environment (Jiang et al., 2014a; Jiang and Ilag, 2014; Lage et al., 2014; Réveillon et al., 2015). Therefore, we screened 23 species of marine microalgae to assess if other phytoplanktonic species contained BMAA, including 4 species of bacillariophyceae and 6 species of dinophyceae but also other classes that have not been screened so far, *i.e.* 6 species of cryptophyceae, 3 of chlorophyceae, 2 of prymnesiophyceae and one of both euglenophyceae and rhodophyceae (table 3).

Several microalgal species screened in this study were observed in different phytoplankton communities within the framework of the REPHY phytoplankton monitoring. While most species were only present at small cell concentrations, others were observed at much higher concentrations, *i.e. Prorocentrum micans*, *Asterionellopsis glacialis*, *Scrippsiella* spp. and the *Pseudo-nitzschia delicatissima* complex (blooms of 170 000 to 2 million cells.L^{-1}). BMAA was not quantified in any of these 23 additionally screened microalgal species. However, a trace of BMAA (confirmed by injecting twice the regular volume) was observed in the diatom *Halamphora coffeaeformis*, another globally distributed common diatom. Two of the known isomers of BMAA, namely DAB and AEG, were respectively found in all microalgae (0.42 - 5.1 µg g^{-1} DW) and in 9 (0.23 - 2.1 µg g^{-1} DW) out of the 23 lab-cultured species.

Table 3: Concentrations of total form of BMAA, DAB and AEG in the lab-cultured microalgae. The presence of similar species observed within the framework of REPHY is mentioned as well as the number of sites in which the species were counted.

Strain	Species observed within REPHY	Concentration (µg g^{-1} DW)			Study
Bacillariophyceae		**BMAA**	**DAB**	**AEG**	
H. coffeaeformis (CCAP 1001/2)	No	Trace	0.66	0.25	This study
A. glacialis (CCMP 139)	Yes (n=8)	< LD	2.9	0.6	
Odontella aurita (AC815)	Yes (n=6)	< LD	5.1	0.35	
P. delicatissima (P5C1)	Yes (n=7)	< LD	3.5	< LD	
C.calcitrans (CCMP 1315)	Genus *	0.32	3.9	< LD	Réveillon et al., 2015
Chaetoceros sp. (France)	Yes (n=8)	0.58	29	< LD	
P. tricornutum (CCAP 1055/1)	No	0.51	1.3	< LD	
T. pseudonana (CCMP 1015)	Family **	0.75	18	< LD	
Dinophyceae		**BMAA**	**DAB**	**AEG**	This study
A. minutum (AMP99PZ)	Yes (n=8)	< LD	0.63	< LD	
H. triquetra (HT99PZ)	Yes (n=7)	< LD	5.1	< LD	
P. micans (PM85BV)	Yes (n=8)	< LD	1.2	2.1	
P. noctulica (CCMP 732)	No	< LD	5.0	0.23	
S. trochoïdea (ST97PZ)	Yes (n=8)	< LD	1.5	< LD	
S. microadriaticum (CCMP 828)	No	< LD	1.1	< LD	
Cryptophyceae		**BMAA**	**DAB**	**AEG**	
Hemiselmis sp. (RCC 659)	Yes (but grouped as Cryptophyceae)	< LD	3.9	< LD	
Proteomonas sp. (RCC 3072)		< LD	0.45	< LD	
Rhinomonas sp. (RCC 821)		< LD	0.9	< LD	
R. salina (RCC 1506)		< LD	1.1	< LD	
Rhodomonas sp. (RCC 1978)		< LD	0.85	< LD	
Unidentified cryptomonad (France)		< LD	1.7	< LD	
Microalgae of other classes		**BMAA**	**DAB**	**AEG**	
C. reginae (CCAP 11/78)	No	< LD	0.95	0.66	
C. vulgaris (CCAP 211/25)	No	< LD	3.5	0.76	
D. salina (CCAP 19/18)	No	< LD	3.1	< LD	
E. huxleyi (CCMP 371)	No	< LD	1.6	< LD	
E. gymnastica (CCMP 1594)	Yes (n=4)	< LD	2.6	< LD	
P. purpureum (CCAP 1380/5)	No	< LD	0.42	0.29	
T. lutea (CCAP 927/14)	No	< LD	2.7	< LD	

< LD: no peak or a peak inferior to the limit of detection (0.23 µg g^{-1} DW) was observed at a retention time corresponding to the standard; * as *Chaetoceros* sp.; ** as *Thalassiosira* sp. or *Thalassiosira* + *Porosira*.

