Failure to Detect the Neurotoxin β-n-methylamino-l-alanine in Samples Collected during an Avian Vacuolar Myelinopathy (AVM) Epornitic in J. Strom Thurmond Lake

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PURPOSE: The neurotoxin β-N-methylamino-L-alanine (BMAA), which is produced by wide genera of cyanobacteria, has been proposed as a factor in the development of amyotrophic lateral sclerosis (ALS), Parkinsonism-dementia complex (PDC), and Alzheimer's Disease (AD). This compound was previously detected in epiphytic alga and vegetation samples collected from a reservoir with frequent outbreaks of avian vacuolar myelinopathy (AVM). The purpose of this work was to evaluate samples of aquatic vegetation with the associated cyanobacterium and waterbird tissues collected during an AVM epornitic and from a novel site. Samples were tested for the presence of BMAA using a recently updated analytical technique, high performance liquid chromatography with tandem mass spectrometry LC-MS/MS. Identifying the putative toxin will be important in determining the etiology of AVM and evaluating risks to fish, wildlife, and humans using these aquatic systems.

INTRODUCTION: Avian vacuolar myelinopathy (AVM), a neurologic disease of unknown etiology, has been linked to a toxin produced by a previously undescribed cyanobacterium in the order Stigonematales (Birrenkott et al. 2004; Wilde et al. 2005; Williams et al. 2007). This often-lethal disease affects waterbirds and their avian predators, most notably American coots, *Fulica Americana,* and the bald eagle, *Halieetus leucocephalus*, and has been documented in reservoirs from Texas to North Carolina (Thomas et al. 1998; Rocke et al. 2002; Fischer et al. 2006). Affected birds lack coordination, exhibit limb paresis, and lose their righting reflex (Thomas et al. 1998). There are no gross abnormalities or infectious agents associated with AVM; diagnosis is confirmed by damage to the central nervous system (CNS), which is the only microscopic abnormality apparent in diseased birds. This damage manifests as an intramyelinic edema, which is caused by splitting and swelling of the myelin sheath creating vacuoles within the myelinated tissues of the CNS (Thomas et al. 1998).

Tissues from diseased birds, sediments, invertebrates, water, and submerged aquatic vegetation (SAV) from affected sites were analyzed for an array of chemicals and toxicants known to elicit this condition, but no compound was detected consistently or in significant amounts (Thomas et al. 1998; Dodder et al. 2003; Rocke et al. 2005). A previously undescribed Stigonematalean cyanobacterium (now formally named Hydrillicola aetokthonos (Wilde et al. 2014)) was detected in SAV from all AVM sites and not in SAV samples from sites with no history of the disease (Wilde et al. 2005). Furthermore, feeding trials with SAV, specifically hydrilla, *Hydrilla verticillata*, containing *Hydrillicola aetokthonos* and a methanol extract of this material caused AVM in mallard ducks, *Anas platyrhynchos* (Birrenkott et al. 2004; Haynie

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et al. 2013). *H. aetokthonos* can be reliably detected from field samples both morphologically and with a real-time polymerase chain reaction (PCR) assay (Williams et al. 2007). The authors hypothesize that herbivorous waterbirds feeding on SAV with the associated cyanotoxin become affected by AVM. Secondary intoxication occurs when raptors feed on these moribund birds or carcasses.

Attempts to isolate and characterize the putative toxin have been stymied because of difficulty establishing an axenic toxic culture of *H. aetokthonos*. Once in culture, many cyanobacteria do not produce toxin because exogenous influences that may stimulate or facilitate toxin production are no longer a factor. *H. aetokthonos*, an adnate epiphyte, is not easily removed from its plant host, and therefore, solvent extraction and subsequent cleanup techniques still produce a mixture too complex for analysis (Wiley 2007). Therefore, the putative toxin remains uncharacterized.

The neurotoxin β-n-methylamino-L-alanine (BMAA), which is produced by numerous genera of cyanobacteria, has received increasing attention for its potential role in human diseases (Banack et al. 2007. This nonprotein amino acid has been proposed as a putative environmental factor in the onset idiopathic neurodegenerative diseases, including Amyotrophic lateral sclerosis-Parkinson's dementia complex (ALS/PDC) and Alzheimer's disease (AD) **(**Murch et al. 2004a, 2004b; Pablo et al. 2009). BMAA functions as an excitotoxin, which is mediated by its role as a glutamate agonist (Lobner et al. 2007; Rao et al. 2006). This causation-linkage was first proposed in the classic case study of the Chamorro people of Guam, who exhibited a 100-fold increase in the prevalence of ALS/PDC to that of comparable populations elsewhere (Cox and Sacks 2002a, 2002b; Banack and Cox 2003; Murch et al. 2004a, 2004b). BMAA was first isolated from the seeds of the cycad tree, *Cycas micronesica,* which are used to make flour and are a favorite food of the flying fox, *Pteropus sp.*, both dietary staples of these indigenous people (Vega and Bell 1967). BMAA was shown to accumulate and magnify, up to 10,000-fold, in ascending levels of the traditional Chamarro diet (Cox et al. 2003; Banack and Cox 2003). *Nostoc sp.*, a cyanobacterium living as an endosymbioant on the roots of the cycad tree, was later identified as the source of this neurotoxic compound (Cox et al. 2005).

