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Four Fish Kills Spanning 2011 – 2013 in the Red River Watershed Beaver Creek to Lake Texoma, Oklahoma

RESEARCH AND DEVELOPMENT

Four Fish Kills Spanning 2011 – 2013 in the Red River Watershed Beaver Creek to Lake Texoma, Oklahoma

Prepared by

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FOREWORD

The U.S. Environmental Protection Agency (EPA) is charged by Congress to protect the nation's natural resources. Under the mandate of national environmental laws, the EPA strives to formulate and implement actions leading to a compatible balance between human activities and the ability of natural systems to support and nurture life. To meet this mandate, the EPA's Office of Research and Development (ORD) provides data and scientific support that can be used to solve environmental problems, build the scientific knowledge base needed to manage ecological resources wisely, understand how pollutants affect public health, and prevent or reduce environmental risks.

The National Exposure Research Laboratory (NERL) is the Agency's center for investigation of technical and management approaches for identifying and quantifying exposures to human health and the environment. Goals of the laboratory's research program are to: (1) develop and evaluate methods and technologies for characterizing and monitoring air, soil, and water; (2) support regulatory and policy decisions; and (3) provide the scientific support needed to ensure effective implementation of environmental regulations and strategies.

The USEPA/ORD-National Exposure Research Laboratory-Environmental Sciences Division (USEPA/ORD-NERL-ESD) assisted USEPA Region 6 and the State of Oklahoma Department of Environmental Quality (OKDEQ) in identifying unknown contaminant(s) that were present during four fish kills in the Red River watershed. These environmental samples were unique in that they were collected during the active phase of the fish kills along the Red River (Oklahoma, United States) in 2011, 2012 and 2013. Using liquid chromatography-time-of-flight high-resolution mass spectrometry (LC-TOFMS), LC-Fourier transform mass spectrometry (LC-FTMS) and/or liquid chromatography-ion trap mass spectrometry (LC-ITMS), the conditional assignments of the molecular weights and chemical formulas of the significant unknown contaminants were determined.

EXECUTIVE SUMMARY

This research was conducted under the auspices of the US Environmental Protection Agency's Office of Research and Development (USEPA ORD) Safe and Sustainable Water Resources Research (SSWR) program: Theme 1, Q7 (Highly Targeted Programmatic Support). Since December 2011, the USEPA ORD, National Exposure Research Laboratory-Environmental Sciences Division (USEPA ORD-NERL-ESD) has assisted EPA Region 6 and the State of Oklahoma Department of Environmental Quality (OKDEQ) in identifying unknown contaminants that were present during four fish kills in the Red River watershed. These environmental samples were unique in that they were collected during the active phase of the fish kills along the Red River (Oklahoma, United States). There were a total of four fish kills: two occurred in July and September 2011, one in June 2012, and one in January 2013; they will hereafter be referred to as fish kill I, II, III and IV.

Using liquid chromatography-time-of-flight high-resolution mass spectrometry (LC-TOFMS), LC-Fourier transform mass spectrometry (LC-FTMS) and/or LC-ion trap mass spectrometry (LC-ITMS), the conditional assignments of the molecular weights and chemical formulas of the significant unknown contaminants were determined. Environmental water samples were extracted using a solid phase extraction (SPE) method. Sediment samples were extracted using a modified sonication liquid extraction method. All extracts were screened and analyzed by LC-ITMS, LC-TOFMS, and/or LC-FTMS. Subsequently, the extracts were then re-analyzed using collision induced dissociation (CID) (either in the ion trap, or in-source CID for TOFMS and FTMS) for product ion formation to elucidate chemical structural components, and re-analyzed by LC-TOFMS and LC-FTMS for accurate mass assignments. Many chromatographic peaks were present, but most could be attributable to ambient background contamination (e.g., surfactants and phthalates). From the screening analyses of the samples, two major unknowns were discovered in three of the four fish kills, detected at masses *m/z* 624.3 Da and *m/z* 639.3 Da. The unknown at mass *m/z* 639.3 Da has been unequivocally identified as a porphyrin, specifically, chlorin e6 trimethyl ester. In fish kill III samples there was no evidence of chlorin e6 trimethyl ester. Instead, in fish kill III samples, there were two large chromatographic peaks detected at different masses. The peaks were identified at masses, *m/z* 562.3760 Da $(M+H)^+$, $C_{33}H_{48}N_5O_3$, and *m/z* 564.3898 Da $(M+H)^+$ $C_{33}H_{50}N_5O_3$. At this time, it would be speculation to suggest which chemical class these two compounds belong to, whether a porphyrin, a mycotoxin (as suspected from earlier identification efforts), or some other unknown chemical class. Another significant unknown was detected in only one sample from the fish kill IV. This unknown eluted earlier than the porphyrin series and was assigned the chemical formula: $C_{46}H_{94}N_6O_6$, with an accurate mass of *m/z* 826.72275 $(M^{+\cdot})$ [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.