4 Discussion

To date, at least three isomers of BMAA, *i.e.* DAB, AEG and BAMA (β-amino-*N*-methyl-alanine) have been reported in different matrices including microalgae and mollusks (Banack et al., 2012; Beach et al., 2015; Jiang et al., 2013; Rosen and Hellenas, 2008). Thus, the chemical analysis of BMAA is challenging (Faassen, 2014; Faassen et al., 2012) and requires highly selective and sensitive methods to distinguish all analogs (Cohen, 2012;

Combes et al., 2013; Jiang et al., 2013). In the present study, we used an HILIC-MS/MS method that had been optimized in a previous study (Réveillon et al., 2014). This method allowed us to unambiguously identify and accurately quantify BMAA, DAB and AEG in microalgae and mollusk matrices, using D_5DAB as internal standard.

Previously, we had reported the existence of a compound eluted just before BMAA in hydrolyzed mollusk samples (Réveillon et al., 2014). In this study, that interfering compound was, as expected, detected in the digestive gland tissues of mollusks (Appendix) confirming the complexity of BMAA analysis and the requirement of highly selective methods. Considering the recent work of Beach et al (2015), the nature of the interfering peak may be BAMA (β-amino-*N*-methylalanine) but this hypothesis should be further confirmed. Indeed, using standards, they showed a BAMA-BMAA resolution with a HILIC chromatography very similar to what we obtained for our hydrolyzed mollusk extracts between the interfering compound and BMAA. In that study, BMAA and BAMA were only separated thanks to the implementation of DMS (differential mobility spectrometry) to the original HILIC-MS/MS method; making DMS a promising approach to improving the reliability of BMAA analysis (Beach et al., 2015).

As an alternative and to cope with the possible misidentification of BMAA, the ratio of specific product ions *m/z* 76/88 was successfully used to confirm BMAA identity in mollusk extracts and therefore applied to all biological samples. The quantification of BMAA with the product ion *m/z* 76 prevented any overestimation as no interfering compound was detected for the mass spectral transition *m/z* 119 > 76. Both the mean total recovery of D_5DAB and the relative standard deviations were similar to previous studies (Jiang et al., 2013; Réveillon et al., 2014). Finally, correct retention times, satisfactory ion ratios and reproducible extraction recoveries indicated adequate performance of the sample preparation and reliability of the analytical procedure.

According to the 2008-2009 data from the French national shellfish farming committee (CNC, 2010), France is the second European shellfish producer (up to 200 000 t annually, 65% of oyster *C. gigas*). To date, BMAA had only been screened in mollusks from Thau lagoon in France (Masseret et al., 2013; Réveillon et al., 2014), even though this production area accounts only for ca. 8% of the French production of shellfish (CNC, 2010). Samples of mussels and oysters collected monthly in 2013 as part of the vigilance program were analyzed to assess the distribution of BMAA in other shellfish production areas. These samples corresponded to nine geographically representative French shellfish growing areas and were analyzed for total BMAA and analogs. BMAA and DAB were always detected while AEG was observed in 88% of the mollusk samples.

298 Concentrations of BMAA reported in this study are very similar to the concentrations that have been reported in
299 mollusks worldwide (Table 4), considering only highly selective methods (*i.e.* no fluorescence detection).
300

