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

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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-D₅ (D₅DAB), 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 (NH₄OH, 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 ≥ 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).

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

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

- Si, without silicate
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$_5$DAB 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 Chromatography (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) 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 D₃DAB.

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 D₃DAB 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 D₃DAB as internal standard. In this study, the total recovery of D₃DAB 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).
3.2 Temporal and spatial distribution of BMAA and isomers in environmental samples

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

3.2.1 Monitoring of BMAA and isomers in mussels from Thau lagoon

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

![Figure 2: Extracted ion chromatograms of periphyton of March 2014](image)
BMAA, DAB and AEG ranged from below the limit of detection to 1.2, 6.2 and 0.31 µg.g\(^{-1}\) DW, respectively, while maximal concentrations of their total form were 9.7, 10.7 and 1.2 µg.g\(^{-1}\) DW, respectively. In a previous study (Reveillon et al., 2014), mollusks from Thau lagoon had been screened as a cluster of ALS had recently been reported in this important bivalve farming area, concurrently with the presence of BMAA and DAB in reared mollusks (Masseret et al., 2013). In that study, oysters collected in 2009 and mussels collected at different seasons between 1995 and 2008 contained similar amounts of BMAA and DAB (0.6-6 µg.g\(^{-1}\) DW), suggesting that these two isomers were repeatedly present in mollusks over several decades. Here, we report the presence of BMAA, DAB and AEG in all samples of Thau lagoon mussels (*Mytilus galloprovincialis*) collected monthly between July 2013 and October 2014.

The presence of BMAA in seafood (as well freshwater foodstuffs) was reported worldwide. Indeed, blue crabs from Chesapeake Bay, USA (< 115.2 µg.g\(^{-1}\) DW) (Field et al., 2013), lobsters collected in Florida Bay (< 27 µg.g\(^{-1}\) DW) (Banack et al., 2014), fishes from Nebraska reservoirs (< 2.6 µg.g\(^{-1}\) DW) (Al-Sammak et al., 2014), dietary supplements from shark cartilage (< 352 µg.g\(^{-1}\) DW) (Mondo et al., 2014), cockles from Portuguese coasts (< 0.43 µg.g\(^{-1}\) DW) (Lage et al., 2014), mussels, oysters and different fish species from the Baltic Sea (< 1.3 µg.g\(^{-1}\) DW and < 1.6 µg.g\(^{-1}\) wet weight) (Jonasson et al., 2010; Salomonsson et al., 2013), even seafood purchased in Swedish markets (< 0.9 µg.g\(^{-1}\) wet weight) (Jiang et al., 2014b) and fishes and invertebrates from Gonghu Bay, China (< 36 µg.g\(^{-1}\) DW) (Jiao et al., 2014) were shown to contain various amounts of BMAA, depending on the matrix and the analytical method used. DAB and/or AEG were only analyzed for in few of these studies but DAB concentrations were generally similar to those of BMAA.

The survey from July 2013 to October 2014 provided insight into the distribution of BMAA and isomers in mussels from Thau lagoon, including seasonal variations. Concentrations of DAB were relatively constant throughout the sampling period, irrespective of the free or total form and the organ considered (Appendix A). The overall mean concentration of total DAB in remaining flesh (RF) and digestive gland (DG) tissues was 7.2 µg.g\(^{-1}\) DW, with a relative standard deviation of only 22%. On the opposite, the concentration of total BMAA varied and showed two time-dependent increases in summer/autumn seasons in 2013 and 2014. High levels of total BMAA (from 3.8 to 14.4 µg.g\(^{-1}\) DW) has also been observed throughout the summer of 2009 in mussels from Thau in a previous study (Reveillon et al., 2014). Similarly, Masseret et al., 2013 also reported higher concentrations of BMAA in mussels collected at the same location during summer. The pattern of BMAA presence in mussels of the lagoon could be due to cycles of accumulation/depuration, as a result of feeding, in analogy to the hypothesis formulated for contaminated cockles in Portugal, feeding at least partially on the
dinoflagellate *Gymnodinium catenatum* that was shown to contain bound BMAA (Lage et al., 2014). The higher seasonal variability in BMAA concentrations compared to DAB may also be due to the generally much wider presence of DAB in all phytoplankton and cyanobacterial species analyzed.