There is evidence that the presence of chlorin e6 trimethyl ester is relational to the dying fish, but this is just a hypothesis. While the unequivocal identification of one unknown emerging contaminant was made in fish kill I, II, and IV samples, there are many other unidentified chromatographic peaks present in both the water and sediment extracts. Only those chromatographic peaks and masses that were substantially above the chromatographic baseline, and not detected in the blank samples, were scrutinized.

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LIST OF ACRONYMS AND ABBREVIATIONS

ACN	acetonitrile
C	centigrade
CID	collision induced dissociation
CSS	Chemical Safety for Sustainability
CWA	Clean Water Act
ECs	emerging contaminants
EPA	Environmental Protection Agency
ESD	Environmental Sciences Division
ITMS	ion trap mass spectrometer
FTMS	Fourier transform mass spectrometer
GC	gas chromatograph
g	gram
HPLC	high performance liquid chromatography
kg	kilogram
L	liter
LC	liquid chromatograph
$(M^{+\cdot})$	intact molecule minus an electron
$(M+H)^{+}$	intact molecule plus a proton (H^{+})
MeOH	methanol
mL	milliliter
MOE-Ontario	Ministry of the Environment-Ontario (Canada)
MS	mass spectrometer
MTBE	methyl tertbutyl ether
NERL	National Exposure Research Laboratory
NaCl	sodium chloride
NH_4OH	Ammonium hydroxide
OKDEQ	State of Oklahoma Department of Environmental Quality
ORD	Office of Research and Development
ppb	part-per-billion
PPE	personal protective equipment
ppt	part-per-trillion
rpm	revolutions per minute
SIM	single ion monitoring
SPE	solid phase extraction
SSWR	Safe and Sustainable Water Research
TOFMS	time-of-flight mass spectrometer
μL	microliter
UPLC	ultra performance liquid chromatography
WWTP	wastewater treatment plant

Acknowledgements

I'd like to thank my colleagues, Dr Wayne Sovocool, Dr Don Betowski, Dr Patrick DeArmond, Mrs Charlita Rosal, and Dr Vince Taguchi (Canadian Ministry of the Environment-Ontario) for all of their hard work in analyzing and deciphering the Red River samples. None of this research would have been possible without their dedication and assistance. I'd also like to thank my students, Mr Trevor Nance Jr. and Mr Matt Ward for their assistance in extracting numerous water and sediment samples, sometimes repeatedly for precision and accuracy.

1.0 Introduction and Background

Since December 2011, the USEPA ORD-NERL-ESD has assisted EPA Region 6 and the OKDEQ in identifying unknown contaminants that were present during four fish kill events in the Red River watershed.

The Red River is a tributary of the Mississippi River, with headwaters in the Texas panhandle, flowing for 917 kilometers between the borders of Oklahoma (OK) and Texas (TX), before eventually emptying into the Mississippi River. The fish kills were located in the Red River, or its tributaries, from Ryan, OK to Lake Texhoma, OK. Three of the fish kills (I, II and III) were located near the confluence of Red Creek (at Ketchum's Bluff) and the Red River, and one (IV) was localized to Beaver Creek, which runs alongside Ryan, OK, and flows into the Red River, north of Ketchum's Bluff. In the first three fish kills, only large bottom feeder fish (i.e., catfish and buffalo), were observed dead or dying. The last fish kill IV was unique in that not only fish, but also other animals (i.e., hardshell and softshell turtles) were affected.

