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Novel contaminants identified in fish kills in the Red River watershed, 2011–2013

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Abstract

Provisional molecular weights and chemical formulas were assigned to 4 significant previously unidentified contaminants present during active fish kills in the Red River region of Oklahoma. The provisional identifications of these contaminants were determined using high-resolution liquid chromatography–time-of-flight mass spectrometry (LC-TOFMS), LC-Fourier transform ion cyclotron resonance mass spectrometry (LC-FTICRMS), and LC-ion trap mass spectrometry (LC-ITMS). Environmental water samples were extracted using a solid-phase extraction (SPE) method, and sediment samples were extracted using a modified sonication liquid extraction method. During screening of the samples, 2 major unknown chromatographic peaks were detected at m/z 624.3 and m/z 639.3. The peak at m/z 639.3 was firmly identified, through the use of an authentic standard, as a porphyrin, specifically chlorin-e6-trimethyl ester, with m/z 639.31735 ($M + H$)⁺ and molecular formula C₃₇H₄₃N₄O₆. The other major peak, at m/z 624.3 ($M + H$)⁺, was identified as an amide-containing porphyrin. It was discovered that the amide compound was an artifact created during the SPE process by reaction of ammonium hydroxide at 1 of 3 potential reaction sites on chlorin-e6-trimethyl ester. Other unique nontargeted chemicals were also detected and the importance of their identification is discussed.

Supplemental Data

The Supplemental Data are attached

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Data availability

Data and associated metadata will be available on publication of this article in the publically available USEPA Science Hub database.

INTRODUCTION

Fish kills are fairly common throughout the world [1–5] and can range from a few fish to millions dead 1–6. Shortly after the implementation of the United States Clean Water Act in 1972, the US Environmental Protection Agency (USEPA) issued a report that summarized a 15-yr history, from 1961 to 1975, of fish kills in the United States 6. Several historical incidents of more than 1 million dead fish occurred in the United States before implementation of the Clean Water Act. For example, in 1962 in San Diego Harbor, California, nearly 38 million fish were reported dead 6. In 1974, 4 separate fish kills were reported in the Back River near Essex, Maryland, with the largest fish kill numbering well over 47 million dead fish and the other 3 incidents totaling more than 53.6 million dead fish 6. However, decades after implementation of the Clean Water Act, US waters are cleaner and massive fish kills are not as prevalent. As an example, in 2014 only 51 fish kills were reported in Maryland, with mortality totals less than 28 500 fish, the second lowest total since 1984 5. Most fish kills that occur today are attributable to known environmental stressors such as hypoxic conditions caused by overgrowth of algae, extreme warm temperatures, lack of fresh water, and accidental releases of industrial and municipal pollutants 4, 5. Other fish kills can originate from exposure to naturally occurring environmental toxins produced by harmful algal blooms 2, 5, 7–13, and frequently no readily identifiable causes can be attributed to the fish kills 3, 5.

The research in the present study resulted from an emergency response request from the state of Oklahoma Department of Environmental Quality and USEPA Region 6 for investigation of 4 significant fish kills that occurred from 2011 to 2013 in a small segment of the Red River. The Red River, located in the midwestern part of the United States, has headwaters in the Texas Panhandle and flows for 917 km between the borders of Oklahoma and Texas before emptying into the Mississippi River. A USEPA report was prepared, per agency procedure, at that time and sent to Oklahoma Department of Environmental Quality and USEPA Region 6 with the initial findings surrounding the fish kill events 14. The present study versus the USEPA report provides new information on the potential source of the novel contaminants discovered during the fish kills and discussion of the advanced environmental analytical techniques used to detect and identify the contaminants.

MATERIALS AND METHODS

Sample collection, preservation, and storage

Environmental samples (water, sediment, and fish) were collected, using grab sampling procedures, from multiple sites along the Red River during active phases of the fish kills. Environmental background samples (water, sediment, and fish) were also collected during nonfish kill periods for quality control purposes. The Oklahoma Department of Environmental Quality collected all water and sediment samples according to their sampling protocols.

