RESEARCH ARTICLE SUMMARY

WILDLIFE DISEASE

Hunting the eagle killer: A cyanobacterial neurotoxin causes vacuolar myelinopathy

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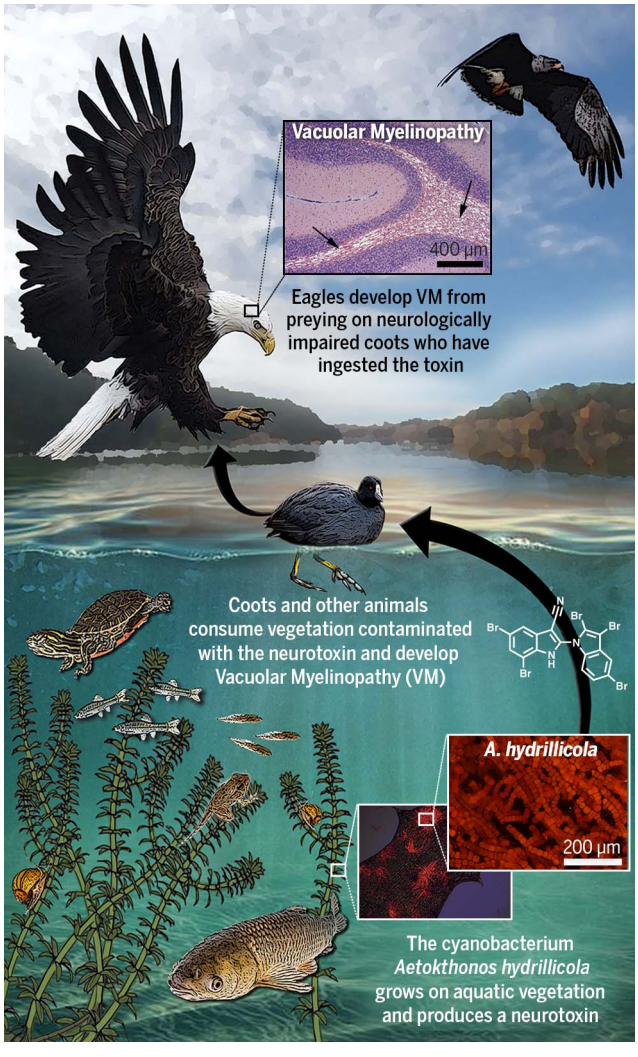
INTRODUCTION: Vacuolar myelinopathy (VM) is a neurological disease characterized by widespread vacuolization in the white matter of the brain. First diagnosed in 1994 in bald eagles, it has since spread throughout the southeastern United States. In addition to avian species such as waterfowl and birds of prey, VM has also been found to affect amphibians, reptiles, and fish. Despite intense research efforts, the cause of this mysterious disease has been elusive. Neither contagious agents nor xenobiotics were detected in deceased animals, but field and laboratory studies demonstrated that VM can be transferred through the food chain from herbivorous fish and wildlife to birds of prey.

RATIONALE: Occurrence of VM has been linked to a cyanobacterium (*Aetokthonos hydrillicola*) growing on an invasive plant (*Hydrilla verticillata*) in man-made water bodies. Cyanobacteria are known to produce potent toxins, so we hypothesized that a neurotoxin produced by the epiphytic cyanobacterium causes VM.

RESULTS: Field studies in the southeastern United States confirmed that *H. verticillata* was colonized with *A. hydrillicola* in more than half of the watersheds. Wildlife VM deaths occurred only in reservoirs with dense *H. verticillata* and *A. hydrillicola* populations. Laboratory bioassays confirmed the neurotoxicity of crude extracts of *A. hydrillicola*–*H. verticillata* biomass collected during VM outbreaks, but neurotoxicity was not detected in samples from VM-free sites. Laboratory cultures of the cyanobacterium, however, did not elicit VM. *A. hydrillicola* growing on *H. verticillata* collected at VM-positive reservoirs was then analyzed by mass spectrometry imaging, which revealed that cyanobacterial colonies were colocalized with a brominated metabolite. Supplementation of an *A. hydrillicola* laboratory culture with potassium bromide resulted in pronounced biosynthesis of this metabolite. *H. verticillata* hyperaccumulates bromide from the environment, potentially supplying the cyanobacterium with this biosynthesis precursor. Isolation and structure elucidation of the metabolite revealed a structurally unusual pentabrominated biindole alkaloid, which we called aetokthonotoxin (AETX). Genome sequencing of *A. hydrillicola* allowed the identification of the AETX biosynthetic gene cluster. Biochemical characterization of a halogenase detected in the cluster demonstrated that it brominates tryptophan with the expected substitution pattern. AETX is highly toxic to the nematode *Caenorhabditis elegans* [median lethal concentration (LC_{50}) 40 nM] and zebrafish (*Danio rerio*; LC_{50} 275 nM). Leghorn chickens (*Gallus gallus*) gavaged with AETX developed brain lesions characteristic of VM, whereas no lesions were observed in control chickens. VM diagnosis in treated chickens was verified using transmission electron microscopy of brain tissue.



CONCLUSION: We confirmed that AETX is the causative agent of VM. AETX biosynthesis relies on the availability of bromide. Seasonal environmental conditions promoting toxin production of *A. hydrillicola* are watershed specific. The consequences of elevated bromide from geologic and anthropogenic sources (e.g., water treatment and power plants) on VM should be further investigated. Notably, integrated chemical plant management plans to control *H. verticillata* should avoid the use of bromide-containing chemicals (e.g., diquat dibromide). AETX is lipophilic with the potential for bioaccumulation during transfer through food webs, so mammals may also be at risk. Increased monitoring and public awareness should be implemented for *A. hydrillicola* and AETX to protect both wildlife and human health. ■

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From the cyanobacterium to the bald eagle—toxin transmission through the food chain. *A. hydrillicola*, growing in colonies on aquatic vegetation, produces the neurotoxin AETX. Waterbirds, tadpoles, aquatic turtles, snails, and fish consume this contaminated vegetation and develop VM. Predators develop VM when they consume animals that have been grazing on *A. hydrillicola*–covered plants.

