New Fish-Killing Alga in Coastal Delaware Produces Neurotoxins

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Ten fish mortality events, involving primarily Atlantic menhaden, occurred from early July through September 2000 in several bays and creeks in Delaware, USA. Two events involved large mortalities estimated at 1–2.5 million fish in Bald Eagle Creek, Rehoboth Bay. Samples from Indian Inlet (Bethany Beach), open to the Atlantic, as well as from an enclosed area of massive fish kills at nearby Bald Eagle Creek and Torque Canal were collected and sent to our laboratory for analysis. Microscopic examination of samples from the fish kill site revealed the presence of a single-cell Raphidophyte alga *Chattonella cf. verruculosa* at a maximum density of 1.04×10^7 cells/L. Naturally occurring brevetoxins were also detected in the bloom samples. Besides the *Chattonella* species, no other known brevetoxin-producing phytoplankton were present. Chromatographic, immunochemical, and spectroscopic analyses confirmed the presence of brevetoxin PbTx-2, and PbTx-3 and -9 were confirmed by chromatographic and immunochemical analyses. This is the first confirmed report in the United States of brevetoxins associated with an indigenous bloom in temperate Atlantic estuarine waters and of *C. cf. verruculosa* as a resident toxic organism implicated in fish kills in this area. The bloom of *Chattonella* continued throughout September and eventually declined in October. By the end of October *C. cf. verruculosa* was no longer seen, nor was toxin measurable in the surface waters. The results affirm that to avoid deleterious impacts on human and ecosystem health, increased monitoring is needed for brevetoxins and organism(s) producing them, even in areas previously thought to be unaffected. *Key words:* **brevetoxins, *Chattonella cf. verruculosa,* Delaware, fish kills, harmful agal blooms.** *Environ Health Perspect* **110:465–470 (2002). [Online 1 April 2002]**
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Harmful algal blooms continue to be a focus of attention in virtually all coastal regions of the United States (*1–2*). Scientists have clearly demonstrated microalgae associated with very specific symptoms of human poisoning. These symptoms, often associated with the consumption of toxic seafood, are known as paralytic shellfish poisoning (saxitoxin ingestion), neurotoxic shellfish poisoning (exposure to brevetoxin), diarrheic shellfish poisoning (ingestion of okadaic acid), amnesic shellfish poisoning (ASP; domoic acid ingestion), and ciguatera fish poisoning (with ciguatoxin ingestion), respectively (*3–7*). These syndromes are common to temperate, subtropical, and tropical environments where the microalgae associated with each toxin are found.

New harmful algae continue to be identified in expanding geographic areas, the most recent being the heterotrophic dinoflagellates *Pfiesteria piscicida* and *P. shumwayae* (*8,9*). These lesion-causing fish killers are reported to produce a narcotizing material that, when airborne, elicits human neurologic impairment and reversible memory deficits (*10*). Discoveries of toxic blooms in regions previously thought to be free of toxic phytoplankton have increased markedly over the past three decades, further supporting the notion of a definite global spread and increase in occurrence (*11,12*). Along with the elevated frequency, organisms thought previously to be nontoxic are being demonstrated to produce potent toxins. Notable recent events of this type are the diatom blooms of *Pseudo-nitzschia multiseries* on the Canadian Atlantic coast in 1987 and *P. australis* on the California coast in 1991, 1998, and 1999, causing human ASP and marine animal deaths, respectively (*7,13,14*).

Fish kills have long been an indicator of the presence of toxic phytoplankton species. During Florida red tides, dead fish are often associated with a bloom of the dinoflagellate *Karenia brevis* [= *Gymnodinium breve* (*15*)]. This organism produces a series of 10 polyether toxins (brevetoxins); the three most abundant forms are shown in Figure 1 (*16–20*). Brevetoxins bind with high affinity to site five of voltage-gated sodium channels in nerves, producing membrane depolarization (*21*,*22*). In addition to the historical record of dead fish in Florida, recent episodes of *K. brevis* blooms caused the deaths of 149 West Indian manatees in 1998 (*23*) and bottlenose dolphins in 1999. Human exposure to brevetoxins can produce neurologic symptoms including dizziness, numbness, muscle spasms, respiratory failure, and in extreme cases, death. Humans are affected annually in Florida by the inhalation of airborne brevetoxins (*24*,*25*) and occasionally by the ingestion of contaminated shellfish (*26,27*).