Water samples.—Water samples were collected in summer and fall 2011 during the active phases of fish kills I and II (Figure 1A). These samples were refrigerated and stored by Oklahoma Department of Environmental Quality at $< 4^{\circ}\text{C}$. Several months after fish kills I

and II, the archived water samples were sent to the USEPA Office of Research and Development's National Exposure Research Laboratory in Las Vegas, Nevada, to perform mass spectral screening analyses for nontargeted compounds. During fish kills III and IV (Figure 1B), in summer 2012 and winter 2013, respectively, Oklahoma Department of Environmental Quality collected water samples during the active phases of the fish kills, and the samples were shipped overnight to the USEPA's Office of Research and Development's National Exposure Research Laboratory in Las Vegas for chemical contaminant screening and analysis. The samples were refrigerated at $< 4^{\circ}\text{C}$ until extractions could occur, usually within 1 to 2 d of receipt.

Sediment samples—The Oklahoma Department of Environmental Quality collected sediment samples during, or shortly after, fish kill events over the span of 2011–2013. Other sediment samples were collected during nonevents upstream from the fish kill sites to be used as background quality control samples.

Fish samples—The Oklahoma Department of Environmental Quality and the Texas Department of Fish and Wildlife collected dead fish from the events for subsequent fish necropsies at their state laboratories.

Reagents and standards

Chlorin-e6-trimethyl ester ($> 95\%$ purity) was obtained from Frontier Scientific. The deuterated surrogates were acquired from either US Biological: d_3 -azithromycin ($\sim 90\%$ purity) and d_3 -clarithromycin ($\sim 90\%$ purity), or Santa Cruz Biotechnology: d_3 -azithromycin ($> 98\%$ purity) and d_3 -clarithromycin ($> 95\%$, $2\% \text{d}_0$), and d_5 -MDMA ($> 99\%$ purity) came from Cerilliant.

High-performance liquid chromatography (HPLC)-grade methanol was secured from multiple sources (e.g., Burdick and Jackson, EK Industries, and JT Baker). Reagent-grade acetic acid (glacial) and HPLC-grade methyl tert butyl ether (MTBE) were obtained from VWR. Reagent-grade formic acid ($96 + \%$) was acquired from Alfa Aesar. High-performance liquid chromatography-grade acetonitrile (ACN) came from Burdick and Jackson. American Chemical Society reagent-grade formic acid and ammonium hydroxide (28%) were obtained from Anachemia. Deionized water was produced on-site using a Nanopure filtration system (Barnstead). All reagents used were of highest purity as designated by the vendors.

A stock standard solution of chlorin-e6-trimethyl ester was prepared in HPLC-grade methanol from the neat compound and stored in darkness at $< 4^{\circ}\text{C}$. Spiking and calibration standards were prepared from the stock standard, diluted in methanol, and stored in darkness at $< 4^{\circ}\text{C}$ until use. Stock standards of the labeled standards were also prepared in HPLC-grade methanol and stored in darkness at $< 4^{\circ}\text{C}$. Spiking and calibration standards were prepared from the stock standards, diluted in methanol, and stored in darkness at $< 4^{\circ}\text{C}$ until use.

Extraction

Water samples—For water samples, 500 mL each were extracted using a solid-phase extraction (SPE) method and the standard operating procedure described in the USEPA report 14. Briefly, 3 g of NaCl were added to each water sample and then acidified to < pH 3; the samples were passed through conditioned Oasis MCX SPE cartridges at a 7-mL/min⁻¹ flow rate. The cartridges were dried and eluted with 5 mL of 90% MTBE/10% methanol, followed by 10 mL of 95% methanol/5% NH₄OH, at a flow rate of 1 mL/min⁻¹. The eluents were reduced to 0.5 mL and transferred to autosampler vials for liquid chromatography/mass spectrometry (LC/MS) analyses.

Sediment samples—Sediment samples were extracted using a simple manual liquid extraction procedure as described in the USEPA report 14. Briefly, 1 g of sediment was weighed into a small beaker, labeled internal standards were added, and the samples were placed under a hood to air dry. After drying, 5 mL of solvent (5% NH₄OH/95% MeOH) were added to each sample and sonicated for 5 min; after 5 min the solvent layer was transferred to capped, glass centrifuge vials. The 3 steps (addition, sonication, and removal of 5 mL of solvent [5% NH₄OH/95% MeOH]) were repeated 2 more times until approximately 15 mL of solvent had been collected. Vials (containing solvent layers) were placed in the centrifuge and spun for 5 min at 670 rpm. After the 5 min, the centrifuge was increased to 1675 rpm for an additional 5 min. Each sample was then rinsed with 4 mL of hexane that was removed and discarded. The supernatants were concentrated to 0.5 mL, and then transferred to autosampler vials for analysis by liquid chromatography–time-of-flight mass spectrometry (LC-TOFMS) or LC-ion trap mass spectrometry (LC-ITMS). The Supplemental Data describes the extraction procedures in greater detail.

