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A cluster of amyotrophic lateral sclerosis in New Hampshire: A possible role for toxic cyanobacteria blooms

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Abstract

Cyanobacteria produce many neurotoxins including β-methylamino-L-alanine (BMAA) that has been liked to amyotrophic lateral sclerosis (ALS) and neurodegenerative disease. A number of ALS cases have been diagnosed among residents of Enfield, NH, a town encompassing a lake with a history of cyanobacteria algal blooms. To investigate an association between toxic cyanobacterial blooms in New Hampshire and development of ALS, we reviewed records from our institution and other community databases to obtain demographic information on patients diagnosed with ALS within New England. We identified nine ALS patients who lived near Lake Mascoma in Enfield, NH, an incidence of sporadic ALS that is 10 to 25 times the expected incidence of 2/100,000/year. We suggest that the high incidence of ALS in this potential cluster could be directly related to chronic exposure to cyanobacterial neurotoxins such as BMAA. Possible routes of toxin exposure include inhalation of aerosolized toxins, consuming fish, or ingestion of lake water. Further investigation, including analysis of brain tissue for cyanobacterial toxins, will be helpful to test for an association between BMAA and ALS.

Key words: *Amyotrophic lateral sclerosis/epidemiology/etiology, β-methylamino L-alanine, amino acids, diamino/analysis/metabolism/toxicity, cyanobacteria*

Introduction

Cyanobacteria are common in lake water, oceans and even deserts, occurring in high concentrations or 'blooms' under optimal environmental and nutrient-rich conditions (1–6). Cyanobacteria produce many neurotoxins and cytotoxins (1,7) including β-methylamino-L-alanine (BMAA) (8–10), anatoxin-a (AXT-a), AXT-a(s), saxitoxin (STX), microcystin (MC), cylindrospermopsin (CYL), and curacin. The natural role of these toxins is unknown. Many of these toxins have well established links to human and animal illness (2,5,11–19), often in the setting of acute exposure during blooms. For this reason, cyanobacterial blooms are a public health concern and many states have methods in place for monitoring and managing algal blooms (2,20). MC, an established hepatotoxin (11,12,21,22), is often implicated in acute illness and is currently the only cyanotoxin monitored by public health agencies in the United States (20).

BMAA, a non-protein amino acid, elicits neurotoxicity through several mechanisms: by acting as an agonist for glutamate receptor subtypes NMDA and AMPA, by inducing oxidative stress, and potentially through misincorporation into proteins causing protein misfolding (23–26). Motor neurons are particularly susceptible to the effect of toxins due to their great length and high metabolic demand. Intraneuronal dysregulation by impairment of axonal transport can affect the distant neuromuscular

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junction and vice versa. Both motor neurons and their supporting astrocytes appear to be particularly vulnerable to BMAA in vitro at concentrations as low as 10 μM. BMAA also appears to increase the potency of other neurotoxins (23). BMAA has been linked to the amyotrophic lateral sclerosis/Parkinsonism-dementia complex (ALS/PDC) that has been remarkably prevalent among the Chamorro people of Guam (27–32), where intake of BMAA occurs through multiple dietary inputs. Initial epidemiological studies on Guam found that the Chamorro diet was the only statistical correlate to the high incidence of neurodegenerative disease (33). BMAA is concentrated in the seeds of the cycad plant Cycas micronesica, which contains cyanobacteria living as symbionts within its roots (28). It was thought that consumption of flour made from cycad seeds might be the environmental trigger for ALS/PDC. The relationship between BMAA and neurological disease was initially rejected because the concentration of BMAA present in indigenous foods such as cycad flour was thought to be too low to cause disease in humans, but the discovery of the protein-bound form of BMAA in washed cycad flour along with evidence suggesting that the toxin could be biomagnified through the food chain supports a potential role for BMAA in ALS/PDC (10,34).