An unusual event involving *K. brevis* occurred along the southeastern North Carolina coast during September 1987. A bloom of this organism originated on the southwest Florida shelf and was entrained by the loop current in the Gulf of Mexico. The Gulf Stream transported the bloom from the west of Florida around the peninsula northward to southern North Carolina (*28*), necessitating closure of shellfish beds. No dead fish were observed with this bloom, but shellfish beds became toxic and remained closed for several months. Although *K. brevis* is known to occur at low abundances in the Gulf Stream, the 1987 event was exceptional in that the stream meandered so that an anomalous thread of warm water veered and brought bloom organisms toward the shore in Carteret County, North Carolina (*29*). This event has not occurred again since 1987. Thermal satellite imagery for the 1987 bloom confirmed the unusual transport, but examination of imagery from August–September 2000 did not support a similar event happening much farther north in coastal and estuarine Delaware (*30*).

From July through August 2000, personnel of the Delaware Department of Natural Resources and Environmental Control (DNREC) and local boaters, including members of the citizen environmental group Surfrider Foundation, recorded 10 separate fish kills in inland bays of Delaware, USA (Figure 2). Between 1.0 to 2.5 million Atlantic menhaden were found dead in inland areas of Bald Eagle Creek/Torquay Canal, Arnell and Pepper Creeks, and Indian River Acres (*31*). Most fish were free of

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J. Lancaster, C. Tomas, A. Weidner, S. Campbell, and T. Neely provided technical assistance. Seawater samples from the bloom area were provided by staff of the Delaware Department of Natural Resources and Environmental Control, members of the Surfrider Foundation, and our personnel from the Center for Marine Science. J. Wright provided laboratory space and editorial comments. Mass spectral analyses were contracted to an independent laboratory (K. Schey at the Medical University of South Carolina).

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lesions, and molecular probes for *Pfiesteria* failed to detect the presence of this species. The cause of death for Bald Eagle Creek was attributed, presumably, to low dissolved oxygen measured during the day at the site of the dead fish. However, water samples that we examined had a substantial phytoplankton bloom dominated by *Chattonella cf. verruculosa* that was previously reported to kill fish elsewhere (*32,33*). The presence of this alga during the fish mortality events and suggestion in the literature that some raphidophytes produce brevetoxins (*34,35*) prompted further investigation. Two water samples taken within 0.5 m of the surface were collected on 17 August, a day before the last fish kill, and the following day (18 August) at the time of a fish kill at Bald Eagle Creek. Each sample was forwarded via overnight courier to the Center for Marine Science (CMS) for examination. Both samples were examined within 20 hr of collection. Subsequent live samples were then taken by personnel from DNREC, Surfrider, and CMS researchers. The complete set of samples (Table 1) observed included archived unpreserved and preserved samples from the Bald Eagle area and Arnell Creek provided by DNREC (E. Humphries); fresh, live whole-water samples as well as sediments were also collected by DNREC (B. Anderson), Surfrider, and our CMS staff during September–December in Rehoboth Bay. All live samples not collected by our staff were shipped overnight and examined or analyzed the day after collection. An independent study using molecular probes specifically designed for detecting *P. piscicida, P. shumwayae*, and *K. brevis* did not detect the presence of these toxic species from the DNREC samples collected during the period of fish mortalities (*36*).

One water sample from an open Atlantic beach (Bethany Beach) was collected on 11 August and kept refrigerated. We received this sample after ≥ 2 weeks of cold storage in the dark. Observations on this sample were also made but remain tentative because during the prolonged storage changes had occurred. In reporting this event for the inland bays, we document the concurrent presence of high concentrations of brevetoxins, high concentrations of a Raphidophyte toxic species, and dead fish.

Materials and Methods

Chemicals. All chemicals were from Fisher Scientific (Pittsburgh, PA) unless otherwise noted.

Field samples. The initial field samples for Bald Eagle Creek and Torquay Canal (Figure 2), where massive fish kills occurred, were collected by N. Carter, coordinator of the Delaware chapter of Surfrider Foundation, a citizen environmental group interested in investigating the cause of fish kills. Surface water samples were taken at this site on 27 August, where a massive fish kill had occurred 5 days previously. Two 100-mL sterile bottles were used to collect the fresh surface (upper 0.5 m) water sample from Bald Eagle Creek and sent to us. In addition, an unpreserved sample taken previously on 11 August at the Atlantic Bethany Beach site by a private citizen (W. Winkler) and stored under refrigeration was forwarded to us. Both were sent via overnight mail. Upon receipt and examination of the Bald Eagle Creek sample, we asked the Surfrider Foundation personnel to obtain a larger sample. Two 1-L surface water samples were taken on 28 August at Bald Eagle Creek, during a time of the last fish kill. The unpreserved samples of subsurface water were taken directly below dead or dying menhaden, placed in sterile bottles, and shipped via overnight courier to our laboratory. These samples were used for microscopic examination and for the extraction, detection, and characterization of the toxins. After complete analysis of these sample, we contacted DNREC and with their cooperation