LC/MS analysis

Briefly, mass spectrometric analyses were performed using the following complementary mass spectrometry techniques: a Varian 500MS LC-ITMS configured with a Varian 212-LC electrospray ion source; a Waters LCT Premier LC-TOFMS configured with an electrospray source and a Waters Acquity LC; and a Varian 901 LC-Fourier transform ion cyclotron resonance mass spectrometer (LC-FTICRMS) configured with an electrospray ion source and a Varian 240 LC. Further details are found in the Supplemental Data.

The total ion chromatograms of each sample were screened for prominent mass spectra that were not present in the blank control samples. Figure 2 shows an example of 2 extracted ion chromatograms of prominent mass spectra, m/z 639 and m/z 624, which were detected in the screened total ion chromatograms but not in the blank control samples. Those prominent mass spectra were further investigated for clues to structural information in the fragment ions that were produced by using LC-ITMS/MS with a collision energy of 60 to 80 V and a 2.5-V collision-induced dissociation voltage in the ion trap. Samples then were analyzed for accurate mass and chemical formula calculations using LC-TOFMS and LC-FTICRMS. For LC-TOFMS, the search criteria for chemical formulas were set at monoisotopic mass, even electrons, 3 peaks for i-FIT, tolerance 100.0 mDa/Double Bond Equivalents (DBE): min = -1.5, max = 50, C₀₋₅₀ H₀₋₁₀₀ N₀₋₂₀ O₀₋₂₀; and the mass resolutions were 25 ppm tolerance for the molecular formulas—and the same elemental composition search parameters for the

in-source collision-induced dissociation product ions. For LC-FTICRMS, the search criteria for chemical formulas were C₀₋₅₀ H₀₋₂₀₀ N₀₋₂₀ O₀₋₂₀; the mass resolutions were 100 000 for the molecular formulas and 50 000 for the in-source collision-induced dissociation product ions. In-source collision-induced dissociation was performed in the LC-TOFMS and LC-FTICRMS to help assign accurate mass and structural information to fragment ions initially detected by LC-ITMS.

RESULTS

Timelines and sampling

On 9 July 2011, a major fish kill (fish kill I) on the Red River near the confluence of Red Creek (a Red River tributary), Ketchum's Bluff, Oklahoma (circles 5, 6, and 7 in Figure 1A), was reported to the Oklahoma Department of Environmental Quality. During fish kill I, hundreds of large bottom-feeder fish (e.g., flathead catfish [*Pylodictis olivaris*], blue catfish [*Ictalurus furcatus*], and smallmouth buffalo fish [*Ictiobus bubalus*]) were observed to be dead, struggling, or actively dying. After 2 d, the fish kill I event was no longer observed to be occurring. Two months later, however, on 14 September 2011 another fish kill (fish kill II) was reported.

Fish kill II occurred farther south along the Red River, approximately 130 km downstream from Ketchum's Bluff near Lake Texoma (Figure 1A). Again it was observed that hundreds of large bottom-feeder fish only were being affected by an unknown toxin(s). Environmental authorities in the area (Oklahoma Department of Environmental Quality, USEPA, and the US Fish and Wildlife Service) hypothesized that the 2 fish kills were related, whereby the unknown toxicant(s) was traveling downstream from the first fish kill (9 July 2011) and causing fish mortality nearly 60 d later (14 September 2011). These phenomena of spatial and temporal delays in fish kills have previously been observed and reported in Spain 3. In the Spanish fish kill, mortalities were attributed to an unidentifiable toxic pollutant rather than a fish virus, hypoxia, or fluctuating temperature 3. In those events, a delay of 3 mo occurred between the start of the first observed fish kill and subsequent ones downstream near the Spain/Portugal border. Muñoz et al. 3 hypothesized that the unknown toxicant was absorbed onto suspended particulate matter that was churned up by going through hydroelectric dams, allowing for new exposures to occur and subsequent fish mortality.