In July 2011, the first fish kill (fish kill I) was observed to occur in the Red River near Ketchum's Bluff, OK. Nearly two months later, in September 2011, another fish kill (fish kill II) was observed happening further south along the Red River, approximately 130 km downstream from Ketchum's Bluff near Lake Texhoma. In December of 2011, Region 6 asked the ORD-NERL-ESD laboratory in Las Vegas, Nevada, for assistance in possibly identifying the unknown toxicant(s) potentially causing the fish kills. During the active phases of fish kills I and II, multiple sites were sampled. Originally, the water samples from fish kills I and II had been sent to various laboratories (i.e., USEPA Region 6, USEPA National Enforcement Investigations Center-Denver, Oklahoma State Environmental Laboratory Services, and the Texas Parks and Wildlife Inland Fisheries Environmental Contaminants Laboratories) for routine traditional analyses (e.g., volatiles, semi-volatiles, metals), and fish necropsies and tissue analyses. With no reasonable cause identified with these analyses, the archived water and sediment samples were sent to ORD-NERL-ESD laboratory to perform analyses for emerging contaminants. A preliminary report of the findings was submitted to USEPA Region 6 and OKDEQ in March 2012.

On June 12, 2012, USEPA Region 6 and OKDEQ notified ORD-NERL-ESD that another fish kill (fish kill III) was in progress on the Red River, and again assistance was requested. This fish kill started at almost exactly the same location (Ketchum Bluff area) as fish kill I. On June 20, 2012, ORD-NERL-ESD received 8 water samples (6 water samples and two trip blanks). The water samples were collected during the observed fish kill (fish dead or actively dying) in the Red River on June 12 and 13, 2012. ORD-NERL-ESD performed the analyses for emerging contaminants and a preliminary report on the initial findings was delivered to Region 6 and OKDEQ in August 2012.

In early February 2013, ORD-NERL-ESD was notified by USEPA Region 6 and OKDEQ that a fourth fish kill was in progress on Beaver Creek, a small tributary of the Red River, near Ryan, OK. It was reported to ORD that this fish kill (fish kill IV) was dissimilar in nature to the previous observed fish kills, reported in the Red River in 2011 and 2012 (Red River fish kills I, II and III), in that a diversity of animals were adversely affected, not only fish, but also dead and

dying hard- and soft-shell turtles. On February 4, 2013, four water samples, including one travel blank and three environmental water samples, were sent for analyses to ORD-NERL-ESD.

The primary objective of this report is to present what was detected in water samples from fish kill IV, and relate how these findings impact the conclusions from the earlier fish kills I-III. The second objective is to discuss what was detected in the sediment samples, and their possible relationship to the water samples. Also attached to this report, is a standard operating procedure (SOP): "Extraction and Detection of Emerging Contaminants using solid-phase extraction and liquid chromatography-ion trap mass spectrometry" (Appendix A). The SOP is provided for USEPA Region 6 and OKDEQ, such that they can repeat the experiments that were performed and continue the surveillance of the Red River for possible toxicants.

2.0 Experimental

2.1 Sampling. OKDEQ collected and shipped all water and sediment samples according to their sampling protocols. All water and sediment samples were collected as grab samples. The initial water samples sent to ORD-NERL-ESD from fish kills I and II were archived samples, refrigerated and stored by OKDEQ at < 4^{o}C. The other samples collected during fish kills III and IV, and during non-fish kill events (background) were stored on ice, or refrigerated, and sent to ORD-NERL-ESD within one to four days from sampling event.

The sediment samples were collected at various times throughout the last two years. Some of the sediment samples were collected during the fish kill events, and other sediment samples were collected at sites upstream from the fish kills during non-events, to be used as background samples.

2.2 Water extraction and analysis. Briefly, four water samples (a travel blank and three environmental waters) were extracted using a solid phase extraction (SPE) method. All Oklahoma fish kill water samples were extracted at a pH < 3. This 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.