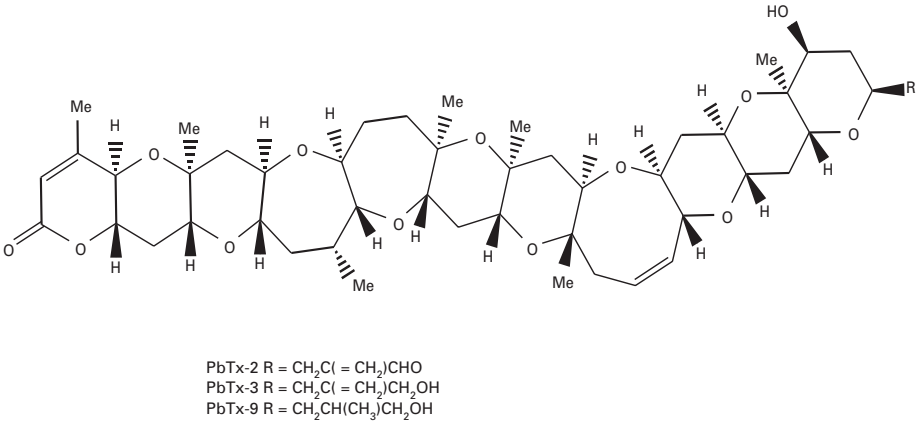

Figure 1. Structures of the three most abundant brevetoxins previously isolated from *K. brevis* (= *Gymnodinium breve).*

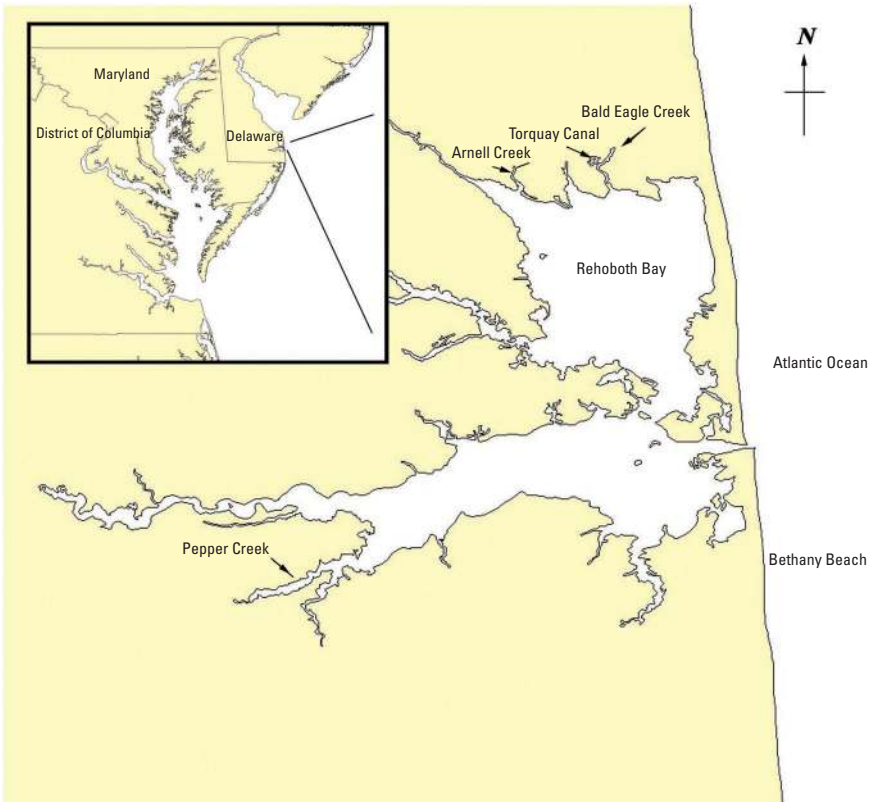

Figure 2. Area of Delaware where August 2000 blooms were observed and samples taken. Torque Canal is a subsection of Bald Eagle Creek.

received additional samples from the Bald Eagle and Torquay Canal sites where fish kills had occurred. We also received unpreserved and preserved archived samples from periods earlier in August and July of stations where fish kills were observed. Samples taken by our local (CMS) staff, Surfrider Foundation, and DNREC were received from September through November, allowing us to follow the progression of the phytoplankton blooms and toxin in seawater.

Microscopic observations. Upon receipt, all water samples were immediately examined with a Nikon Diaphot inverted microscope equipped with a Nikon N2000 and Optronix digital camera (Nikon, Melville, NY) and a Zeiss Photomicroscope III (Zeiss, Thornwood, NY). Observations for species composition and abundance were made under brightfield, epifluorescence, and differential interference contrast. Samples were observed live because phytoflagellates are particularly sensitive to fixatives, and observations of color, motion, and general morphology are essential for proper identification. A portion of each sample was preserved in 2% glutaraldehyde at pH 7.9 and each was concentrated using Üttermohl settling chambers (Hydrophobias, Kiel, Germany) and standard counting techniques described by Hasle (*37*). Observations as to the identification and abundance of species were made on live and fixed settled samples for those species that were easily preserved.

