Quantification of neurotoxin BMAA (β-N-methylamino-L-alanine) in seafood from Swedish markets

Liying Jiang1, Nadezda Kiselova1, Johan Rosén2 & Leopold L. Ilag1

1Department of Analytical Chemistry, Stockholm University, SE-10691 Stockholm, Sweden, 2National Food Administration, SE-751 26 Uppsala, Sweden.

The neurotoxin β-N-methylamino-L-alanine (BMAA) produced naturally by cyanobacteria, diatoms and dinoflagellates can be transferred and accumulated up the food chain, and may be a risk factor for neurodegenerative diseases. This study provides the first systematic screening of BMAA exposure of a large population through the consumption of seafood sold in metropolitan markets. BMAA was distinguished from known isomers by liquid chromatography tandem mass spectrometry after acidic hydrolysis and derivatization. Using deuterium-labeled internal standard, BMAA was quantified as 0.01–0.90 mg/g wet weight of tissues in blue mussel, oyster, shrimp, plaice, char and herring, but was undetectable (<0.01 mg/g) in other samples (salmon, cod, perch and crayfish). Provided that the content of BMAA detected is relevant for intake calculations, the data presented may be used for a first estimation of BMAA exposure through seafood from Swedish markets, and to refine the design of future toxicological experiments and assessments.

The non-protein amino acid β-N-methylamino-L-alanine (BMAA) is naturally produced in the environment and putatively associated with neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS), Parkinson’s disease (PD) and Alzheimer’s disease (AD). BMAA can be produced in nature by cyanobacteria, diatoms and dinoflagellates. These planktonic organisms are ubiquitous in both aquatic and terrestrial environments and serve as primary producers in the food web. BMAA is not expected to be bio-accumulated in fatty tissues through the food chain, a common bio-accumulation mechanism, because of its water-soluble and non-lipophilic properties. However, it has been reported that in biological samples, BMAA is present not only as free form but also in a protein-bound fraction that can function as an endogenous reservoir, being transferred and accumulated between trophic levels. An earlier study proposed a transfer and bio-magnification pathway for BMAA through the food chain in a terrestrial ecosystem in Guam, where BMAA was first discovered. This pathway has been supported by three later studies on aquatic ecosystems in: Florida Bay and Biscayne Bay in South Florida, which are eutrophic habitats with blooms of cyanobacteria caused by human activities; the Baltic Sea, which is a temperate brackish ecosystem with dramatically increased eutrophication over the past few decades primarily caused by human land-based activities; and Gonghu Bay in Lake Taihu, which is another highly eutrophic freshwater ecosystem, mainly caused by the excessive growth of Microcystis. Although BMAA was detected in many samples from these three ecosystems, the high eutrophication that occurs in all of them makes these sites less economically important as seafood sources.

The health risk of BMAA is not just a matter of inherent toxicity, but also the degree of exposure. Commercial seafood in markets is regularly consumed by the public and thus directly relevant to the BMAA exposure of humans. There have been several studies dealing with BMAA contaminated seafood that are used for human consumption, such as blue crabs from Chesapeake Bay in Annapolis, MD, USA; mussels (Mytilus galloprovincialis) and oysters (Crassostrea gigas) from Thau lagoon in France; cockles (Gerastoderma edule) from two Portuguese water bodies; and commercial shark cartilage supplements in USA. However, these studies only investigated one or two organisms, mainly shellfish, and the population exposed to those seafood in some studies was either small or not specified. To our best knowledge, no study has systematically investigated commercial seafood that are sold in metropolitan markets and consumed regularly by a large population. This study aims to fill this gap and provide the first such survey by profiling BMAA in 10 popular commercial seafood (fishes, shellfishes and crustaceans) sold in mainstream markets, the end of the seafood supply chain, in the Swedish capital Stockholm with an estimated population of 897,000 in 2014.
Results
This study was designed to estimate the degree of exposure to BMAA of the Swedish public through the consumption of seafood sold in markets. Therefore, five of the most consumed fish species (salmon, cod, char, perch and herring), together with molluscs (blue mussel and oyster) and crustaceans (shrimp and crayfish), which are popular in Stockholm, were purchased from five mainstream supermarkets (referred to as S1–5) and two popular local open markets (labeled S6–7) in the centre of Stockholm, sampled in different seasons (winter, summer and autumn in 2013 and 2014), as shown in Table 1. Detailed sample information, such as species name, capture site, production mode (e.g., farmed or naturally caught), and process/preservation method, was obtained from supermarkets or their suppliers. Raw and fresh seafood (mussel, oyster and shrimp with shell, and fish with skin) was chosen to reduce the chance of spoilage and to avoid uncertain contamination during commercial handling. However, all shrimps and crayfishes included in this study were cooked since this is the prevalent form sold in markets in Stockholm. The samples were transported on ice to the laboratory, cut and prepared immediately on the same day. Only the common edible parts, such as muscles from fish, shrimp and crayfish, soft body from blue mussel and oyster, were analyzed by ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) to estimate the BMAA exposure of humans via oral intake.