The environmental samples from the first 2 fish kills (I and II) were measured initially in the field for routine chemical parameters (e.g., dissolved oxygen content, pH) and then sent to laboratories for routine chemical analyses to identify potential contaminants (e.g., volatile and semi-volatile organic compounds, toxic algae, and heavy metals) that could have contributed to the fish kills. The fish necropsies and tissue analyses performed on the dead fish showed evidence of liver damage (i.e., hepatic injury) in the fish. Nevertheless, routine field and laboratory testing results revealed no readily recognizable cause for the fish mortality. In January 2012, archived water and sediment samples were sent from Oklahoma Department of Environmental Quality to Office of Research and Development's National Exposure Research Laboratory in Las Vegas where nontargeted mass spectral screening analyses were performed for potential unknown contaminants.

In the following 2 yr, 2 more fish kills occurred in the same area of the Red River: fish kill III in June 2012 and fish kill IV in January 2013. Fish kill III occurred in the same location as fish kill I, at the confluence of Red Creek (Red River tributary) and the Red River near Ketchum's Bluff (Figure 1B). Fish kill IV occurred in Beaver Creek, another Red River tributary slightly upstream from Ketchum's Bluff (Figure 1B). The Oklahoma Department of Environmental Quality collected water and sediment samples while the fish were actively dying and shipped the samples overnight to Office of Research and Development's National Exposure Research Laboratory in Las Vegas for nontarget mass spectral screening analyses. The water samples were extracted immediately on receipt and sediment samples were processed at a later date.

Identification of major unknown chemical contaminants in water and sediment

Liquid chromatography-ion trap mass spectrometry and LC-TOFMS were used to screen the water and sediment samples collected during the fish kills. Two masses were detected in the total ion chromatograms (m/z 624.3 and m/z 639.3) of some of the samples collected from fish kills I, II, and IV, in which the extracted ion chromatogram masses had > 1 000 000 height counts (Figure 2). The observed masses initially were hypothesized to be mycotoxins because a review of the scientific literature found an article by Uhlig et al. 15 regarding a newly discovered mycotoxin, ergosedmine, that has a theoretical mass of m/z 624.3202 ($M + H$)⁺, and molecular formula of C₃₆H₄₂N₅O₅. One of the unknown extracted ion chromatogram peaks observed in the Office of Research and Development's National Exposure Research Laboratory in Las Vegas' laboratory using LC-TOFMS had a measured mass at m/z 624.3175 ($M + H$)⁺ with a molecular formula of C₃₆H₄₂N₅O₅. The difference between the measured accurate masses of the unknown in the fish kill water samples and Uhlig's ergosedmine 15 was 2.7 mmu (< 4 ppm). However, this prospective identification of ergosedmine in the fish kill water samples was later proven to be inconsistent with the accurate mass in-source collision-induced dissociation data.

During fish kill IV, enough water sample (2 L) was collected to allow for 2 sets of extractions, whereby the second set of extracts was sent to another laboratory (Ministry of the Environment, Ontario, Canada) for further high-resolution mass spectrometric analyses using LC-FTICRMS. The ultra-high-resolution LC-FTICRMS generated more accurate masses of m/z 639.31735 ($M + H$)⁺ and m/z 624.31794 ($M + H$)⁺, with molecular formulas of C₃₇H₄₃N₄O₆ and C₃₆H₄₂N₅O₅, respectively. For m/z 639.31735 there were 2 possible formulas within 1 ppm error. One formula (C₃₇H₄₃N₄O₆) was consistent with the in-source collision-induced dissociation product ions, whereas formula C₂₁H₄₃N₁₂O₁₁ was not consistent with the in-source collision-induced dissociation product ions. Although the molecular formulas did not change (with regard to the ones originally generated from the LC-TOFMS data), the more accurate masses of the in-source collision-induced dissociation product ions generated from LC-FTICRMS allowed for generation of more precise compositions of the unknown compound(s). The LC-FTICRMS in-source collision-induced dissociation of the accurate mass at m/z 639.31736 gave very stable high-mass ions that retained the nitrogen atoms (N₄). The proposed fragmentation schemes shown in Figure 3A include m/z 607 = [$M + H - CH_4O$]⁺, m/z 579 = [$M + H - C_2H_4O_2$]⁺, and m/z 566 = [$M + H - C_3H_5O_2$]⁺. Using the accurate mass fragments, and neutral losses to form fragments, we

