Harmful Algae
September 2016, Volume 58 Pages 45-50
http://dx.doi.org/10.1016/j.hal.2016.07.008
http://archimer.ifremer.fr/doc/00348/45911/

Production of BMAA and DAB by diatoms (*Phaeodactylum tricornutum, Chaetoceros* sp., *Chaetoceros calcitrans* and, *Thalassiosira pseudonana*) and bacteria isolated from a diatom culture

Réveillon Damien [1], Séchet Veronique [1], Hess Philipp [1], Amzil Zouher [1, *]

[1] Ifremer, Laboratoire Phycotoxines, rue de l'Ile d'Yeu, BP 21105, F-44311 Nantes, France

* Corresponding author : Zouher Amzil, email address : zouher.amzil@ifremer.fr

damien.reveillon@ifremer.fr ; veronique.sechet@ifremer.fr ; philipp.hess@ifremer.fr

Abstract :

Microalgae have previously been reported to contain β-*N*-methylamino-l-alanine (BMAA), and the global presence of these primary producers has been associated with the widespread occurrence of BMAA in marine organisms. It has been repeatedly shown that filter-feeding bivalves accumulate phytoplankton species and their toxins. In this study, the concentrations of total soluble BMAA and DAB as a function of growth phase were observed for four non-axenic diatom species (*i.e. Phaeodactylum tricornutum*, *Chaetoceros* sp., *Chaetoceros calcitrans* and *Thalassiosira pseudonana*). These strains had previously been shown to contain BMAA using a highly selective HILIC-MS/MS method. BMAA cell quota appeared to be species-specific, however, highest BMAA concentrations were always obtained during the stationary growth phase, for all four species, suggesting that BMAA is a secondary metabolite. While DAB was detected in a bacterial culture isolated from a culture of *P. tricornutum*, the presence or absence of a bacterial population did not influence production of BMAA and DAB by *P. tricornutum*, *i.e.* no significant difference was noted for BMAA and DAB production between axenic and non-axenic cultures. The presence of DAB in bacteria had previously been shown, and raised the question as to whether DAB observed in many species of microalgae may arise from the non-axenic culture conditions or from the microalgae themselves.

Keywords : BMAA, DAB, Diatoms, *Phaeodactylum tricornutum*, Bacteria

1. Introduction

The non-proteinogenic amino acid β-*N*-methylamino-l-alanine (BMAA) is a putative nutritional factor involved in the etiology of amyotrophic lateral sclerosis-Parkinsonism dementia complex (ALS-PDC) (Banack et al., 2010 and Spencer et al., 1987). This specific neurodegenerative disease was observed on the Island of Guam in the 1950s and was suspected to be linked to the consumption of BMAA-containing foodstuff (Banack et al., 2006 and Murch et al., 2004). Since the discovery of BMAA (Vega and Bell, 1967), the role of BMAA in the ALS syndrome is still under debate. It has gained increased attention over the last decade from the observation of BMAA presence in many different primary producers in both freshwater and marine environments. While cyanobacteria were the first organisms suspected to produce BMAA, some diatom and dinoflagellate species have recently been recognized as potential BMAA-producers as well (Cox et al., 2005, Jiang et al., 2014, Jiang et al., 2013, Lage et al., 2014 and Réveillon et al., 2015). Phytoplankton species, including those containing BMAA, are at the base of food chains and their general presence in aquatic ecosystems might suppose a widespread exposure for aquatic organisms, through classic trophic interactions. Hitherto, the production of BMAA by primary producers is still poorly understood. A group recently noted that BMAA was always detected

in several diatom cultures while its production by cyanobacteria seemed to be fluctuating (Jiang et al., 2014). Nevertheless, BMAA concentrations that were reported by that group were ca. 1000 times lower than those reported by two other groups (Lage et al., 2015; Réveillon et al., 2015).

The aims of the present study were to examine total soluble BMAA concentrations (*i.e.* free plus bound to not-precipitated proteins, as well explained in Faassen et al. (2016)) in four diatom species as a function of growth phase and to investigate the role of bacteria in the production of BMAA and DAB by *Phaeodactylum tricornutum*. For this purpose bacteria were isolated from *P. tricornutum* culture. Meanwhile, antibiotic treatments were performed to rule out the impact of bacteria on BMAA and DAB productions by this diatom species. All samples were analyzed using a highly selective and sensitive HILIC-MS/MS method (Réveillon et al., 2014). Both labeled D_3BMAA and D_5DAB internal standards were used to correct BMAA and DAB concentrations, thus allowing for further increase of both reliability and accuracy of the previously published method (Réveillon et al., 2014).

