Oklahoma Fish Kill Study: Looking for a Toxic Needle in an Environmental Haystack

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OVERVIEW

Purpose

To determine unknown contaminants in water samples during an active fish kill.

Methods

A combination of solid-phase extraction (SPE), LC-ion trap-MS/MS and high resolution LC-MS.

Results

An unknown contaminant was uniquely identified as chlorin-e6-trimethyl ester, using both LC-ion trap-MS/MS and high resolution LC-MS.

INTRODUCTION

On July 9, 2011, a major fish kill (fish kill I) was observed by the Oklahoma Department of Environmental Quality (OKDEQ) in the Red River, near Ketchum's Bluff, Oklahoma. The Red River, with headwaters in the Texas panhandle, flows for 917 kilometers, between the borders of Oklahoma (OK) and Texas (TX), before emptying into the Mississippi River. During this fish kill, hundreds of large bottom feeder fish (i.e., catfish and buffalo) were observed as either dead, struggling, or actively dying. Nearly two months later, on September 14, 2011, another fish kill (fish kill II) occurred further south along the Red River, approximately 130 km downstream from Ketchum's Bluff near Lake Texhoma. Again, it was observed that hundreds of only the large bottom feeder fish were affected by an unknown toxin(s). OKDEQ believed that the two fish kills were related, with the unknown toxicant(s) traveling further downstream from the first fish kill (July 9, 2011), but causing fish mortality 60 days later downstream. The following year, on June 13, 2012, another fish kill (fish kill III) occurred, again near the area of Ketchum's Bluff and Red Creek confluence. And a final fish kill (fish kill IV) occurred on January 31, 2013, in the same watershed, near Red River and Beaver Creek confluence. Environmental samples (i.e., water, sediment, and fish) were collected, by OKDEQ and the United States Environmental Protection Agency's (USEPA) Region 6 on-scene coordinators, from multiple sites along the Red River during the active phases of these fish kills. Archived water and sediment samples from fish kills I and II were sent January 2012 from OKDEQ to the USEPA's Office of Research and Development-National Exposure Laboratory in Las Vegas, Nevada (ORD-NERL-Las Vegas), to perform mass spectral screening analyses for unknown emerging contaminants. During fish kills III and IV, OKDEQ and Region 6 collected samples as the fish kills were occurring and shipped immediately to ORD-NERL-Las Vegas for chemical contaminant analysis.

METHODS

Water extraction. Water samples, 500 mL each, were extracted using a solid phase extraction (SPE) method. Water samples were prepped for extraction by adding labeled pharmaceutical standards (for quality control purposes), 3 grams of NaCl, and small volumes of HCl were added to each sample until a pH < 3 was achieved. The lower pH was necessary as OKDEQ reported that the water samples formed a cloudy colloidal suspension when a base was added to the initial samples from fish kills I and II. OASIS MCX SPE cartridges were conditioned with each extracting solvent. The samples were loaded at 7 mL min^{-1} flow rates, cartridges were dried for 40 min, unknowns and surrogates (labeled pharmaceutical standards) were eluted from the cartridges with 5 mL 90% methyl tert butyl ether/10% methanol, followed by 10 mL 95% methanol/5% NH_4OH, at a flow rate of 1 mL min^{-1}. Eluants were qualitatively transferred to 50 mL Turbovap tubes, tubes were rinsed, and solvent exchanged, with methanol/1% acetic acid. The eluant was subsequently reduced under a steady, gentle stream of nitrogen, to 0.5 mL.

Liquid Chromatography-Mass Spectrometry.

LC conditions: Column: Phenomenex Fusion RP 150 cm x 2.1 mm column, or a Sigma-Aldrich Ascentis C_{18} 100 cm x 2.1 mm column, coupled with a Varian guard column, MetaGuard 2.0 mm Pursuit XRs 3µm C_{18}; compositions of the mobile phases were as follows: (A) DI water/0.5% formic acid, and (B): 82% methanol/18% acetonitrile/0.5% formic acid.