reconstructed a correct chemical structure with a stable core containing the 4 nitrogen atoms, and it was discerned that the unknown detected at m/z 639.31736 ($M + H$)⁺ was not a mycotoxin. Instead the unknown at m/z 639.3 was identified as a porphyrin, specifically chlorin-e6-trimethyl ester (Figure 4), with a theoretical molecular weight of 638.31043 Da and a molecular formula of C₃₇H₄₂N₄O₆. A chemical formula search in ChemSpider, the United Kingdom Royal Society of Chemistry's free online chemical database, retrieved 50 chemicals of which chlorin-e6-trimethyl ester was the top in terms of number of references. To be indisputably certain that this was the correct identification, a standard of chlorin-e6-trimethyl ester was obtained from Frontier Scientific. Using the collision energy function of the ion trap, product ion mass spectra of the standard were obtained and compared with the product ion spectra detected at m/z 639.3 ($M + H$)⁺ in fish kill IV extracts. A positive confirmation was made through matching the exact mass of the molecular ion and fragment ions and the retention time of the standard to the unknown (Figure 3B).

The other extracted ion chromatogram mass present at < 1 000 000 height counts in fish kills I, II, and IV water and sediment samples was detected at m/z 624.3 ($M + H$)⁺. This compound was determined to be an artifact created during the SPE process. Tandem mass spectrometry (MS/MS) experiments showed that the compound observed at m/z 624.3 was chemically related to chlorin-e6-trimethyl ester. Through a series of simple experiments, 1 ng/μL of chlorin-e6-trimethyl ester was made up in methanol, 95% methanol/5% NH₄OH, and 95% ACN/5% NH₄OH stages: 1) no m/z 624.3 was present in the standard before the addition of the 95% methanol/5% NH₄OH, and 2) 1 h after the addition of the basic methanolic solution, an ion was detected in the solution at m/z 624.3 ($M + H$)⁺, thereby confirming that the compound detected at m/z 624.3 ($M + H$)⁺ was indeed an artifact accidentally created during the SPE process. Whereas the water samples were extracted at pH < 3, one of the eluting solvents was 95% methanol/5% NH₄OH, the use of which inadvertently created the m/z 624.3 ($M + H$)⁺ ion. A tentative identification was then assigned to m/z 624.3 ($M + H$)⁺ as an amide-containing porphyrin by comparing the product ion spectra from the LC-ITMS data, the LC-TOFMS data, and the LC-FTICRMS data. The molecular formula, as determined by LC-FTICRMS, was C₃₆H₄₂N₅O₅ at m/z 624.31794 ($M + H$)⁺. Three methyl ester groups on chlorin-e6-trimethyl ester are potential sites for amide formation, and the detection of 2 new LC-MS peaks corresponding to 2 major reaction products suggests that 2 of the 3 possible sites are more sterically accessible to ammonolysis-type reactions. Figure 5 presents one of the possible structures hypothesized as one of the isomeric amides formed by ammonolysis of chlorin-e6-trimethyl ester. Under the mild conditions of the extraction (i.e., 95% methanol/5% NH₄OH at ambient temperature), it is unlikely that a carboxylic acid can be converted to the corresponding amide. Reaction of ammonium hydroxide with a carboxylic acid will result in the formation of the ammonium salt. This salt must be heated and the water removed (e.g., via azeotropic distillation) to generate the corresponding amide. The fact that only 2 mono-amide analogues were detected at 2 different retention times suggests that the parent compound was the trimethyl ester having one methyl ester sterically hindered from ammonolysis.