Detection analyses were performed using mass spectrometry, LC-ITMS (in-house) and LC-TOFMS (in-house), or LC-FTMS [analyses performed by Canadian Ministry of the Environment-Ontario (MOE-Ontario)]. Splits of the extracts were sent to MOE-Ontario for analysis by LC-FTMS in order to obtain greater mass accuracy than the in-house LC-TOFMS could assign.

For in-depth aqueous extraction method details, see Appendix A: SOP on "Extraction and detection of emerging contaminants using solid-phase extraction and liquid chromatography-ion trap mass spectrometry."

2.3 Sediment extraction and analysis. Over the last three years, several sediment samples were received and archived in ORD-NERL-ESD walk-in refrigerator, < 4^{o}C. The sediment samples were extracted using a crude extraction method. One gram of sediment was weighed into a small

(250 mL) beaker, and labeled internal standards were added. The samples were placed under a hood and allowed to dry (approx. 2hrs). After drying, 5 mL of solvent (5% NH_4OH/95% MeOH) was added to each sample. The samples were then placed in a sonicator and sonicated for 5 minutes. Samples were removed from the sonicator and the solvent layer was transferred to 15 mL capped glass centrifuge vials. These three steps [addition, sonication, and removal of 5 mL of solvent (5% NH_4OH/95% MeOH)] were repeated two more times until approximately 15 mL of solvent had been collected. Vials (containing solvent layers) were placed in the centrifuge and spun for 5 minutes at 670 revolutions per minute (rpm). After the 5 minutes the centrifuge was increased to 1675 rpm for an additional 5 minutes. Each sample was then rinsed with 4 mL of hexane, a hexane layer was allowed to form, which was then removed and discarded. The supernatant was poured into 50-mL concentrator tubes, and the solid remaining in the centrifuge vials was discarded. The concentrator tubes were placed in a semi-automated evaporator (TurboVap^{TM}), the nitrogen stream was set to approx. 7 psi, and the supernatants were concentrated to 0.5 mL. The concentrated supernatant was subsequently transferred to autosampler vials for analysis by LC-TOFMS or LC-ITMS.

Screening analyses of sediment extracts were performed using mass spectrometry, LC-ITMS and LC-TOFMS. See Appendix A for further mass spectrometric analytical details.

3.0 Results and Discussion

3.1 Major chromatographic unknowns observed in fish kill IV. From the screening analyses of fish kill IV samples two major polar non-volatile unknowns, that were discovered in fish kills I and II (detected at masses *m/z* 624.3 Da and *m/z* 639.3 Da), were again present in significant amounts in the three environmental water samples. Fortunately 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 to Dr. Vince Taguchi, at Canada's Ministry of the Environment-Ontario (MOE-Ontario) for further mass spectrometric analyses. MOE-Ontario was able to obtain more detailed accurate mass and structural information using MOE-Ontario's LC-FTMS than was possible on ORD-NERL-ESD mass spectrometers.

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 searching web resources, it was discerned that the unknown, at mass *m/z* 639.31735 $(M+H)^+$, was not a mycotoxin, as had been previously hypothesized from fish kills I and II. Instead the unknown at mass *m/z* 639.3 Da was identified as a geoporphyrin, specifically chlorin e6 trimethyl ester (Figure 1), mw 638.310425 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 ORD-NERL-ESD 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, and a positive confirmation was made (Figures 2a and 2b). To ensure accuracy of the identification, verification was completed on a second mass spectrometer. In-source CID experiments were carried out using the LC-TOFMS, and a comparison of the spectra in Figure 2d to the spectra in Figure 2c, confirmed the identification.

Figure 3 shows the identification of the major fragmentation pathways of the three major product ions that were formed during CID.