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

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

![Graph showing relative abundance of free and bound BMAA, DAB, and AEG in DG and RF tissues.](image)

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

Relative abundance of BMAA, DAB and AEG all differed significantly between digestive glands and remaining flesh of mussels (P < 0.05). Indeed, free and bound BMAA were more present in RF tissues and accounted respectively for 69.3% ± 17% and 59.5% ± 6% (mean ± RSD) of the BMAA concentrations in mussels. On the opposite, concentration of bound AEG was significantly higher in DGs. Concerning DAB, while free concentration was significantly higher in RFs, the tendency was reversed for bound DAB which was significantly more present in DGs. This differential distribution is somewhat surprising, considering the small differences at molecular level of these amino acids. However, differences between analogs had already been observed in an *in vitro* model for protein incorporation (Glover et al., 2014a).

The distribution of BMAA and DAB within mussel tissues was unusual in comparison to other cyanotoxins and phycotoxins. Toxins accumulating in filter-feeding bivalves as a function of consumption of microalgae were 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 $\mu$g 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 $\mu$g g$^{-1}$ DW).
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).
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.
Table 2: Concentrations of total BMAA, DAB and AEG in the 15 lab-cultured microalgae.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
<th>Concentration of total form (µg.g(^{-1}) DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diatoms</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chaetoceros calcitrans</td>
<td>CCMP 1315</td>
<td>0.32, 3.9, &lt; LD</td>
</tr>
<tr>
<td>Chaetoceros sp.</td>
<td>Argenton (France)</td>
<td>0.58, 2.9, &lt; LD</td>
</tr>
<tr>
<td>Chaetoceros pumilum</td>
<td>Argenton (France)</td>
<td>&lt; LD, 5.5, &lt; LD</td>
</tr>
<tr>
<td>Phaeodactylum tricornutum</td>
<td>CCAP 1055/1</td>
<td>0.51, 1.3, &lt; LD</td>
</tr>
<tr>
<td>Skeletonema marinoi</td>
<td>CCMP 1332</td>
<td>&lt; LD, 1.1, &lt; LD</td>
</tr>
<tr>
<td>Skeletonema pseudocostatum</td>
<td>Bouin (France)</td>
<td>&lt; LD, 1.6, 0.28</td>
</tr>
<tr>
<td>Thalassiosira pseudonana</td>
<td>CCMP 1015</td>
<td>0.75, 18, &lt; LD</td>
</tr>
<tr>
<td>Thalassiosira weiss flogii</td>
<td>CCMP 1336</td>
<td>&lt; LD, 1.1, &lt; LD</td>
</tr>
<tr>
<td><strong>Dinoflagellates</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alexandrium catenella</td>
<td>Thau lagoon (C2-4)</td>
<td>&lt; LD, 1.3, 0.53</td>
</tr>
<tr>
<td>Alexandria sp. catenella</td>
<td>Thau lagoon (C11-4)</td>
<td>&lt; LD, 3.7, 0.61</td>
</tr>
<tr>
<td><strong>Cyanobacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synechococcus sp.</td>
<td>Thau lagoon (TES 206V)</td>
<td>&lt; LD, 1.3, &lt; LD</td>
</tr>
<tr>
<td>Synechococcus sp.</td>
<td>Thau lagoon (TES 206R)</td>
<td>&lt; LD, 2.0, &lt; LD</td>
</tr>
<tr>
<td>Synechococcus sp.</td>
<td>Thau lagoon (TES 206H6)</td>
<td>&lt; LD, 1.9, &lt; LD</td>
</tr>
<tr>
<td>Synechococcus sp.</td>
<td>Thau lagoon (TES 206D8)</td>
<td>&lt; LD, 4.7, &lt; LD</td>
</tr>
<tr>
<td><strong>Prasinophyceae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ostreococcus tauri</td>
<td>Thau lagoon (H95)</td>
<td>&lt; LD, 8.7, 4.7</td>
</tr>
</tbody>
</table>

< 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.