Surprisingly, BMAA was found to be present in about half of the food we analyzed, including blue mussel, oyster, shrimp, plaice, herring and char as shown in Fig. 1 b–g. The contents of BMAA in these species were determined to be 0.01–0.90 μg/g wet weight of the sample tissues. To our best knowledge, plaice and char were for the first time reported in this study to contain BMAA. No BMAA was detected in the other types of samples, as listed in Table 1.

The calibration curve for quantification exhibited good linearity over the examined range (Fig. 2). Results for the quality control (QC) samples demonstrated that the method used for BMAA quantification had good accuracy and precision (Table 2). The limit of detection had good accuracy and precision (Table 2).

Table 1 | BMAA contents in the seafood purchased in eight supermarkets in Stockholm, Sweden during 2013 and 2014

| Name (Species/ *Infraorder) | Supermarket | Purchase Date | Capture Site | Production Mode | Process and Preservation Method | BMAA/Tissue (μg/g) |
|----------------------------|-------------|---------------|--------------|----------------|---------------------------------|-------------------|
| Blue mussel (Mytilus edulis) | S1          | Feb. 14, 2013 | Sweden (west coast) | farmed | raw | 0.90 |
|                            | S2-L1       | Sep. 03, 2013 | Sweden (west coast) | raw | raw | 0.34 |
|                            | S1          | Sep. 04, 2013 | Sweden (west coast) | farmed | raw | 0.42 |
|                            | S3          | Oct. 08, 2013 | Sweden (west coast) | farmed | raw | 0.25 |
|                            | S6          | Oct. 09, 2013 | Sweden (west coast) | farmed | raw | 0.19 |
|                            | S7          | Oct. 09, 2013 | Sweden (west coast) | farmed | raw | 0.08 |
| Oyster (Ostrea edulis, **Crassostrea gigas) | S1 | Sep. 04, 2013 | Greece | farmed | raw | 0.32 |
|                            | S3          | Oct. 08, 2013 | France** | farmed | raw | 0.66 |
|                            | S6          | Oct. 09, 2013 | Sweden (west coast) | farmed | raw | 0.10 |
|                            | S7          | Oct. 09, 2013 | Sweden (west coast) | farmed | raw | 0.28 |
| Shrimp (*Caridea) | S1          | Sep. 04, 2013 | Sweden | caught | cooked | 0.20 |
|                            | S2-L2       | Oct. 08, 2013 | Northern Atlantic | caught | smoked | 0.11 |
|                            | S3          | Oct. 08, 2013 | Northern Atlantic | caught | cooked | 0.20 |
|                            | S4          | Oct. 08, 2013 | Sweden | caught | cooked/frozen | 0.17 |
|                            | S6          | Oct. 09, 2013 | Sweden | caught | cooked/frozen | 0.46 |
|                            | S7          | Oct. 09, 2013 | Sweden | caught | cooked | 0.13 |
| Plaice (Pleuronectes platessa) | S1          | Sep. 04, 2013 | Northeast Atlantic | caught | raw | 0.01 |
|                            | S1          | Feb. 14, 2013 | Northeast Atlantic | caught | raw | 0.01 |
|                            | S5          | Oct. 09, 2013 | Baltic Sea | caught | raw | 0.02 |
| Baltic Herring (Clupea harengus) | S1          | Feb. 14, 2013 | Baltic Sea | caught | raw | ND |
|                            | S1          | Sep. 04, 2013 | Baltic Sea | caught | raw | 0.02 |
|                            | S5          | Oct. 09, 2013 | Baltic Sea | caught | raw | ND |
| Salmon (Salmo salar) | S2-L1       | Sep. 03, 2013 | Norway | farmed | raw | ND |
|                            | S1          | Sep. 04, 2013 | Norway | farmed | raw | ND |
|                            | S2-L1       | Aug. 07, 2014 | Norway | farmed | raw | ND |
|                            | S1          | Aug. 07, 2014 | Norway | farmed | raw | ND |
| Char (Salvelinus alpinus) | S1          | Feb. 14, 2013 | Sweden | farmed | raw | ND |
|                            | S2-L1       | Sep. 03, 2013 | Sweden | farmed | raw | 0.01 |
|                            | S1          | Sep. 04, 2013 | Sweden | farmed | raw | ND |
| Cod (Gadus morhua) | S2-L1       | Sep. 03, 2013 | Norway | caught | raw | ND |
|                            | S1          | Sep. 04, 2013 | Norway | caught | raw | ND |
|                            | S2-L1       | Aug. 07, 2014 | Norway | caught | raw | ND |
|                            | S1          | Aug. 07, 2014 | Norway | caught | raw | ND |
| Perch (Perca fluviatilis) | S1          | Feb. 14, 2013 | Northeast Atlantic | caught | raw | ND |
|                            | S1          | Sep. 04, 2013 | Northeast Atlantic | caught | raw | ND |
|                            | S2-L1       | Aug. 07, 2014 | Sweden (lake) | caught | raw | ND |
|                            | S2-L1       | Aug. 07, 2014 | Sweden (lake) | caught | raw | ND |
| Crayfish (Astacus leptodactylus) | S2-L1      | Sep. 03, 2013 | Turkey | unknown | cooked/frozen | ND |
|                            | S1          | Sep. 04, 2013 | Sweden (lake) | caught | raw | ND |
|                            | S2-L1       | Aug. 07, 2014 | Turkey | caught | cooked | ND |
|                            | S1          | Aug. 07, 2014 | Turkey | caught | cooked/frozen | ND |
|                            | S2-L1       | Aug. 07, 2014 | Sweden (lake) | caught | raw | ND |
|                            | S7          | Aug. 07, 2014 | Sweden | caught | cooked | ND |