Isolation of brevetoxins from water samples. Samples from the bloom sites were extracted three times with ethyl acetate (1:2, ethyl acetate:water). The extracts were combined and reduced *in vacuo*. Preliminary removal of nontoxic materials including pigments was achieved by solid-phase extraction using a Supelco Supelclean (silica LC 1 g) column (Supelco, Bellefonte, PA). The sample was adsorbed to the normal-phase column and washed with dichloromenthane followed by toxin elution using dichloromethane: methanol (97.5:2.5). The toxin fraction was then subjected to reverse-phase solid-phase extraction (Supelco Supelclean C_{18} silica). Toxin adsorbed to the column in this phase was washed using acetonitrile: water (40:60) and eluted with a mobile phase of acetonitrile: water (85:15). The toxin-containing fraction was then separated into its components using reverse-phase HPLC (Bio-Rad 1330 dual piston pump, reverse-phase Microsorb C_{18} 4.6 × 250 mm column; Varian, Walnut Creek, CA) with a mobile phase consisting of 80% aqueous acetonitrile (1.4 mL/min) and detection (V4 Ultraviolet detector; ISCO, Lincoln, NE) of the toxin at 215 nm. An automated fraction collector (Isco Foxy Jr.) collected fractions from the HPLC separation at 30-sec intervals. At each step of the isolation procedure (i.e., crude extract, partially purified fractions from the extraction columns, and purified fraction from the HPLC), we tested a portion of the sample using a brevetoxin-specific enzyme-linked immunosorbent assay (ELISA). Positive fractions were compared to PbTx-2, -3, -9 standards (CalBiochem/Nova Biochem, La Jolla, CA). To prevent sample contamination, we performed all preparations and analyses in a new laboratory using equipment and supplies that were toxin-free.

ELISA methodology. The ELISA used in this study is a multistep competitive biotin-strepavidin coupled immunoperoxidase technique recently refined in our laboratory (*38,39*). The method is conducted in flat-bottomed 96-well polystyrene immunoplates. Step 1 of this assay involves preparation of sensitized immunoplates produced by incubating plates for 1 hr with 100 µL of PbTx-3–bovine serum albumin (BSA) conjugate (250 ng/mL in phosphate-buffered saline) as a primary adsorbent; in a separate vessel goat anti-brevetoxin serum [1:2,000 final dilution, gαPbTx (*38*)] was mixed and incubated 1 hr with equal volumes of serial dilutions ($\log_2$ dilutions from 1:1 to 1:64) of the seawater unknowns as potential inhibitors. All incubations of this initial step were conducted at room temperature. During step 2, 0.1 mL of antibody–seawater extract mixtures were placed into individual wells of 96-well microtiter plates and incubated for 1 hr at room temperature. In step 3, after incubation, plates were emptied and washed three times with BSA, and the antibodies associated with the plates were visualized with a commercial three-step amplification method using rabbit antigoat biotinylated secondary antibody (1/10,000 final dilution) followed by streptavidin-horseradish peroxidase conjugate (1/1,000 final dilution) and *o*-phenylene diamine substrate (Sigma Chemical, St. Louis, MO). Absorbance in each well was measured at 492 nm after 15 min of incubation. The differences in absorbance from tests with the inhibitor and without the inhibitor (maximal signal) are referred to as B and B_o, respectively. All results are expressed as percent inhibition (100%–% $B/B_o \times 100$). We used the ELISA at various stages of purifying water samples to guide us as to those components having brevetoxin epitopes. This process is called immunoassay-guided fractionation.

Spectroscopic determination of brevetoxins in the samples. We examined purified fractions of PbTx-2 from HPLC using nuclear magnetic resonance (NMR) spectroscopy. ^{1}H NMR spectra were run on a Bruker Avance DRX 400 MHz spectrometer (Bruker Biospin, Billerica, MA) with a 5-mm broadband probe. NMR spectra were recorded in deuterated chloroform and referenced to $CHCl_3$ (^{1}H δ 7.24). For the fish kill sample containing abundant purified PbTx-2, we analyzed samples by low-resolution electrospray, tandem mass spectrometry (MS-MS), and matrix-assisted laser desorption ionization (MALDI) mass spectra performed by the mass spectrometry facility at the Medical University of South Carolina.

Results

We examined samples for known toxic phytoplankton using light microscopy. Due to the age and cold storage of the 11 August sample (≥ 2 weeks old), we could identify no living phytoplankton and observed only cellular debris. Although we detected brevetoxin PbTx-3 by HPLC in this sample, encouraging further examination, its uncertain history with potential changes in composition obviated the use of sample for further study. The

Table 1. Delaware Samples for *Chattonella*, August–November 2000.