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. Deuterium labeled BMAA (BMAA-4,4,4-d_3, referred-to D_3BMAA, purity 98%), at a certified concentration of 1 mg mL^{-1} was purchased from Novakits (Nantes, France). Deuterium labeled DAB (D-2,4-diaminobutyric acid-2,3,3,4,4-d_5 dihydrochloride, referred-to D_5DAB, purity > 99%) was obtained 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 *Diatom culture conditions*

Four diatom species were grown for growth phase experiments, namely *Phaeodactylum tricornutum* CCAP 1055/1, *Chaetoceros* sp. isolated from Argenton, English Channel, France, *Chaetoceros calcitrans* CCMP 1315 and *Thalassiosira pseudonana* CCMP 1015.

Experiments were carried out with the Conway culture medium (Walne, 1970) at a temperature of 22 °C, an irradiance of 80 µmol m^{-2} s^{-1} under a photoperiod of 16 h of light and 8 h of dark. The culture media were prepared in 10 L borosilicate round flasks with filtered seawater (0.2 µm) at a salinity of 35. At day 0, non-axenic starter cultures in mid-exponential growth phase were diluted in fresh medium (8 L) aerated with filtered air (0.2 µm), at a final concentration of ca. 150 000 cells mL^{-1}. Cellular concentration was regularly assessed from day 0 to 31 by image analysis on Malassez slides using specific image analysis software (Samba Technologies, Meylan, France) after Lugol dying. The growth was also monitored by measuring chlorophyll fluorescence (excitation 450 nm and emission 685 nm) and absorbance (A_{680}) with a TECAN Infinite® 200 Multi-Mode Microplate Reader and Tecan i-control 1.5.14.0 software.

For *Phaeodactylum tricornutum*, the experiment was performed in duplicate and cells were sampled nine times between day 4 and 31. For the other three strains, single experiments were performed and the cells were sampled eight times between day 4 and 31. Aliquots of 200 – 400 mL of cultures were harvested via centrifugation at 4000 *g* for 30 min at 4 °C. Supernatant was carefully discarded and the resulting pellet was stored at -20 °C until lyophilisation.

2.3 *Antibiotic treatments of P. tricornutum culture*

To reduce bacterial community, a non-axenic *P. tricornutum* culture was treated with a mixture of antibiotics. The penicillin-streptomycin solution (10 000 units of penicillin and 10 mg streptomycin per mL, P0781, Sigma, France) was used at 15 and 30 mL L^{-1} on cultures in mid-exponential growth phase. After five days of treatment, the cultures were centrifuged twice 7 min at 500 *g* and once 10 min at 600 *g* then the procedure was fully repeated (*i.e.* antibiotics treatment, centrifugation and inoculation in fresh medium). Efficacy of the procedure after the two antibiotic treatments was checked by growth on Marine Agar plates (Difco™ 2216), no bacterial growth was found following the two treatments. Then, fresh autoclaved Conway medium (500 mL) was inoculated with both treated and untreated mid-exponential cultures of *P. tricornutum* at 150 000 cells mL^{-1}. The growth was monitored with fluorescence and absorbance of chlorophyll. Cells were harvested at day 16 and analyzed in duplicate for BMAA and isomers content.

2.4 *Isolation of bacteria*

In parallel, supernatant of *P. tricornutum* culture was spread on Marine Agar plates. Some of the heterotrophic marine bacteria colonies obtained were inoculated in 400 mL of Marine Broth (Difco™ 2216) and agitated at 100 rpm. Bacteria were harvested as for microalgae after 11 days of growth and analyzed for BMAA and isomers.

2.5 *Extraction procedure for total soluble BMAA and DAB*

All matrices were lyophilised before extraction. BMAA and DAB were extracted and analyzed as previously described (Réveillon et al., 2014), with both D_3BMAA and D_5DAB as internal standards. Briefly, freeze-dried material (10 mg) was ground in TCA 0.1 M containing the internal standards. The supernatant was collected, evaporated to dryness and hydrolyzed in HCl 6 M at 99 °C for 24 h before SPE clean-up on Bond Elut® Plexa PCX cartridges (Agilent Technologies, VWR, France). Therefore, only free and

TCA-soluble bound forms of BMAA and DAB were analyzed (*i.e.* total soluble fraction), as the pellets containing cell debris and any precipitated proteins were not considered.

Instrumentation and analytical method for BMAA and DAB

Liquid chromatography and tandem mass spectrometry were performed as described in Réveillon et al. (2014). The three isomers BMAA, DAB and AEG were unambiguously distinguished thanks to chromatographic resolution, specific mass spectral transitions and qualitative to quantitative ion ratios. The common transition *m/z* 119 > 102 was used to quantify BMAA, DAB and AEG. The internal standards were quantified with the transitions *m/z* 122 > 105 and *m/z* 124 > 47 for D_3BMAA and D_5DAB, respectively.