注：ND: not detectable (i.e., below the method LOD at 0.01 μg BMAA/g wet weight of sample tissue); S1–7 refers to the seven supermarkets where the samples were purchased; L1–2 refers to locations 1 and 2. The part analyzed was the whole soft body for blue mussel and oyster, and muscle for others.
Figure 1 | LC-MS/MS chromatograms of (a) a standard solution of BMAA (β-N-methylamino-L-alanine) and its isomers, BAMA (β-Amino-N-methyl-alanine), AEG (N-(2-aminoethyl) glycine) and DAB (2,4-diaminobutyric acid), (b) blue mussel (Mytilus edulis), (c) oyster (Crassostrea gigas), (d) shrimp (Caridea), (e) plaice (Pleuronectes platessa), (f) herring (Clupea harengus) and (g) char (Salvelinus alpinus).
Discussion

As the old adage says: "What you can’t measure, you can’t manage.", the quality of measurements using analytical chemistry techniques has important implications for food safety and environmental monitoring. To date, only five reports have quantified BMAA in environmental samples on a relatively large scale. For comparison, the methodology and quantification results from them and the present study are listed in Table 3. The quantitative data reported by the previous studies showed large variation of up to a couple of orders in magnitude. Considering the difference between dry and wet weight, which can affect the final value (the dry/wet weight ratio is about 0.25 for fish and shrimp muscle, and 0.20 for blue mussel and oyster bodies15), our values are in good agreement with those about 0.25 for fish and shrimp muscle, and 0.20 for blue mussel and oysters as reported previously10,11,15,16,20. A blue mussel (Mytilus edulis) or oyster (Ostrea edulis or Crassostrea gigas) with typical shell length of 5 cm can filter water containing algae at a rate of around 3–6 liters per hour11. Their filter-feeding life style means they may have a large intake of algae, and thus are capable of accumulating toxic substances produced by algae, probably including BMAA. The caridean shrimps in our study, mainly collected from the Northern Atlantic Ocean, were all found to contain BMAA. Another shrimp (Peneaus duorarum), one species of penaeid shrimps that are commercially important, was previously reported to contain BMAA28. However, those penaeid shrimps were caught from a highly eutrophied unnatural ecosystem and it was not mentioned if shrimps caught from this area are constituents of regular diets of inhabitants. Given that shrimps are one of the top five consumable seafood in Sweden and eutrophication is usually to a lesser degree in Northern Atlantic Ocean, our unexpected finding, i.e. 100% of coverage of BMAA in caridean shrimp raised some concern. It should be mentioned that caridean shrimps feed mainly on algae and zooplankton during their larval and mysis stages, and live primarily on the sea floor as adults. These features of their lifestyle make them highly likely to be exposed to the BMAA producers: cyanobacteria, diatoms and dinoflagellates. Plaice (Pleuronectes platessa, also called the European Plaice) was also found to contain BMAA. It is a commercially important flatfish and one of the most commonly eaten fishes. One noteworthy feature of this organism is that it lives on the sandy bottom of the sea22 and has a life-span of up to 50 years23, although it currently has an apparently diminishing longevity due to over-fishing. Nevertheless, it is possible for this bottom-living organism to be exposed to BMAA producers such as diatoms, eukaryotic organisms enclosed within a silica cell wall which makes them sink down easily to the seafloor21 and bioaccumulate this toxin during their long life span. Char (Salvelinus alpinus) was also found to be positive for BMAA although these fish were farmed, making exposure to BMAA not so obvious. However, farmed char is commonly fed with fish meal and fish oil that are made up of about 20 different species of fishes caught from nature. This provides a potential route for char to be exposed to BMAA from natural sources. Interestingly, there is a tendency towards including more grain as source for fish feed which can attenuate this apparent exposure24. The herring samples (Clupea harengus, also called Baltic herring) analyzed in this study were all fished from the Baltic Sea, a eutrophic water body that has regular annual algal blooms, and BMAA was detected in one out of three samples. This species of herring from this water body was also earlier reported to contain BMAA in muscle from one out of three replicates but with about ten times lower BMAA concentration28. The crayfish

RSD: relative standard deviation.