Although the link between ALS/PDC and the presence of BMAA in Chamorro diets is striking, it was not initially clear how the connection between BMAA and neurological disease could apply to other regions, since the use of cycads and cycad products for food and medicine in other regions such as North America and Europe is rare. Given that the background annual incidence rate of ALS, approximately 2–3 per 100,000, is relatively constant worldwide, any putative environmental factor that triggered the disease would have to be global. An environmental factor that triggers sporadic ALS is supported by geographic clusters of ALS and also by the development of the disease in conjugal couples who are genetically unrelated (35–38). Cyanobacteria and their toxins are ubiquitous inhabitants of aquatic and terrestrial environments and therefore could provide a common mechanism for chronic exposure to toxins that cause neurodegeneration. Analysis of brain tissues from deceased Chamorro ALS patients (28), along with Canadian AD patients as a comparison, identified BMAA in both of these study groups but not in tissues from control patients who died of causes unrelated to neurodegenerative illness. These results have recently been replicated independently by researchers at the Department of Neurology, the Miller School of Medicine at the University of Miami, who showed that a high concentration of BMAA was present in the brains of North American ALS and AD patients, while brain tissue from control patients free of neurodegenerative disease and from a group of patients with Huntington's disease generally had no detectable BMAA (39).

A number of cases of ALS were diagnosed among residents of Enfield, NH over the past several years. Many of the cases reside on Lake Mascoma, a large water body with documented blooms of cyanobacteria previously reported as part of a state monitoring program (20). Given the association of BMAA with ALS in other parts of the world (28,29,40,41), we investigated a possible correlation between cyanobacterial blooms in Lake Mascoma and adjoining Crystal Lake and development of ALS.

Methods

Case mapping

We reviewed electronic patient records at Dartmouth-Hitchcock Medical Center in Lebanon, New Hampshire to identify patients diagnosed with sporadic ALS between 1990 and 2007. We obtained the exact dwelling address, family history and relevant medical and social histories. Data on additional ALS patients within New England were obtained from other community databases (1999–2007). Google Earth 5.0 software was used to find and verify the spatial coordinates of each patient's home address, which was then used to create de-identified patient location points. Spatial data for current and past cyanobacterial blooms were obtained from the New Hampshire Department of Environmental Services (20). ArcGIS 9.3 software was used for spatial analysis. The underlying population density was adjusted for by using 2000 Census data from the US Census Bureau (42). The ArcToolbox 'spatial join' function was implemented to assign each patient location point to the Census Block Group in which it was located, and the density of ALS patients was calculated for each Census Block Group. The case density was represented in maps, along with the locations of water bodies larger than 0.5 km^2 for visual comparison of areas with high ALS patient proportions to the spatial locations of water bodies. To determine if there was a spatial association between cyanobacterial blooms and ALS, ArcGIS was used to calculate the incidence of ALS within a 0.5 mile (805 meter) buffer zone around lakes with documented blooms, and was compared to the incidence of ALS for persons living further than 0.5 miles from lakes with cyanobacterial blooms.

Cyanobacteria collection and toxin analysis

Lake Mascoma was surveyed both vertically and horizontally in July 2008 using a multi-parameter probe (YSI Incorporated, Yellow Springs, Ohio) with sensors including chlorophyll (estimates algal biomass) and phycocyanin (estimates cyanobacterial biomass) fluorescence. Phycocyanin concentrations were measured along horizontal transects at a depth of 0.5 m. Data were interpolated through kriging (2D) spatial analysis and mapped using ArcGIS software to demonstrate the density of cyanobacteria across the

lake surface (Figure 3A). Water samples were obtained from Lake Mascoma, initially in November 2007, then again in July–September 2008 and June 2009. Samples were also collected from regional lakes with past and/or current documented algal blooms. Bloom samples were collected from the water surface with an 80-μm phytoplankton net. Core integrated whole water samples were also obtained at a depth of 3–6 m and filtered through glass fiber filters (Whatman GAC-F) and cellulose filters (Millipore 0.45 μm, 0.22 μm). Lake water volumes of 1500 ml, 500 ml, and 250 ml were passed through each filter, respectively, using a vacuum. Cellulose filters were stored with desiccant, and all filters were stored in a dark container at −80°C. Presence of cyanobacteria (primarily Microcystis and Anabaena species) was confirmed with microscopy, using unfiltered phytoplankton samples preserved with Lugol's solution.