The other major unknown present in fish kill IV extracts, at *m/z* 624.3 Da $(M+H)^+$, was also previously detected in fish kill samples I and II. This unknown is chemically related to chlorin e6 trimethyl ester and is an artifact that was accidentally created during the SPE elution process. The core chemical structure is very similar to chlorin e6 trimethyl ester. 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 $C_{36}H_{42}N_5O_5$, *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 [Figure 5(b)], 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, $C_{36}H_{42}N_5O_5$, was an artifact of extracting the samples containing the porphyrin, chlorin e6 trimethyl ester, with the 95% MeOH/5% NH_4OH solution. Figure 5 shows a (a) chromatogram of unreacted chlorin e6 trimethyl ester, and (b) chromatogram of reacted chlorin e6 trimethyl ester with 95% MeOH/5% NH_4OH solution. The *m/z* 624.3 Da $(M+H)^+$ ions are nonexistent in the ion chromatogram (a) of the unreacted standard of chlorin e6 trimethyl ester, while the ion chromatogram (b) clearly shows the presence of two ions at two different retention times with the mass *m/z* 624.3 Da $(M+H)^+$. Figure 4 is just one possible structure hypothesized of one of the isomeric amides that was formed by ammonolysis of the chlorin e6 trimethyl ester. There is no commercial chemical standard available at this time to compare and confirm with the unknown.

Originally, the masses *m/z* 624.3 Da $(M+H)^+$ and *m/z* 639.3 Da $(M+H)^+$, detected in fish kill samples I and II, were misidentified as mycotoxins. This misinterpretation came about because the accurate mass that was measured in-house using high-resolution TOFMS, was *m/z* 624.3175 Da, $(M+H)^+$, and a molecular formula of $C_{36}H_{42}N_5O_5$ was generated. At that time a search of the scientific literature generated a newly discovered mycotoxin, ergosedmine, by Uhlig (Uhlig et al. 2011), assigned molecular formula of $C_{36}H_{42}N_5O_5$, but a measured exact mass of 624.3202, $(M+H)^+$. The difference of 2.7 mmu between the two mass spectral measurements for that chemical formula equated to a < 4 ppm difference; this is well within acceptable limits for those types of measurements made by the LC-TOFMS. However, as discussed earlier, enough water sample during fish kill IV was collected such that a second set of extracts were generated and sent to MOE-Ontario laboratory for further confirmation. MOE-Ontario has a LC-FTMS, which is capable of measuring even greater mass accuracy than ORD-NERL-ESD's LC-TOFMS. The measured mass from the FTMS was *m/z* 624.31805 Da, generating the molecular formula of $C_{36}H_{42}N_5O_5$. The measured mass from FTMS of the other compound was *m/z* 639.31736 Da, generating the molecular formula, $C_{37}H_{43}N_4O_6$. Although the molecular formula didn't change (with regards to the ones originally generated from the TOFMS data), the more accurate mass allowed for the generation of a more accurate composition of the compound(s), as well as specific generation of rings plus bonds calculations. Also, FTMS in-source CID of the accurate mass at *m/z* 639.31736 Da gave very stable fragment ions, like the porphyrin nucleus with their ester groups. Using the accurate mass fragments and neutral losses to form fragments, allowed for re-constructing an accurate chemical structure. Both pieces of information, accurate mass and accurate mass fragments, from the FTMS data, allowed for the re-computed identification of

the unknown emerging contaminant at mass *m/z* 639.31736 Da $(M+H)^+$ as chlorin e6 trimethyl ester. It was also fortuitous that a standard of the hypothesized contaminant was commercially available, leading to an unequivocal identification of the unknown at mass *m/z* 639.31736 Da $(M+H)^+$ as chlorin e6 trimethyl ester.

3.2 Other unknowns tentatively identified in fish kill IV sample(s). Another significant polar non-volatile unknown was detected in only one fish kill IV sample (Beaver Creek Main Street site) by both ORD-NERL-ESD and MOE-Ontario. This unknown eluted early (before the porphyrin series) in the total ion chromatogram (TIC), and was assigned the chemical formula: $C_{46}H_{94}N_6O_6$, with an accurate mass of *m/z* 826.72275 ($M^{+\cdot}$) [the doubly charged ion was also detected at: *m/z* 413.36039 (M^{+2})]. From the mass spectra obtained, this chemical has been identified as belonging to the chemical class of diquaternary ammonium compounds. Using the accurate mass provided by the LC-FTMS [*m/z* 826.72275 ($M^{+\cdot}$)] and a search of relevant chemical databases, it 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 (ChemSpider, a free on-line chemical data base, www.chemspider.com). There is no commercial chemical standard available for purchase at this time for confirmation.