Quantitation was performed relative to pure standards of BMAA, DAB and AEG. The limit of detection (LOD) equaled the limit of quantification (LOQ) and was 0.23 µg g^{-1} dry weight (DW). The method used to determine the LOQ was reported in Réveillon et al (2014). Briefly, LOQ corresponded to the lowest concentration in a spiked microalgal matrix giving a signal-to-noise ratio of three and ten for the qualitative and quantitative mass spectral transitions of BMAA and DAB, respectively. Results were expressed as <LOQ when a peak was detected at a retention time corresponding to the internal standard but identity could not be verified by all criteria (*i.e.* specific mass spectral transitions and/or ion ratios could not be used). Corrective factors derived from both internal standard recoveries were applied to compensate for losses during samples preparation and matrix effects. The software Analyst 1.5.1 was used to analyze acquired raw data.

Statistical analysis

Statistical analyses (t-tests) were carried out using SigmaPlot 11 (Systat Software Inc., Chicago, IL, USA). Differences were considered significant at $p<0.05$.

Results

Kinetics of BMAA and DAB production by diatoms

The results of growth and BMAA and DAB concentrations obtained for the four diatom species are shown in figure 1.

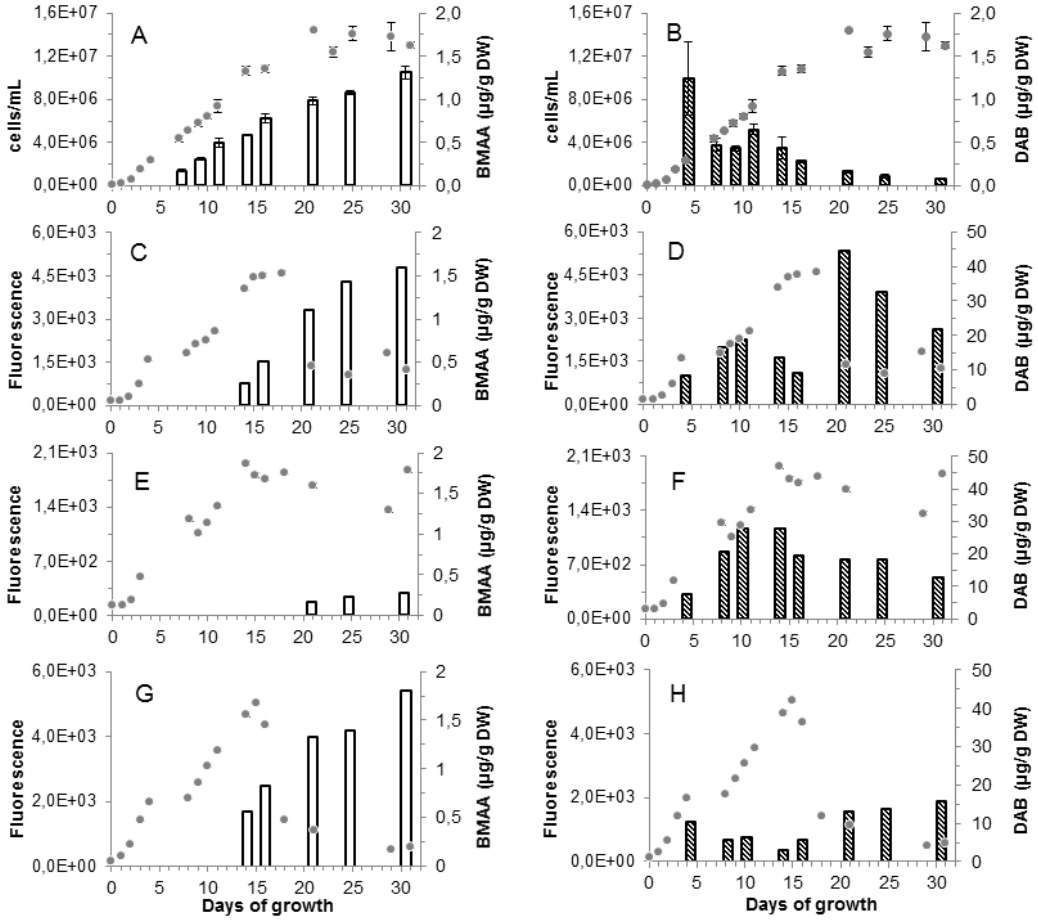
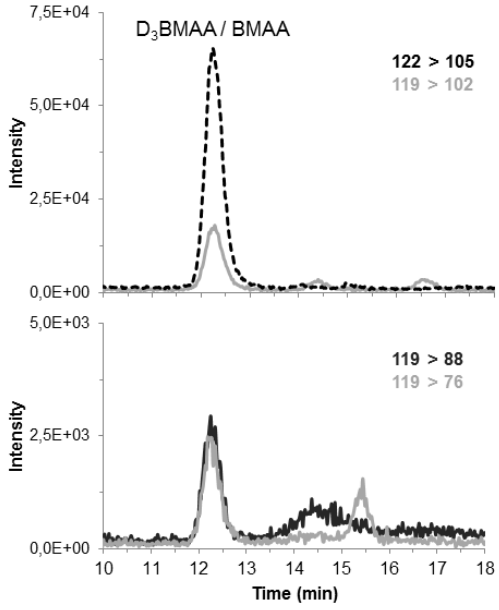


