ANSES Opinion
Request No 2016-SA-0012

The Director General

Maisons-Alfort, 19 May 2017

OPINION
of the French Agency for Food, Environmental and Occupational Health & Safety

on the acute and chronic toxicity of BMAA (beta-methylamino-L-alanine)

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It also contributes to the protection of the health and welfare of animals, the protection of plant health and the evaluation of the nutritional characteristics of food.

It provides the competent authorities with the necessary information concerning these risks as well as the requisite expertise and technical support for drafting legislative and statutory provisions and implementing risk management strategies (Article L.1313-1 of the French Public Health Code).

Its opinions are published on its website.

This opinion is a translation of the original French version. In the event of any discrepancy or ambiguity the French language text dated 19 May 2017 shall prevail.

On 11 January 2016, ANSES received a formal request from the Directorate General for Food (DGAL) and the Directorate General for Health (DGS) to undertake the following expert appraisal: Request for an opinion on the acute and chronic toxicity of BMAA (beta-methylamino-L-alanine).

1. BACKGROUND AND PURPOSE OF THE REQUEST

Beta-methylamino-L-alanine (BMAA) is a non-proteinogenic amino acid. It is a neurotoxin and is suspected of being associated with neurodegenerative diseases. Among the indigenous population of the island of Guam (in the Pacific Ocean), suspected cases of amyotrophic lateral sclerosis/Parkinsonism-dementia complex (ALS/PDC) syndrome observed in the 1950s could be attributable to a biomagnification of BMAA in the food chain from the seeds and fruits of a local tree (the cycad), contaminated with cyanobacteria from its root system. It has been suggested that the traditional consumption by this indigenous population of bats feeding on cycad seeds could be the main vector of exposure (Cox et al., 2002, 2003).

In France, recent work carried out by Ifremer (French Research Institute for Exploitation of the Sea) has highlighted the presence of BMAA in bivalve molluscs (mussels, oysters) collected in several shellfish-producing areas in 2013 (Réveillon, 2015). This study was complementary to a first study which only concerned shellfish from the Thau Lagoon in France (Réveillon et al., 2014).

In addition, areas of excessive incidence of amyotrophic lateral sclerosis (ALS) have been reported in some French regions, including one where shellfish are exploited and consumed (Masseret et al., 2013). This raises the question of the possible implication of shellfish contaminated by BMAA in the onset of neurodegenerative diseases.

Since then, a wide study funded by the French National Research Agency (ANR) was conducted between 2012 and 2016 in order to investigate a possible link in France between ALS and exposure to BMAA (ANR project entitled BMAALS). The findings of this study were presented in February 2016 at the closing meeting.

Against this background, on 11 January 2016, ANSES received a formal request from the DGAL and the DGS to answer the following questions:

1) How robust is the suggested epidemiological link between chronic exposure to BMAA and neurodegenerative diseases such as "ALS/Parkinson/dementia"? (To be conducted via a literature review, which could include among other elements the information concerning Guam.)
2) What toxicological data are available on BMAA?
3) In view of the toxicological data, and taking into account the levels observed by Ifremer in some shellfish, are there health concerns related to BMAA as regards the ingestion of aquatic products? (Preferably with realistic scenarios of shellfish consumption.)
4) In addition to the answers to the previous questions, can the Agency identify areas of research and studies to be pursued as a priority?

2. ORGANISATION OF THE EXPERT APPRAISAL

The expert appraisal was carried out in accordance with French Standard NF X 50-110 "Quality in Expert Appraisals – General Requirements of Competence for Expert Appraisals (May 2003)".

It falls within the sphere of competence of the Expert Committee (CES) on "Assessment of the physical and chemical risks in food" (ERCA). The Agency entrusted examination of this request to the Working Group on BMAA, set up by a decision taken on 2 June 2016 following a public call for candidates.

The methodological and scientific aspects of this WG's work were submitted to the CES ERCA during plenary meetings on 23 June and 15 December 2016, and 15 February and 12 April 2017. The completed work was adopted by the CES ERCA at its plenary meeting on 12 April 2017. The report produced by the Working Group takes account of the observations and additional information provided by the members of the CES ERCA, especially those of two experts appointed to review the document. This work was therefore conducted by a group of experts with complementary skills.

ANSES analyses the links of interest declared by the experts prior to their appointment and throughout the work, in order to avoid potential conflicts of interest with regard to the matters dealt with as part of the expert appraisal.
The experts' declarations of interests are made public via the ANSES website (www.anses.fr).

In order to perform the most exhaustive literature search possible concerning articles dealing with (i) analytical methods for detection and quantification and (ii) the occurrence (measurement of the concentration) of BMAA and its isomers in aquatic produce (marine or freshwater) that can be consumed by humans, the WG BMAA decided to undertake a systematic review. For toxicity data (human, animal and mechanistic), the WG BMAA conducted a thorough analysis of the literature.

3. ANALYSIS BY THE WG BMAA AND THE CES ERCA

The Expert Committee on "Assessment of the physical and chemical risks in food" (CES ERCA) adopted the report of the collective appraisal carried out by the Working Group on BMAA, a summary of which is presented below.

3.1. Physico-chemical analysis of BMAA and its isomers

BMAA is a non-proteinogenic amino acid. Three isomers have been described so far: 2,4-diaminobutyric acid (DAB), *N*-(2-aminoethyl)glycine (AEG) and β-amino-*N*-methylalanine (BAMA) (Figure 1).