Since this discovery, BMAA has been detected, using a variety of methods, in marine, brackish, and freshwater cyanobacteria from around the world (Cox et al. 2003, 2005; Murch et al. 2004a, b; Banack et al. 2007; Metcalf et al. 2008; Esterhuizen and Downing 2008). Biomagnification of this compound has been demonstrated in marine, freshwater, and terrestrial foodwebs (Banack and Cox 2003; Murch et al. 2004b; Brand et al. 2010; Jonasson et al. 2010). Cyanobacteria are ubiquitous in the environment; it is plausible that BMAA is accumulated and magnified in many aquatic trophic systems and could explain the etiopathenogenesis of other neurologic diseases.

BMAA was detected in pure samples of an axenic culture of *H. aetokthonos* and in field-collected samples of *H. aetokthonos* on hydrilla using high performance liquid chromatography/fluorescence detection (HPLC/FD). The identification was confirmed by liquid chromatography/mass spectrometry (LC/MS) (Bidigare et al. 2009). BMAA was not detected in hydrilla without *H. aetokthonos* collected from a reservoir with no history of AVM (Bidigare et al. 2009). Target organ (brain/CNS), clinical signs, and certain aspects of the AVM food-chain-linkage paradigm are akin to published laboratory studies on BMAA toxicity. Young chicks, *Gallus domesticus,* dosed with BMAA displayed similar clinical signs to those associated with AVM, but brain tissues from this study were not evaluated (Polsky et al. 1972**)**. Diet is one route of BMAA exposure and subsequent accumulation in fish and wildlife (Banack et al. 2003; Brand et al. 2010; Jonasson et al. 2010). A recent study showed rapid

uptake and protein association of BMAA, originating from free-living cyanobacteria, by *Ceratophyllum demersum*, a type of SAV similar in structure and function to species associated with AVM (Esterhuizen and Downing 2011). Uptake of this toxin by SAV presents a possible dietary route of bioaccumulation, and possibly biomagnification, and exposure for herbivorous waterbirds and their avian predators. It is epidemiologically plausible that BMAA may be the uncharacterized toxin responsible for AVM or a potential factor in the disease.

The objectives of this study were to

- determine if BMAA or its structural isomer was present in samples of aquatic vegetation with the associated cyanobacterium and waterbird tissues collected during an AVM epornitic and from a naïve site
- evaluate methanol extracts produced from these collections, and tested in an avian bioassay, for the presence of these compounds.

METHODS

Sample collection, preparation. Aquatic vegetation for extracts. Although other species of native and exotic SAV can host *H. aetokthonos*, hydrilla, *Hydrilla verticillata,* was used because it was the most plentiful species in the study region. Treatment hydrilla, which supports epiphytic colonies of *H. aetokthonos*, was collected in December 2010 during an AVM epornitic from J. Strom Thurmond Lake (JSTL: (33.706112, -82.343082). This 28,700 hectare (ha) reservoir is located in the piedmont on the Savannah River between Georgia and South Carolina. Hydrilla, first discovered in JSTL in 1994, is the most prevalent SAV species, and AVM epornitics have occurred annually since 1998 (Fischer et al. 2006 Southeastern Cooperative Wildlife Disease Study (SCWDS), unpublished data). *H. aetokthonos* was identified from JSTL hydrilla samples in 2002, and this species has been documented yearly in SAV samples from around the reservoir (Wilde, unpublished data). Control hydrilla, without *H. aetokthonos*, was collected in March 2011 from Lake Hatchineha, FL (28.021663, -81.405260), a 2,697 ha lake where no AVM epornitics have ever been documented.

For each collection, hydrilla was collected at depths of ~0–1 meter (m) using a rake. Excess water was squeezed from the vegetation prior to placing it in large (38-liter (L)) coolers. The samples were transported with cold packs to the UGA Whitehall Fisheries Laboratory, transferred to zip-top plastic bags (3.79 L) and frozen (–20 °C). Before freezing, subsamples were selected from each large collection to confirm the presence or absence of *H. aetokthonos*. Three slides were prepared from each large collection. For each slide, five randomly selected leaves were removed from the stems, mounted on glass slides, and viewed using light and epifluorescent microscopy (Nikon Eclipse Ti, Nikon Corp., Melville, NY). Using a Rhodamine red filter set, *H. aetokthonos* is identified morphologically and other species present (e.g., cyanobacteria, diatoms, green alga) are recorded. To confirm the presence or absence of *H. aetokthonos* based on microscopy, PCR analysis was conducted on each sample using modifications to a previously validated method (Williams et al. 2007).