The concentrations of BMAA in the four diatom species ranged from 0.32 to 0.75 µg g\(^{-1}\). BMAA has recently been detected in all diatom strains analyzed by a Swedish group (Jiang et al., 2014a), including one Thalassiosira sp. (CCAP 1085/15) and two Skeletonema marinoi isolates. In contrast to our results, this group reported concentrations < 0.003 µg.g\(^{-1}\) DW, i.e. ca. 100-fold lower than concentrations in the present study. That group focused on the concentration of bound BMAA while we analyzed the total form. As sample preparation in case of the Swedish study included a protein precipitation step (not used in our study), the difference in results may derive from this difference. The Swedish study introduced this step due to column effects observed when injecting hydrolyzed crude extracts. We did not observe such effects in our method and did also not observe any interference for BMAA in diatom extracts (Figure 5). Ion ratios for the different transitions in diatom extracts were equivalent to those of standards for all samples in which BMAA was detected.
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 µg.g\(^{-1}\) DW, which is in the same order of magnitude (between 0.5 and 370 µg.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 (Figure 7). 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
species analyzed. BMAA concentrations in the remaining flesh of mussels never decreased to less than 2 µg.g\(^{-1}\) DW between July 2013 and October 2014. The long accumulation and retention of BMAA in mussels may be coherent with the strong association with (and potentially incorporation into) proteins. Furthermore, the increase of BMAA concentrations was relatively slow and lasted for a few months after the peak of diatom blooms, which suggests that additional uptake routes via zooplankton might play a significant role in the BMAA food chain of Thau lagoon. As suspension-feeding aquatic animals, bivalve mollusks have specialized in grazing on particles of a wide range of size, ranging from bacteria (< 2µm) to zooplankton (> 200µm) (Riisgard and Larsen, 2010). Thus, mussels may accumulate BMAA from diatoms as well as from zooplankton. This hypothesis is supported by the occurrence of BMAA in fractions of plankton dominated by either diatoms or zooplankton. BMAA has already been reported in zooplankton, in 6-fold higher concentrations than in cyanobacterial samples from the Baltic Sea, suggesting a bioaccumulation through feeding processes (Jonasson et al., 2010). This bioaccumulation may have also occurred in Thau lagoon as zooplankton is assumed to feed on microalgae and some dinoflagellate and diatom species present in this ecosystem are reported to contain BMAA. In view of our results confirming the presence of BMAA in cultured diatoms, periphyton and seston sampled in Thau lagoon, we propose a theoretical bioaccumulation pathway within Thau lagoon based on accumulation from these three compartments (figure 7). Hitherto, only one study focused on bioaccumulation and depuration of BMAA in four freshwater mussel species (Downing et al., 2014). This study used isotopically labeled BMAA, yet could not provide any evidence of either degradation of BMAA nor incorporation into or association of BMAA with proteins. Thus, further work is required to better understand accumulation and metabolism of BMAA in mussels and to confirm the validity of the hypothesized BMAA bioaccumulation pathway in Thau lagoon.
Figure 8: Hypothesized bioaccumulation pathway within the Thau lagoon ecosystem. Mean concentrations of total BMAA and DAB are expressed as µg.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.

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REFERENCES


Appendices

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

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

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

<table>
<thead>
<tr>
<th>Date</th>
<th>Plankton &gt; 20 µm and &lt; 125 µm</th>
<th>Abundance</th>
<th>Plankton &gt; 63 µm</th>
<th>Abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>08-Jul-14</td>
<td>- 80% diatoms (70% <em>Chaetoceros</em> sp., <em>Nitzschia</em> sp., <em>Navicula</em> sp.)&lt;br&gt;- 15% dinoflagellates (<em>Prorocentrum micans, Peridinium</em> sp.)&lt;br&gt;- 5% zooplankton</td>
<td>+++</td>
<td>- 80% zooplankton (copepods)&lt;br&gt;- 20% diatoms</td>
<td>++</td>
</tr>
<tr>
<td>18-Jul-14</td>
<td>- 90% zooplankton (tinninnids)&lt;br&gt;- 5% dinoflagellates (<em>Peridinium</em> sp.)&lt;br&gt;- 5% diatoms</td>
<td>++++</td>
<td>- 90% zooplankton (copepods)&lt;br&gt;- 10% diatoms</td>
<td>++++</td>
</tr>
<tr>
<td>20-Aug-14</td>
<td>- 90% diatoms (45% <em>Chaetoceros</em> sp.)&lt;br&gt;- 10% zooplankton (copepods)</td>
<td>+++</td>
<td>- 60% zooplankton (copepods)&lt;br&gt;- 40% diatoms (Chaetoceros sp., <em>Navicula, Nitzschia</em>)</td>
<td>++</td>
</tr>
<tr>
<td>03-Sep-14</td>
<td>- 60% dinoflagellates (<em>Peridinium</em> sp. and <em>Prorocentrum</em> sp.)&lt;br&gt;- 40% zooplankton (tinninnids)</td>
<td>++</td>
<td>- 99% zooplankton (copepods)&lt;br&gt;- 1% diatoms</td>
<td>++++</td>
</tr>
<tr>
<td>06-Oct-14</td>
<td>- 85% diatoms (<em>Nitzschia</em> sp. and <em>Pseudo-nitzschia</em> sp.)&lt;br&gt;- 10% zooplankton&lt;br&gt;- 5% dinoflagellates (<em>Prorocentrum</em> sp.)</td>
<td>+</td>
<td>- 60% zooplankton (copepods and tinninnids)&lt;br&gt;- 40% diatoms (<em>Nitzschia</em> sp. and <em>Pseudo-nitzschia</em> sp.)</td>
<td>++</td>
</tr>
</tbody>
</table>