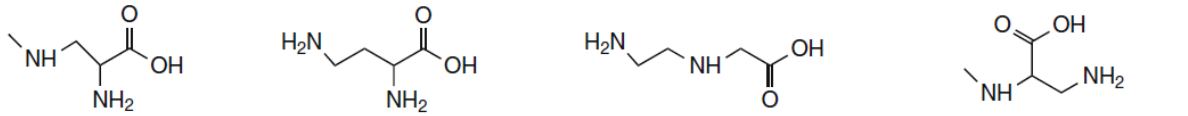

Figure 1: Structure of ß-*N***-methylaminoalanine (BMAA), 2,4-diaminobutyric acid (DAB), *N*-(2-aminoethyl)glycine (AEG) and ß-amino-*N*-methylalanine (BAMA) (Jiang et al., 2012)**

BMAA was observed for the first time in 1967 in cycad seeds (Vega and Bell, 1967). From 2003, Cox's team reported the widespread production of BMAA by cyanobacteria (Cox et al., 2003, 2005). Nevertheless, with the development of new analytical methods, more mixed results on the presence and concentrations of BMAA have been appearing and are the subject of considerable controversy in the scientific literature (Krüger et al., 2010; Banack et al., 2011; Faassen, 2014). In particular, recent work by Ifremer reports the absence of any BMAA production under experimental conditions by strains of cyanobacteria that had nonetheless been reported as producing it (Réveillon et al., 2015).

The WG BMAA observed that the question of the reliability of the analytical method is central for this issue. It was therefore decided to carry out an inventory of all the methods of analysis used in the occurrence and toxicity studies cited in the literature and then to classify these methods according to the degree of confidence that can be assigned to the results, from a qualitative (presence) and quantitative (concentration) point of view.

For the qualitative aspects, the following criteria were chosen:

- Whether a retention time is given, to attest the separation of BMAA and its isomers;
- Whether a limit of detection is mentioned;
- Whether specific transitions of BMAA and its isomers are used with LC-MS/MS, in order to differentiate them from each other. Ion ratios can also provide a way of confirming the identification of toxins.

For the quantitative aspects, the following criteria were chosen:

- In the first place, whether or not the qualitative criteria were correctly taken into consideration;
- Whether a limit of quantification in the matrix is included;
- The calculation of the recovery rate during extraction, with its corresponding standard deviation;
- Other optional quantitative criteria to increase confidence in the analytical approach, such as the use of an internal benchmark or the presence of information concerning linearity, reliability, etc.

On the basis of all these criteria, a code was assigned to each of the publications assessed:

Qualitative **and** quantitative criteria Not Satisfactory (NS)

Qualitative criteria satisfactory and quantitative criteria Moderately Satisfactory (MS)

Qualitative **and** quantitative criteria Totally Satisfactory (TS)

Using this classification, 48% of the publications were rated as NS, 27% as MS and 25% as TS.

It should be mentioned that BMAA may be present in matrices in a free form or a protein-bound form. Extraction protocols thus vary according to the analytical strategy chosen. Levels of total BMAA (free and protein-bound) can be determined by directly hydrolysing the matrix to be analysed. Figure 2 shows the chief stages in the analysis of BMAA and its isomers.

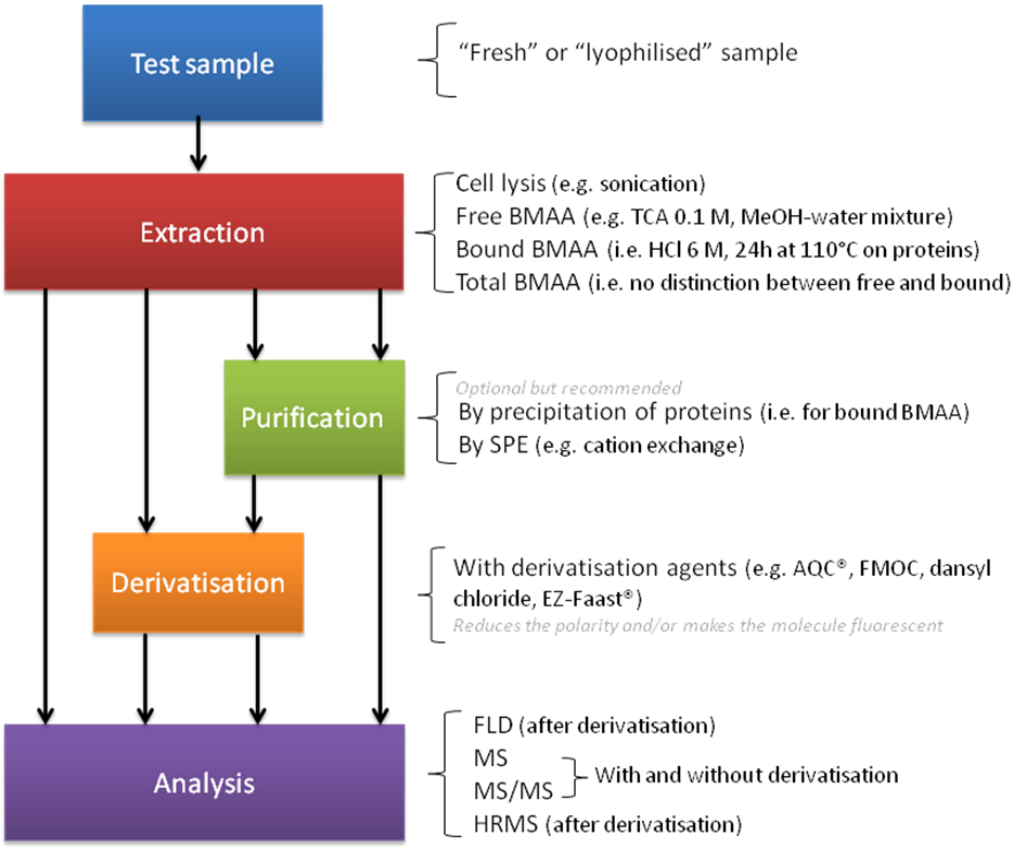

Figure 2: Schematic overall diagram of the analysis of BMAA and its isomers (from Réveillon, 2015, PhD dissertation). TCA: trichloroacetic acid; FMOC: 9-fluorenylmethyl chloroformate; SPE: solid-phase extraction; HRMS: high-resolution mass spectrometry.