An avian bioassay was conducted according to a standard protocol to determine whether the etiologic agent was present in the respective collections (Birrenkott et al. 2004). Because the putative toxin remains uncharacterized, an avian bioassay is the only means to demonstrate toxicity in a given collection.

Extract preparation. A methanol extract was prepared from both the treatment and control hydrilla collections according to a previously validated method (Wiley et al. 2008). This validated method yielded a concentrated extract (10 grams per milliliter (g/mL)) that elicited AVM when administered to mallards via oral gavage (Wiley et al. 2008). Briefly, a known volume of the coarsely ground lyophilized material was extracted using a series of nonpolar to polar solvents, hexane, acetone, and methanol. After filtration, the methanol was removed using a rotary evaporator. Once dry, the extracts were resuspended in methanol to achieve a concentration of 10 g vegetation/mL based on the initial dry weight of the total vegetation extracted. Frozen samples (2 mL) of both the crude extracts were shipped on ice, overnight, to two independent laboratories for BMAA analysis (Center for Marine Microbial Ecology and Diversity- CMMED, University of Hawaii, Honolulu, and the Institute for EthnoMedicine- IEM, Jackson, WY).

Epornitic samples. Tissues were harvested from fresh American coot (n=18) carcasses that were collected by U.S. Army Corps of Engineers (USACE) biologists during a December 2010 AVM epornitic. This epornitic took place on JSTL in the same vicinity as the aforementioned hydrilla *H. aetokthonos* collection. The coot carcasses were transported to the UGA Whitehall Fisheries Laboratory where necropsies were performed immediately. Tissues (brain, liver, muscle, crop/GI tract, kidney) from each bird were collected, wrapped in foil, and frozen ($-20\ °C$). The tissue samples ($n = 90$) were then lyophilized, ground to a coarse powder, and stored in parafilmed microcentrifuge tubes. Whole brains from a subset ($n = 5$) were reserved and processed for histopathology: the entire brain was fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 3 μm, and stained with hematoxylin and eosin. The slides were examined by light microscopy for the diagnostic AVM lesions by SCWDS pathologists.

Hydrilla samples ($n = 0$) were also collected during this event from the region where the birds were recovered. The hydrilla samples were first screened for the presence of *H. aetokthonos* in the same manner(s) as previously described. Each sample was frozen ($-20\ °C$) and then lyophilized, ground to a coarse powder, and stored in parafilmed microcentrifuge tubes. The hydrilla and coot tissue samples were shipped to CMMED for BMAA analysis.

BMAA detection

Extracts. Each sample was analyzed separately and in triplicate. Methanol was dried off the extracts with a stream of nitrogen gas and the material was resuspended in 0.1 N HCl with sonication and filtered through 0.22-um spin filters. Samples were then diluted to several different strengths with 0.1 N HCl and derivatized with Waters AccQ-Tag Ultra.

Amino acids were separated using a Waters XBridge C18 column (4.6×150 millimeter (mm)) on an Agilent 1200 LC system equipped with an Agilent 6410 triple quadrupole mass spectrometer. Detection was done in multiple reaction monitoring (MRM) mode on the double derivatized BMAA molecule at mass-to-charge ratio (m/z) 459 with transitions to its product ions at m/z 289 (single derivatized BMAA), m/z 258 (BMAA specific ion), and m/z 119 (BMAA).

Epornitic samples. All samples were analyzed using high-performance liquid chromatography with tandem mass spectrometry (LC-MS/MS). Each sample was evaluated separately and in triplicate. Samples consisted of 10 to 20 milligrams (mg) of the dried, homogenized material, which was weighed

out into 2 mL glass vials. Material was acid hydrolyzed with 1 mL of 6 N HCl for 18 hours at 110 °C. A subsample (200 µL) was filtered (0.22 µm Ultrafree-MC, Millipore) and dried down completely using a Thermo-Savant SPD131DDA speed vac. The sample was then reconstituted with 0.1 N HCl, diluted appropriately, and derivatized according to the Waters AccQ-Tag Ultra protocol for protein hydrolysates (Grumbach et al. 2006).

The derivatized amino acids were separated using an Agilent 1200 LC system with a Waters Xbridge C18 column (3.5 µm, 4.6 × 150 mm) at 55 °C. Eluents are Waters AccQ-Tag Ultra Eluents A and B (proprietary compositions). The gradient was a modification of the standard Waters AccQ-Tag Ultra method (for UPLC), scaled up and optimized for use with a larger column on a traditional HPLC. Separation of BMAA from the protein amino acids and from its structural isomer 2,4-diaminobutyric acid (DAB) was achieved.