RESEARCH ARTICLE

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Hunting the eagle killer: A cyanobacterial neurotoxin causes vacuolar myelinopathy

Steffen Breinlinger[1]*, Tabitha J. Phillips[2]*, Brigette N. Haram[2], Jan Mareš[3,4,5], José A. Martínez Yerena[3,5], Pavel Hrouzek[4,5], Roman Sobotka[4,5], W. Matthew Henderson[6], Peter Schmieder[7], Susan M. Williams[8], James D. Lauderdale[9], H. Dayton Wilde[10], Wesley Gerrin[2], Andreja Kust[3], John W. Washington[6], Christoph Wagner[11], Benedikt Geier[12], Manuel Liebeke[12], Heike Enke[13], Timo H. J. Niedermeyer[1]†‡, Susan B. Wilde[2]†‡

Vacuolar myelinopathy is a fatal neurological disease that was initially discovered during a mysterious mass mortality of bald eagles in Arkansas in the United States. The cause of this wildlife disease has eluded scientists for decades while its occurrence has continued to spread throughout freshwater reservoirs in the southeastern United States. Recent studies have demonstrated that vacuolar myelinopathy is induced by consumption of the epiphytic cyanobacterial species *Aetokthonos hydrillicola* growing on aquatic vegetation, primarily the invasive *Hydrilla verticillata*. Here, we describe the identification, biosynthetic gene cluster, and biological activity of aetokthonotoxin, a pentabrominated biindole alkaloid that is produced by the cyanobacterium *A. hydrillicola*. We identify this cyanobacterial neurotoxin as the causal agent of vacuolar myelinopathy and discuss environmental factors—especially bromide availability—that promote toxin production.

Over the winter of 1994 to 1995, the largest undiagnosed mass mortality of bald eagles (*Haliaeetus leucocephalus*) in the United States occurred at DeGray Lake in Arkansas (*1*). More than 70 dead eagles were found in the next 2 years. The mysterious mortalities were characterized by a spongiform myelinopathy that had never been documented in wild avian populations (*2*, *3*). Investigators of the Arkansas die-off began to notice eagles and waterbirds with similar neurological impairment throughout the southeastern states. By 1998, the emerging disease was termed avian vacuolar myelinopathy (AVM) and had been confirmed at 10 sites in six states (*1*). AVM has since been documented in numerous avian species across the southeastern United States during the fall and winter, most notably in waterbirds such as American coots (*Fulica americana*), ringnecked ducks (*Aythya collaris*), mallards (*Anas platyrhynchos*), and Canada geese (*Branta canadensis*) and in various birds of prey (*3*–*7*). All documented AVM cases were recovered on or near man-made water bodies with abundant aquatic vegetation that senesces during the late fall and winter months (*3*–*7*). The abundance of fish and avian prey associated with these aquatic plants attracts overwintering and nesting bald eagles and other birds of prey (*8*–*10*). AVM-afflicted wildlife are prone to injury and become easy prey for predators, as clinical signs of the disease include the severe loss of motor functions (movie S1 shows affected American coots) (*2*, *10*). Although neurological impairment is a visual indication of disease, AVM diagnosis relies on histological confirmation of widespread vacuolization of the myelinated axons (intramyelenic edema) in the white matter of the brain and spinal cord (*1*).

Early sentinel field trials documented neuropathy and vacuolar lesions in wild coots and mallards within 5 days of release into a lake with an ongoing AVM epizootic (*4*–*6*). Initial chemical analysis of sediment and dead birds recovered from reservoirs where AVM cases were documented revealed no xenobiotic compounds known to induce intramyelenic edema in mammals and birds—e.g., hexachlorophene, triethyltin, or bromethalin (*1*, *11*, *12*). Early laboratory feeding trials using plants, water, and sediment collected from disease sites failed to induce the neuropathy and vacuolar lesions seen in wild birds (*13*, *14*). Additionally, no contagious transfer, pathogens inducing myelinopathy, or neuroinflammation were documented in wild or experimental AVM-affected animals (*12*, *13*). These early studies suggested that an unknown, seasonal, and environmental neurotoxin could be responsible (*14*, *15*).

The search for the elusive source of this disease then focused on environmental conditions in AVM-positive water bodies, which revealed that all of them supported invasive submerged aquatic vegetation, primarily *Hydrilla verticillata*, with a previously unidentified epiphytic cyanobacterium—*Aetokthonos hydrillicola*—colonizing up to 95% of the plant leaves (*16*–*18*). Field and laboratory studies demonstrated that AVM could be transferred up the food chain. It is induced in herbivorous waterbirds after ingestion of *H. verticillata* colonized by *A. hydrillicola* and in birds of prey that consume the affected waterfowl (*9*, *16*, *19*, *20*). Not only does AVM present an emerging threat to the Southeast's avian species (*5*, *6*), but subsequent field and laboratory *H. verticillata*–*A. hydrillicola* feeding trials have confirmed neuropathy and mortality in a wide variety of taxa, including amphibians, reptiles, and fish, as well as secondary disease transfer through the food chain. Thus, the disease is now referred to as vacuolar myelinopathy (VM) (*21*–*24*).

Cyanobacteria have long been associated with the production of toxins and other specialized metabolites (*25*–*29*). We hypothesized that a neurotoxin produced by the epiphytic cyanobacterium *A. hydrillicola* is the causative agent of VM. Here, we present our evidence that VM is caused by a cyanobacterial neurotoxin with notable structural features. In addition to discovering the neurotoxin, we have identified its biosynthetic gene cluster and present toxicity data on model birds, fish, nematodes, and crustaceans. Finally, we discuss environmental factors that promote toxin production.