3.2. Definition of Amyotrophic Lateral Sclerosis (ALS)

The WG BMAA chose to restrict its investigation to ALS alone, because ALS is the common denominator of all neurodegenerative diseases suspected of being associated with exposure to BMAA.

ALS is defined by its effect on both the upper motor neurons (cellular bodies located in the cortex) and the lower motor neurons (cellular bodies in the medulla or the anterior horn of the spinal cord). The progressive death of the motor neurons which innervate striated muscle tissue leads to the

inability of the peripheral muscles to contract. At its onset, the disease can take two distinct forms, (i) a bulbar form affecting the motor neurons of the brain stem, whose symptoms are difficulty in articulating or swallowing or (ii) a spinal form affecting the lower motor neurons of the spinal cord, characterised by difficulty in moving the limbs (INSERM, 2015). Patients retain all of their mental faculties, but lose the use of muscles under voluntary control, and the outcome is fatal after 3 to 5 years of evolution, because of the effect on muscles related to breathing or swallowing. ALS is not a notifiable disease. There is currently no specific biological marker for the disease. Diagnosis is based on a range of arguments and the elimination of differential diagnoses (inclusion body myositis, multifocal motor neuropathy with conduction blocks, cervical thoracic myelopathy, etc.) (www.has.fr.).

ALS is rare before the age of 40 and the risk increases exponentially with time after that age. Onset of sporadic ALS is reported most commonly between the ages of 58 and 63, and between 40 and 60 for familial ALS, with peak incidence between the ages of 60 and 69 for the sporadic forms; 20% of cases are in the bulbar form. Men have a higher risk of ALS than women, with a men/women ratio of 1.2-1.5. The progression of the disease is not linear but curvilinear, with more rapid deterioration in the early and late stages of the disease. Average survival is 3 years. From 5 to 10% of the forms are familial, 70% of which have a genetic explanation (35% of forms related to mutations in the C9 gene). The average standardised incidence of ALS for the entire world is 1.68 (1.50 to 1.85) per 100,000. Standardised incidences differ slightly between those for Northern Europe [1.89 (1.46 to 2.32) per 100,000] and Eastern Asia [0.83 (0.42 to 1.24) per 100,000] or Southern Asia [0.73 (0.58 to 0.89) per 100,000]. Homogeneous rates have been reported in the populations of Europe, North America and New Zealand [standardised incidence of 1.81 (1.66 to 1.97) per 100,000].

One of the working assumptions relating to the pathophysiology of ALS is an excessive release of glutamate in the glutamatergic networks, causing excitotoxicity. Glutamate is the principal excitatory neurotransmitter of the central nervous system, and plays a crucial role in cognitive functions, synaptic plasticity and neuronal growth. This over-stimulation of post-synaptic neurons classically leads to neuronal death.

The report by the WG BMAA gives more detail about the criteria of the disease, the anatomopathological aspects, the pathophysiological mechanisms, genetic factors, the incidence and prevalence of the disease, the associated risk factors, the Guam cohort, epidemiological studies exploring the link between ALS and BMAA, and the BMAALS study (funded by the ANR).

3.3. Toxicological data

3.3.1 Toxicokinetic and toxicodynamic data

The oral bioavailability of BMAA was studied in two animal models, the rat (Duncan et al., 1991[NS]) and the monkey (Duncan et al., 1992[NS]). Although the data were produced using analytical methods classified not satisfactory, they suggest significant and rapid absorption.

For the purpose of this expert appraisal, the data of a kinetics study conducted by the National Toxicology Program (NTP) were transmitted to ANSES in the form of a poster presented at a scientific congress. The study had not been published in a peer-reviewed journal. The results, based on the oral administration of radiolabelled BMAA (^{14}C-BMAA), confirm the strong oral bioavailability of BMAA in rats (Garner et al., 2013[TS]).

It was observed that the blood-brain barrier was crossed in newborn rats after subcutaneous administration of BMAA, and in newborn mice exposed via milk from mothers who had received BMAA intravenously (Andersson et al., 2013[MS]; Karlsson et al., 2015[MS]).

The presence of BMAA in tissues (including the brain) was studied in adult rats after oral, intravenous or subcutaneous administration, but according to analytical methods deemed

unreliable from a quantitative point of view (Duncan et al., 1991[NS]; Andersson et al., 2013[MS]; Karlsson et al., 2015[MS]). In the study by Garner et al. (2013[TS]) with a single administration of ^{14}C-BMAA by the oral route, radioactivity was observed in the liver, adipose tissue, muscles and skin. Less than 0.01% of the dose was found in the brain. After a single or repeated administration, it would seem that 50 to 60% of the radioactivity is present in the tissues in the protein-bound form.

Concerning the metabolism of BMAA, there are considerable gaps in the data, meaning that it was not possible to identify the degradation products or measure their proportions relative to the parent compound.