Date	Location	Cells per liter	Toxin
11 August	Bethany Beach	No cells seen	—[a]
13 August	Bald Eagle Creek	2.33×10^5	Fixed sample
13 August	Torquay Canal	1.45×10^6	Fixed sample
27 August	Bald Eagle Creek	2.20×10^5	—[a]
28 August	Bald Eagle Creek	1.04×10^7	—[a]
28 August	Bald Eagle Creek	4.39×10^6	Fixed sample
28 August	Torquay Canal`	7.80×10^6	Fixed sample
06 September	Torquay Canal	1.72×10^6	Fixed sample
28 September	Bald Eagle Creek	1.45×10^6	Fixed Sample
29 September	Bald Eagle Creek 1	5.00×10^3	ND
29 September	Bald Eagle Creek 2	0.96×10^6	—[a]
29 September	Torquay Canal	1.20×10^6	—[a]
03 October	Bald Eagle Creek	7.09×10^5	—[a]
03 October	Torquay Canal	1.73×10^6	—[a]
12 October	Torquay Canal	1.72×10^3	ND
25 October	Bald Eagle Creek	No cells seen	ND
25 October	Torquay Canal	2.84×10^3	ND
11 November	Bald Eagle Creek	No cells seen	ND
11 November	Torquay Canal	No cells seen	ND

ND, no toxin detected. Fixed samples collected by DNREC and made available for cell counts.
[a]Brevetoxin detected.

17 August sample from Bald Eagle Creek, however, did have a substantial phytoplankton bloom dominated by a previously unrecorded Raphidophyte species *C. cf. verruculosa* (Figure 3).

A notable variation in the morphology of this species in samples throughout the study period required our taxonomic placement as the form *C. cf. verruculosa*. The major differences were observed in flagellar structure (length and orientation), cell shape varying from spherical to the typical pyriform shapes of other species of *Chattonella,* as well as a coloration dominated by green instead of the golden brown pigments of other marine raphidophytes. Details regarding the morphology, ultrastructure, and genetic sequence will be presented elsewhere (*40*). The cell density of *C. cf.* verruculosa in the 27 August sample was 10^5 cells/L (Table 1), and no other known toxic phytoplankton species were seen. A larger (2 L) seawater sample from Bald Eagle Creek collected on 28 August—the day of a massive fish kill—contained 1.04×10^7 cells/L of *C. cf. verruculosa* with few other species present. The presence of this flagellate accompanying the dead fish prompted further investigation.

Following microscopic examination and documentation, the whole water samples were tested using the ELISA assay, chemically extracted, and purified with HPLC. The ELISA screening indicated that the Bald Eagle Creek sample of 27 and 28 August, respectively, had 8 and 60 ng/L brevetoxins (Figure 4). Water samples taken from Gulf Stream offshore water (North Carolina) and from the Intracoastal Waterway site at CMS were used as controls and were treated in a manner identical to that used for the Delaware samples. These control samples were uniformly negative for *C. cf. verruculosa* and brevetoxins. The Bethany Beach sample had > 200 ng/L brevetoxins that proved to be all PbTx-3.

The symptoms of fish kills with few or no lesions and the notable absence of other toxic organisms like *Pfiesteria* led us to suspect known fish-killing toxins such as the brevetoxins. The Delaware Bay samples (i.e., two initial 100-mL as well as the 1-L volumes) all tested positive for brevetoxins by ELISA. Immunoassay-guided fractionation consisting of flash column chromatography and reverse-phase HPLC-UV led to the isolation of three compounds that cross-reacted with the antibodies in the ELISA and had HPLC retention times identical to standards of brevetoxins PbTx-2, -3, and -9 (Figure 5). The most abundant of these fractions was PbTx-2.

We performed mass spectrometry and nuclear magnetic resonance spectroscopy on the fraction with the highest concentration and identified as PbTx-2 by HPLC and ELISA. Electrospray ionization (ESI; Figure 6) and MALDI (Figure 7) mass spectrometry produced ion signals characteristic of those of PbTx-2, and ESI MS-MS had a fragmentation pattern that matched standard PbTx-2. The proton NMR spectrum (Figure 8) of the isolated material also matched published data for PbTx-2 (*19*), including clearly resolved resonances for all seven methyls, as well as for the aldehydic hydrogen and the five olefinic hydrogens. We isolated PbTx-2 (20 µg) as a white amorphous solid: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.00 (3H, d, *J* = 7.1), 1.48 (3H, s), 1.20 (3H, s), 1.27 (6H, s), 1.28 (3H, s), 1.94 (3H, s), 1.5-2.5 (m), 3.0-4.2 (m), 5.71 (1H, s), 5.76 (2H, m), 6.07 (1H, s), 6.30 (1H, s), 9.51 (1H, s); ESI MS-MS *m/z* 895.8 (100), 893.8 (35), 835.7 (65), 806.6 (15), 538.3 (25); MALDI-MS *m/z* 917.2 (70) (H, hydrogens; s, d, m, singlet, doublet, or multiplet in spectra; *J*, coupling constant in hertz).