Results

A. hydrillicola *distribution*

Sampling of submerged aquatic vegetation in lakes, reservoirs, and other water bodies throughout the southeastern United States has revealed a complex and widespread pattern of *A. hydrillicola* distribution (*16*–*18*). As of fall 2019, we documented *H. verticillata* colonized with *A. hydrillicola* in 31 of 69 sampled watersheds (Fig. 1 and table S1). Water bodies include large (>10,000 ha) hydropower or water-supply reservoirs, county water-source reservoirs, suburban recreational lakes, and farm ponds. Given the difficulty of documenting animals dying from VM and the extensive spread of invasive *H. verticillata*, our current

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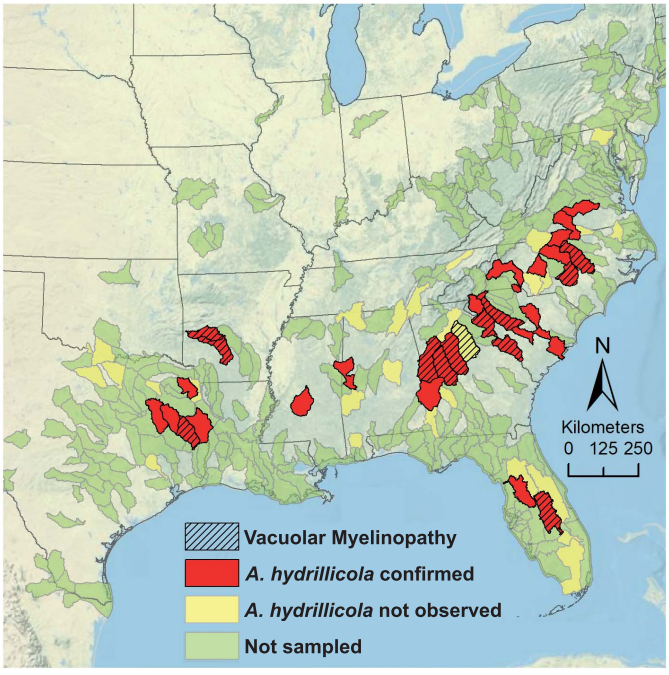

Fig. 1. VM occurs in watersheds where *A. hydrillicola* colonizes *H. verticillata*. Watersheds where VM has been diagnosed (indicated by black crosshatching). Watersheds where *H. verticillata* has been confirmed to be colonized with *A. hydrillicola* are shown in red, and watersheds where *A. hydrillicola* has not yet been observed on *H. verticillata* are shown in yellow. Watersheds not yet screened for *A. hydrillicola*, but where *H. verticillata* occurs, are shown in green. Base map: copyright 2013 from the National Geographic Society.

map of *A. hydrillicola* distribution is certainly underestimating the prevalence of the cyanobacterium and its threat to endemic wildlife, fish, and freshwater resources.

Discovery and production of the putative toxin

In 2011, we collected *H. verticillata* with epiphytic *A. hydrillicola* from the J. Strom Thurmond Reservoir (in Georgia and South Carolina) to isolate the cyanobacterial strain for mass cultivation and subsequent isolation of the putative cyanotoxin. As a result of challenges in establishing culture conditions for this epiphytic colonizer, it took 2 years to generate sufficient *A. hydrillicola* biomass for a first feeding trial. The identity of the strain was confirmed as *A. hydrillicola* by 16*S* ribosomal RNA sequencing. However, chickens gavaged with this *A. hydrillicola* biomass did not develop VM, which failed to support that *A. hydrillicola* was producing a VM-inducing toxin.

Hypothesizing that the cyanobacterium produces the hypothetical toxin only when growing on *H. verticillata*, but not in laboratory culture, we collected additional samples of *A. hydrillicola* growing on *H. verticillata* at confirmed VM sites. The cyanobacterial colonies on the *H. verticillata* leaves were analyzed by atmospheric-pressure matrix-assisted laser desorption/ionization mass spectrometry imaging (AP-MALDI-MSI) to detect cyanobacteria-specific metabolites in situ. Using AP-MALDI-MSI, we could colocalize the cyanobacterial colonies with a metabolite with the sum formula $C_{17}H_6Br_5N_3$ (Fig. 2, A to D), which was not detectable in laboratory cultures by high-performance liquid chromatography–mass spectrometry (HPLC-MS). Neither commercial (Dictionary of Natural Products 28.2, SciFinder) nor in-house natural product databases revealed an entry for this elemental composition, which suggests that it is a novel natural product. The fact that the metabolite contains five bromine atoms is notable, as polyhalogenated synthetic compounds, such as bromethalin or hexachlorophene, are known to induce VM-like brain lesions in birds and mammals (*1*, *5*, *11*, *30*).

The presence of bromine in the putative toxin presented a potential explanation for why our laboratory cultures of the cyanobacterium did not cause VM. Our standard cultivation medium, BG11, does not contain any bromide, which is critical for the biosynthesis of the toxin. Supplementation of the cultivation medium with potassium bromide resulted in the pronounced biosynthesis of this pentabrominated metabolite. We found a nonlinear relation between bromide concentration in the medium and the production of the pentabrominated metabolite by the cyanobacterium. We determined that the optimum bromide concentration for productivity is between 0.1 and 0.5 mM KBr (fig. S1).

Although a minor production can readily be detected by HPLC-MS once the medium is supplemented with bromide, we observed a substantial increase in production under stress conditions. A drop in temperature (from cultivation at 28°C down to 21°C) or enhanced culture movement (shear stress) trigger metabolite production to an extent (>100-fold) that it becomes easily detectable by HPLC-UV (ultraviolet, detection at 286 nm) (figs. S2 and S3).