It has been suggested that urinary excretion of BMAA is low (Duncan et al., 1992[NS]; Andersson et al., 2013[MS]; Garner et al., 2013[TS]).

Significant excretion in the milk of lactating females is reported in mice with ^{14}C-BMAA (Andersson et al., 2013[MS]).

3.3.2 Toxicity data from laboratory animals

The WG BMAA identified and analysed five studies by the oral route, two in mice and three in monkeys.

- In adult CD-1 mice, the two studies showed no behavioural abnormality, nor any symptom characteristic of a neurodegenerative pathology similar to ALS. In one of the studies (Perry et al., 1989[NS]), female mice were exposed by gavage for 11 weeks to very high doses of BMAA (500 to 1000 mg/kg bw/d), while in the other (Cruz-Agado et al., 2006), male mice were exposed by food to a dose of BMAA of 28 mg/kg bw/d for one month.
- In adult male cynomolgus monkeys (macaques), different doses of BMAA (100 to 350 mg/kg bw/d) were administered in the framework of a complex protocol with exposure durations of up to 12 weeks but in a sequential manner. In certain cases, individual animals developed muscle weakness, Parkinson's-like malfunctions of the upper motor neurons and behavioural abnormalities with degenerative changes to the cerebral cortex and spinal cord. These motor malfunctions were mitigated by the administration of L-DOPA, a precursor of dopamine (Spencer, 1987; Spencer et al., 1987).
- In young vervet monkeys fed with BMAA for 140 days (210 or 21 mg/kg bw/d) administered in bananas, the development of a neurofibrillary disease with amyloid plaques and hyper-phosphorylated Tau proteins was observed in the brain. Simultaneous administration of serine (210 mg/kg/d) by the oral route significantly decreased the density of neurofibrillary tangles. Vervets are less likely than macaque monkeys to develop the clinical picture (Cox et al., 2016[MS]).

The WG BMAA identified and analysed three studies by the intraperitoneal route, one in mice and two in rats.

- In outbred adult NIH Swiss mice, 5 doses of BMAA were tested by single administration (30, 300, 1000, 2000, 3000 mg/kg bw, and a control group) to groups of males and females, with an observation period of 14 days. Following a qualitative appreciation (without statistical analysis of the whole dose-response relationship), the authors defined a LD_{50} of 3000 mg/kg bw on the basis of the following symptoms that led to the euthanasia of animals: myoclonus[1], convulsions, and uncontrolled micturition/defecation in the 20 minutes post-injection. The Lowest Observed Adverse Effect Level (acute LOAEL) was 2000 mg/kg bw (1 female moribund out of 6). A histological examination of six organs (brain, liver, lung, heart, spleen, kidney) from a single animal per group showed no lesions (Al Sammak et al., 2015[NS]). BMAA

[1] Myoclonus: involuntary rapid muscle contractions

was detected in the brain and liver of treated mice and not in those of the controls, but according to a method deemed non-reliable by the WG BMAA.

- In adult Wistar rats, signs of acute neurotoxicity (circular or swinging movements and, ultimately, ataxia) were observed a few hours after a single administration or repeated administration for up to 21 days of BMAA in the form of L-BMAA (at doses between 500 and 2000 mg/kg bw) or racemic mixture of D- and L-BMAA (up to 4000 mg/kg bw) to males and females. Histological changes were only reported in the cerebellum, the result of excitotoxic activity (Seawright et al., 1990).
- In another study, young male Wistar rats received a dose of 300 mg/kg bw of BMAA for 5 consecutive days. Behavioural tests were carried out every week for 14 months. Biological and histological examinations were also performed on the brain. A unilateral (left) loss of muscle volume of up to 17% was observed, becoming bilateral after 8 months. In the brain, the volume of the cerebral cortex also decreased bilaterally by 10 to 18%. An enlargement of the lateral ventricles was also observed. The neurochemical analyses showed increased levels of glutamate and taurine as well as a decline in γ-aminobutyric acid (GABA) in the motor cortex. The authors believe that the rat has been found to be a good model for reproducing the symptoms observed in ALS (de Munck et al., 2015; Munoz-Saez et al., 2015).

The WG BMAA identified and analysed three studies by the subcutaneous route, all in newborn or very young rats.

- Dawson et al. (1998) studied three groups of newborn male and female Sprague Dawley rats. Group A received a dose of 500 mg/kg bw on Day 5 post-natal, Group B a dose of 500 mg/kg bw on Day 2 and Day 5 post-natal, and Group C a dose of 100 mg/kg bw on Day 2 and Day 5 post-natal. Psychomotor tests were performed between Day 6 and Day 84 post-natal, and hormonal and neurochemical levels were measured. The usual behavioural tests revealed only slight variations, at the limit of significance. No disturbance of the motor functions was observed, with the exception of splayed hind limbs, and increased locomotor activity. Regarding organ weight, only the weight of the ovaries was greater (Groups A and B) and that of the cerebellum was lower (Groups A Male and B Female). Regarding hormone levels, TSH was higher (Group A Male), as well as IGF1 (Group B Male) and free T4 was lower (Group B Female). Concerning the neurochemical aspect, the only changes observed were in the spinal cord, with aspartic acid and glutamate being increased (Group C Female) in the cerebellum, aspartic acid being reduced (Group A Male) in the hippocampus, and serotonin increased (Groups A and B Female, Groups A, B, C Male) and reduced (Group C Female).
- Karlsson et al. (2009a[MS], b[MS]) administered BMAA at doses of 200 or 600 mg/kg bw/d to male Wistar rats at Days 9 and 10 post-natal. At the higher dose, the rats showed severe neurological motor disorders. After weaning, behavioural tests were performed between Day 13 and Day 27 post-natal. The results showed hyperactivity with locomotive difficulty and a loss of the righting reflex without great effect on muscle performance. The cognitive functions appeared to be impaired. In testing in Weeks 1 to 22 post-natal (in order to assess the long-term effects in adult animals), spatial learning but not long-term spatial memory tested 2 weeks after the acquisition phase were disrupted, but were unrelated to the alterations to motor skills.
- The same team (Karlsson et al., 2011[MS]) completed its study protocol with a behavioural component for animals exposed to lower doses of 50 and 200 mg/kg bw on Days 9 and 10 post-natal (as well as at the high dose of 600 mg/kg bw). Observations were made periodically when the animals were between the ages of 13 and 26 weeks. The two lowest doses tested resulted in an alteration of the spatial memory but no alteration in the other types of memory, and no histological anomaly, including in the hippocampus, was observed. At the highest dose, neuronal losses were observed in the cingulate cortex and the