Samples were initially analyzed using liquid chromatography/mass spectrometry for the presence of microcystins, cylindrospermopsin and anatoxin-a (LC/MS; Gregory Boyer, University of New York – Syracuse) (1). Samples were also sent for BMAA analysis using liquid chromatography mass spectrometry/mass spectrometry (LC/MS/MS, Institute for Ethnomedicine, Jackson WY). Microcystins were also measured using an enzyme-linked immunosorbent assay (ELISA, Envirologix, Portland, Maine) following the manufacturer's instructions after a 10-fold concentration of water samples by lyophilization. The environmental bloom samples were freeze-dried, weighed, and hydrolyzed with 6.0 *M* HCl (0.169 mg sample/μl HCl) at 110°C for 16 h. After hydrolysis the samples were filtered through a centrifuge filter (Ultrafree-MC 0.22 μm) at 12,000 rpm for 3 min (Spectrafuge 16M, Labnet) and dried in a speed vacuum (Thermo-Savant SC250DDDA Speed Vac Plus with a Savant refrigerator trap RVT 4104) on medium heat. All samples tested were reconstituted in 20 mM HCl (2.5× dilution) before derivatization, and the one sample of a surface bloom collected from Locke Lake was derivatized in a large batch (10 μl reconstituted sample + 190 μl of 20 mM HCl + 600 μl borate buffer + 200 μl 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, AQC), vortexed, and set at room temperature for 1 min before heating for 10 min at 55°C. This Locke Lake solution was then concentrated by drying in a speed vacuum under medium heat for 3 h and then resuspended in 100 μl of borate buffer (10× concentration), vortexing, and LC/MS/MS analysis. All samples were run alongside a derivatized BMAA standard (10 ng BMAA/ml, Peter Nunn, Univ. Portsmouth, UK, triple crystallized BMAA standard) and an AQC derivatized negative blank, and a derivatized L-2, 4-diaminobutyric acid (DAB) standard (10 ng DAB/ml, Sigma D8376).

The samples and standards were then injected into a Thermo-Finnigan Ultra AM triple quadrupole mass spectrometer using a Waters Acquity UPLC and a Waters AccQTag Ultra column (no. 186003837, 2.1×100 m) at 55°C with a flow rate of 0.65 ml/min. Separation was achieved using a gradient elution with Eluent A 0.1% formic acid (Thermo Scientific Formic Acid 99+%, 46.02 g/mole, no. 28905) in Fisher Optima LC/MS water (W6-4) and Eluent B 0.1% formic acid in acetonitrile (Honeywell, Burdick & Jackson LC/MS grade, LC441-2.5): 0.0 min=99.1% A; 0.5 min=99.1% A; 2.0 min=95% A; 3.0 min=95% A; 5.5 min=90% A; 6.0 min=15% A; 6.5 min=15% A; 6.6 min=99.1% A; 8.0 min=99.1% A all with a curve 6. Nitrogen gas (NitroFlow laboratory, Parker-Balston) was supplied to the H-ESI probe (heated electrospray ionization) and the instrument run with the following instrument settings: vaporizer temperature of 400°C, capillary temperature of 270°C, sheath gas at 40, aux gas at 35, spray voltage of 3500 V, capillary offset of 35, tube lens offset of 110, source collision energy of 5, and a multiplier voltage of −1789. The second quadrupole was pressurized to 0.5 mTorr with 100% argon.

The derivatized BMAA molecule after ionization (MW 459) via HESI was selected as the precursor ion for analysis. Collision induced dissociation (CID) was achieved in the second quadrupole using the following parameters: 459–119 transition, CE=21; 459–171 transition, CE=38; 459–289 transition, CE=17 following optimization for these dominant product ions. The resultant three product ions of the derivatized BMAA (MW 119, 289, 171) were scanned by the third quadrupole, subsequently detected, and their relative abundances quantified. The ratios of these three product ions were compared to the ratios of the product ions created by injections of AQC derivatized standards under the same conditions.

Results

Epidemiology

Epidemiological evaluation of the geographical area of Lake Mascoma and a small adjoining Crystal Lake in Enfield, NH (population 4854 in 2006; 120.5 persons per square mile of land area), together encompassing an area of approximately 7.75 square miles (2.75 square miles of inland water excluded) shows an incidence of sporadic ALS that is approximately 10–25 times the expected incidence of 2/100,000/year. We identified nine ALS patients who lived on or near the lakes (on average less than 0.15 miles from the shore) for a minimum of nine years, and were diagnosed between the years 1990 and 2007. One patient was diagnosed in 1990; all other patients were diagnosed between the years 2000–2006.