Another extracted ion chromatogram mass was also present in fish kill IV at < 1 000 000 height counts at m/z 826.7 M⁺, with a doubly charged ion at m/z 413.8 M⁺². This unknown compound had not been observed in any of the other water samples, and this compound

eluted earlier in the total ion chromatogram before m/z 624.3 and m/z 639.3. This compound was tentatively assigned the chemical formula $C_{46}H_{94}N_6O_6$, with an accurate mass of m/z 826.72275 (M^+) and a doubly charged ion at m/z 413.36039 (M^{+2}). From the mass spectra obtained, this chemical has been identified as belonging to the chemical class of polyamino compounds. Using the accurate mass, m/z 826.72275 (M^+), provided by LC-FTICRMS, and a search of relevant chemical databases (e.g., ChemSpider), only one structure was tentatively identified: N,N,N,N',N',N'-hexamethyl-4,20,27,43-tetraoxo-3,44-dioxa-6,19,28,41-tetraazaahexatetracontane-1,46-diaminium, with a theoretical monoisotopic mass of 826.722412 Da. No commercial chemical standard was available for confirmation. The early retention time was consistent with an ionic compound having little retention on the LC column. However, this particular diquatery compound seems to be an unlikely match to the LC-MS data because of a discrepancy between its structure and the LC-TOFMS collision-induced dissociation fragmentation pattern: it lacked the expected abundant fragment ions related to the trimethylamine neutral losses 16, while exhibiting sequential ammonia losses (m/z 809 $\rightarrow$ m/z 792; m/z 749 $\rightarrow$ m/z 732) characteristic of primary amines or amides (Table 1).

Water samples from fish kill III showed no evidence of m/z 624.3 and m/z 639.3. Nevertheless, 2 very distinctive and large chromatographic peaks were detected at m/z 562.4 ($M + H$)⁺ and m/z 564.4 ($M + H$)⁺. The fish kill III samples were then screened using LC-TOFMS, and the chemical formulas were determined for the masses detected at m/z 562.4 ($M + H$)⁺ and m/z 564.4 ($M + H$)⁺ to be $C_{33}H_{48}N_5O_3$ and $C_{33}H_{50}N_5O_3$, respectively. At this time, no potential chemical structural candidates have been identified that correlate with these masses and chemical formulas. Considering the C, N, and O atom content solely, these 2 compounds might possibly possess the geoporphyrin structure with an additional fused ring. The high hydrogen atom content within this molecule, however, necessitates an unusual degree of C-C bond saturation and the absence of the stable conjugated double bond system that characterizes porphyrins.

DISCUSSION

Tracing chlorin-e6-trimethyl ester in sediments to potential source of fish kills

During the period spanning the 4 fish kills, 15 sediment samples were collected from various creeks and riverbeds in the watershed of Red Creek and Red River. Of the 15 sediment samples collected, the compound chlorin-e6-trimethyl ester was positively identified in 8 samples (Table 2). A map showing the positive sediment collection sites points to a potential source of chlorin-e6-trimethyl ester as emanating from the east tributary of Red Creek (Figure 6).

Geoporphyryns

The most widely detected and positively identified nontargeted chemical in the water and sediment samples was chlorin-e6-trimethyl ester, which belongs to the porphyrin chemical class. Some porphyrins termed geoporphyryns are chemically fingerprinted to global oil and oil shale deposits. One specific group of geoporphyryns is unique to the Ordovician Viola and Arbuckle formations found underneath south central Oklahoma 17. It is possible that the

porphyrin detected in the environmental samples belonged to these geological formations. We hypothesize that chlorin-e6-trimethyl ester originated from an organism unique to these formations, *Gloeocapsamorpha priscas*, which has been suggested to be a blue-green alga or large bacterium existing millions of years ago in the primeval ocean 17. Underlying our hypothesis is the lack of the phytyl group (the chemical side chain for chlorophyll) on the porphyrin. Pickering gives a very good explanation of the possible formation of these compounds in his dissertation and in 2 more recently published articles 18–20. Experiments performed by Pickering and Keely showed that the microenvironments present in the water–sediment (porewater) interface can provide both sulfide and oxygen to form de-alkylated chlorophylls 20. They hypothesized that during early diagenesis pH and oxidation-reduction potential, as well as any claylike materials present, played a role in the transformation of chlorophylls to geoporphyrins 20.