hippocampus. The authors consider that these observed changes in the rat do not coincide with the *post-mortem* observations on tissues from patients who died of ALS.

In conclusion, the following points stand out concerning the toxicological effects:

- It is difficult to draw clear conclusions from the data in the studies available in the literature: the concentrations in BMAA tested are very high, many used a non-oral route of administration, the number of animals per group is low, and the period of administration (focused on the most sensitive period of brain development) does not reflect the reality of consumer exposure.
- The neurotoxicity of BMAA has been demonstrated in rats and monkeys but the toxicological data currently available do not allow the identification of a no-effect dose applicable to humans.
- There seems to be a considerable difference in sensitivity between species, the adult mouse appearing to be resistant to the neurotoxicity induced by BMAA, even at very high doses. It would be well worth investigating the origin of this specific feature.

3.3.3 Mechanistic data

Excitotoxicity and dependence on bicarbonate

The neurotoxicity of BMAA has also been observed *in vitro* on cells from rodents and leeches, and in human cell lines. Its excitotoxic character, that is to say the process of cell death occurring as a result of the activation of excitatory amino acids (glutamate), is the mechanism most often proposed (Chiu et al., 2013). The interaction of BMAA with bicarbonate is assumed to produce a molecule "resembling" glutamate, and playing the role of glutamate receptor agonist, in particular ionotropic receptors such as NMDA (N-methyl-D-aspartic acid) (Weiss et al., 1989). These studies demonstrated the central role of bicarbonate ions in the mechanism of action of BMAA.

Intracranial injections of BMAA in the striata of mice have shown that the toxin induces the death of hippocampal neurons *in situ*. These results have been confirmed *in vitro*, because BMAA exerts a dose-dependent cytotoxic effect (50 µM to 1 mM) on a spinal cell line close to motoneurons (Buenz & Howe, 2007). Other *in vitro* results have shown that, at lower concentrations (10 µM), BMAA potentiates neuronal death induced by other neurotoxic molecules (NMDA, kainate, amyloid-β, and MPP+). This observation suggests that BMAA is capable of acting at low concentrations as co-actor of a neurodegenerative phenomenon involving other molecules. This neurotoxicity involves both ionotropic (NMDA) and metabotropic (mGluR5) glutamate receptors, which suggests that BMAA is excitotoxic via different molecular targets (Lobner et al., 2007).

Hyperphosphorylation of Tau[2]

Various studies have shown that BMAA plays a role in the phosphorylation of the Tau protein, either by inhibiting the activity of its main phosphatase, PP2A, or by increasing the activity of the kinase GSK3β. BMAA thus acts as an agonist of certain metabotropic glutamate receptors such as mGluR5 (Liu et al., 2009; Arif et al., 2014).

Overexpression of TDP-43[3]

Several *in vitro* and *in vivo* studies suggest that BMAA causes an overexpression of TDP-43 as well as the formation of aggregates of this protein (de Munck et al., 2013; Munoz-Saez et al., 2013, 2015; Karlsson et al., 2015; Yin et al., 2014).

[2] Tau (Tubulin-Associated Unit) is a neuronal microtubule-associated protein that has been identified as the major component of the Paired Helical Filaments (PHF) that lead to neurofibrillary degeneration (NFD), assumed to be the cause of Alzheimer's disease. The Tau proteins of NFD are aggregated and abnormally phosphorylated.

[3] TDP-43 (TAR DNA-binding protein 43, i.e. with a transactivation response of 43 kDa) is a protein located in the cell nucleus in most tissues, which binds to DNA and participates in the regulation of transcription. This protein can also bind to RNA to ensure its stability. By cutting and rearranging mRNA by alternative splicing, TDP-43 controls the production of different versions of certain proteins. At least 60 mutations in the TARDBP gene have been found in patients with ALS.

Protein incorporation

One mechanism by which BMAA is thought to exercise its neurotoxic effects is its ability to be incorporated into protein during protein synthesis (Glover et al., 2014; Karlsson et al., 2014). It seems that this incorporation only occurs to take the place of alanine and serine residues (Glover et al., 2014; Dunlop et al., 2013).

This phenomenon of the protein incorporation of BMAA thus results in faults in the structure of these proteins (misfolding) and therefore an accumulation of protein in the lysosome (Dunlop et al., 2013). This anomalous protein synthesis associated with a massive input of Ca^{2+} also leads to stress phenomena in the endoplasmic reticulum, to deregulation of the reduction/oxidation ("redox") systems, and to an activation of certain pro-apoptotic caspases such as caspase-12 and therefore to cell death (Perry et al., 1989; Buenz & Howe, 2007; Lobner et al., 2007; Santucci et al., 2009; Liu et al., 2010; Karlsson et al., 2012; Chiu et al., 2013; de Munck et al., 2013; Karlsson et al., 2013; Okle et al., 2013).