Table 2 | The accuracy and precision of the quantification method for BMAA demonstrated by QC samples (n = 3)

| BMAA content (µg/g) | Accuracy (%) | Precision (RSD%) |
|---------------------|--------------|------------------|
| 0.05                | 108          | 14               |
| 0.5                 | 115          | 11               |
| 0.8                 | 119          | 10               |

Figure 2 | Calibration curve (0.1–10 ng BMAA, n = 3) for quantification of BMAA in seafood samples.
and all other fishes contained no detectable BMAA using the method employed here.

It is noteworthy that studies that only investigate the ecosystems near/in Sweden cannot comprehensively reflect the real situation of BMAA exposure of Swedes via seafood consumption. The influence of imported seafood has to be considered as well because of current globalization. Indeed, around half of the seafood we purchased in Stockholm were found to be imported. Moreover, one should care for the long-term effects of BMAA in the Baltic Sea.

Whereas there have been many reports analyzing environmental and commercial samples for BMAA, toxicological assessments, especially via an oral route that is relevant to human exposure, have so far been limited. Various studies have suggested that BMAA is bioavailable to humans in seafood via oral exposure. In two recent studies, no neurochemical changes or behavioral anomalies were observed in mice after oral administration of BMAA for 11 weeks or 30 days, respectively. No studies have examined the long-term effects of BMAA in animals.

Most degenerative diseases have very long periods of latency that can last decades and late-onset, usually after 65 years old in humans. The significant presence of BMAA in seafood revealed in the present study provides a regular route for BMAA exposure directly affecting the public. Although the concentration of BMAA detected in seafood was not very high, between 0.01–0.90 µg/g wet weight of tissues, others in µg BMAA/g dry weight of tissues.

In addition, two recent studies indicated that BMAA can replace serine and alanine in human proteins during protein synthesis.

There have been three animal studies investigating the toxic potential of BMAA using oral administration, which is a route relevant to human exposure. Chronic behavioral anomalies and degenerative changes of motor neurons in the cerebral cortex and spinal cord were observed in Macaque monkeys (Macaca fascicularis) after administration of a relatively high dose of BMAA for up to 12 weeks via gavage. The symptoms were attenuated 30 min after treatment with an anti-Parkinson's drug known to be an N-methyl-D-aspartate receptor antagonist. In two other studies, no neurochemical changes or behavioral anomalies were observed in mice after oral administration of BMAA for 11 weeks or 30 days, respectively. No studies have examined the long-term effects of BMAA in animals.

The average life expectancy of the Swedish population is above 80, which is higher than that in most other countries. The prevalence of ALS and other dementia-related diseases in 2010 was estimated to be 7.2% of people aged above 60 among western Europeans including Sweden, which is the highest in the world. The incidence of ALS disease in Sweden increased by 2% annually during 1991–2005. Food-derived neurotoxins might be one of several risk factors for neurodegenerative diseases, acting

### Table 3: Comparison of the methodologies and BMAA quantitative results of environmental and food samples used in this study and previous publications

| Analytical method | Isomers included | Quantification method | Instrument LOD/LOQ | Method LOD/LOQ | Reference |
|-------------------|------------------|----------------------|--------------------|---------------|-----------|
| LC-FD             | DAB              | BMAA                 | NR                 | 2.0 ng/8.8 ng | Brand 2010 |
| LC-MS/MS          | DAB              | BMAA                 | 70 fmol, i.e., 8.3 pg (S/N = 9.2)/NR | NR           | Jonasson 2010 |
| LC-FD and LC-MS/MS| DAB and AEG      | BMAA in matrix       | NR                 | 2.7 ng/7.0 ng [LC-FD method] | Mondo 2012 |
| LC-MS/MS          | DAB and AEG      | BMAA in matrix       | 5 pg (S/N = 3)/17 pg (S/N = 10) | 0.5 µg/g (S/N = 3)/1.7 µg/g (S/N = 10) | Christensen 2012 |
| LC-MS/MS          | No               | BMAA                 | NR                 | <0.01 µg/g (S/N = 14)/<0.01 µg/g (S/N = 44) | this study |
| LC-MS/MS          | BAMA, AEG and DAB| d3-BMAA              | NR                 |              |           |