Figure 1: Growth (grey circle symbols) and concentrations of total soluble BMAA (white bars) and DAB (black hatched bars), for respectively (A, B) *Phaeodactylum tricornutum* CCAP 1055/1, (C, D) *Chaetoceros* sp. (E, F) *Chaetoceros calcitrans* CCMP 1315 and (G, H) *Thalassiosira pseudonana* CCMP 1015. Error bars correspond to standard deviation. Cellular concentration was used as a proxy of growth only for *P. tricornutum* while fluorescence of chlorophyll was used as an alternative for the three other strains (*i.e.* formation of aggregates led to underestimated concentrations of cells).

188 The four species showed comparable growth curves with a short lag phase
189 (≤ two days), an exponential phase starting from day 2, up to day 21 for *P.*
190 *tricornutum* and to day 14-15 for the three other species, then a stationary
191 phase up to day 31 (*i.e.* the end of the experiment). The drop of fluorescence
192 signal observed for *Chaetoceros* sp. and *T. pseudonana* coincided with the
193 stationary phase and may be related to loss of chlorophyll, as a result of
194 nutrient limitation (Ruivo et al., 2011).
195 BMAA in *P. tricornutum* was observed from day 7 until the end of the
196 experiment, with increasing concentrations ranging from 0.20 to 1.4 µg g^{-1}
197 DW. An inverse relationship was observed for DAB (from 1.2 µg g^{-1} to
198 < LOQ). A chromatogram of *P. tricornutum* extract at day 25 can be seen
199 in figure 2.



200

201 Figure 2: Extracted ion chromatogram of *P. tricornutum* culture at day 25
202 of the growth curve experiment. Black dotted line represent transition *m/z*
203 122 > 105 used to quantify D_3BMAA. It can be seen that retention time of
204 the internal standard matched the one of BMAA detected in the culture of *P.*
205 *tricornutum.*

206

207 Similar pattern was observed for BMAA concentrations in the three other
208 diatom species. Nevertheless, BMAA was first detected on day 14 for both

Chaetoceros sp. and *T. pseudonana* and on day 21 for *C. calcitrans*. The BMAA concentrations were between 0.26 – 1.6, 0.17 – 0.28 and 0.56 – 1.8 µg g^{-1} DW, respectively. DAB was always detected in these strains, at higher concentrations varying from 8.5 to 44 for *Chaetoceros* sp., 7.5 to 28 for *C. calcitrans* and 3.0 to 16 µg g^{-1} for *T. pseudonana*.

3.2 *Effects of antibiotic treatments on BMAA and DAB production by P. tricornutum*

Bacterial population in *P. tricornutum* cultures was significantly reduced after the two cycles of antibiotic treatments (*i.e.* no growth observed on marine agar plates for the treated cultures, at both 15 and 30 mL L^{-1}). Growth of treated and untreated cultures was very similar as were the BMAA and DAB concentrations after 16 days of growth. Indeed no significant difference ($p>0.05$) was observed for BMAA and DAB concentrations as 0.62, 0.62 and 0.56 µg g^{-1} of BMAA and 0.55, 0.53 and 0.59 µg g^{-1} DW of DAB were quantified in the untreated culture, and the cultures treated with 15 and 30 mL L^{-1} of antibiotic solution, respectively.

3.3 *LC-MS/MS analysis of bacteria isolated from P. tricornutum*

One type of bacterial colony was obtained from *P. tricornutum* culture. After growth, the bacterial biomass was screened for BMAA and isomers. While no BMAA was detected, DAB was quantified at 18.6 µg g^{-1} DW.

4 Discussion

The selective and sensitive HILIC-MS/MS method used in this study had previously been optimized (Réveillon et al., 2014). The reliability of BMAA identification and accuracy of quantification were, however, further increased by the use of D_3BMAA as isotopically labeled internal standard. The mean total recoveries ± SD of both internal standards were in agreement with previous studies (65 ± 7% and 61% ± 8% for D_3BMAA and D_5DAB respectively) (Réveillon et al., 2014; Réveillon et al., 2015).