It would be interesting to know the nature of the proteins in which BMAA may incorporate and to study the role of this misfolding in the pathophysiology of ALS.

Interaction with neuromelanin

Neuromelanin shares the same synthesis pathway as the catecholamines, which include dopamine, a neurotransmitter essential for the control of the striatum. An accumulation of neuromelanin modified by BMAA could disrupt the functioning of dopaminergic neurons (Krone et al., 2016). BMAA may induce an accumulation of catabolic intermediates of catecholamines involved in oxidation processes combining a mitochondrial dysfunction, an accumulation of free radicals, lipid peroxidation, protein aggregation and microglial activation, leading to early neural aging common to many neurodegenerative pathologies (McManus et al., 2011; Heitz et al., 2012; Lastres-Becker et al., 2012; Dariani et al., 2013; An et al., 2014; Eleuteri et al., 2015; Tan et al., 2015). Finally, by interacting with this pigment but without being incorporated therein, BMAA could be stored for many years with the possibility of leaching throughout the subject's lifetime, which could lead to chronic damage to the brain, including permanent inflammation and a reactive gliosis contributing to the development of neurodegenerative pathologies (Krone et al., 2016).

It has been shown that BMAA is capable of binding to melanin in various tissues, including the central nervous system, and accumulating in the cells rich in this pigment (Karlsson et al., 2009c). The accumulation of BMAA in specific brain structures could induce a targeted neurotoxicity, only in neurons rich in neuromelanin and nearby tissues during any leaching of BMAA. This could take place over many years. This hypothesis could explain (i) the difficulty of finding any correlation between plasma and brain levels of BMAA, as many studies have been carried out on brain areas largely devoid of neuromelanin, (ii) the unusual neurotoxicity of BMAA – as described in the syndrome found on Guam – combining a Parkinsonian syndrome, ALS, Alzheimer's disease and a rare pigmentary retinopathy, and (iii) the impact in the very long term of BMAA on exposed populations.

Under this hypothesis, it could therefore be interesting to (i) focus on the structures rich in melanin and neuromelanin when assaying for BMAA in the central nervous system including the *locus coeruleus*, the *substantia nigra* and the retinal tissue, (ii) preferentially use species rich in neuromelanin during animal experimentation (Marsden, 1961) and avoid the murine model, which is practically devoid of this substance in the brain (Barden & Levine, 1983). These important inter-species differences in their regional expression of neuromelanin could thereby provide the start of an explanation for the contradictory findings of studies concerning the effect of BMAA on the brain when they compare the mouse model (poor in neuromelanin) and the simian model (rich in neuromelanin), and (iii) monitor the retinal function of humans or animals exposed to BMAA.

3.3.4 Genotoxicity

An Ames test performed on 5 strains of *Salmonella* Typhimurium (TA97a, TA98, TA100, TA102 and TA1535) with 5 concentrations ranging between 11 and 900 µg of BMAA per plate, with and without metabolic activation, showed no significant rise in the number of revertants. Another test (the SOS/umuC assay) was carried out with 6 concentrations between 0.32 and 1000 µg of BMAA/ml on the strain of *Salmonella* Typhimurium TA1535/pSK1002, with and without metabolic activation, which also gave a negative result (Novak et al., 2016).

3.4. Assessment of the weight of evidence for a link between chronic exposure to BMAA and ALS

In its Opinion of 25 July 2016, based on the recommendations of the Working Group on the "Methodology of risk assessment" (WG MER), ANSES defined the concept of the weight of evidence as "a formalised summary of lines of evidence, which may be of heterogeneous quality, for the purpose of determining the level of plausibility of assumptions." A line of evidence is "a set of information of the same nature, integrated in order to assess an assumption". The integration of lines of evidence then makes it possible to express the weight of evidence. They can be integrated on a qualitative or a quantitative basis.

The WG BMAA decided to follow a qualitative method without quantitative scoring, taking care to give as much detail as possible about the criteria used. The criteria chosen for this analysis were those used by Hill (1965), as these are the most widely recognised and used in epidemiology. The nine criteria for providing evidence in favour of cause in the relationship studied are: temporality, strength of association, consistency (reproducibility) of the results, biological gradient (the dose-response relationship), experimental evidence of the type of parallelism between the cause and effect (including the effect of the removal of exposure), specificity, plausibility, biological coherence, and analogy.

Temporality: this criterion is difficult to judge because of the following:

- A long time can elapse between exposure to BMAA and the appearance of neurotoxic symptoms in humans (analogy with prion diseases or neurolathyrism);
- There is obviously no such thing as a "human model", and no case studies;
- The only data available in laboratory animals are derived from an acute or subacute model (administration over a short period of time).

The strength of the association between the environmental risk factor and the emergence of ALS: even in the historical example of Guam and its Chamorro people, where the incidence of ALS was 50 to 100 times higher than the average world level in the 1950s, the association with exposure to BMAA cannot be calculated because the analyses conducted on the cycad seeds, bats and human brains followed a methodology deemed not satisfactory by the WG, in terms of both detection (confirmation of presence) and quantification (concentration)[4].