301 Table 4: BMAA levels in bivalve mollusks used in this study and in previous articles

Organism	**Origin**	**Total BMAA concentration (µg g^{-1} DW)**	**Reference**
Oyster (*Crassostrea virginica*) (n=15)	Louisiana and Mississippi	6.8 – 47	Christensen et al., 2012
Mussel (*Mytilus edulis*) (n=3) *Ostrea edulis* (n=3)	West coast of Sweden, Baltic Sea*	0.15 – 0.2 0.006 – 0.14	Jonasson et al., 2010
Mussel (*Mytilus edulis*) (n=6) Oyster (*O. edulis* or *C. gigas*) (n=4)	West coast of Sweden, Baltic Sea and imported from Greece and France	0.40 – 4.5** 0.50 – 3.3**	Jiang et al., 2014b
Cockle (*Cerastoderma edule*) (n=19)	Rias de Aveiro and Formosa, Portugal	< LD – 0.43	Lage et al., 2014
Mussel (*M. galloprovincialis*) (n=7) Oyster (*C. gigas*) (n=5)	Thau lagoon, France	1.8 – 6 0.60 – 1.6	Masseret et al., 2013
DG[a] of mussel (*M. galloprovincialis*) (n=17) RF[b] of mussel (n=17)	Thau lagoon, France	1.0 – 6.6 1.2 – 9.7	Réveillon et al., 2015
Mussel (*M. edulis* or *galloprovincialis*) (n=74) Oyster (*C. gigas*) (n=23)	French coasts (Channel, Atlantic and Mediterranean sites)	0.44 – 6.7 0.19 – 2.4	This study

302 * Fully marine ecosystem; ** concentrations expressed originally in µg g^{-1} wet weight thus estimated here
303 assuming that the dry/wet weight ratio is 0.2 for bivalve mollusks (Jiang et al., 2014b). [a] Digestive Gland and [b]
304 Remaining Flesh.
305

306 Within the vigilance program, only the digestive gland tissues of mollusks are conserved as they are known to
307 more efficiently accumulate toxins (Blanco et al., 2007; Jauffrais et al., 2012; Lassus et al., 2007). However,
308 significant amounts of BMAA (*i.e.* 60 ± 6.2%) have been detected in non-visceral tissue of *Mytilus*
309 *galloprovincialis* from Thau lagoon (Réveillon et al., 2015) suggesting that total concentration of BMAA in the
310 whole tissue of mollusks could be higher than those reported in the present study. It seems that BMAA presence
311 is widespread among filter-feeding bivalves. Indeed, almost all studies reported shellfish to contain BMAA (and
312 isomers, when included in the analysis), except some burrowing bivalves (*i.e. Cerastoderma edule* sampled from
313 Portuguese transitional water bodies and from the Baltic Sea (Lage et al., 2014)). The feeding behavior of
314 shellfish has been suggested to explain the apparently global observation of BMAA in these organisms (Jiang et
315 al., 2014b; Lage et al., 2014). Actually, as filter-feeding bivalves, shellfish are known to concentrate phycotoxins

(Amzil et al., 2007; Jauffrais et al., 2012; Lassus et al., 2007), bacterial or viral particles (Cavallo et al., 2009; Le Guyader et al., 2006; Love et al., 2010) as well as chemicals (Chu et al., 2003; Jeon et al., 2010; Ueno et al., 2010) during the process of filter-feeding. Therefore, they may accumulate BMAA and isomers from ingested organisms. The reported BMAA producers include species of some ubiquitous phytoplankton classes that are largely represented on the French coast, *i.e.* species belonging to diatoms and dinoflagellates (Jiang et al., 2014a; Lage et al., 2014; Réveillon et al., 2015). These primary producers are at the base of aquatic food webs and assumed to constitute the major food source for bivalve mollusks.