Fish kill IV extracts were also split in-house for gas chromatography-mass spectrometry (GC-MS) analysis of the semi-volatile fraction. The results of those analyses are reported in Appendix B – "GCMS Analysis on water extracts dated 2.22.2013." The main finding from the GC-MS analyses was the discovery of the pharmaceutical gabapentin, and low-levels of several alkyl organophosphate flame retardants. None of these compounds were totally unexpected due to reports in the literature of these types of compounds in global surface waters (Kasprzyk-Horden et al. 2009; Regnery et al. 2010).

3.3 Relevance of new data observations to earlier fish kills I and II. Two water samples from fish kill I (OK ID 506352 – LV12wat004, and OK ID 506353 – LV12wat008) were re-extracted and re-analyzed by LC-ITMS, using the optimized CID conditions for detecting chlorin e6 trimethyl ester. These water samples had been archived by ORD-NERL-ESD since January 2012. They were stored in their original ½ gallon plastic jugs in a walk-in refrigerator at ~ 4° C. The unknown mass, *m/z* 639.3 Da $(M+H)^+$, that had previously been detected during the first analyses back in February 2012, was again re-detected in these samples and now positively confirmed as chlorin e6 trimethyl ester using the CID mass spectrum of the unknowns at *m/z* 639.3 Da $(M+H)^+$ and comparing them to the chlorin e6 trimethyl ester CID spectra. For the other archived water samples, from fish kill I and II, the archived CID spectra (if available, LC-ITMS or LCTOFMS), were compared to the current CID spectra of chlorin e6 trimethyl ester for affirming confirmation (Table 1).

3.4 Sediment samples. Over the course of the last three years, fifteen sediment samples were collected and received from OKDEQ, and archived in the ORD-NERL-ESD walk-in refrigerator, < 4°C. The sediment samples were recently extracted and analyzed by LC-ITMS, see methods section 2.3. Of the fifteen sediment samples, chlorin e6 trimethyl ester was positively confirmed in seven sediments (Table 2).

4.0 Conclusions

The major unknown identified from Fish Kills I, II, and IV, was chlorin e6 trimethyl ester. Chlorine6 trimethyl ester belongs to the porphyrin chemical class; for example, chlorophyll and hemoglobin are considered porphyrins. 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 water samples may belong to these Oklahoma formations. The particular geoporphyrin that was detected is thought to 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."

There was no evidence of chlorin e6 trimethyl ester in fish kill III samples. Instead two other large chromatographic peaks were detected, and the masses were identified at *m/z* 562.3760 Da $(M+H)^+$, $C_{33}H_{48}N_5O_3$, and *m/z* 564.3898 Da $(M+H)^+$ $C_{33}H_{50}N_5O_3$. At this time it would be speculation to suggest which chemical class these two compounds belong to, whether a geoporphyrin, a mycotoxin, or some other unknown chemical class.

It can only be hypothesized as to whether the chlorin e6 trimethyl ester was responsible, or just relational, to fish kills I, II, and IV. There is some evidence, Figure 6, that the presence of chlorin e6 trimethyl ester is relational to the dying fish, but it is just a hypothesis at this point in time.

While the unequivocal identification of one emerging contaminant unknown has been made in fish kill I, II, and IV 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 masses that were substantially above the chromatographic baseline and not detected in the blank samples.