The derivatized amino acids were detected using an Agilent 6410 triple quadrupole mass spectrometer. Detection occurred in the MRM mode on the derivatized molecule at m/z 459 with transitions to its product ions at 119 (molecular ion), 289 (single derivatized molecule), and 258 (specific fragment ion).

RESULTS

Extracts. *H. aetokthonos* was identified morphologically and genetically in the JSTL (treatment) hydrilla collection. *H. aetokthonos* was not observed visually or detected with PCR in the Hatchineha (control) hydrilla collection.

Two of five (2/5) treatment birds displayed clinical signs of AVM during the avian bioassay. Lesions, diagnostic for AVM, were present in the optic tectum, brain stem, and cerebellum in all five (5/5) birds that received vegetation from the treatment collection (Robertson 2012). No vacuolar lesions were present in any region of the brain tissues of birds (0/5) that received hydrilla from the control collection.

Neither of the laboratories detected BMAA in treatment or control extracts using LC-MS/MS analysis. The IEM laboratory detected trace levels of 2,4-diamino-butyric acid (DAB), BMAA's neurotoxic structural isomer, in both control and treatment extracts. The CMMED laboratory did not initially detect DAB in either extract. However, following a solid phase extraction procedure, CMMED reported trace levels of DAB in both treatment and control extracts.

Epornitic samples. AVM was confirmed in all the wild birds evaluated (5/5) based on the presence of characteristic lesions in the optic tectum, optic chiasm and intercerebellar tracts (Figure 1). *H. aetokthonos* was identified morphologically and genetically in the hydrilla collection made during the 2010 JSTL epornitic. BMAA was not detected in any avian tissue fraction analyzed (n=50) or in the hydrilla samples (n=10). It is highly unlikely that this compound is present above detection limits (5 pmoles per injection) because none of the three target molecules were detected at the correct retention times (Figure 2). Very low concentrations of DAB were detected in 3/10 hydrilla samples; however, an additional MS analysis was not performed to quantify these levels.
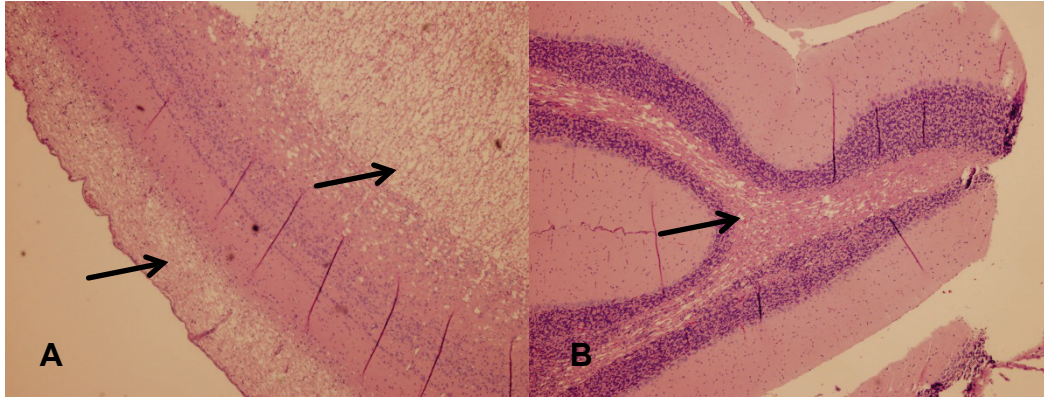
Figure 1. Light micrograph images of brain sections from a coot collected during the epornitic. AVM was diagnosed in all coots (n=5) based on the definitive lesions (arrows) within the (A) optic tegmentum and optic tectum and (B) intercerebellar tracts (arrow).
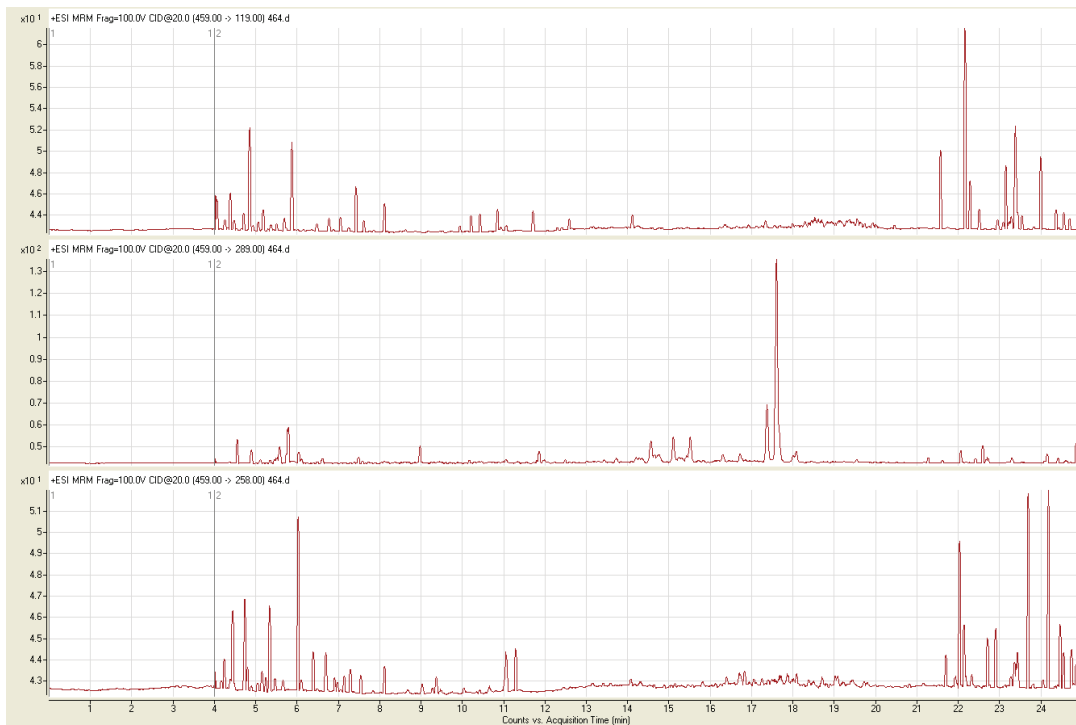