LC gradient (flow rate 0.3 mL/min):

Time (min)	%A	%B
Initial	100	0
2	100	0
5	30	70
10	30	70
13	100	0
15	100	0

Mass Spectrometric Detection. Analyses were performed using the following complementary mass spectrometry techniques: LC-ITMS (in-house) and LC-TOFMS (in-house), or LC-FTMS [Canadian Ministry of the Environment-Ontario (MOE-Ontario)].

All samples were initially screened by LC-ITMS. Large unknown chromatographic peaks were further investigated using LC-ITMS/MS. Subsequently, samples were analyzed for accurate mass and chemical formula calculations using LC-TOFMS and LC-FTMS. In-source CID was performed in the LC-TOFMS and LC-FTMS to help assign accurate mass and structural information to fragment ions initially detected by LC-ITMS.

RESULTS

Major chromatographic unknowns observed in fish kills. During the screening analyses of the first two fish kills I and II, two major polar non-volatile unknowns were detected at masses *m/z* 624.3 Da and *m/z* 639.3 Da. In fish kill III samples there was no evidence of masses *m/z* 624.3 Da and *m/z* 639.3 Da. Instead, there were two large chromatographic peaks detected at masses, *m/z* 562.3760 Da $(M+H)^+$, $C_{33}H_{48}N_4O_3$, and *m/z* 564.3898 Da $(M+H)^+$ $C_{33}H_{50}N_4O_3$. However, in fish kill IV water samples, masses *m/z* 624.3 Da and *m/z* 639.3 Da were again present in significant amounts. Initially, these masses (*m/z* 624.3 Da and *m/z* 639.3 Da) were hypothesized to be a mycotoxin, ergosedmine (Uhlig et al, 2011). However, enough water sample (2 L) had been collected with fish kill IV to allow for two sets of extractions. The second set of extracts was sent MOE-Ontario for further high resolution mass spectrometric analyses using LC-FTMS. The information obtained from LC-FTMS gave the following accurate masses: *m/z* 639.31735 $(M+H)^+$, generating the molecular formula, $C_{37}H_{43}N_4O_6$, and *m/z* 624.31794 $(M+H)^+$, generating the molecular formula, $C_{36}H_{42}N_5O_5$. By piecing together accurate mass fragment ions, calculating rings and bonds, and searching web resources, it was discerned that the unknown, at mass *m/z* 639.31735 $(M+H)^+$, was not a mycotoxin. Instead, the unknown at mass *m/z* 639.3 Da was identified as a geoporphyrin , specifically

chlorin-e6-trimethyl ester (Figure 1), MW 638.31043 Da, $C_{37}H_{42}N_4O_6$. In order to be indisputably certain that this was the correct identification, a standard of chlorin-e6-trimethyl ester was obtained from Frontier Scientific (Logan, Utah). Using the collision induced dissociation (CID) function of the LC-ITMS, a CID mass spectra of the standard was obtained and compared to the unknown spectra detected at mass *m/z* 639.4 Da $(M+H)^+$ 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 (Fig. 2a and 2b).

The other major unknown present in fish kill IV extracts at *m/z* 624.3 Da $(M+H)^+$ (previously detected in fish kill samples I and II) is chemically related to chlorin-e6-trimethyl ester. This compound was an artifact accidentally created during the SPE elution process. A tentative identification was assigned as an amide-containing porphyrin by comparing the CID spectra from the LC-ITMS data, the LC-TOFMS data, and the LC-FTMS data. The molecular formula, as calculated by LC-FTMS, is $C_{36}H_{42}N_4O_5$, *m/z* 624.31794 $(M+H)^+$. There are three methyl ester groups that are potential sites for amide formation, and the detection of two major products suggests that two of the three possible sites are more accessible to ammonolysis-type reactions. A series of chemical synthesis experiments were performed to test the hypothesis that this compound, $C_{36}H_{42}N_4O_5$, was an artifact of extracting the samples containing the porphyrin, chlorin-e6-trimethyl ester, with the 95% MeOH/5% NH_4OH solution. Figure 3 is just one possible structure hypothesized of one of the isomeric amides that was formed by ammonolysis of the chlorin-e6-trimethyl ester.