Figure 1. Chlorine6 trimethylester, $C_{37}H_{42}N_4O_6$

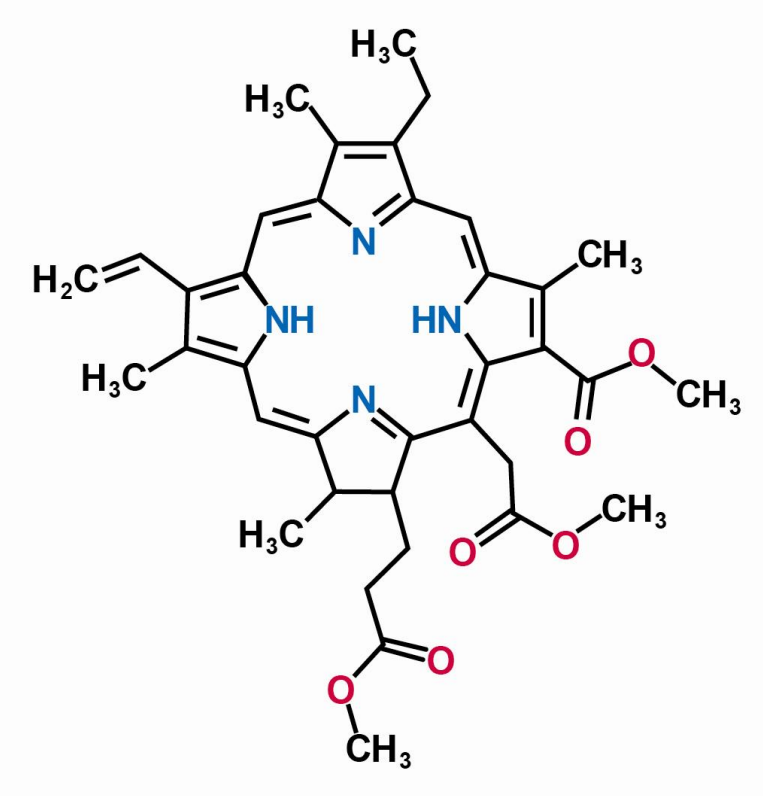

Figure 2a. CID MSMS LC-ITMS: Chlorine6 trimethylester standard, *m/z* 639.3 $(M+H)^+$