Figure 2.   MS/MS detection, in MRM mode, for a pectoral muscle sample from an AVM-positive coot. No peaks were detected at the correct retention times for any of the three target molecules present for BMAA identification: 119 (molecular ion), 289 (single derivatized molecule), and 258 (specific fragment ion).

DISCUSSION: Failure to detect BMAA in avian tissues, hydrilla samples, or extracts produced from this material does not immediately rule out its potential etiologic role. Based on AVM diagnoses in both wild and laboratory birds, the putative toxin was present in the treatment hydrilla but *not* in the control hydrilla samples that were analyzed. However, failure to detect BMAA may be attributed to matrix effects and/or dilution of the compound by the vegetation biomass (Banack et al. 2007). Studies have demonstrated that BMAA does not always follow the classic biomaginification pattern with increasing concentrations detected in ascending trophic levels (Brand et al. 2010; Jonasson et al. 2010).

Organisms that routinely feed on BMAA-tainted prey items may have evolved a mechanism to detoxify and/or depurate this compound, resulting in nondetection. Researchers have also hypothesized that a metabolite of BMAA may be the acutely toxic agent, explaining why the parent compound would escape detection (Zeevalk and Nicklas 1989).

Conversely, BMAA is not present in these samples, and the use of a less specific detection method (HPLC-FD) in earlier analyses (i.e., Bidigare et al. 2009) overestimated the amount present or actually measured levels of a structurally similar compound. The amount of BMAA detected in cyanobacterial isolates or more complex organismal matrixes varies depending on the detection method used (Faassen et al. 2012). Earlier reports relying on HPLC-FD with LC-MS confirmation detected BMAA in nearly all laboratory and field samples (Banack et al. 2007; Metcalf et al. 2008). More recent analyses using LC-MS/MS either failed to detect BMAA (Kruger et al. 2010) or detected low concentrations (Jonasson et al. 2010). The use of LC-MS/MS, which was performed by both laboratories in the study, is considered optimal for detection of this compound; a comparison of three commonly used methods determined that HPLC-FD overestimated BMAA concentrations (Faassen et al. 2012). Despite this apparent consensus, there are conflicting reports in the literature regarding whether LC-MS/MS analysis of derivitized versus underivitized samples is more suitable for BMAA detection (Banack et al. 2011 Faassen et al. 2012). Basically, detection of this compound is difficult, and the optimum sample treatment and analytical method remains the subject of scientific debate.

At the time of this report, neither laboratory had quantified the amount of DAB present in the samples analyzed. This compound was first detected in the seeds of the legume, *Lathyrus latifolius, in an attempt to determine the* causative agent of the debilitating human neurologic disease neurolathyrism (Ressler et al. 1961). This neurotoxic nonprotein amino acid has been detected in seeds of other legumes, and multiple routes of neurotoxicity have been demonstrated (Ressler et al. 1961; Iverson and Kelley 1975). Co-occurrence of several cyanotoxins, including BMAA, DAB, saxitoxin and anatoxin, has been reported from both urban and rural water sources with cyanobacteria blooms (Craighead et al. 2009; Faassen et al. 2009). Synergistic effects and/or potentiation among these co-occurring toxins have been proposed (Craighead et al. 2009). It is plausible that DAB and the uncharacterized AVM toxin may exert a synergistic effect when they co-occur, thus enhancing toxicity of one or both compounds.