Reproducibility of the results of the association: cases of ALS potentially associated with BMAA have been reported in several parts of the world (Guam, Japan, Indonesia), but the link with an exposure to BMAA could be due to confounding factors (such as genetic factors or other environmental factors).

Dose-effect relationship in humans: neither the epidemiological data from the historical cases on Guam nor the experimental data from laboratory animals are capable of supporting this criterion.

[4] It is regrettable that analyses have not been updated with the new methodologies selected by the WG. This is also true of the study by Pablo et al. (2009[NS]), which suggests that BMAA has been detected in the brains of U.S. patients (outside Guam) with ALS, with no correlation with the environment. More generally, there is a controversy on the presence of intracerebral bound BMAA (in both humans and animals).

The effect of the removal of exposure: the historical examples (Guam, Japan, Indonesia) show that a change in lifestyle (in particular the diet) decreased the incidence of ALS to a level comparable to the average level in the world for subsequent generations. However, it is not certain that this development is associated with the decrease or removal of exposure to BMAA.

Specificity of the effect: no specificity of effect, as BMAA is not the only potential factor for explaining the onset of ALS, which is a multifactorial disease.

Analogy: other toxins similar to BMAA (e.g. β-N-oxalylamino-L-alanine, designated by the 2 acronyms BOAA and β-ODAP) are known to be responsible for neurological disorders of the motor pathways, such as neurolathyrism.

Plausibility and **biological coherence**: from a mechanistic point of view, the *in vivo* and *in vitro* experimental data confirm the neurotoxicity of BMAA and suggest several potential, and possibly complementary, mechanisms of action (excitotoxicity, protein incorporation, interaction with neuromelanin) and the presence of markers of neurodegenerative diseases (hyperphosphorylation of Tau protein, aggregated form of TDP-43).

Besides the Hill criteria, the WG BMAA notes that, although ALS is multifactorial, it has a well-documented genetic component, which means that such cases can be excluded, thus enabling the identification of a link with environmental factors. The presence of clustered cases of ALS, including the presence of non-genetic familial cases, reinforces the hypothesis of exposure related to lifestyle (e.g. eating habits) and to the environment. However, to date it has not been possible to identify a causal link between the clustered cases and exposure to BMAA.

In the light of these criteria, the WG BMAA concludes that the causal link between exposure to BMAA and the occurrence of ALS is not proven, in the current state of knowledge.

Generally speaking, it is difficult to establish an epidemiological link according to the Hill criteria in the case of multifactorial neurodegenerative diseases.

However, the hypothesis that exposure to BMAA is a factor favouring neurotoxic phenomena is highly probable, primarily via its ability to activate mechanisms leading to neurodegeneration.

3.5. Sources and occurrence of BMAA

3.5.1 BMAA-producing organisms

In aquatic environments, it is currently considered that the presence of BMAA is the result of the proliferation of certain phytoplankton or phytobenthic species. Although cyanobacteria were initially considered as the main producers of BMAA in fresh water (Cox et al., 2005[NS]), a more recent study has shown that few species of cyanobacteria produce it in experimental conditions (Réveillon et al., 2015[TS]). However, various diatoms and dinoflagellates of marine origin or found in saline environments have recently been described as producers of BMAA and its isomers (Jiang et al., 2014[TS]; Lage et al., 2014[MS]; Réveillon et al., 2015[TS]).

3.5.2 Contamination of aquatic and other environments

The report by the WG BMAA presents detailed information on the contamination of water resources, of aquatic animals (freshwater and marine), plants and aerosols by BMAA and its isomers, DAB, AEG and BAMA.

The information relating to aquatic animals (freshwater and marine) likely to be consumed by humans was entered in a database, including the results of the analysis of each sample, taking into account only those obtained by analytical methods considered satisfactory for detection and quantification.

Table 1 gives the number of results collected by category of food that enabled the calculation of consumer exposure to total BMAA according to various scenarios.

Table 1: List of the species of marine or freshwater produce and the categories of food to which they correspond

Produce	Number of results	Number of results below the limit of detection*	Category of food
Bream	2	2 [a]	Freshwater produce
Coregonus	2	2 [a]	Freshwater produce
Crab	2	1 [a]	Crustaceans, molluscs and shellfish
Shrimp	9	3 [a]	Crustaceans, molluscs and shellfish
Crayfish	7	7 [a, b]	Freshwater produce
Herring	3	2 [a]	Sea fish
Oyster	35	0	Crustaceans, molluscs and shellfish
European bass	2	2 [a]	Sea fish
Cod	4	4 [a]	Sea fish
Mussel	138	0	Crustaceans, molluscs and shellfish
Arctic char	4	3 [a]	Freshwater produce
Perch	4	4 [a]	Freshwater produce
Scallop	3	0	Crustaceans, molluscs and shellfish
Plaice	3	0	Sea fish
Pike-perch	2	2 [a]	Freshwater produce
Salmon	6	6 [a, b]	Sea fish
TOTAL	226		

* The value of the limit of detection varies in different studies
[a] Limit of detection = 0.10 mg/kg fresh weight; [b] Limit of detection = 0.01 mg/kg fresh weight

3.6. Estimating dietary exposure in humans

On the basis of data concerning individual consumption for the French general population (INCA2, AFSSA, 2009) and for French high consumers of seafood products (CALIPSO, AFSSA, 2006), as well as contamination data, exposure was calculated according to the following equation:

$$E_i = \sum_{k=1}^{n} \frac{C_{i,k} \times L_k}{PC_i}$$

where:

- E_i is the total daily exposure of the individual i (µg/kg of body weight per day),
- $C_{i,k}$ is the daily average consumption of the food k by the individual i (g/d),
- L_k is the estimated level of the studied contaminant in the food k (mg/kg fresh weight),
- BWi is the body weight of the individual i (kg),
- and n is the total number of foods consumed b $_{BWi}$ individual i.