A total of 278 cases of ALS were identified in NH between 1990–2007, the majority of which were diagnosed between 1998 and 2007 (Figure 1). The calculated rate of ALS per 100,000 was elevated in the area encompassing Lake Mascoma (roughly

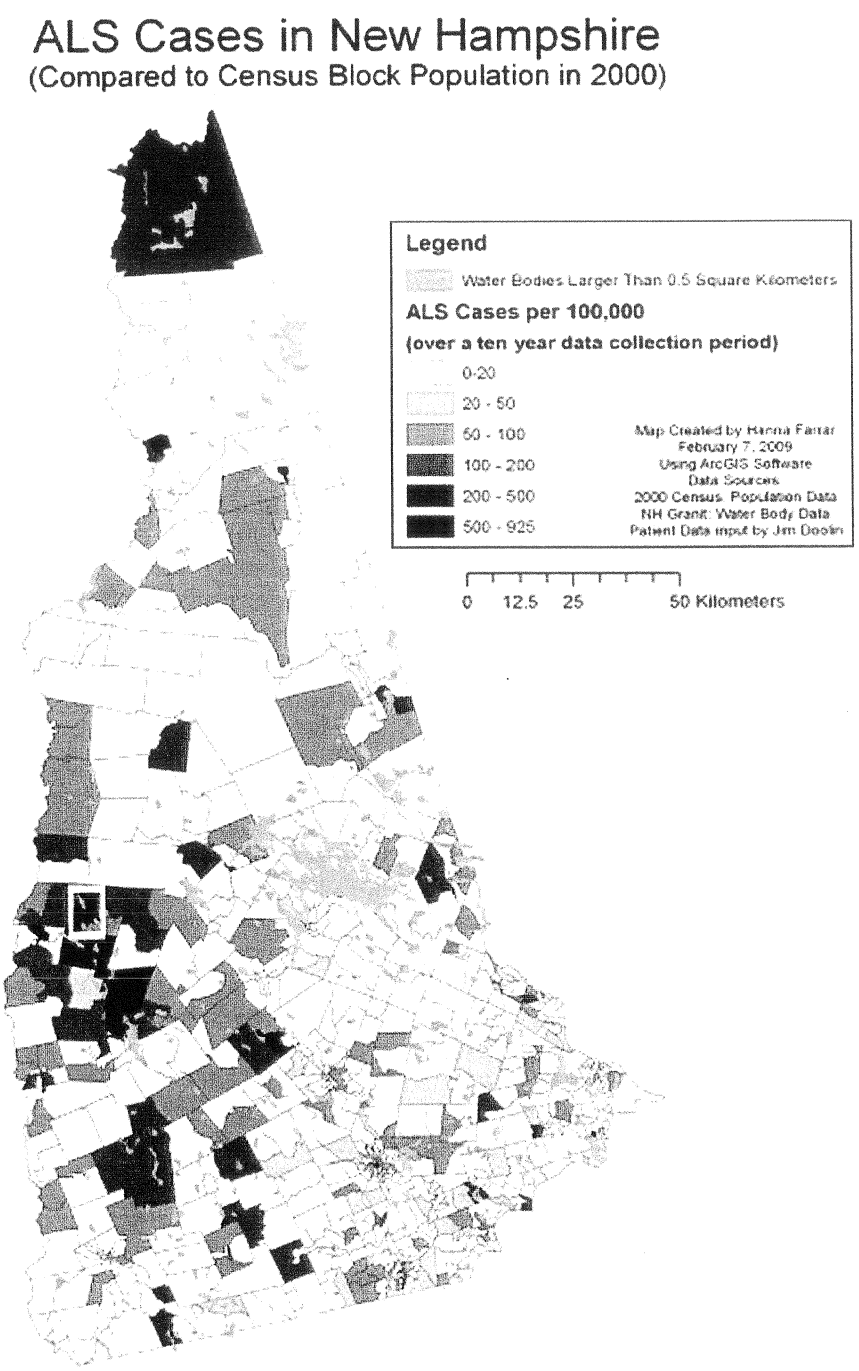

Figure 1. ArcGIS mapping of ALS cases in NH adjusted for population density using U.S. Census 2000 Block Group data. The white rectangle approximates the Enfield area.

estimated at 20–50 cases per 100,000 per year) compared with the remainder of the state. We then examined for a spatial association between cyanobacterial blooms and ALS. The odds ratio of developing ALS for persons living within 0.5 miles of a lake with current or past cyanobacterial blooms, compared to the odds for persons living further away from cyanobacterial blooms, was 2.32 (95% CI 1.42–3.80). Since population density was corrected for using ArcGIS, the distribution of ALS cases is not thought to be an artifact of population density. We were unable to map incidence of ALS by year because of the small number of cases and in some instances the exact year of diagnosis was unknown.