The results of these analyses suggest that BMAA is likely not the etiologic agent of AVM. Vigilance and further investigation into isolation and identification of the uncharacterized putative toxin are still prudent. Although there remains a level of scientific uncertainty regarding the toxin's precise mode of action and chemical composition, AVM presents a serious risk to threatened and endangered raptors. The impacts to avifauna have been documented in active AVM sites, and the disease continues to spread in the absence of invasive SAV control. Recent monitoring and feeding trials have indicated that the AVM toxin may be present in the critical habitat of another highly endangered raptor, the Florida snail kite, *Rhostrahmus socialibilis* (Robertson 2012). This disease has already affected the recovery of bald eagles in regions with frequent epornitics, including piedmont Georgia (Ozier 2013).[1] Furthermore, the association between AVM and environmental health is alarming considering the potential role of cyanotoxins in the emerging etiopathogenesis of many human neurologic disorders

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[1] Personal Communication. 2013. Jim Ozier, Non-Game Program Manager, Wildlife Resources Division, Georgia Department of Natural Resources, Social Circle, GA.

(Cox and Sacks 2002a, b; Lobner et al. 2007). The public regularly consumes wildlife and fish and obtains drinking water from these reservoirs. Risks to public health should be critically evaluated, and it is imperative that a water resources management strategy be developed to address the presence of these toxins.

POINTS OF CONTACT: For additional information, contact Michael Netherland (352-392-0335) (*Michael.D.Netherland@usace.army.mil*) or the Program Manager of the Aquatic Nuisance Species Research Program (ANSRP), Dr. Linda Nelson (601-634-2656) (*Linda.S.Nelson@usace. army.mil*). This technical note should be cited as follows:

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REFERENCES

Augspurger, T. A., J. R. Fischer, N. J. Thomas, L. Sileo, R. E. Brannian, J. G. Miller, and T. E. Rocke. 2003. Vacuolar myelinopathy in waterfowl from a North Carolina impoundment. *Journal of Wildlife Diseases* 39: 412–417.

Banack , S. A., H. E. Johnson, R. Cheng, and P. A. Cox. 2007. Production of the neurotoxin BMAA by a marine cyanobacterium. *Marine Drugs* 5:180–196.

Banack, S. A., and P. A. Cox. 2003. Biomagnification of cycad neurotoxins in flying foxes: Implications for ALS–PDC in Guam. *Neurology* 61(3): 387–389.

Banack, S. A., J. S. Metcalf, Z. Spacil, T. G. Downing, A. Long, P. B. Nunn, P. A. Cox. 2011. Distinguishing the cyanobacterial neurotoxin b-N-methylamino-L-alanine (BMAA) from other diamino acids. *Toxicon* 57: 730–738.

Bidigare, R. R., S. J. Christensen, S. B. Wilde, and S. A. Banack. 2009. Cyanobacteria and BMAA: Possible linkage with avian vacuolar myelinopathy (AVM) in the south-eastern United States. *Amyotrophic Lateral Sclerosis* 10**:** 71–73.

Birrenkott, A. H., S. B. Wilde, J. J. Hains, J. R. Fischer, T. M. Murphy, C. P. Hope, P. G. Parnell, and W. W. Bowerman. 2004. Establishing a food-chain linkage between aquatic plant material and avian vacuolar myelinopathy in mallard ducks (*Anas platyrhynchos*). *Journal of Wildlife Diseases* 40: 435–492.

Brand, L. E., J. Pablo, A. Compton, N. Hammerschlag, and D. C. Mash. 2010. Cyanobacterial blooms and the occurrence of the neurotoxin, beta-N-methylamino-L-alanine (BMAA), in South Florida aquatic food webs. *Harmful Algae* 9:620–635.

Cox, P. A., S. A. Banack, S. J. Murch, U. Rasmussen, G. Tien, R. R. Bidigare, J. S. Metcalf, L. F. Morrison, G. A. Codd, and B. Bergman. 2005. Diverse taxa of cyanobacteria produce beta-N-methylamino-L-alanine, a neurotoxic amino acid. In *Proceedings of the National Academy of Sciences of the United States of America* 102 (14): 5074–5078.

Cox, P. A., S. A. Banack, and S. J. Murch. 2003. Biomagnification of cyanobacterial neurotoxins and neurodegenerative disease among the Chamorro people of Guam. In *Proceedings of the National Academy of Sciences of the United States of America* 100 (23): 13380–13383.

Cox, P. A., and O. W. Sacks. 2002a. Cycad neurotoxin, consumption of flying foxes, and ALS/PDC disease in Guam. *Neurology* 59 (10): 1664–1665.

Cox, P. A., and O. W. Sacks. 2002b. Cycad neurotoxins, consumption of flying foxes, and ALS–PDC disease in Guam. *Neurology* 58 (6): 956–959.