Hypothesizing that this pentabrominated metabolite was the putative toxin, we screened *A. hydrillicola–H. verticillata* assemblages collected from lakes during VM outbreaks using HPLC-MS and compared them with biomass from VM-free sites (where *H. verticillata* was not colonized with *A. hydrillicola*). We could detect this metabolite only in biomass collected from VM-affected lakes, which strengthened our hypothesis (fig. S4). Furthermore, the compound could be detected in the tissues of two deceased wild American coots collected during an AVM outbreak at the J. Strom Thurmond Reservoir (Georgia) in November 2014 (fig. S5), which confirmed that the compound is absorbed from the gut and accumulates in wild waterfowl. Additionally, we observed a seasonal variation of the toxin concentration in *A. hydrillicola–H. verticillata* biomass collected from J. Strom Thurmond Reservoir, with the peak concentration detected in November (fig. S6). This observation agrees with the finding that VM occurrences have been documented in late autumn in reservoirs, coinciding with seasonal water temperature declines and lake turnover.

As the biosynthesis of the pentabrominated metabolite requires bromide, we investigated bromide availability in *A. hydrillicola* habitats. Total bromine content in *H. verticillata* and sediments as well as the bromide concentration in water from VM-positive (containing *H. verticillata* colonized by *A. hydrillicola*) and VM-negative (containing only uncolonized *H. verticillata*) reservoirs was monitored seasonally. Colonized and uncolonized *H. verticillata* leaves contain significantly ($P < 0.001$) higher concentrations of bromine than sediments (~20-fold) and water (500- to 1000-fold) (fig. S7). In late summer, southeastern U.S. reservoirs are stratified, with warm, sunlit, and oxygenated water above and cool, dark, and anoxic water trapped below. During late fall, surface water temperatures cool, water layers mix, and *H. verticillata* senesces. We propose that this seasonal shift provides a bromide-enriched local environment, which ultimately triggers *A. hydrillicola*'s production of the elusive toxin.

Isolation and structure elucidation of aetokthonotoxin (AETX)

Cultivation of *A. hydrillicola* with bromide supplementation as well as field collections of *A. hydrillicola–H. verticillata* assemblages allowed us to isolate the compound in sufficient amounts for structure elucidation and bioactivity characterization. Because of the proton deficiency of the compound, extensive nuclear magnetic resonance (NMR) and infrared spectroscopic as well as high-resolution mass spectrometric analyses were required to elucidate its structure, which was confirmed by x-ray crystallography (Fig. 3, supplementary

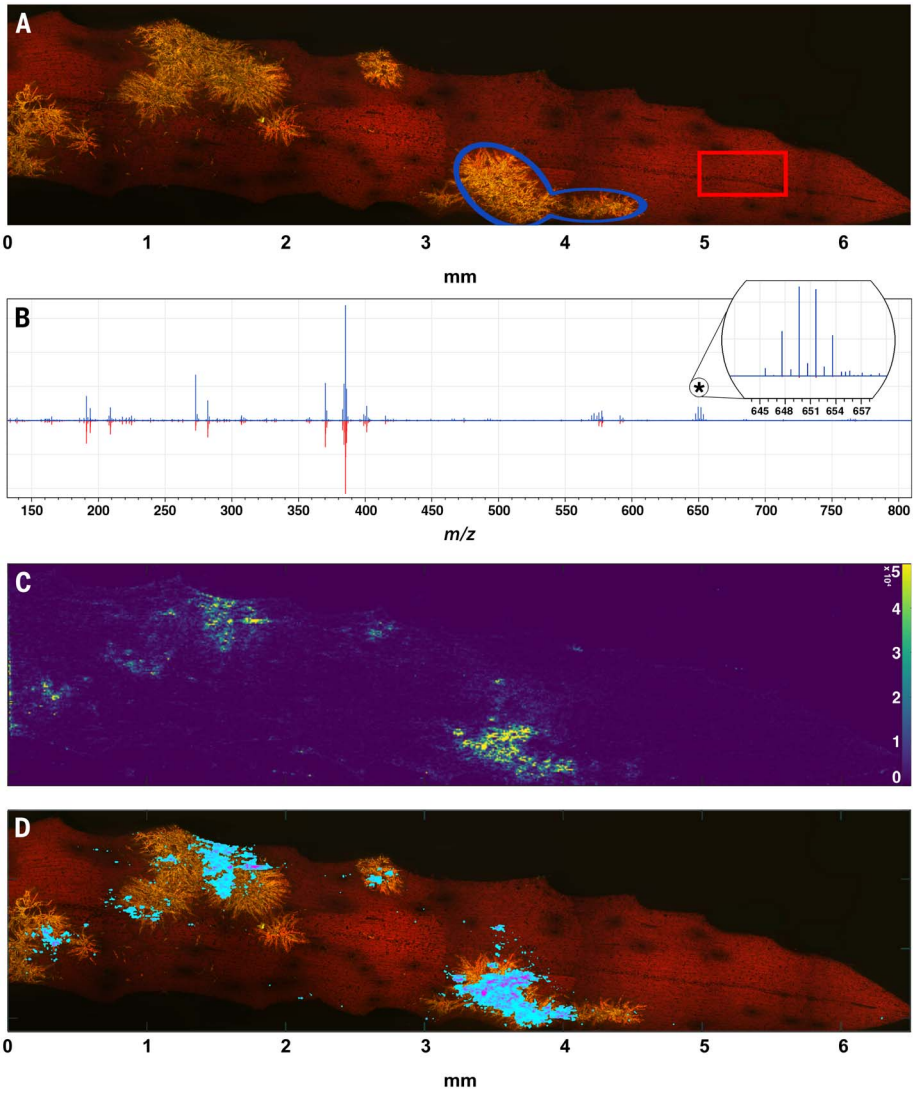

Fig. 2. AP-MALDI-MSI of *A. hydrillicola* colonies growing on *H. verticillata* reveals a cyanobacterium-specific metabolite. (**A**) Micrograph of *A. hydrillicola* colonies on *H. verticillata* leaf. Autofluorescence (excitation, 395 to 440 nm; emission, 470 nm) was used to acquire the image. Regions of interest for evaluation of the subsequent MSI experiments are shown bordered in red (leaf without cyanobacterium) and blue (cyanobacteria colony on the leaf). (**B**) Comparison of mean mass spectra of the two regions of interest. The enlarged region shows the characteristic isotope pattern of the pentabrominated metabolite at a mass/charge ratio (*m/z*) of 645 ($[M - H]^-$). This molecule is exclusively found to be associated with the cyanobacterial colony. (**C**) AP-MALDI image showing the spatial distribution of the distinct feature *m/z* 649.6382 ± 2 parts per million (ppm) ($[C_{17}H_6{}^{79}Br_3{}^{81}Br_2N_3 - H]^-$). Intensity is scaled from 0 (violet) to 5×10^4 (yellow). (**D**) Overlay of micrograph and *m/z* feature 649.6382 ± 2 ppm.