Qualitative data were collected through interviewing residents of Enfield at local lake association meetings. Several participants endorsed frequent consumption of fish and shellfish from Lake Mascoma. One resident reported that his neighbor used lake water for drinking that was boiled prior to consumption, and that lake water was not infrequently used by many residents for daily household activities such as washing dishes and showering. Similar reports of using lake water for drinking and household

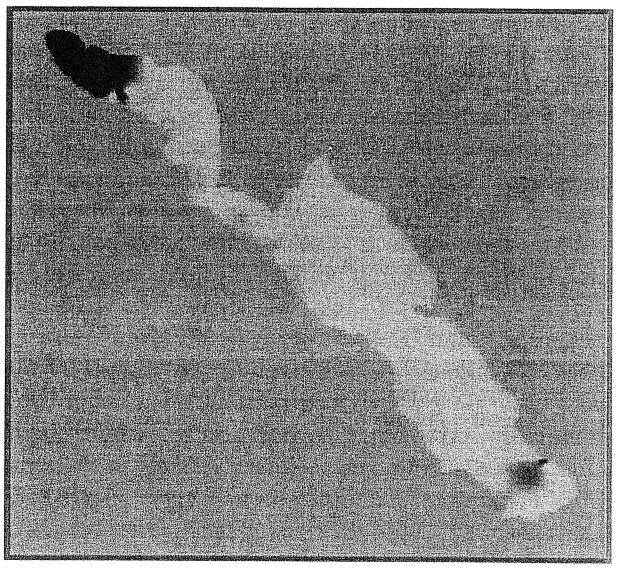

Figure 2. Phycocyanin levels at depth of 0.5 m in Lake Mascoma were mapped using GIS software to demonstrate the density of cyanobacteria. Range is 1000 cells/ml (light grey) to 270,000 cells/ml (black).

activities were cited by the New Hampshire Department of Environmental Services (20).

Cyanobacteria and toxin analysis in water and collected samples

Water samples collected from Lake Mascoma in November 2007 were found to contain cyanobacteria, predominantly Microcystis and Anabaena species that are known to produce BMAA (10,44). We were able to identify MC in our samples, but we were unable to detect BMAA, CYL, or ATX using LC/MS and this was thought to be in part due to low biomass collected on a filter. Sampling was again performed through the summer of 2008. During the 2008 sampling period no significant cyanobacterial blooms were observed on Lake Mascoma. Vertical profiling of Lake Mascoma indicated phycocyanin levels to be highest at the surface of the lake, equivalent to about 200,000 cyanobacteria cells per ml (Figure 2). BMAA was not detected in Lake Mascoma with LC/MS/MS in either surface samples or core integrated samples; however, it should be noted that the analysis was made on extremely small and insufficient quantities of cyanobacteria collected on the filters and the results should be repeated with a greater quantity of sample for proper analysis. Using the ELISA technique, MCs were detected in the water of Lake Mascoma. Notably, MC concentrations in the lake sediments were 10–100 times higher than found in the water, further indicating the presence of toxic cyanobacteria in Lake Mascoma.