**Sample Origin**

| Sample Origin | BMAA contents in organisms | Reference |
|---------------|----------------------------|-----------|
| Florida       | ND-305                     | Brand 2010 |
| Baltic Sea    | 0.006–0.201**              | Jonasson 2010 |
| South Florida | NA                         | Mondo 2012 |
| Mississippi   | 4.7–46.9                   | Christensen 2012 |
| Tai Lake      | 0.63–6.72                  | Jiao 2013  |
| Stockholm     | 0.08–0.90*                 | this study |

*Molluscs*  

| Florida       | ND-305                     | 20–2559   | Brand 2010 |
| Baltic Sea    | 0.006–0.201**              | 0.0019–1.29 | Jonasson 2010 |
| South Florida | NA                         | 144–1836* | Mondo 2012 |
| Mississippi   | NA                         | NA        | Christensen 2012 |
| Tai Lake      | 0.63–6.72                  | 0.07–35.91| Jiao 2013  |
| Stockholm     | 0.08–0.90*                 | ND–0.02*  | this study |

**Crustaceans**

| Florida       | ND-305                     | 20–2559   | Brand 2010 |
| Baltic Sea    | 0.006–0.201**              | 0.0019–1.29 | Jonasson 2010 |
| South Florida | NA                         | 144–1836* | Mondo 2012 |
| Mississippi   | NA                         | NA        | Christensen 2012 |
| Tai Lake      | 0.63–6.72                  | 0.07–35.91| Jiao 2013  |
| Stockholm     | 0.08–0.90*                 | ND–0.02*  | this study |

**Fishes**

| Florida       | ND-305                     | 20–2559   | Brand 2010 |
| Baltic Sea    | 0.006–0.201**              | 0.0019–1.29 | Jonasson 2010 |
| South Florida | NA                         | 144–1836* | Mondo 2012 |
| Mississippi   | NA                         | NA        | Christensen 2012 |
| Tai Lake      | 0.63–6.72                  | 0.07–35.91| Jiao 2013  |
| Stockholm     | 0.08–0.90*                 | ND–0.02*  | this study |

*Values in µg BMAA/g wet weight of tissues, others in µg BMAA/g dry weight of tissues.
**Samples from Swedish west coast.
***For comparison with other studies, the method LOD/LOQ in µg/g used in these two studies can be converted into BMAA amount by multiplying by the tissue weight, about 10 mg.
ND: not detectable; NA: not analyzed; NR: not reported; FD: fluorescent detector.
synergistically with aging. Swedes usually regard seafood as a healthy alternative to meat, thus there is an increasing trend of seafood consumption. The average value of per capita consumption (2005–2007) of seafood products in Sweden was estimated to be 28.7 kg/year (cf. 16.9 kg/year for the world average), i.e., 0.6 kg/week (according to the Food and Agriculture Organization of the United Nations). This situation, together with the findings of the present study, strengthens the need for a risk assessment of this neurotoxin. Unfortunately, due to lack of available neurotoxicological data on BMAA, it is currently not possible to estimate a dose-response relationship from which a Tolerable Daily Intake for humans can be derived. Moreover, the proposed link between BMAA and neurodegenerative diseases has not unequivocally been verified. Three studies have reported the presence of BMAA in brain tissues of patients who died of neurodegenerative diseases from Canada6,34, Guam34 and North America35, while there were also several studies that failed to detect BMAA in any brain tissues of patients with neurodegenerative diseases36–38. Therefore, the link between BMAA and neurotoxicity needs to be further elucidated to understand the actual mechanism of BMAA neurotoxicity. Nevertheless, caution and vigilance must be exercised without causing alarm.

In conclusion, given the potential risk that wide variations in the published quantitative data on BMAA in food samples and other matrices are due to erroneous analyses, it would be valuable when comparing different studies to consider, e.g., ring or proficiency tests, and the establishment of reference materials, etc. The data in the present study provides an important initial insight into possible BMAA exposure from commercial seafood in Stockholm. Confirmatory studies should preferably be broader regarding species, number of samples, places of sampling etc. Also other food types need to be tested in order to determine other sources before BMAA exposure can be more accurately estimated for the population. Given the ubiquity of BMAA producers, i.e., cyanobacteria, diatoms and dinoflagellates, in the environment, these observations are probably not unique in Sweden and BMAA may also be present in seafood available in other countries. Provided that the present study has covered the most significant sources of exposure and that the resulting data is relevant for intake calculations, the findings can be used as a basis for refining the design of toxicological experiments.