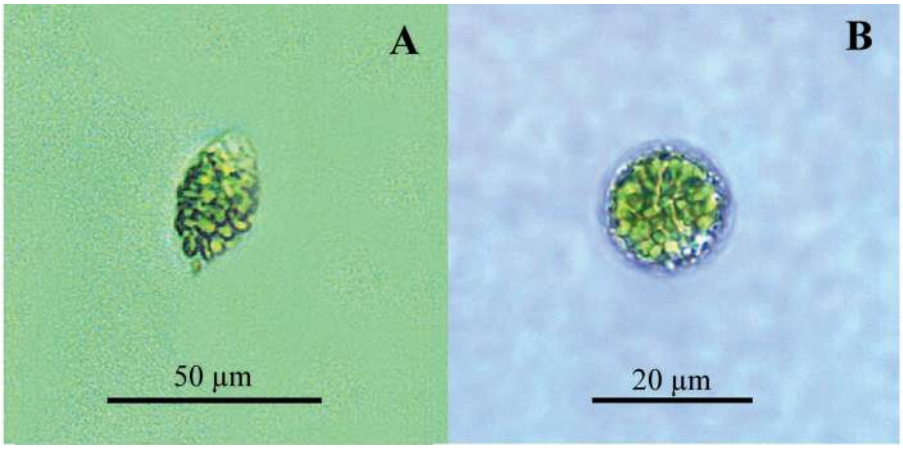

Figure 3. *C. cf. verruculosa* isolated from toxic bloom waters in Delaware on 28 and 29 August 2000. (*A*) Typical pyriform *Chattonella* morphology. (*B*) rounded, nonmotile cyst.

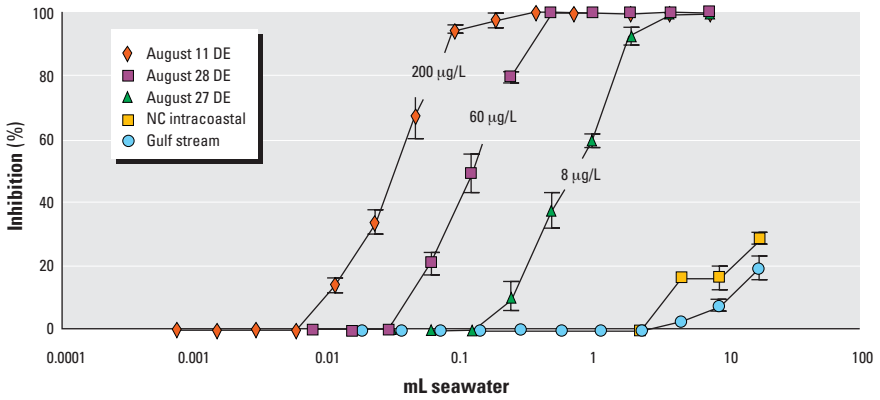

Figure 4. Competitive ELISA results for brevetoxins in seawater extracts from three Delaware (DE) sites. Triplicate assays (error bars = 1 SD) for August 11 Bethany Beach sample and August 27 and 28 samples from fish kill site at Bald Eagle Creek, Rehoboth Bay, were compared to control samples of bloom-free seawater from North Carolina Intracoastal and Gulf Stream sites.

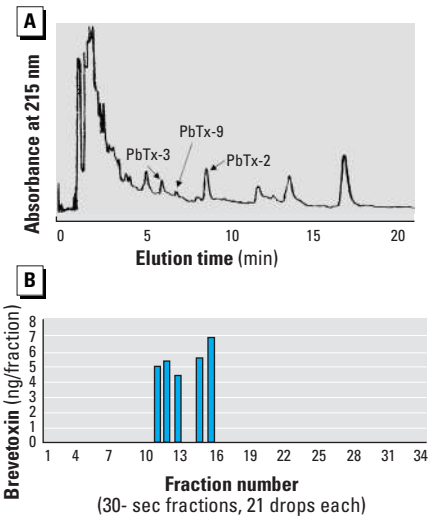

Figure 5. Immunoassay guided fractionation. Thirty fractions were collected and assayed. (*A*) Positive ELISAs were found only on those fractions associated with the HPLC peaks corresponding to brevetoxins PbTx-2, -3, and -9. All other fractions were negative. (*B*) ELISA assays taken from 30 HPLC fractions of extracts of samples from Delaware.