BMAA was systematically detected in French mollusks but specific patterns (*i.e.* cycles of accumulation/depuration) were not obvious. The BMAA level was not correlated to total or specific classes of phytoplankton abundances considered here (figures 2 and 3). Indeed, similar BMAA concentrations were observed at locations with contrasted abundances of phytoplankton, e.g. between “Pointe de St Quentin” and “Banc Arguin sud” for mussels or between “Ronce” and “Parc Leucate” for oysters. The phytoplankton communities observed in the water samples were composed of species that mollusks can feed on (Brown et al., 1997; Dunstan et al., 2005; Fernandes et al., 2013; Ren et al., 2006), even though *Phaeocystis* and *Leptocylindrus* spp. may have less significance as food sources when blooming (Lauringson et al., 2014a; Peperzak and Poelman, 2008; Smaal and Twisk, 1997). Several hypotheses could explain the absence of correlation between phytoplankton and BMAA concentration in digestive gland of mollusks. Firstly, phytoplankton communities observed within REPHY are restricted to the cells ≥ 20µm present at sub-surface (0 – 1m) and sampled at distances up to several kilometers away from the location of mollusk sampling. Thus the observed phytoplankton populations may not be totally representative of the food directly available for mollusks. Furthermore, different feeding behavior depending on the season, type and abundance of plankton have been reported (Kreeger and Newell, 2001; Marín Leal et al., 2008; Wong and Levinton, 2004). Actually, the composition of stomach contents of mussels may not reflect the occurrence and abundance of phytoplankton communities (Rouillon et al., 2005; Sidari et al., 1998). For example, in the Baltic Sea, *Mytilus trossulus* never consumed *Leptocylindrus*, yet the most abundant genera in the water column while most of the benthic diatoms consumed appeared not to be present in simultaneously taken water samples (Lauringson et al., 2014b). This selective feeding behavior (Shumway et al., 1985) strengthens the possibility that the observed phytoplankton communities may not well represent the diet of mussels and oysters. In spite of their similar feeding processes and capacity for particle selection (Riisgard and Larsen, 2010), oysters appeared capable of greater trophic plasticity than mussels (Dubois et al., 2007) which may at least partly explain some differences in BMAA and

DAB levels between these two shellfish species. Finally, bivalves are opportunistic filter-feeders thus, additional sources that are not sampled by REPHY cannot be excluded, *i.e.* picocyanobacteria, microphytobenthos, aggregated bacteria, detritus (microalgal or terrestrial) and zooplankton (Kach and Ward, 2008; Lefebvre et al., 2009; Masseret et al., 2013; Page and Lastra, 2003; Peharda et al., 2012; Pernet et al., 2014). Indeed, as suspension-feeding aquatic animals, bivalve mollusks have specialized in grazing on particles of a wide range of size (Riisgard and Larsen, 2010). Available studies using stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) and focusing on food webs of some ecosystems, in which mussels and oysters were sampled in this study, revealed that mollusks preferentially consumed marine particulate organic matter, mainly composed of phytoplankton. However, microphytobenthos represented a significant proportion in most of the areas while terrestrial or riverine organic matter and detritus from macroalgae were less relevant (Carlier et al., 2009; Dubois et al., 2014; Lefebvre et al., 2009; Mortillaro et al., 2014; Riera and Richard, 1996; Rigolet et al., 2014). For example, in Lingreville near Agon, the area exhibited a high marine influence and the diet of mussels was composed of 70-90% of phytoplankton, with the rest consisting of half microphytobenthos and half riverine organic matter (Lefebvre et al., 2009). On the opposite, in Bay of Concarneau (near Le Scoré), mats of *Navicula* sp. (benthic diatoms consisting of epibionts) can contribute up to 50% to the diet of the suspension feeder *Polititapes virgineus* (Rigolet et al., 2014).

The number of recorded BMAA-producing and potentially producing species has increased over the past few years. In Thau lagoon, BMAA and DAB were detected in plankton collected with nets, indicating that mussels were exposed to organisms that contained both BMAA and DAB (Réveillon et al., 2015). However, specific species producing BMAA have not been identified in that ecosystem. Nevertheless, (Jiang et al., 2014a) hypothesized that BMAA production might be common among diatoms after they reported the detection of BMAA in five cultures of axenic diatoms species. We therefore assessed the capacity of some microalgae species, including diatoms, to contain BMAA. For this purpose, 23 microalgae belonging to seven classes were screened and if we consider the species analyzed in a previous study (Réveillon et al., 2015), a total of 38 species representing potential food sources for mollusks have been evaluated for their BMAA production. BMAA was only detected in 5 out of the 13 diatoms species (0.32 – 0.75 µg g^{-1} DW) and only at trace level in microalgae in this study. None of the other species, independent of their classes, contained BMAA. The systematic detection of DAB and the observation of AEG in only some species (*i.e.* in 8 out of the 23 screened species) are in agreement with previous studies, suggesting that DAB is always detected in lab-cultured microalgae (Jiang et al., 2014a;