text, fig. S13, and tables S4 to S9). The structure has notable chemical features. Most prominent—also evident from the isotope pattern observed in mass spectrometry analyses of the compound—are the five bromo substituents. Brominated organic compounds are often found to be produced by marine organisms but are also found in plants, fungi, lichen, bacteria, and even humans (*31*–*33*). Several bromoindoles have been isolated from natural sources (*34*). Tyrian purple, one of the first brominated indole alkaloids to have been discovered, is not only the most famous example, it is also a 2,2′-biindole (*35*). Many brominated organic compounds exhibit strong bioactivity, ranging from antifungal to antimicrobial to antioxidant activity (*36*). Synthetic representatives have lately become infamous as environmental pollutants (*37*): Because of their lipophilicity, they tend to accumulate in sediment and biota, where they can pose a serious threat to ecosystems (*38*, *39*). Another notable chemical feature of the compound is the connection of the two indole moieties via N1 and C2′; to date, no natural 1,2′-bi-1H-indole has been described. The two indole substructures present in AETX are rare in natural products. The 2,3,5-tribromoindole substructure has only been described from red algae of the genera *Laurencia* and *Nitophyllum* (*40*), the mollusk *Aplysia dactylomela* (likely because of its red-algal diet) (*41*), and the cyanobacterium *Rivularia firma*, which produces numerous structurally related brominated 1,3′-, 3,3′-, and 3,4′-bi-1H-indoles (*42*). 5,7-Dibromoindole-3-carbonitrile has not yet been found as a natural product substructure. Indole-3-carbonitrile without additional substituents has only been described once as a natural product, isolated from a halophilic bacterium probably belonging to the genus *Bacillus* (*43*). On the basis of the systematic name of the cyanobacterium, *A. hydrillicola* (which is Greek for "eagle killer residing on *Hydrilla*"), we called this compound aetokthonotoxin (AETX), or "poison that kills the eagle" [from the Greek ἀετός (áetós), eagle; κτείνω (kteínō), to kill; and τοξικόν (toxikón), toxin].

AETX biosynthesis

Because of the specific structure of AETX, which illustrates several chemical features not previously observed in nature, we investigated its biosynthesis. Biosynthetic machineries of most cyanotoxins are organized in compact gene clusters, which can be linked to the resulting structures by functional annotation. Thus, we sequenced the whole genomes of two independent *A. hydrillicola* strains isolated from J. Strom Thurmond Reservoir in 2011 (confirmed to produce AETX, as described above) and 2014 and subsequently performed a bioinformatic analysis. Both sequenced genomes contain an identical gene cluster consisting of six deduced genes (*aetA* to *aetF*), whose annotated functions imply their involvement in AETX biosynthesis (Fig. 4A). BLAST searches against all available genomes in the National Center for Biotechnology Information (NCBI) database did not show the presence of a similar gene cluster sequenced to date (as of October 2020). The genomic regions adjacent to the candidate gene cluster contained other cyanobacterial genes, and the deduced proteins within the cluster had closest relatives in cyanobacteria (table S10), corroborating its cyanobacterial origin.

The gene *aetE* was predicted to encode a tryptophanase (fig. S37 and table S10)—a well-studied enzyme responsible for the conversion of tryptophan to indole (*44*). This provides a viable hypothesis to explain the origin of the two indole cores in AETX from tryptophan (or a tryptophan-derived intermediate of the pathway). Two genes, *aetA* and *aetF*, were predicted to encode NAD(P)/FAD-dependent halogenases related to known halogenases involved in the biosynthesis of cyanobacterial

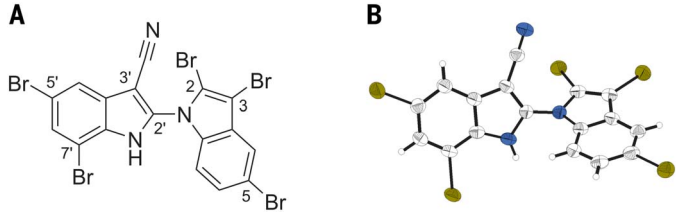

Fig. 3. AETX is a pentabrominated biindole alkaloid. (**A** and **B**) Structure (A) and x-ray crystallography structure (B) of AETX.