Several other New Hampshire lakes were also sampled. Surface samples were collected from three lakes during active blooms (Locke Lake in Barnstead, NH; Goose Pond in Caanan, NH; and Webster Lake in Franklin, NH). In addition, we obtained core integrated samples from three other lakes with a history of current and past blooms but without active blooms during our sampling period (Winnesquam Lake, Laconia, NH; Little Sunapee Lake, New London, NH; and Otter Pond in George's Mills, NH). BMAA was not detected in the integrated core samples; however, one sample of a surface bloom collected from Locke Lake was positive for BMAA and DAB using LC/MS/MS. The protonated AQC derivative fragment m/z 171 is the quantitation ion, while the protonated BMAA AQC fragment m/z 289 is the first qualifier ion and the protonated BMAA fragment m/z 119 is the second qualifier ion. The ratios for two runs of the Locke Lake bloom at 4.61 min retention time were 10.62 +/– 0.45% of the quantitation ion for the m/z 119 fragment and 19.43 +/– 1.03 % of the quantitiation ion for the m/z 289. These values are within two standard deviations of six BMAA standard values, which were 14.22 +/– 1.92% of the quantitiation ion for the m/z 119 fragment and 23.43 +/– 4.76% of the quantitiation ion for the m/z 289 at retention time 4.69. Likewise, the ratios at retention time 4.74 min for two runs of the Locke Lake bloom were 4.85 +/– 0.4% of the quantitation ion for the m/z 119 fragment and 6.49 +/– 0.57% of the quantitation ion for the m/z 289, which matches the ratios for four DAB standard runs, 3.79 +/– 1.12% of the quantitation ion for the m/z 119 fragment and 5.37 +/– 1.65% of the quantitiation ion for the m/z 289 at retention time 4.89 min. These sample values are also within two standard deviations of the standard values.

Discussion

ALS appears to occur in the area surrounding Mascoma and Crystal Lakes at a higher frequency than expected based on our preliminary analysis. Limitations in our spatial analysis include the possibility of bias due to the modifiable area unit problem, and U.S. Census blocks may not be the most appropriate boundaries within which to analyze rates of ALS. Because of the low overall incidence of ALS, our data may also be biased because of the impact of missed cases not included in our data set and the sparse population density in some areas of rural New Hampshire that can distort the calculated ALS rate. Our finding of a higher rate of ALS proximal to lakes with known cyanobacterial blooms indicates only a spatial association and does not infer causality. During the course of mapping ALS patients near Lake Mascoma we identified other potential ALS clusters also located in proximity to lakes with documented algal blooms. More sophisticated analytic measures will be undertaken to determine whether any of these clusters are statistically significant. We were unable to map incidence of disease by year of onset as the exact year of diagnosis was unclear for many cases; the majority of our reported cases were diagnosed between