Réveillon et al., 2015). Our results did not confirm the global production of BMAA by diatoms under our culture conditions. It is noteworthy that we did not detect BMAA in *Tisochrysis lutea* CCAP 927/14 (formerly *Isochrysis* aff. *galbana*) since it is frequently used in aquaculture to feed farmed bivalve mollusks (Marchetti et al., 2012). Even though we could not detect any BMAA in most of the microalgal species, further studies are required to better understand the conditions allowing the production of BMAA and isomers by primary producers, like the role of nitrogen (Downing et al., 2011; Scott et al., 2014). The absence of detectable BMAA in many cultured species that represent possible or important food sources for the mollusks raised questions about the origin of BMAA detected in the shellfish. However, BMAA-producing species have not been clearly identified yet and due to the high diversity of potential primary producers that mollusks can feed on, as well as their trophic plasticity, there is a great uncertainty in determining which sources make important contributions to both their diets and BMAA content. In the future, particular attention should be paid to benthic diatoms belonging to microphytobenthos as they may represent a significant food source for mollusks.

According to Christensen et al (2012), knowledge of the concentration and variability of BMAA in seafood species is essential for calculating human exposure and designing animal model studies for human risk assessment. The variation in BMAA and DAB levels in mollusks across the sampling sites and seasons was small in this study, and very similar to those reported by Jiang et al. (2014b) for different seafood sold in Swedish markets. However, only few studies reported the screening of BMAA in seafood on a relatively large scale (Christensen et al., 2012; Jiang et al., 2014b; Jonasson et al., 2010). The results of the present study highlight the fact that BMAA and DAB are continuously detected in both mussels and oysters that are farmed in different ecosystems along the three French coasts. Regarding these observations and the results obtained with mussels of Thau lagoon (Réveillon et al., 2015) BMAA accumulation and depuration might be slow processes. As non proteinogenic amino acid, BMAA exist in two forms, as a free amino acid and bound to proteins (Glover et al., 2014). However, the exact nature of molecular interaction between BMAA and proteins should be thoroughly studied to better understand both the mechanism of bioaccumulation and the metabolization by mollusks.

The results of the present study, outlining the widespread occurrence of BMAA in shellfish from all French mainland coasts, also contrast the hypothesis made by Masseret et al. (2013) of a potential correlation of the ALS cluster observed in Southern France and BMAA in shellfish from Thau Lagoon. We therefore strongly suggest to re-examine the causative agents for ALS in French patients and also to evaluate more closely the levels of BMAA in foodstuffs likely to trigger ALS.

5 Conclusion

This study was performed to assess the occurrence of BMAA and isomers in mollusks reared in nine representative bivalve farming areas located on the three French coasts (Channel, Atlantic and Mediterranean sites). Digestive gland tissues of mollusks were collected monthly in 2013 as part of the vigilance surveillance program of the French phytoplankton and phycotoxins monitoring network (REPHY). A highly selective and sensitive HILIC-MS/MS method allowed the unambiguous identification and accurate quantification of BMAA in biological samples, using D_5DAB as internal standard. Despite their different origins, mollusks were systematically shown to contain BMAA, DAB and to a lesser extent AEG. Considering only results obtained with highly selective methods, concentrations were similar to those reported worldwide in filter-feeding bivalves. However, small variations of BMAA and DAB levels were noted, irrespective of the season and sampling location. Thus no specific contamination pattern could be identified. Unlike for Thau lagoon, no correlation between phytoplankton population recorded and BMAA/DAB concentration in digestive glands was observed. However, diatoms and dinoflagellates species, some being reported as BMAA-producers, largely dominated the phytoplankton populations in the areas of the bivalve farming areas.

Finally, food web structures are complex and the primary producers containing BMAA and consumed by mollusks have not been totally identified yet. Further studies are required about the origin, accumulation and metabolization of BMAA and DAB in mollusks.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

AKNOWLEDGMENTS

This study was carried out under the RISALTOX project (Ifremer) and co-funded by the Regional Council of the "Pays de la Loire" through the PhD program (Damien Réveillon) and the COSELMAR project. 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, especially Michèle Bardouil and Virginie Raimbault for the cultures of microalgae. They also would like to thank the members of Ifremer involved in the French phytoplankton and phycotoxins monitoring network (REPHY) for all the data and samples available.