Even though cyanobacteria were the first organisms suspected to produce BMAA (Cox et al., 2005), only few species were confirmed to contain BMAA using highly selective methods (Jiang et al., 2013). On the opposite, some marine phytoplankton species belonging to diatom and dinoflagellate groups were recently reported to contain BMAA (Jiang et al., 2014; Lage et al., 2015; Lage et al., 2014; Réveillon et al., 2015). Nevertheless, production of BMAA and isomers as a function of growth of marine microalgae has not been studied so far. For this purpose, four diatom strains that had previously been reported to contain BMAA were used in this study (Réveillon et al., 2015). Two *Chaetoceros* species (CCMP 1315 and isolated from Argenton, France), one *Phaeodactylum tricornutum* (CCAP 1055/1) and one *Thalassiosira pseudonana* (CCMP 1015) strains were cultured under conditions previously reported for each species.

Both appearance and concentration of BMAA varied between strains. *P. tricornutum* showed a more prolonged BMAA production than the three other species, with BMAA detected from day 7 (*i.e.* mid exponential phase). The concentrations at the end of the growth curve experiments were similar for three out of the four strains (*i.e.* from 1.3 to 1.8 µg g^{-1} DW), except in *C. calcitrans* in which BMAA concentration was about 5 times lower. Therefore, BMAA production by diatoms may be species-specific as for other toxin-microalgae combinations (Anderson et al., 2012; Reguera et al., 2012; Thessen et al., 2009). It should be mentioned that the growth curve experiments were not replicated, except for *P. tricornutum* which showed low variability of BMAA concentrations. Therefore the species-specific hypothesis of BMAA production should be further confirmed as the variability within- and consequently the variability between strains are not well characterized for the three other diatom strains. Moreover, while we only analyzed total soluble BMAA, the presence of BMAA in pellets (*i.e.* as bound to precipitated proteins) should be investigated, even though the

soluble bound fraction accounted for the majority of BMAA concentration in other studies (Faassen et al., 2016; Rosén et al., 2016).

Data about BMAA production by primary producers are scarce in literature. While Downing and coworkers postulated that cyanobacteria may preferentially produce BMAA under nitrogen limiting conditions (Downing et al., 2011; Scott et al., 2014), such limitation was not necessary for *P. tricornutum* to produce BMAA at significant levels in the present study. Indeed, BMAA production increased as a function of growth, as for other toxins produced by microalgae, (e.g. Hwang and Lu, 2000; Jauffrais et al., 2013; Thorel et al., 2014).

DAB, an isomer of BMAA largely reported in microalgal species (Jiang et al., 2014; Krüger et al., 2010; Lage et al., 2014; Réveillon et al., 2014) was also detected in the four diatom strains. In *P. tricornutum*, the two isomers were present in similar concentrations (*i.e.* < 1.4 µg g^{-1} DW), and while DAB concentration decreased as a function of growth, an inverse relationship was noted for BMAA. Nonetheless, much higher DAB concentrations were found in the other species but no particular patterns could be defined. The concentrations, from 7.5 to 44 µg g^{-1} DW, are in agreement with those reported by other groups, but relatively high in comparison to the concentrations that the authors group had previously observed in cyanobacteria (McCarron et al., 2014; Réveillon et al., 2014; Rosén and Hellenas, 2008). Biosynthetic pathways of the two non proteinogenic amino acids are still lacking, neither a common precursor nor BMAA-DAB or DAB-BMAA conversions have been reported to explain the relative variation of concentrations in the different diatom species and especially for *P. tricornutum*.

As xenic cultures of diatoms were used, the hypothesis that BMAA and DAB productions by diatoms may be directly or indirectly influenced by bacteria cannot be excluded. Interactions between bacteria and diatoms are

296 common (Amin et al., 2012; Doucette, 1995; Paul et al., 2013). They are
297 recognized as an important factor in the physiology of diatoms (Grossart et
298 al., 2005; Rooney-Varga et al., 2005) and might be involved in the
299 production of the neurotoxin domoic acid by *Pseudo-nitzschia* (Bates et al.,
300 1995; Sison-Mangus et al., 2014). A potential bacterial BMAA production
301 has not been reported yet, while DAB is commonly present in bacteria.
302 Indeed, it has been detected in the peptidoglycan cell wall of actinomycetes
303 and had been used as a diagnostic diamino acid for identifying new strains
304 (Groth et al., 1996). Moreover, a gene encoding DAB has been identified in
305 *Acinetobacter baumannii* (Ikai and Yamamoto, 1997). To assess the impact
306 of bacteria on BMAA and DAB production by *P. tricornutum*, antibiotic
307 treatments and isolation of bacteria were concurrently performed on the
308 same culture. Analysis revealed that both growth and BMAA and DAB
309 contents in *P. tricornutum* cells were not influenced by the presence of
310 bacteria, suggesting that *P. tricornutum* did produce these compounds as no
311 significant difference was observed between treated and untreated cultures.
312 While BMAA was not directly detected in bacteria isolated from *P.*
313 *tricornutum* culture, high concentration of DAB was found. This
314 observation may be relevant for DAB production by diatoms, especially for
315 *Chaetoceros* and *Thalassiosira pseudonana* strains on which no antibiotic
316 treatment was performed. Moreover, these three diatom strains displayed
317 high concentrations of DAB (*i.e.* in comparison to *P. tricornutum*) that was
318 not correlated to the growth. The presence of a bacterial community might
319 explain the high concentration of DAB as well as its behaviour in
320 *Chaetoceros* and *Thalassiosira pseudonana* cultures. Thus, further work is
321 required to disentangle to which proportion bacteria (including symbiotic
322 bacteria) are involved in DAB production by diatoms and more broadly by
323 microalgae, including cyanobacteria. Moreover, antibiotic treatments should
324 be performed on all non-axenic microalgal strains to confirm that they are
325 the actual BMAA producers.