UHPLC-MS/MS Analysis. The method for UHPLC-MS/MS analysis was the same as described previously27. Briefly, a liquid chromatographic system consisting of an Acclaim column, a degasser and an Accela auto-sampler was coupled with a TSQ Quantum triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, USA) for LC-MS/MS analysis. A Rheos 6000 pump (Flux instruments) was used to deliver an additional post-column flow at 600 μl/min before the LC flow entered the MS ion source to improve the electrospray ionization efficiency. BMAA and its isomers, BAMA, AEG and DAB were separated on an ACQ-QTAG Ultra C18 column (100 x 2.1 mm, 1.7 μm particle size, Waters, Ireland) with a binary mobile phase (solvent A: 5% acetonitrile in water with 0.3% acetic acid; solvent B: acetonitrile with 0.3% acetic acid) delivered at a flow rate of 200 μl/min for 10 min and then 400 μl/min for the remaining 6 min. The linear gradient elution program used was as follows: 0 min, 0% B; 10 min, 10% B; 11 min, 80% B; 12.1 min, 0% B; and 16.0 min, 0% B. The LC flow was directed to a waste container during the first one minute to eliminate the non-volatile borate that was introduced in the samples during AQC derivatization. The MS/MS analysis was performed in positive-ion detection mode using multiple reaction monitoring (MRM). Diagnostic transitions in total for BMAA identification and quantification simultaneously, as shown in Fig. 1a. For identification of BMAA and its three isomers, one general transition (459.19 > 119.08) and three diagnostic transitions (459.18 > 258.09 for BMAA and BAMA, 459.18 > 188.08 for DAB, and 459.18 > 214.10 for AEG) were monitored. The chromatographic retention time, diagnostic transition and peak area ratio between the general and diagnostic transitions for each analyte were used for unambiguous identification of BMAA and its isomers. For quantification of BMAA, two transitions (459.18 > 119.08 for BMAA and 462.20 > 122.10 for d1-BMAA internal standard) were monitored. The peak area ratios of these two transitions determined from a dilution series of BMAA (0.1, 0.2, 1.0, 2.5, 5.0, 7.5, 10 ng) each containing a fixed amount of d1-BMAA (1.0 ng), were plotted as a function of BMAA amount in the standard solutions to construct a seven-point calibration curve, from which the BMAA amount in unknown samples could be calculated (Fig. 2). All other instrument parameters were the same as reported previously27.

Analytical Method Validation. The method of crayfish, which was analyzed and confirmed to contain no BMAA in this study was used as a sample matrix for method validation. The LOD and LOQ of BMAA for the method were measured experimentally by spiking 10 mg wet weight of crayfish muscle matrix with a certain amount of BMAA standard and processing it in the same way as the unknown samples for detection of BMAA. The LOD and LOQ were calculated according to the signal-to-noise ratio (S/N = 3) of the analytical signal. The accuracy and precision of the quantification method were evaluated by using triplicate QC samples containing low, medium and high levels of BMAA (0.05, 0.5 and 0.8 μg/g, respectively) in the quantification range and a fixed amount of d1-BMAA (1.0 ng) in 10 mg wet weight of crayfish muscle matrix.

Methods

Chemicals. β-N-methylamino-L-alanine (L-BMAA) hydrochloride (B107, Germany) and L-2,4-diaminobutyric acid (L-DAB) dihydrochloride (B387, Switzerland) were purchased from Sigma Aldrich. N2 (aminoethyl) glycine (AEG) was purchased from TCI (A1153, TCI, Japan). An AccQ Tag kit was purchased from Waters (WAT052880, Milford, MA, USA). β-Amino-N-methyl-alanine (BAMA) and deuterium-labeled BMAA (d1-BMAA) were synthesized as recently reported10.16. Sample Preparation. The sample preparation procedure was similar to the method used previously27. Briefly, the samples were rinsed with water, cut into thin strips, frozen in liquid nitrogen, ground into small pieces using a mortar and pestle, and homogenized using a homogenizer (Janke & Kunkel KG, IKA-WERK RW 18). A portion (about 50–100 mg wet weight) of the obtained homogenate was placed into a plastic tube, mixed with 600 μl water and lysed in an ice-water bath by ultrasonication (VibraCellTM, Sonics & Materials Inc. Danbury CT, USA) for 5 min at 70% power with one second on/off pause. Afterwards, an aliquot corresponding to 10 mg of wet weight tissue was transferred into a glass tube, mixed with water and concentrated hydrochloric acid to a final concentration of 6 M HCl, and hydrolyzed at 110 °C in an oven for 20 h. For the samples used for quantification, 10 μl of deuterated-BMAA internal standard solution (100 μg/ml) was added to the sample solution before acid hydrolysis. The hydrolysate was filtered, dried and subsequently cleaned up by liquid-liquid extraction using water and chloroform and solid-phase extraction using an Isolute HCX-3 column (905-0010-a, Biotage Sweden AB), using the same protocol as described previously27. The samples were reconstituted in 20 μl of 20 mM HCl, buffered with 60 μl of borate, then derivatized with 60 μl of 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) reagent, and then again derivated and reconstituted in 30 μl of 5% acetonitrile in water. Finally, a 10 μl aliquot of this sample solution was injected into a liquid chromatographic instrument for UHPLC-MS/MS analysis.