The combination of physical data, chromatographic properties, and immunochemical reactivity unequivocally confirmed the identity of the isolated material as PbTx-2. Lesser quantities of PbTx-3 and PbTx-9 were also identified from their chromatographic (HPLC) and immunochemical (ELISA) properties, although because of the small quantity, physical data such as mass and NMR spectra were not acquired. Using information derived from ELISA and *C. cf. verruculosa* cell densities, we calculated the concentration of brevetoxins in these cells to be approximately 6 pg/cell on 28 August, a day for which precise cell and toxin measurements were made at a fish kill event. This is similar to the brevetoxin concentration in *K. brevis* of 10–12 pg/cell (*41*).

We observed additional samples from Delaware. One series supplied by DNREC consisted of archived preserved and unpreserved samples (Table 1) dating to early August. The unpreserved samples were not useful for phytoplankton composition or toxin analyses because they had deteriorated due to long storage at room temperature. The fixed samples, however, clearly showed the development of the *Chattonella* bloom at Bald Eagle Creek/Torquay Canal region as well at Arnell Creek. Subsequent samples were taken during September and October (Table 1). The bloom of *C. cf. verruculosa* persisted throughout September, with densities near or at 10^6 cells/L, as did the presence of brevetoxins as measured by HPLC and ELISA. In late October, both cells and toxin diminished in these waters. By mid-November, no cells could be observed nor could toxins be detected in surface waters.

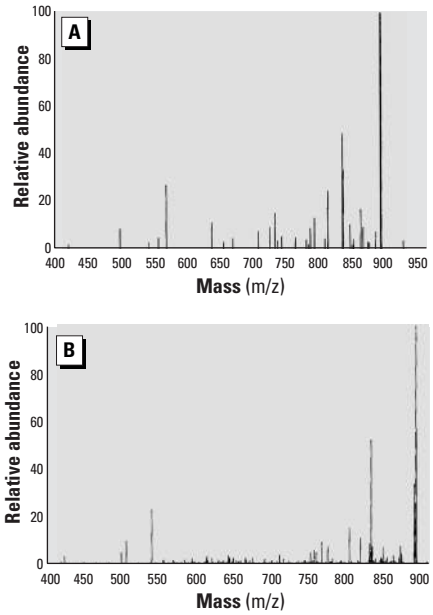

Figure 6. ESI-MS of (*A*) brevetoxin (PbTx-2) standard and HPLC-purified toxin from (*B*) Delaware sample. Identical fragmentation peaks (mass + 1 of 895) for PbTx-2 were found in both samples.

Discussion

All seawater samples collected from Delaware during August through mid-October from this bloom contained brevetoxins. Neither *K. brevis* nor any of the newly described potentially toxic "breve-like" species such as *K. brevisculcatum, K. selliformis, K. papilionacea*, or *K. mikimotoi* possibly containing brevetoxins were observed in any microscopic examination of this sample. There was no positive indication of *K. brevis* by the sensitive molecular probe designed specifically for this species when applied to any of the DNREC samples from the Delaware sites (*36*). Nor were other toxic species identified. *C. cf. verruculosa* was the most consistent and prominent organism observed in samples from the fish kill area, remaining at 10^7–10^6 cells/L throughout the August–October period.

Chattonella has been implicated in the mortality of cultured fish in Japan (*32,33*). Blooms of *Chattonella* were first reported from the North Sea in 1998, and *C. cf. verruculosa* killed 400 tons of cultured salmon in southern Norway as well as some wild fish (*42*). During May 2000, extensive blooms of *C. cf. verruculosa* were reported from the German Bight (North Sea) and western Denmark at densities of 8.7–11.0 × 10^6 cells/liter. These blooms were so expansive that they could be mapped by satellite imagery. During March 2001, a similar bloom of *C. aff. verruculosa* was observed off the southern coast of Norway at concentrations greater than when it killed 400,000 farmed fish (*43*). Again, losses to fish farmers were expected to be high. Blooms of *C. verruculosa* are documented for European and Scandinavian coastal waters, but this species to date was not reported from coastal U.S. waters. The findings reported here are the first sightings for *C. cf. verruculosa* for the United States.

Chattonella species kill fish likely by either of two mechanisms. One is the copious production of mucus on fish gills, causing physical blocking or clogging. The actual mechanism of the mucus accumulation is not well documented, but brevetoxins

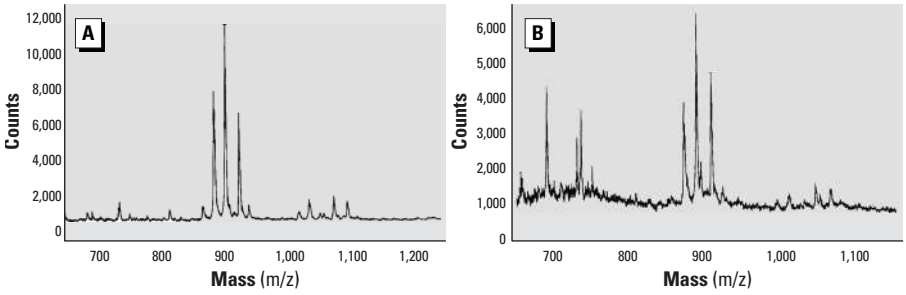

Figure 7. MALDI-MS indicating identical fragmentation patterns and major peaks (mass +1) at 895 for (*A*) PbTx-2 standard and (*B*) purified toxin from Deleware samples.