The control of toxin production by microalgae is thought to be complex and triggered by many environmental factors, as for azaspiracids or domoic acid, two phycotoxins produced by the dinoflagellate *Azadinium spinosum* and the diatom *Pseudo-nitzschia* (Jauffrais et al., 2013; Trainer et al., 2012). Here, the concentration of BMAA and DAB were assessed over time but in only one and non-limiting condition (*i.e.* conditions for routine maintenance). Therefore, the factors controlling BMAA production by diatoms should be further investigated (e.g. salinity, temperature, irradiance, culture media, nutrient sources and bacteria-diatom interactions) as stressed cells are generally assumed to produce more toxins (Bates and Trainer, 2006). Moreover, it was recently suggested that BMAA was released or formed from the low molecular weight compounds in blue mussels while the amino acid was neither in a free nor a protein-bound form in the extracts (Rosén et al., 2016). Thus, the exact nature of BMAA detected in microalgae, but also in the other aquatic organisms, should be further studied (e.g. degradation product or amino acid released from proteins or other compounds).

5 Conclusion

The prevalent presence of BMAA in aquatic organisms of higher trophic levels in freshwater ecosystems has predominantly been associated with the occurrence of cyanobacteria. In the marine environment, diatoms may represent a more likely primary source of BMAA. It is therefore necessary to study BMAA production by diatoms. The four diatom strains that were used in this study did contain BMAA. Production as a function of growth phase seemed species-specific but should be further confirmed by replicating the growth curve experiments. Maximum BMAA concentrations were observed at the end of the experiment (*i.e.* late exponential phase) as for many toxin-microalgae combination. Nevertheless, the factors that trigger BMAA production should be further investigated.

355 DAB was detected in bacteria isolated from *P. tricornutum* culture. While
356 bacteria did not influence the production of BMAA and DAB by this diatom
357 species, their importance for BMAA and DAB concentrations observed
358 cannot be ruled out for the three other species, and for non-axenic cultures
359 of microalgae on a larger scale.
360

361 **CONFLICT OF INTEREST**

362 The authors declare that there are no conflicts of interest.
363

364 **AKNOWLEDGMENTS**

365 This study was carried out under the RISALTOX project (Ifremer) and co-
366 funded by the Regional Council of the “Pays de la Loire”. The authors
367 would like to thank all of the members of the laboratory Phycotoxins at the
368 Atlantic Centre of Ifremer for their help and advice during this study.
369

REFERENCES

Amin, S.A., Parker, M.S., and Armbrust, E.V. (2012). Interactions between Diatoms and Bacteria. Microbiology and Molecular Biology Reviews *76*, 667-684.

Anderson, D.M., Alpermann, T.J., Cembella, A.D., Collos, Y., Masseret, E., and Montresor, M. (2012). The globally distributed genus Alexandrium: Multifaceted roles in marine ecosystems and impacts on human health. Harmful Algae *14*, 10-35.

Banack, S.A., Caller, T.A., and Stommel, E.W. (2010). The Cyanobacteria Derived Toxin Beta-N-Methylamino-L-Alanine and Amyotrophic Lateral Sclerosis. Toxins *2*, 2837-2850.

Banack, S.A., Murch, S.J., and Cox, P.A. (2006). Neurotoxic flying foxes as dietary items for the Chamorro people, Marianas Islands. Journal of Ethnopharmacology *106*, 97-104.

Bates, S.S., Douglas, D.J., Doucette, G.J., and 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.

Bates, S.S., and Trainer, V.L. (2006). The Ecology of Harmful Diatoms. In Ecology of Harmful Algae, E. Granéli, and J. Turner, eds. (Springer Berlin Heidelberg), pp. 81-93.

Cox, P.A., Banack, S.A., Murch, S.J., Rasmussen, U., Tien, G., Bidigare, R.R., Metcalf, J.S., Morrison, L.F., Codd, G.A., and Bergman, B. (2005). Diverse taxa of cyanobacteria produce β-N-methylamino-l-alanine, a neurotoxic amino acid. Proc Natl Acad Sci U S A *102*, 5074-5078.