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Appendix

Systematic detection of BMAA (β-*N*-methylamino-L-alanine) and DAB (2,4-diaminobutyric acid) in mollusks collected in shellfish production areas along the French coasts

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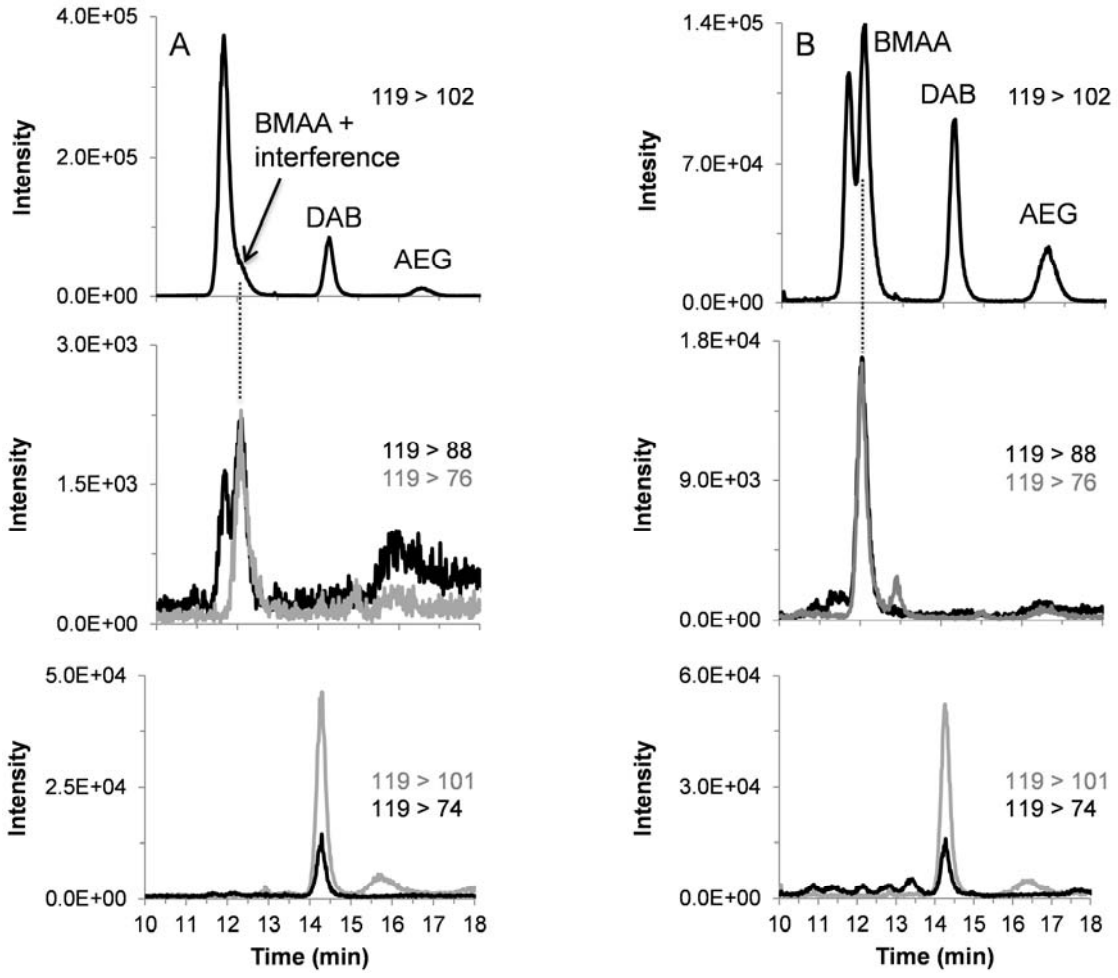

Appendix: Extracted ion chromatograms of BMAA, DAB and AEG of digestive gland tissues of (A) oyster from "Ronce" and (B) mussel from "Kervoyal". For the oyster sample, there is a co-elution between BMAA and an interfering compound leading to false ion ratios. However, the two peaks are partially resolved for mussel sample and the ion ratios are in agreement with those of the standards.