Craighead, D., J. S. Metcalf, S. A. Banack, L. Amgalan, H. V. Reynolds, and M. Batmunkh. 2009. Presence of the neurotoxic amino acids β-*N*-methylamino-L-alanine (BMAA) and 2,4-diamino-butyric acid (DAB) in shallow springs from the Gobi Desert. *Amyotrophic Lateral Sclerosis* 2009(Supplement 2): 96–100.

Dodder, N. G., B. Strandberg, T. Augspurger, and R. A. Hites. 2003. Lipophilic organic compounds in lake sediment and American coot (*Fulica americana*) tissues, both affected and unaffected by avian vacuolar myelinopathy. *Science Total Environment* 311: 81–89.

Eriksson, J., S. Jonasson, D. Papaefthimiou, U. Rasmussen, and B. Bergman. 2009. Improving derivatization efficiency of BMAA utilizing AccQ-Tag((R)) in a complex cyanobacterial matrix. *Amino Acids* 36: 43–48.

Esterhuizen M., and T. G. Downing. 2011. β-N-Methylamino-l-alanine (BMAA) uptake by the aquatic macrophyte *Ceratophyllum demersum*. *Ecotoxicology and Environmental Safety* 74(1):74–77.

Esterhuizen M., and T. G. Downing. 2008. β-N-methylamino-l-alanine (BMAA) in novel South African cyanobacterial isolates. *Ecotoxicology and Environmental Safety* 71(2): 309–313.

Faassen, E. J., F. Gillissen, and M. Lurling. 2012. A comparative study on three analytical methods for the determination of the neurotoxin BMAA in cyanobacteria. *PLoS One* 7(5): e36667.

Faassen, E. J., F. Gillissen, H. A. J. Zweers, and M. Lurling. 2009. Determination of the neurotoxins by LC-MSMS β-N-methylamino-L-alanine (BMAA) and 2,4-diaminobutyric acid (2,4-DAB), in Dutch urban waters with cyanobacterial blooms. *Amyotrophic Lateral Sclerosis* 10: 79–84.

Fischer, J., L. A. Lewis-Weis, C. M. Tate, J. K. Gaydos, R. W. Gerhold, R. H. Poppenga**.** 2006. Avian vacuolar myelinopathy outbreaks at a southeastern reservoir. *Journal of Wildlife Diseases* 42: 501–510.

Grumbach, E. S., T. E. Wheat, and J. R. Mazzeo. 2006. *A novel method for the analysis of amino acids*. Poster (Waters library WA43226). Milford, MA: Waters Corporation.

Haynie, R. S., W. W. Bowerman, S. K. Williams, J. R. Morrison, J. M. Grizzle, J. M. Fischer, and S. B. Wilde. 2013. Triploid grass carp susceptibility and potential for disease transfer when used to control aquatic vegetation in reservoirs with avian vacuolar myelinopathy. *Journal of Aquatic Animal Health* 25(4): 252–259.

Iverson, L., and J. S. Kelly. 1975. Uptake and metabolism of y-aminobutyric acid by neurones and glial cells. *Biochemical Pharmacology* 24: 933–938.

Jonasson, S., J. Eriksson, L. Berntzon, Z. Spacil, L. L. Ilag, L. O. Ronnev, U. Rasmussen, and B. Bergman. 2010. Transfer of a cyanobacterial neurotoxin within a temperate aquatic ecosystem suggests pathways for human exposure. *Proceedings of the National Academy of Sciences* 107(20): 9252–9257.

Kruger, T., B. Monch, S. Oppenhauser, and B. Luckas. 2010. Lc-MS/MS determination of the isomeric neurotoxins β-N-methylamino-L-alanine (BMAA) and 2,4-diaminobutyric acid (2,4-DAB) in cyanobacteria and seeds of Cycas revoluta and Lathyrus latifolius. *Toxicon* 55: 547–557.

Lewis-Weis, L. A., J. Fischer, and R. W. Gerhold. 2004. Attempts to reproduce vacuolar myelinopathy in domestic swine and chickens. *Journal of Wildlife Diseases* 40: 476–484**.**

Lobner, D., P. M. T. Piana, A. K. Salous, and R. W. Peoples. 2007. b-N-methylamino-Lalanine enhances neurotoxicity through multiple mechanisms. *Neurobiology of Diseases* 25: 360–366.

Metcalf, J. S, S. A. Banack, J. Lindsay, L. F. Morrison, P. A. Cox, and G. A. Codd. 2008. Co-occurrence of β-*N*-methylamino-l-alanine, a neurotoxic amino acid with other cyanobacterial toxins in British waterbodies, 1990–2004. *Environmental Microbiology* 10(3): 702–708.

Murch, S. J., P. A. Cox, and S. A. Banack. 2004a. A mechanism for slow release of biomagnified cyanobacterial neurotoxins and neurodegenerative disease in Guam. In *Proceedings of the National Academy of Sciences of the United States of America* 101(33): 12228–12231.