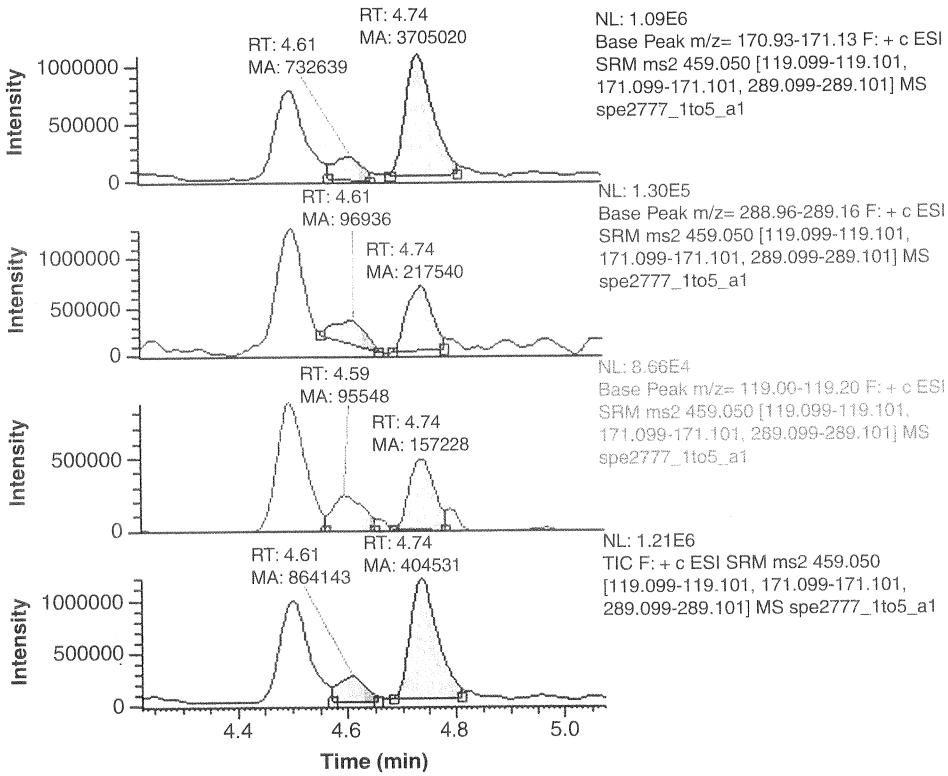

Figure 3. LC/MS/MS identification and verification of L-BMAA in a Locke Lake cyanobacterial bloom sample. Ion chromatograms of product ion from collision induced dissociations of /m/z/ 459. The chromatography of the three major ions produced are from the top-down protonated AQC derivative fragment /(m/z/ 171); protonated-BMAA AQC fragment (/m/z/ 289); protonated-BMAA fragment (/m/z /119); and total ion count for dissociated ions. BMAA was identified at retention time 4.61 min and DAB was identified at 4.74 min. The ion ratios matched within two standard deviations the ion ratios of BMAA and DAB standards run under the same conditions.

2000 and 2007, providing an approximate incidence density for a seven-year time-frame.

The higher rate of ALS in proximity to New Hampshire lakes with known algal blooms may implicate a role of cyanobacterial toxins in the neurodegenerative process. The high incidence of ALS in the Enfield, NH area could be directly related to chronic exposure to cyanobacteria producing neurotoxins such as BMAA. While we have not yet identified BMAA in Lake Mascoma, we have now identified that the neurotoxin is present in at least one New Hampshire lake and our inability to detect the toxin in Lake Mascoma thus far may reflect our sampling technique. The exposure of cyanobacterial toxins to humans is not understood but could occur through ingestion of water, consumption of contaminated food such as fish, which may biomagnify toxins (28,45), or inhaled through aerosolization. We have identified residents of Lake Mascoma who use the lake water for drinking, under the assumption that boiling the water will kill any harmful agents in the water; such treatment will not, however, remove BMAA or other cyanotoxins. There are also many low income families in NH that eat fish several times a week from waters affected by algal blooms because it is a low- or no-cost food source. Compared to much of the U.S., the northern half of NH is very sparsely populated and residents tend not to change domiciles, so the patients in our maps have potentially had many years of chronic BMAA exposure.

Aerosolization and inhalation of toxins is also a plausible route of chronic exposure. For example, certain marine toxins such as brevetoxins, and also Pfiesteria can be aerosolized by wave action or turbulence leading to neurological and respiratory symptoms (46). Algal cells, bacteria, and waterborne toxins can be aerosolized by a bubble-bursting process with a wind-driven white-capped wave mechanism as demonstrated by one recent study using a vacuum aerosol collector to collect aerosolized water containing MC (47). Interestingly, we noted several ALS patients who live in close proximity to dams where aerosolization is common. Water-based recreational activities such as swimming and water-skiing can expose people to very low concentrations of aerosol-borne MC (48). In addition, bathing or showering with lake water represents another source of inhalation exposure. Further studies will investigate whether exposure to cyanotoxins is plausible through each of these routes. In addition, it would be likely that genetic susceptibility must be present in order for chronic exposure to low concentrations of cyanotoxins to be sufficient to cause neurodegeneration.

Our samples predominantly contained Anabaena, previously documented in independent studies to produce BMAA. Our inability to detect toxin levels

in many of our samples may represent a technical issue. Due to lack of fulminate blooms in many of the lakes we sampled (including Lake Mascoma), we obtained only small biomasses of cyanobacteria from filtering core integrated water samples; current methods for analyzing BMAA are not sensitive enough to detect the molecule in such a small sample (25 mg is generally required). Alternatively, this may reflect variation of toxin production between species or the episodic occurrence of high toxin levels during cyanobacterial blooms. Complete analysis of our data will provide more insight, as an inability to identify any toxins in our samples suggests low yield, while identification of other toxins may argue for the absence of BMAA in the lake water. Identification of BMAA in brain samples and hair samples of ALS patients living near lakes with algal blooms is currently being undertaken and a positive correlation would further support our hypothesis.

While it is entirely plausible that BMAA could independently trigger neurodegeneration, another explanation could be that multiple toxins are interacting synergistically to damage motor neurons. We may be unable to detect very low concentrations of BMAA in our samples using currently available techniques, but perhaps low levels of BMAA combined with other unmeasured toxins could be sufficient to disrupt motor neurons. Anatoxin-a's effects on postsynaptic acetylcholine receptors, anatoxin-a(s) acting as an acetylcholinesterase, or the effect of curacin on motor neuron microtubules may work in conjunction with BMAA to synergistically cause sufficient neurodegeneration to produce disease.

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