Doucette, G.J. (1995). Interactions between bacteria and harmful algae: a review. Nat Toxins *3*, 65-74.

398 Downing, S., Banack, S.A., Metcalf, J.S., Cox, P.A., and Downing,
399 T.G. (2011). Nitrogen starvation of cyanobacteria results in the
400 production of β-N-methylamino-L-alanine. Toxicon *58*, 187-194.
401 Faassen, E. J.; Antoniou, M. G.; Beekman-Lukassen, W.; Blahova,
402 L.; Chernova, E.; Christophoridis, C.; Combes, A.; Edwards, C.;
403 Fastner, J.; Harmsen, J.; Hiskia, A.; Ilag, L. L.; Kaloudis, T.; Lopicic,
404 S.; Lurling, M.; Mazur-Marzec, H.; Meriluoto, J.; Porojan, C.; Viner-
405 Mozzini, Y.; Zguna, N. (2016). A collaborative evaluation of LC-
406 MS/MS based methods for BMAA analysis: soluble bound BMAA
407 found to be an important fraction. Marine Drugs *14*, 45.
408 Grossart, H.P., Levold, F., Allgaier, M., Simon, M., and Brinkhoff,
409 T. (2005). Marine diatom species harbour distinct bacterial
410 communities. Environ Microbiol *7*, 860-873.
411 Groth, I., Schumann, P., Weiss, N., Martin, K., and Rainey, F.A.
412 (1996). Agrococcus jenensis gen. nov., sp. nov., a new genus of
413 actinomycetes with diaminobutyric acid in the cell wall. International
414 journal of systematic bacteriology *46*, 234-239.
415 Hwang, D.F., and Lu, Y.H. (2000). Influence of environmental and
416 nutritional factors on growth, toxicity, and toxin profile of
417 dinoflagellate Alexandrium minutum. Toxicon *38*, 1491-1503.
418 Ikai, H., and Yamamoto, S. (1997). Identification and analysis of a
419 gene encoding L-2,4-diaminobutyrate:2-ketoglutarate 4-
420 aminotransferase involved in the 1,3-diaminopropane production
421 pathway in Acinetobacter baumannii. J Bacteriol *179*, 5118-5125.
422 Jauffrais, T., Séchet, V., Herrenknecht, C., Truquet, P., Véronique,
423 S., Tillmann, U., and Hess, P. (2013). Effect of environmental and
424 nutritional factors on growth and azaspiracid production of the
425 dinoflagellate Azadinium spinosum. Harmful Algae *27*, 138-148.

426 Jiang, L., Eriksson, J., Lage, S., Jonasson, S., Shams, S., Mehine, M.,
427 Ilag, L.L., and Rasmussen, U. (2014). Diatoms: A Novel Source for
428 the Neurotoxin BMAA in Aquatic Environments. PLoS One *9*,
429 e84578.
430 Jiang, L., Johnston, E., Åberg, K.M., Nilsson, U., and Ilag, L. (2013).
431 Strategy for quantifying trace levels of BMAA in cyanobacteria by
432 LC/MS/MS. Anal Bioanal Chem *405*, 1283-1292.
433 Krüger, T., Mönch, B., Oppenhäuser, S., and Luckas, B. (2010). LC–
434 MS/MS determination of the isomeric neurotoxins BMAA (β-N-
435 methylamino-l-alanine) and DAB (2,4-diaminobutyric acid) in
436 cyanobacteria and seeds of Cycas revoluta and Lathyrus latifolius.
437 Toxicon *55*, 547-557.
438 Lage, S., Burian, A., Rasmussen, U., Costa, P., Annadotter, H.,
439 Godhe, A., and Rydberg, S. (2015). BMAA extraction of
440 cyanobacteria samples: which method to choose? Environmental
441 Science and Pollution Research, 1-13.
442 Lage, S., Costa, P.R., Moita, T., Eriksson, J., Rasmussen, U., and
443 Rydberg, S.J. (2014). BMAA in shellfish from two Portuguese
444 transitional water bodies suggests the marine dinoflagellate
445 Gymnodinium catenatum as a potential BMAA source. Aquat
446 Toxicol *152*, 131-138.
447 McCarron, P., Logan, A., Giddings, S., and Quilliam, M. (2014).
448 Analysis of beta-N-methylamino-L-alanine (BMAA) in spirulina-
449 containing supplements by liquid chromatography-tandem mass
450 spectrometry. Aquatic Biosystems *10*, 5.
451 Murch, S.J., Cox, P.A., and Banack, S.A. (2004). A mechanism for
452 slow release of biomagnified cyanobacterial neurotoxins and