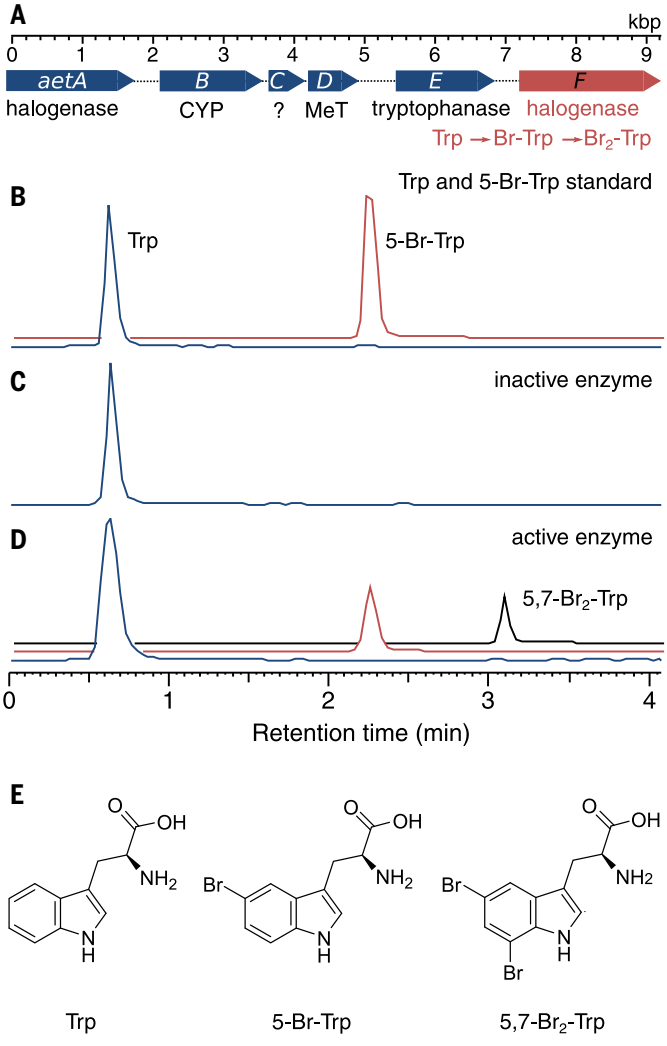

Fig. 4. The halogenase AetF encoded in the AETX gene cluster brominates tryptophan. (**A**) Map of the 9.23–kilobase pair (kbp) AETX biosynthetic gene cluster consisting of six predicted genes coding for two halogenases, a cytochrome P450 (CYP), a tentative methyl-transferase (MeT), a tryptophanase, and a single unknown protein. (**B** to **E**) HPLC-MS analysis (extracted ion chromatograms) of authentic standards compared with the reaction mixtures of assays with fresh and heat-inactivated purified His-AetF (N = 3). (B) L-tryptophan [Trp; retention time (t_r) 0.73 min] and 5-bromo-DL-tryptophan (5-Br-Trp, t_r 2.26 min) standards. (C) Heat-inactivated His-AetF does not brominate L-tryptophan. (D) Functional His-tagged AetF brominates L-tryptophan to 5-bromotryptophan and 5,7-dibromotryptophan (5,7-Br_2-Trp; t_r 3.03 min), proving the tryptophan brominase activity of AetF in vitro. (E) Structures of the detected tryptophan variants (L-tryptophan, 5-bromotryptophan, and 5,7-dibromotryptophan).

metabolites (*45*, *46*) (table S10). Besides *aetA* and *aetF* from the *aet* cluster, bioinformatic analysis of the *A. hydrillicola* genome did not reveal any other homologs to known bacterial tryptophan halogenases organized in a biosynthesis gene cluster, which further supports their involvement in AETX biosynthesis. The presence of two distinct halogenases could explain the different substitution patterns of the two indole cores of the AETX molecule, which could be achieved by two or more subsequent (poly)halogenation reactions.

To test this hypothesis and provide initial mechanistic evidence for the role of the *aet* gene cluster in AETX biosynthesis, we assessed the activity of the putative halogenase AetF in in vitro experiments. Assays containing recombinant AetF and L-tryptophan as substrate showed the formation of two brominated products (Fig. 4, B to E). The high yield of recombinant AetF from *Escherichia coli* (fig. S36) allowed us to conduct a larger-scale in vitro assay with yields of both brominated products sufficient for isolation. Subsequent NMR analysis of the products unambiguously confirmed that AetF is capable of bromination of tryptophan at positions 5 and 7, which exactly matches the substitution pattern observed in one of the AETX indole substructures (figs. S39 to S47). Additional in vitro assays showed that indole is not a substrate of the enzyme, but 5,7-dibromoindole was obtained when 5-bromoindole was provided to AetF (fig. S38). On the basis of these data, we conclude that tryptophan is the primary substrate for AetF, as the enzyme is capable of introducing bromine into unsubstituted tryptophan. The additional AetF bromination activity on 5-bromoindole could result from its steric similarity to 5-bromotryptophan, which we found to be an intermediate of the stepwise bromination of tryptophan. Further in vitro assays with AetA and structural analyses of its products are needed to fully elucidate the chronology of the individual halogenation reactions. The functions of the remaining predicted genes have yet to be experimentally confirmed. Hypothetically, 5-bromotryptophan produced by AetF (or a later, more complex intermediate) could be used for subsequent additional bromination by AetA to generate the 2,3,5-tribrominated indole substructure. Concerning the possible function of AetB, it is noteworthy that cytochrome P450-like proteins have been previously found to play a crucial role in carbonitrile formation in natural products (*47*).

Bioactivity characterization of AETX

Initial HPLC microfractionation of an *A. hydrillicola–H. verticillata* assemblage extract revealed that the microfraction containing the pentabrominated compound is toxic to water fleas (*Ceriodaphnia dubia*), nematodes (*Caenorhabditis elegans*), and larval zebrafish (*Danio rerio*). This again strengthened our hypothesis that this compound might be the toxin responsible for causing VM. After preparative isolation of AETX, we determined the lethal dose for 50% of the population (LC_{50}) of the purified compound for *C. elegans* (40 nM) and larval *D. rerio* (275 nM) (fig. S48 and tables S12 to S14). At sublethal concentrations (>10 nM), an inhibitory effect of AETX on reproduction could be observed for *C. elegans*. Exposure to

purified AETX induced neurological seizure-like behavior in larval zebrafish (movie S2), consistent with behavior seen when larva were exposed to AETX containing *H. verticillata*–*A. hydrillicola* biomass extract that had been confirmed to cause VM lesions in the chicken bioassay. Neurological behaviors observed in zebrafish included twitching, gulping, full-body convulsions, loss of equilibrium, bunching toward the edge of the treatment dishes, rapid pectoral fin movements, and lack of escape response when stimulated with a probe.