Furthermore, two types of exposure were calculated:

- First, **chronic exposure**: taking average concentration as a parameter of contamination. Chronic exposure means the quantity of the substance that individuals can ingest on a daily basis over a long period of their lives, in relation with a late-onset toxicological effect.
- Secondly, **acute exposure**: taking the 95th percentile as a parameter of contamination when the category contains 30 or more data items; or the maximum concentration when the category contains fewer than 30 data items. Acute exposure corresponds to a toxicological effect manifested rapidly, at the very first ingestion.

These two types of exposure were each calculated on the one hand, with all of the available data and on the other hand, with the French data only, from a total of 138 data items. It should be noted that the French data relate only to mussels and oysters.

The detailed results are given in the report by the WG BMAA and are not repeated here. The estimates of dietary exposure (all products combined: molluscs, crustaceans, fish) lie in the following ranges:

For acute exposure to total BMAA in adults:

- on average from 0.295 µg/kg bw/d for the general population to 2.149 µg/kg bw/d for high consumers;
- at the 95th percentile from 1.352 µg/kg bw/d for the general population to 5.124 µg/kg bw/d for high consumers.

For chronic exposure to total BMAA in adults:

- on average from 0.069 µg/kg bw/d for the general population to 0.52 µg/kg bw/d for high consumers;
- at the 95th percentile, from 0.319 µg/kg bw/d for the general population to 1.224 µg/kg bw/d for high consumers.

4. CES ERCA'S CONCLUSIONS AND RECOMMENDATIONS

The toxicological data (both *in vivo* and *in vitro*) demonstrate the neurotoxicity of BMAA, thus making it a danger to humans.

However, these data are insufficient for establishing either a dose-effect relationship or a health-based guidance value (acute or chronic).

It is therefore not possible to characterise the hazard represented by BMAA.

Following their analysis, the WG BMAA and the CES ERCA conclude that a causal link between exposure to BMAA and the occurrence of ALS is not proven, in the current state of knowledge. Like other neurodegenerative diseases, this pathology is multifactorial and develops slowly.

The Ifremer data showed that BMAA was quantified in all of the samples of oysters and mussels from the French coast (n = 138). From these data and other data available in the literature it was possible to calculate the exposure of French consumers according to several scenarios, but it was not possible to compare this with the exposure of consumers in other countries, as the WG BMAA found no other published information.

In the light of these elements, the CES ERCA is unable to issue an opinion on the level of health concern with respect to BMAA in marine and freshwater produce.

<u>Areas of research to be pursued as a priority</u>

- Analyse BMAA in both its free and bound forms in samples of the brains of patients with ALS and control samples, using a reliable analytical method.

- Acquire metabolic and toxicological data to characterise the hazard and define a health-based guidance value (for BMAA and for its isomers, AEG, DAB and BAMA).

- Obtain data concerning the contamination of seafood and freshwater produce (shellfish, fish, crustaceans) using a reliable analytical method capable of taking temporal and spatial variability into account. BMAA could be included in the list of toxins monitored for the emergence of marine biotoxins in shellfish, overseen by the DGAL.

- Undertake an epidemiological case-control study to investigate the possibility of a causal link between exposure to BMAA and the occurrence of ALS.

To be more specific, it is important to:

- Produce reference material to enable the scientific community to characterise the analytical methods implemented and compare the results, without any prior judgement concerning the methods (i.e. derivatisation versus no derivatisation).
- Clarify the production of BMAA by cyanobacteria (which strains produce it, whether constitutively or by induction, at what concentrations).
- Reassess the bioaccumulation pathways of BMAA in marine and freshwater produce. This is because recent work has shown that some species of diatoms (such as *Phaeodactylum tricornutum*, *Chaetoceros* sp, and *Thalassiosira pseudonana*) produce BMAA.
- Ensure that epidemiological studies that aim to establish a link between exposure to BMAA and the occurrence of ALS take better account of information relating to eating habits.
- Define a reliable marker of exposure (BMAA, metabolite or other biomarker).
- Investigate the origin of the low susceptibility of mice to the neurotoxicity of BMAA.
- Study the mechanisms of binding, incorporation or interaction of BMAA and its isomers to proteins in the biological matrices.

5. AGENCY CONCLUSIONS AND RECOMMENDATIONS

The French Agency for Food, Environmental and Occupational Health & Safety endorses the conclusions of the Expert Committee on "Assessment of the physical and chemical risks in foods" (CES ERCA).

The available epidemiological data are insufficient to assess the weight of evidence of a causal link between exposure to BMAA and the occurrence of ALS. According to the classification used by the IARC (International Agency for Research on Cancer), this would belong in Group 3 ("the agent is not classifiable") due to a lack of data confirming exposure to BMAA in populations where excess incidences of ALS have been observed. More specifically, studies on Guam have shown an excessive incidence of ALS cases in the past that is no longer found today, without being able to determine whether this situation is related to dietary exposure to BMAA. As no case-control study has explored this relationship, the hypothesis remains open with no way of providing an answer.

ANSES therefore stresses the importance of undertaking research work to supplement knowledge on BMAA, in particular case-control epidemiological studies to investigate lifestyle and dietary exposure to BMAA.

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KEY WORDS

Beta-methylamino-L-alanine; BMAA; ALS; amyotrophic lateral sclerosis; neurodegenerative diseases

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