1. Spencer, P. S. et al. Guan amyotrophic lateral sclerosis-parkinsonism-dementia linked to a plant excitant neurotoxin. Science 237, 517–522 (1987).
2. Cox, P. A. et al. Diverse taxa of cyanobacteria produce the neurotoxin β-N-methylamino-L-alanine, a neurotoxic amino acid. Proc. Natl. Acad. Sci. USA 102, 5074–5078 (2005).
3. Jiang, L. et al. Diatoms: A Novel Source for the Neurotoxin BMAA in Aquatic Environments. PLoS One 9, e45784 (2014).
4. Jiang, L. & Ilag, L. Detection of endogenous BMAA in dinoflagellates (Heterocapsa triquetra) hints at evolutionary conservation and environmental concern. Pupubl Science 2, 1–8 (2014).
5. Lage, S. et al. BMAA in shellfish from two Portuguese transitional water bodies suggests the marine dinoflagellate Gymnodinium catenatum as a potential BMAA source. Aquatic Toxicology 152, 131–138 (2014).
6. Murch, S. J., Cox, P. A. & Banack, S. A. A mechanism for slow release of biomagnified cyanobacterial neurotoxins and neurodegenerative disease in Guam. Proc. Natl. Acad. Sci. USA 101, 12228–12231 (2004).
7. Cox, P., Banack, S. & Murch, S. Biomagnification of cyanobacterial neurotoxins and neurodegenerative disease among the Chamorro people of Guam. Proc. Natl. Acad. Sci. USA 100, 13380–13383 (2003).
8. Vega, A. & Bell, E. A. alpha -Amino-beta -methylpropionnic acid, a new component from seeds of Cycas circinalis. Phytochemistry 6, 759–762 (1967).
9. Brand, L. E., Pablo, J., Compton, A., Hammerschlag, N. & Mash, D. C. Cyanobacterial blooms and the occurrence of the neurotoxin, beta-N-methylamino-L-alanine, in aquatic ecosystems. Harmful Algae 9, 620–635 (2010).
10. Jonasson, S. et al. Transfer of a cyanobacterial neurotoxin within a temperate aquatic ecosystem suggests pathways for human exposure. Proc. Natl. Acad. Sci. USA 107, 9252–9257 (2010).
11. Jiao, Y. et al. Occurrence and transfer of a cyanobacterial neurotoxin β-methylamino-L-alanine within the aquatic food webs of Gonghua Bay (Lake Taihu, China) to evaluate the potential human health risk. Sci. Total Environ. 468–469, 457–463 (2014).
12. Field, N. C. et al. Linking β-methylamino-L-alanine exposure to sporadic amyotrophic lateral sclerosis in Annanpol, MD. Toxicol 70, 179–183 (2013).
13. Masseret, E. et al. Dietary BMAA Exposure in an Amyotrophic Lateral Sclerosis Cluster from Southern France. *PloS One* **8**, e83406 (2013).

14. Mondo, K. et al. Environmental neurotoxins β-N-methylamino-l-alanine (BMAA) and mercury in shark cartilage dietary supplements. *Food and Chemical Toxicology* **70**, 26–32 (2014).

15. Salomonsson, M. L., Hansson, A. & Bondesson, U. Development and in-house validation of a method for quantification of BMAA in mussels using dansyl chloride derivatization and ultra performance liquid chromatography tandem mass spectrometry. *Analytical Methods* **5**, 4865–4874 (2013).

16. Christensen, S. J., Hemscheidt, T. K., Trapido-Rosenthal, H., Laws, E. A. & Bidigare, R. R. Detection and quantification of β-methylamino-L-alanine in aquatic invertebrates. *Limnol. Oceanogr.: Methods* **10**, 891–898 (2012).

17. Mondo, K. et al. Cyanobacterial neurotoxin β-N-methylamino-alanine (BMAA) in shark fins. *Mar. Drugs* **10**, 509–520 (2012).

18. Rosén, J. & Hellénäs, K.-E. Determination of the neurotoxin BMAA (β-N-methylamino-l-alanine) in cydad seed and cyanobacteria by LC-MS/MS (liquid chromatography tandem mass spectrometry). *Analyst (Cambridge, U. K.)* **133**, 1785–1789 (2008).

19. Banack, S. A. et al. Cyanobacteria produce N-(2-aminoethyl) glycine, a backbone for Peptide nucleic acids which may have been the first genetic molecules for life on Earth. *PloS One* **7**, e94093 (2012).

20. Jiang, L., Aigret, B., De Borggraeve, W. M., Spacil, Z. & Ilag, L. L. Selective LC-MS/MS method for the identification of BMAA from its isomers in biological samples. *Anal. Bioanal. Chem.* **403**, 1719–1730 (2012).