Murch, S. J., P. A. Cox, S. A. Banack, J. C. Steele, and O. W. Sacks. 2004b. Occurrence of beta-methylamino-L-alanine (BMAA) in ALS/PDC patients from Guam. Acta Neurologica Scandinavica 110 (4): 267–269.

Pablo, J., S. A. Banack, P. A. Cox, T. E. Johnson, S. Papapetropoulos, W. G. Bradley, A. Buck, and D. C. Mash. 2009. Cyanobacterial neurotoxin BMAA in ALS and Alzheimer's disease. Acta Neurologica Scandinavica 120: 216–225.

Polsky, F. I., P. B. Nunn, and E. A. Bell. 1972. Distribution and toxicity of α-amino-β-methylaminopropionic acid. *Federation Proceedings* 31:1473–1475.

Rao, S. D., S. A. Banack, P. A. Cox, and J. H. Weiss. 2006. BMAA selectively injures motor neurons via AMPA/kainate receptor activation. *Experimental Neurology* 201: 244–252.

Ressler, C., P. A. Redstone, and R. H. Erenberg. 1961. Isolation and identification of a neuroactive factor from *Lathyrus latifolius. Science 134: 188–190.*

Robertson, S. R. Potential threats of the exotic apple snail (*Pomaceae insularum*) to aquatic ecosystems in Georgia and Florida. 2012. Unpublished Masters thesis. Athens, GA: University of Georgia.

Rocke, T. E., N. J. Thomas, T. Augspurger, and K. Miller. 2002. Epizootiologic studies of avian vacuolar myelinopathy in waterbirds. *Journal of Wildlife Diseases* 38: 678–684.

Rocke, T. E., N. J. Thomas, C. U. Meteyer, C. F. Quist, J. R. Fischer, T. Augspurger, and S.E. Ward. 2005. Attempts to identify the source of avian vacuolar myelinopathy for waterbirds. *Journal of Wildlife Diseases* 41: 163–170.

Rosen J., and K. E. Hellenäs. 2008. Determination of the neurotoxin BMAA (beta-Nmethylamino-L-alanine) in cycad seed and cyanobacteria by LC-MS/MS (liquid chromatography tandem mass spectrometry). *Analyst* (London) 133: 1785–1789.

Thomas, N. J., C. U. Meteyer, and L. Sileo. 1998. Epizootic vacuolar myelinopathy of the central nervous system of bald eagles (*Haliaeetus leucocephalus*) and American coots (*Fulica americana*). *Veterinary Pathology* 35: 479–487.

Vega A., and E. A. Bell. 1967. α-Amino-β-methylaminopropionic acid, a new amino acid from seeds of *Cycas circinalis. Phytochemistry 6(5): 759–762.*

Wilde, S. B., T. M. Murphy, C. P. Hope, S. K. Habrun, J. Kempton, A. Birrenkott, F. Wiley, W. W. Bowerman, and A. J. Lewitus. 2005. Avian vacuolar myelinopathy (AVM) linked to exotic aquatic plants and a novel cyanobacterial species. *Environmental Toxicology* 20: 348–353.

Wilde, S. B. , J. R. Johansen, H. D. Wilde, P. Jiang, B. Bartelme, R. S. Haynie. 2014. Aetokthonos hydrillicola gen. et sp. nov.: Epiphytic cyanobacteria on invasive aquatic plants implicated in Avian Vaculoar Myelinopathy. *Phtotaxa* 181(5).

Wiley, F. E., S. B. Wilde, A. H. Birrenkott, S. K. Williams, T. M. Murphy, C. P. Hope, W. W. Bowerman, and J. R. Fischer. 2007. Investigation of the link between avian vacuolar myelinopathy and a novel species of cyanobacteria through laboratory feeding trials. *Journal of Wildlife Diseases* 43: 337–344.

Wiley, F. E. 2007. Extraction method development and in vivo and in vitro studies of the etiologic agent of avian vacuolar myelinopathy. Unpublished Ph.D. Dissertation. Clemson, SC: Clemson University.

Wiley, F. E., M. J. Twiner, T. A. Leighfield, S. B. Wilde, F. M. Van Dolah, W. W. Bowerman, and J. R. Fischer. 2008. An extract of Hydrilla verticillata and associated epiphytes responsible for avian vacuolar myelinopathy. *Journal of Environmental Toxicology* 10: 362–368.

Williams, S. K., J. Kempton, S. B. Wilde, and A. Lewitius. 2007. A novel epiphytic cyanobacterium associated with reservoirs affected by avian vacuolar myelinopathy. *Harmful Algae* 6: 343–353.

Zeevalk, G. D., and W. J. Nicklas. 1989. Acute excitotoxicity in chick retina caused by the unusual amino acids BOAA and BMAA: Effects of MK-801 and kynurenate. *Neuroscience Letters* 102: 284–290.