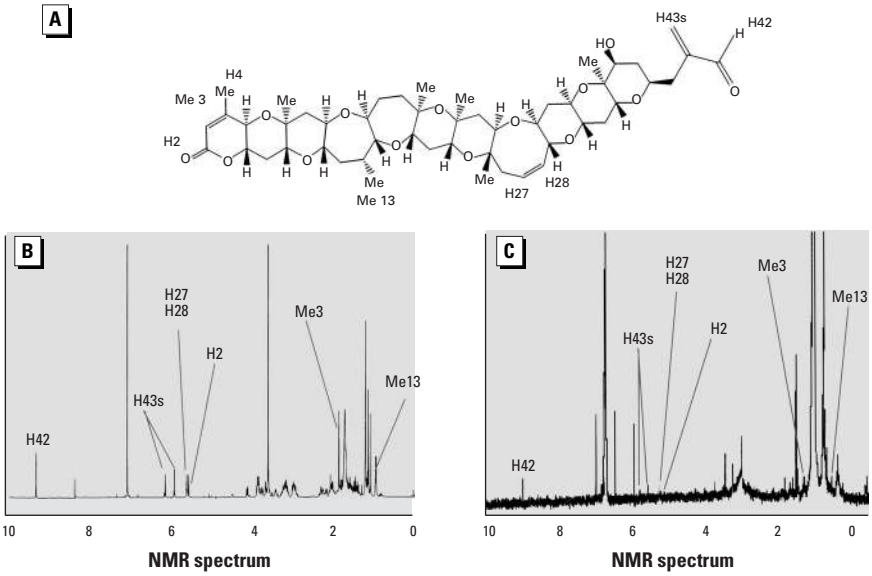

Figure 8. Structure of PbTx-2 (A) and proton NMR spectrum for PbTx-2 (*B*) and purified toxin from Delaware sample (*C*) showing clearly resolved resonances for all seven methyls as well as for aldehydic hydrogen and the five olefinic hydrogens.

have been described as muscarinic stimulants consistent with the observed mucous production (*44*). The second mode is the production of brevetoxin-like compounds from *C. marina* and *C. antiqua*. Although these compounds were not previously fully characterized, they were identified as comigrating on HPLC with brevetoxin standards having similar retention times and chemical characteristics, though no spectrometric analyses of these compounds were reported (*34,35*).

Given the previously reported toxicity of *C. verruculosa (32,33)*, the recent development of extensive blooms in coastal waters, the association of this genus with brevetoxin-like compounds, and the clear lack of known brevetoxin producers, we conclude that *C. cf. verruculosa* was the major causative agent in the Delaware fish mortalities and the source of the toxins. We are currently studying cultures of this organism to define further the nature and synthesis of the brevetoxins and mode of action in killing fish. To our knowledge, the present study is the first report of a confirmed brevetoxin-producing organism other than the Florida red tide dinoflagellate *K. brevis*. The *C. cf. verruculosa* found in Delaware is nearly as toxic as *K. brevis* (*41*) and orders of magnitude more toxic than other suspected brevetoxic *Chattonella* species (*34,35*). Furthermore, although the species closely resembles *C. verruculosa*, several variations in morphology observed warrant further examination. The biosynthesis of brevetoxins by an organism other than *K. brevis* will provide insights regarding the biologic origin and metabolic machinery possible for brevetoxin synthesis.

The significance of this study is severalfold. First, the discovery of a new organism synthesizing brevetoxins indicates that identification and cell counting of only a specific known dinoflagellate is insufficient to provide the sentinel warning system required for this particular group of toxins. Second, finding these toxins associated with *Chattonella* is particularly important, because *Chattonella* is a brackish-water species that can introduce ichthyotoxins far into estuaries, potentially contaminating fisheries (both fin and shellfish) and threatening public health in areas previously thought to be safe from these marine biotoxins. Third, the putative biosynthesis of brevetoxins by a temperate marine organism provides the vehicle for brevetoxins to enter temperate environments of the North Atlantic United States and create new monitoring problems there. The geographic distribution of this *Chattonella* is yet to be determined. Fourth, because of the toxicity of *C. cf. verruculosa* and its dominance in the present bloom, this species must be considered in subsequent episodes of estuarine fish kills.

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