21. Walne, P. The influence of current speed, body size and water temperature on the filtration rate of five species of bivalves. *J. Mar. Biol. Assoc. U. K.* **52**, 345–374 (1972).

22. Riede, K. in Global register of migratory species - from global to regional scales (p. 329). (Federal Agency for Nature Conservation, Bonn, Germany, 2004).

23. Muus, B. J. & Dahlström, P. in Collins guide to the sea fishes of Britain and North-Western Europe (p. 244). (Collins, London, UK, 1974).

24. Webster, C. D. & Lim, C. in Nutrient requirements and feeding of finfish for aquaculture (p. 212) (London UK, 2002).

25. Duncan, M. W. Role of the cycad neurotoxin BMAA in the amyotrophic lateral sclerosis-parkinsonism dementia complex of the western Pacific. *Adv Neurol* **56**, 301–310 (1991).

26. Xie, X., Basile, M. & Mash, D. C. Cerebral uptake and protein incorporation of cyanobacterial toxin β-N-methylamino-L-alanine. *NeuroReport* **24**, 779–784 (2013).

27. Karlsson, O., Jiang, L., Ilag, L. L., Andersson, M. & Brittebo, E. B. Protein association of the neurotoxin and non-protein amino acid BMAA (β-N-methylamino-l-alanine) in the liver and brain following neonatal administration in rats. *Toxicol Lett* **226**, 1–5 (2014).

28. Dunlop, R. A., Cox, P. A., Banack, S. A. & Rodgers, K. J. The Non-Protein Amino Acid BMAA Is Misincorporated into Human Proteins in Place of l-Serine Causing Protein Misfolding and Aggregation. *PloS One* **8**, e75376 (2013).

29. Glover, W. B., Mash, D. C. & Murch, S. J. The natural non-protein amino acid N-β-methylamino-L-alanine (BMAA) is incorporated into protein during synthesis. *Amino Acids*, 1–7 (2014).

30. Perry, T. L., Bergeron, C., Biro, A. J. & Hansen, S. β-N-Methylamino-L-alanine: chronic oral administration is not neurotoxic to mice. *J. Neurol. Sci.* **94**, 173–180 (1989).

31. Cruz-Aguado, R., Winkler, D. & Shaw, C. A. Lack of behavioral and neuropathological effects of dietary β-methylamino-L-alanine (BMAA) in mice. *Pharmacol., Biochem. Behav.* **84**, 294–299 (2006).

32. Kaplan, W. & Laing, R. in Priority Medicines for Europe and the World: A Public Health Approach to Innovation (World Health Organization, Drugs, E. & Policy, M, Geneva, Switzerland, 2004).

33. Fang, F. et al. Amyotrophic lateral sclerosis in Sweden, 1991–2005. *Arch Neurol* **66**, 515–519 (2009).

34. Murch, S. J., Cox, P. A., Banack, S. A., Steele, J. C. & Sacks, O. W. Occurrence of β-methylamino-L-alanine (BMAA) in ALS/PDC patients from Guam. *Acta Neurol. Scand.* **110**, 267–269 (2004).

35. Pablo, J. et al. Cyanobacterial neurotoxin BMAA in ALS and Alzheimer’s disease. *Acta Neurol. Scand.* **120**, 216–225 (2009).

36. Montine, T. J., Li, K., Perl, D. P. & Galasko, D. Lack of beta-methylamino-L-alanine in brain from controls, AD, or Chamorros with PDC. *Neurology* **65**, 768–769 (2005).

37. Snyder, L. R. et al. Lack of cerebral bmaa in human cerebral cortex. *Neurology* **72**, 1360–1361 (2009).

38. Snyder, L. R. et al. Parkinson-dementia complex and development of a new stable isotope dilution assay for BMAA detection in tissue. *Toxicol. Appl. Pharmacol.* **240**, 180–188 (2009).

39. Jiang, L., Johnston, E., Aaberg, K. M., Nilsson, U. & Ilag, L. L. Strategy for quantifying trace levels of BMAA in cyanobacteria by LC/MS/MS. *Anal. Bioanal. Chem.* **405**, 1283–1292 (2013).

Acknowledgments
The authors gratefully acknowledge the Henning and Johan Throne-Holst Foundations for their financial support of this study.

Author contributions
L.J., J.R. and L.L.I. designed the project. L.J. and N.K. performed the experiments and analyzed the data. L.J. wrote the manuscript. J.R. and L.L.I revised the manuscript.

Additional information
Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Jiang, L., Kiselova, N., Rosén, J. & Ilag, L.L. Quantification of neurotoxin BMAA (β-N-methylamino-L-alanine) in seafood from Swedish markets. *Sci. Rep.* **4**, 6931; DOI:10.1038/srep06931 (2014).

This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivs 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder in order to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-nd/4.0/