453 neurodegenerative disease in Guam. Proc Natl Acad Sci U S A *101*,
454 12228-12231.
455 Paul, C., Mausz, M.A., and Pohnert, G. (2013). A co-
456 culturing/metabolomics approach to investigate chemically mediated
457 interactions of planktonic organisms reveals influence of bacteria on
458 diatom metabolism. Metabolomics *9*, 349-359.
459 Reguera, B., Velo-Suárez, L., Raine, R., and Park, M.G. (2012).
460 Harmful Dinophysis species: A review. Harmful Algae *14*, 87-106.
461 Réveillon, D., Abadie, E., Sechet, V., Brient, L., Savar, V., Bardouil,
462 M., Hess, P., and Amzil, Z. (2014). Beta-N-Methylamino-L-Alanine:
463 LC-MS/MS Optimization, Screening of Cyanobacterial Strains and
464 Occurrence in Shellfish from Thau, a French Mediterranean Lagoon.
465 Marine Drugs *12*, 5441-5467.
466 Réveillon, D., Abadie, E., Séchet, V., Masseret, E., Hess, P., and
467 Amzil, Z. (2015). β-N-methylamino-l-alanine (BMAA) and isomers:
468 Distribution in different food web compartments of Thau lagoon,
469 French Mediterranean Sea. Mar Environ Res *110*, 8-18.
470 Rooney-Varga, J.N., Giewat, M.W., Savin, M.C., Sood, S.,
471 LeGresley, M., and Martin, J.L. (2005). Links between
472 Phytoplankton and bacterial community dynamics in a coastal marine
473 environment. Microb Ecol *49*, 163-175.
474 Rosén, J., and Hellenas, K.E. (2008). Determination of the
475 neurotoxin BMAA (beta-N-methylamino-L-alanine) in cycad seed
476 and cyanobacteria by LC-MS/MS (liquid chromatography tandem
477 mass spectrometry). Analyst *133*, 1785-1789.
478 Rosén, J., Westerberg, E., Schmiedt, S., and Hellenäs, K.-E. (2016).
479 BMAA detected as neither free nor protein bound amino acid in blue
480 mussels. Toxicon *109*, 45-50.

481 Ruivo, M., Amorim, A., and Cartaxana, P. (2011). Effects of growth
482 phase and irradiance on phytoplankton pigment ratios: implications
483 for chemotaxonomy in coastal waters. Journal of Plankton Research
484 *33*, 1012-1022.
485 Scott, L.L., Downing, S., Phelan, R.R., and Downing, T.G. (2014).
486 Environmental modulation of microcystin and beta-N-methylamino-
487 l-alanine as a function of nitrogen availability. Toxicon : official
488 journal of the International Society on Toxinology *87*, 1-5.
489 Sison-Mangus, M.P., Jiang, S., Tran, K.N., and Kudela, R.M. (2014).
490 Host-specific adaptation governs the interaction of the marine
491 diatom, Pseudo-nitzschia and their microbiota. ISME J *8*, 63-76.
492 Spencer, P.S., Nunn, P.B., Hugon, J., Ludolph, A.C., Ross, S.M.,
493 Roy, D.N., and Robertson, R.C. (1987). Guam amyotrophic lateral
494 sclerosis-parkinsonism-dementia linked to a plant excitant
495 neurotoxin. Science *237*, 517-522.
496 Thessen, A.E., Bowers, H.A., and Stoecker, D.K. (2009). Intra- and
497 interspecies differences in growth and toxicity of Pseudo-nitzschia
498 while using different nitrogen sources. Harmful Algae *8*, 792-810.
499 Thorel, M., Fauchot, J., Morelle, J., Raimbault, V., Le Roy, B.,
500 Miossec, C., Kientz-Bouchart, V., and Claquin, P. (2014). Interactive
501 effects of irradiance and temperature on growth and domoic acid
502 production of the toxic diatom Pseudo-nitzschia australis
503 (Bacillariophyceae). Harmful Algae *39*, 232-241.
504 Trainer, V.L., Bates, S.S., Lundholm, N., Thessen, A.E., Cochlan,
505 W.P., Adams, N.G., and Trick, C.G. (2012). Pseudo-nitzschia
506 physiological ecology, phylogeny, toxicity, monitoring and impacts
507 on ecosystem health. Harmful Algae *14*, 271-300.

508 Vega, A., and Bell, E.A. (1967). α-Amino-β-methylaminopropionic
509 acid, a new amino acid from seeds of Cycas circinalis.
510 Phytochemistry *6*, 759-762.
511 Walne, P.R., 1970. Studies on the food value of nineteen genera of
512 algae to juvenile bivalves of the genera Ostrea, Crassostrea,
513 Mercenaria and Mytilus. Fish. Invest., London. II, *26*: 1-61.