AETX causes VM

To test whether AETX was the cause of VM, we used an avian bioassay (*3*, *9*, *15*, *19*, *21*). Specific pathogen-free leghorn chickens (*Gallus gallus*) were gavaged with either a suspension of purified AETX (15 mg per kilogram of body weight), AETX containing *H. verticillata*–*A. hydrillicola* extract as positive control (HTX; 4 mg of AETX per kilogram of body weight), or solvent control [10% dimethyl sulfoxide (DMSO) in deionized water]. All birds appeared normal on physical and neurological exams before the start of the study. During the short duration of the study, chickens did not exhibit pronounced clinical signs of VM in any treatment group (four doses over the course of 8 days). However, histological results revealed that chickens in the HTX (N = 3) and purified AETX (N = 3) treatment groups developed extensive vacuolization throughout the white matter of the brainstem, optic tectum, and cerebellum, whereas no lesions were documented in carrier solvent control chickens (N = 2) (Fig. 5A). Transmission electron microscopy of the optic tectum of birds from both the HTX and AETX groups revealed numerous vacuoles delimited by myelin laminae that had split at the intraperiod line, confirming the diagnosis of VM (Fig. 5B). Thus, we could confirm that AETX is the causative agent of VM.

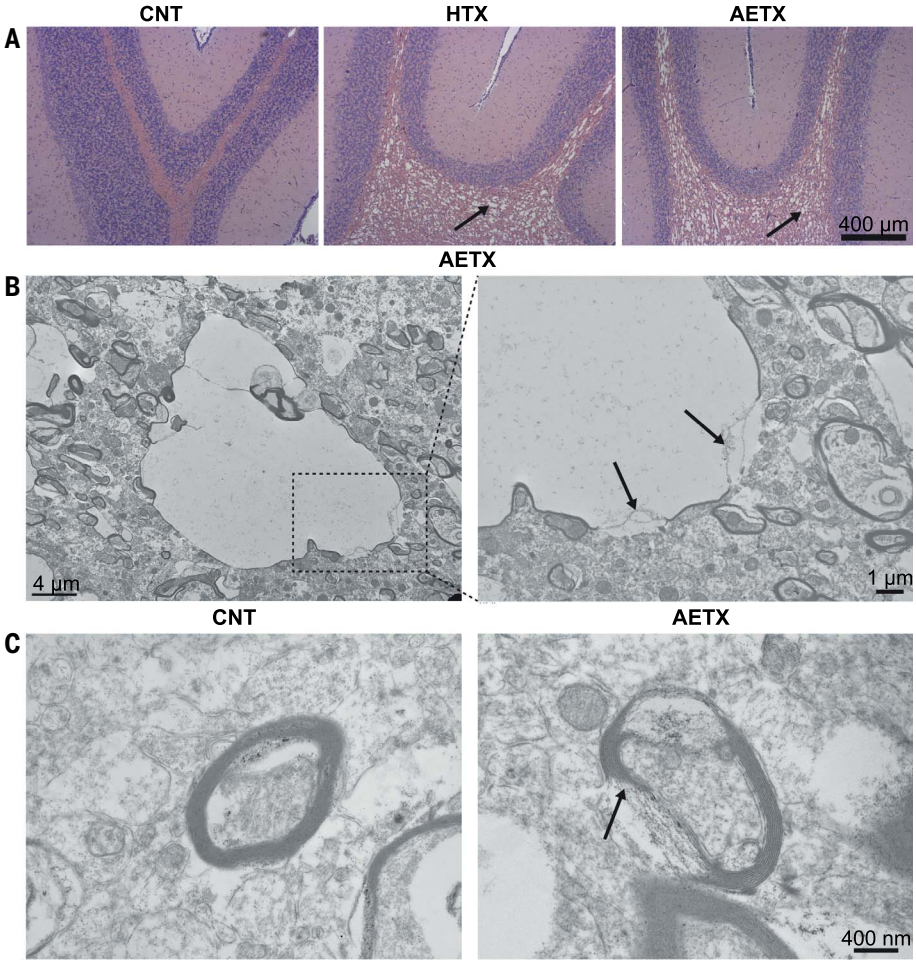

Fig. 5. Light and transmission electron microscopy of brain tissue from chickens exposed to AETX and controls confirm that AETX is causing VM. (**A**) Histologic sections of cerebellum with hematoxylin and eosin stain for solvent control (CNT), aetokthonotoxin containing *H. verticillata* extract (HTX), and pure aetokthonotoxin treatments (AETX). Chickens exposed to HTX and AETX treatments had widespread vacuolization of the white matter myelin tracts compared with controls (arrows). (**B**) Numerous vacuoles delimited by myelin laminae that had split at the intraperiod line (arrows) were observed, confirming the diagnosis of VM in both AETX (shown here) and HTX treatment groups. (**C**) Image of solvent control oligodendrocyte (left) and of oligodendrocyte bursting open (right) because of intramyelenic edema when treated with AETX (arrow). Transmission electron micrographs were taken of the optic tectum of test animals.

Conclusions

The causative agent of VM has eluded scientists for >25 years. Our discovery that VM is induced by a pentabrominated biindole alkaloid produced by an epiphytic cyanobacterium expands the role of cyanobacteria as potentially dangerous toxin producers. Although harmful blooms of planktonic algae have been shown to extensively alter ecosystems, our findings warrant further research into the potential toxins produced by epiphytic and benthic species. Our in vitro cultivation experiments show that the biosynthesis of AETX depends on bromide availability and that physical stressors (e.g., temperature and agitation) enhance production. We need to understand the complex environmental factors that affect the distribution and toxicity of *A. hydrillicola*. Further investigation is also needed on bioavailability of bromide from natural (i.e., geologic origin) and anthropogenic sources (e.g., power plants, fungicides, and gasoline additives) and how they influence AETX production.