Another significant unknown was detected in only one sample from the 2013 fish kill. This unknown eluted earlier than the porphyrin series, and was assigned the chemical formula: $C_{46}H_{54}N_6O_6$, with an accurate mass of *m/z* 826.72275 (M^+) [doubly charged ion detected at: *m/z* 413.36039 (M^{+2})]. This chemical has been tentatively identified as belonging to the chemical class of diquaternary ammonium compounds. The accurate mass, assigned by LC-FTMS, was *m/z* 826.72275 (M^+), and has been tentatively identified as N,N,N,N',N',N'-Hexamethyl-4,20,27,43-tetraoxo-3,44-dioxa-6,19,28,41-tetraazahexatetracontane-1,46-diaminium; with a theoretical monoisotopic mass of 826.722412 Da. No commercial chemical standard is available for confirmation.

CONCLUSIONS

The major unknown identified from the fish kill water samples was chlorin-e6-trimethyl ester. Chlorin-e6-trimethyl ester belongs to the porphyrin chemical class. Some porphyrins are termed geoporphyrins, and many are chemically fingerprinted to global oil and oil shale deposits. There is one specific group of geoporphyrins that are unique to the Ordovician Viola and Arbuckle formations found underneath south central Oklahoma (Michael et al. 1989). It is possible that the geoporphyrin that was detected in the environmental samples may belong to these geologic formations. The particular geoporphyrin that was detected could possibly emanate from an organism unique to this formation, *Gloeocapsamorpha priscas*, which was possibly a blue-green alga or large bacterium present millions of years ago in the primitive oceans (Michael et al. 1989). The reasoning behind this is the lack of the phytyl group (the chemical side chain for chlorophyll) on the geoporphyrin. Pickering (Pickering 2009) gives a very good explanation on the possible formation of these compound in his dissertation "Low temperature sequestration of photosynthetic pigments: Model studies and natural aquatic environments."

It can only be hypothesized as to whether the chlorin-e6-trimethyl ester was responsible for, or just relational to, the fish kills. There is some evidence, Figure 4, that the presence of chlorin-e6-trimethyl ester is relational to the dying fish, but that is a hypothesis at this point in time. While the unequivocal identification of one emerging contaminant unknown has been made in fish kill samples, there are many other unidentified chromatographic peaks present in both the water and sediment extracts. We have focused only on those chromatographic peaks and ions that were substantially above the chromatographic baseline and not detected in the blank samples.

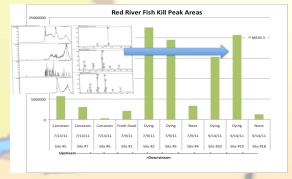
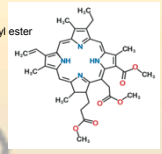


Fig. 4 Relationship of mass *m/z* 639 Da vs timeline of fish kill

Fig 1. Chlorin-e6-trimethyl ester



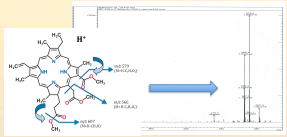
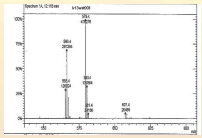
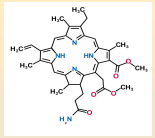


Figure 2a. CID MS/MS LC-ITMS: Chlorin-e6-trimethyl ester standard, *m/z* 639.3 $(M+H)^+$



2b. CID MS/MS LC-ITMS Unknown *m/z* 639.3 $(M+H)^+$

Fig. 3 Likely ammonolysis transformation product (SPE artifact)



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