Fish kills

Without toxicological studies on chlorin-e6-trimethyl ester, we can only speculate whether the chemical was responsible for the fish kills or whether its presence was simply a marker of toxic environmental exposure related to the fish dying. The precise answer might come from further LC-MS/MS and toxicological studies involving any future fish kill episodes in the same geographic area. Figure 7 shows a clear response of high area counts of chlorin-e6-trimethyl ester, as extracted from the water samples and measured by LC-ITMS, to observations of actively dying fish. In addition, numerous papers in the literature cite the use of chlorin-e6-trimethyl ester and its derivatives in oncology as photodynamic therapeutic agents 21, 22 because of the chemical's potential for carrying other compounds across cell barriers. Both the compound's structure and the literature review of geoporphyrins suggest this compound is capable of metal complexation (e.g., of iron, copper, and nickel) 23, and hence it is plausible that the heavy metals could produce cell toxicity caused by the co-transport of such metals across the cell membrane. Heavy metal analyses of the waters collected during the active fish kill cycles I and II showed various metals present. Elevated levels of manganese were detected in most of the waters. Interestingly, the highest level of manganese was detected at site 1, while the fish were observed by the federal and state investigators to be in an active dying state (Supplemental Data, Table S1). Fish necropsies and tissue analyses of the dead fish performed for the present study showed evidence of hepatic injury, although the report was not specific enough to point to heavy metal toxicity as its origin.

CONCLUSIONS

The chemical formulas of 4 environmental compounds and one extraction method artifact that were present during the fish kills, but absent outside the fish-kill time and area, were determined based on accurate-mass, high-resolution LC-FTICRMS data. In addition, the chemical structures of chlorin-e6-trimethyl ester and its methanolysis product were established. The present study illustrates the necessity of employing high-performance, accurate-mass LC-MS and MS/MS instruments for the identification of chemical formulas and structures of unknown chemicals in complex environmental matrices. Similar future studies using modern high-resolution, accurate-mass spectrometers are necessary to achieve

a better understanding at the systemic level of biochemistry, geochemistry, and environmental toxicity. Precise systematic characterization of the unknown compounds and cataloging of chemical fingerprints surrounding environmental toxic events are needed for identification of case-to-case similarities and thus biomarkers of toxic exposure. The collection and reporting of data, such as provided in the present study, can lead to a critical mass of information that will eventually allow for a more precise identification of the mechanisms of toxicity and the potential to link an event to its source.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Red River base map of (A) fish kills I and II, and (B) fish kills III and IV.

Figure 2.
Example of total ion chromatogram and 2 major ions detected in extracted ion chromatogram.

Figure 3. Collision-induced dissociation tandem mass spectrometry liquid chromatography-ion trap mass spectrometry of (A) chlorin-e6-trimethyl ester standard, m/z 639.3 ($M+H$)⁺, and (B) unknown, m/z 639.3 ($M+H$)⁺.

Figure 4.
Chlorin-e6-trimethyl ester.

Figure 5.
One likely ammonolysis transformation product (solid-phase extraction artifact).

Figure 6.
Map of positive detects of chlorin-e6-trimethyl ester in collected sediments.

Figure 7.
Relationship of area of m/z 639 versus timeline of fish kill.

Table 1.

Collision-induced dissociation (CID) source fragment ions from liquid chromatography–time-of-flight mass spectrometry (LC-TOFMS) of proposed polyamino compound

<i>m/z</i>	CID voltage (V)	Fragment ions (<i>m/z</i>)
826.7245 (M ⁺)	80	809.7075
		792.6763
		749.6786
		732.6575
		714.6425
		696.6324
		636.6105
413.8634	80	405.3518

Table 2.Sediment data—collision-induced dissociation ion trap mass spectrometry screening^a

Site identification	MS/MS confirmation
Site #2 ~ 5.72 mi upstream of I-35	nondetect (nd)
Site #3 ~ 3.56 mi upstream of I-35	nd
Site #5 Ketchum's Bluff, OK	nd
Site #6 ~ 2.24 mi downstream of BR	nd
Site #7 primitive BR at Oscar, OK	nd
Hwy 89 near Courtney, OK	Yes
Hwy 81/Ryan, OK (barn stockpile)	nd
Ketchum's Bluff, OK	Yes
County Rd. 2940	Yes
Union Valley Rd. near Oscar, OK	< trace
Lower reach	nd
Upper reach	Yes
Hwy 32	< trace
S11RC east tributary	Yes
S12RC N2900	Yes

^aFor *m/z* 639.3, chlorin-e6-trimethyl ester.

MS/MS = tandem mass spectrometry; BR = boat ramp x; RC = Red Creek; S = site.