Dense infestations of submerged aquatic plants can be managed using chemical, biological, or physical controls. Notably, herbicides containing bromide as counterions (e.g., diquat dibromide) are currently used to combat the spread of invasive submerged aquatic plants, including *H. verticillata*. Benefits and risks of using any bromide-containing chemical control agents within VM reservoir watersheds need to be reassessed. Biological controls, especially triploid grass carp (*Ctenopharyngodon idella*), can remove excess plants, but fishery managers are reluctant to stock these fish for vegetation control because of concerns of overstocking and escape. These triploid sterile grass carp have been effective in eliminating *H. verticillata* in VM reservoirs with a history of eagle deaths (e.g., DeGray Lake, Arkansas, and J. Strom Thurmond Reservoir, Georgia and South Carolina) (*21*, *48*). Physical control of *H. verticillata* is often ineffective because of its ability to reproduce from fragments, tubors, and turions released during mechanical harvesting. Site-specific, integrated management solutions have emerged from more than 50 years of research into invasive-plant control by the Florida Fish and Wildlife Conservation Commission, the U.S. Army Corps of Engineers, and the University of Florida (*49*). Controlling *H. verticillata* in VM reservoirs with toxic *A. hydrillicola* is critical to protect aquatic species and their consumers, but doing so

presents additional complexity and risks. Future vegetation management should prioritize overall ecosystem health and affordable, long-term solutions to control and prevent the expansion of *H. verticillata* and *A. hydrillicola*.

Wildlife are exposed to AETX in their environment for a substantially longer time period compared with the animals in our short-term bioassays. Raptors breeding on VM reservoirs and juveniles returning to their natal territories extend the disease risk over generations. Considering the lipophilicity of AETX and the demonstration of trophic transfer, there is potential for bioaccumulation. The confirmation that additional herbivorous aquatic taxa in VM food webs are susceptible to AETX (i.e., birds, fish, amphibians, reptiles, and invertebrates) also increases the potential risk to their consumers. Because we do not yet fully understand how *A. hydrillicola* and its neurotoxin affect the complex aquatic ecosytems, increased monitoring and public awareness should be implemented for *A. hydrillicola* and AETX. A toxin produced by cyanobacteria that colonize a highly invasive plant, which has the capacity to affect diverse animal phyla, should not be underestimated in its potential impact on our environment. Analytical methods described should facilitate expanded monitoring of AETX in aquatic environments and animal tissues. Moreover, there remains a critical need for research on mammalian susceptibility to VM and on human health risks from the consumption of fish and waterbirds from VM reservoirs.

Materials and methods summary

H. verticillata samples collected from numerous watersheds from 2014 to 2020 were screened for the presence of *A. hydrillicola*. After isolation of *A. hydrillicola* from environmental samples and adaptation to laboratory conditions, the cyanobacterium was cultivated in BG11 medium with or without the addition of potassium bromide. *H. verticillata* leaves colonized with *A. hydrillicola* were analyzed using fluorescence microscopy as well as AP-MALDI-MSI (9-AA as matrix in negative-ionization mode; lateral resolution, 10 μm). Environmental bromide and bromine concentrations in *H. verticillata*, sediment, and water samples were analyzed by x-ray fluorescence spectroscopy and ion chromatography. The structure of AETX was elucidated by NMR spectroscopy, high-resolution tandem mass spectrometry, infrared spectroscopy, and x-ray crystallography after isolation of the compound using flash chromatography, semi-preparative HPLC, and recrystallization. The genome of *A. hydrillicola* was amplified from single filaments using multiple displacement amplification and then sequenced using the Illumina MiSeq platform. The putative AETX biosynthetic gene cluster was identified by BLASTp searches for bacterial halogenases against the *A. hydrillicola* genome. The halogenase AetF was heterologously expressed in *E. coli* and then purified. Biochemical assays to characterize its activity used tryptophans and indoles as substrates. Reaction products were structurally characterized by HPLC-MS and NMR spectroscopy. *A. hydrillicola*–*H. verticillata* extract fractions and pure AETX were tested for activity on *C. dubia*, *D. rerio*, *C. elegans*, and *G. gallus*. Bioassays on *D. rerio* and *G. gallus* were performed in accordance with the National Insitutes of Health Guide for the Care and Use of Laboratory Animals and followed protocol A2017 11-007-Y1-A0, which was reviewed, approved, and overseen by the University of Georgia Institutional Animal Care and Use Committee. VM occurrence in treated birds was confirmed by analysis of the white matter of their optic lobe using light microscopy and transmission electron microscopy. Tissues of deceased wild birds were extracted and analyzed for AETX by HPLC-MS. A full description of the materials and methods used in this study is provided in the supplementary materials.

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The other authors declare no competing interests. **Data and materials availability:** NMR and MS raw data are available at Figshare (*50*). X-ray data and models are available at the Cambridge Crystallographic Data Centre under accession no. CCDC-2018827. The whole-genome assemblies (Whole Genome Shotgun projects) of two *A. hydrillicola* strains, CCALA 1050 and Thurmond2011, have been deposited at DDBJ/ENA/GenBank under the accession nos. JAALHA000000000 and JAAKGC000000000, respectively. The versions described in this paper are JAALHA010000000 and JAAKGC010000000. The sequence of the putative AETX biosynthetic gene cluster can be found at DDBJ/ENA/GenBank under the accession no. MT225528. All other data are available in the main text or the supplementary materials.

SUPPLEMENTARY MATERIALS

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Science

Hunting the eagle killer: A cyanobacterial neurotoxin causes vacuolar myelinopathy

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A lethal combination

Although many human activities have clear negative effects on the natural world, there are also unforeseen consequences. Bald eagle mass death events in the southeastern United States may be one such downstream effect of human activity. After considerable effort, Breinlinger *et al.* identified the cause of these events as an insidious combination of factors. Colonization of waterways by an invasive, introduced plant provided a substrate for the growth of a previously unidentified cyanobacterium. Exposure of this cyanobacterium to bromide, typically anthropogenic in origin, resulted in the production of a neurotoxin that both causes neuropathy in animals that prey on the plants and also bioaccumulates to kill predators such as bald eagles.

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