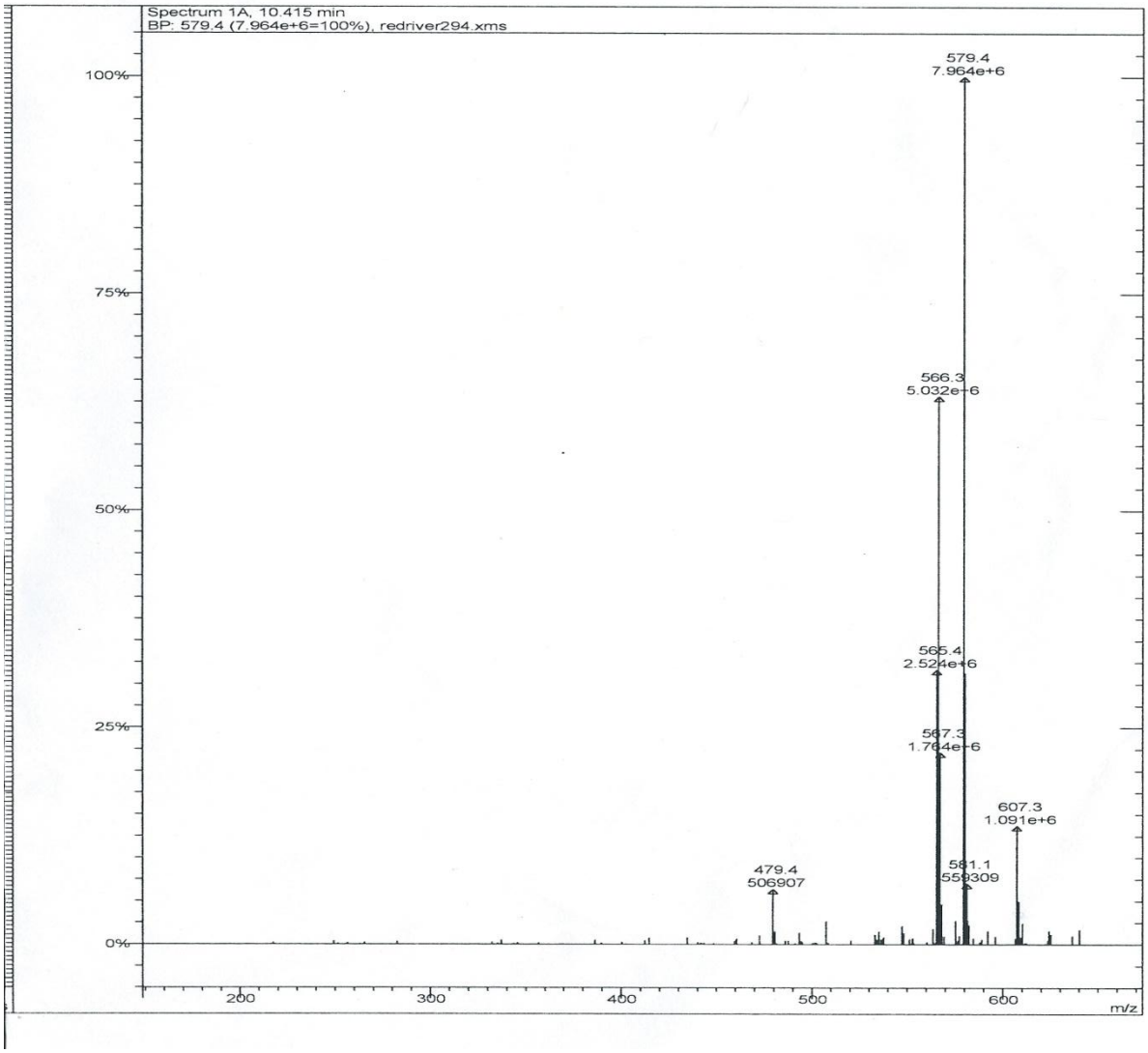

Figure 2b. CID MSMS LC-ITMS: Unknown *m/z* 639.3 $(M+H)^+$ in sample lv13wat008

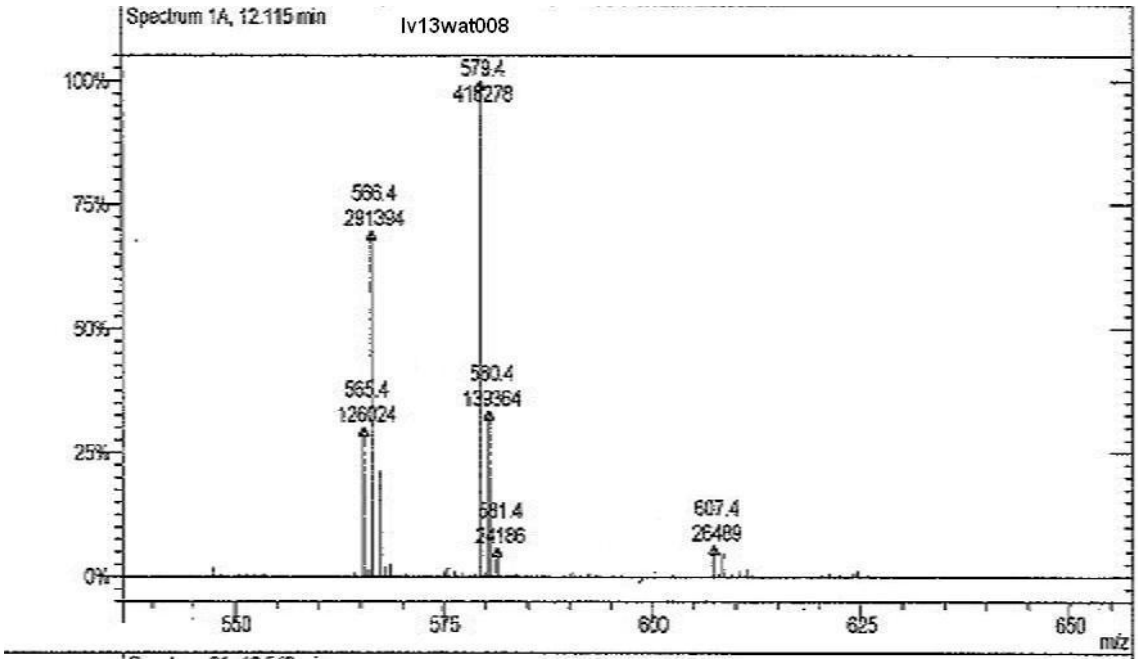

Figure 2. In-source CID TOFMS of: (2c) unknown *m/z* 639 $(M+H)^+$ in Sample lv13wat0082; and (2d) *m/z* 639 $(M+H)^+$ in Chlorine6 trimethylester standard

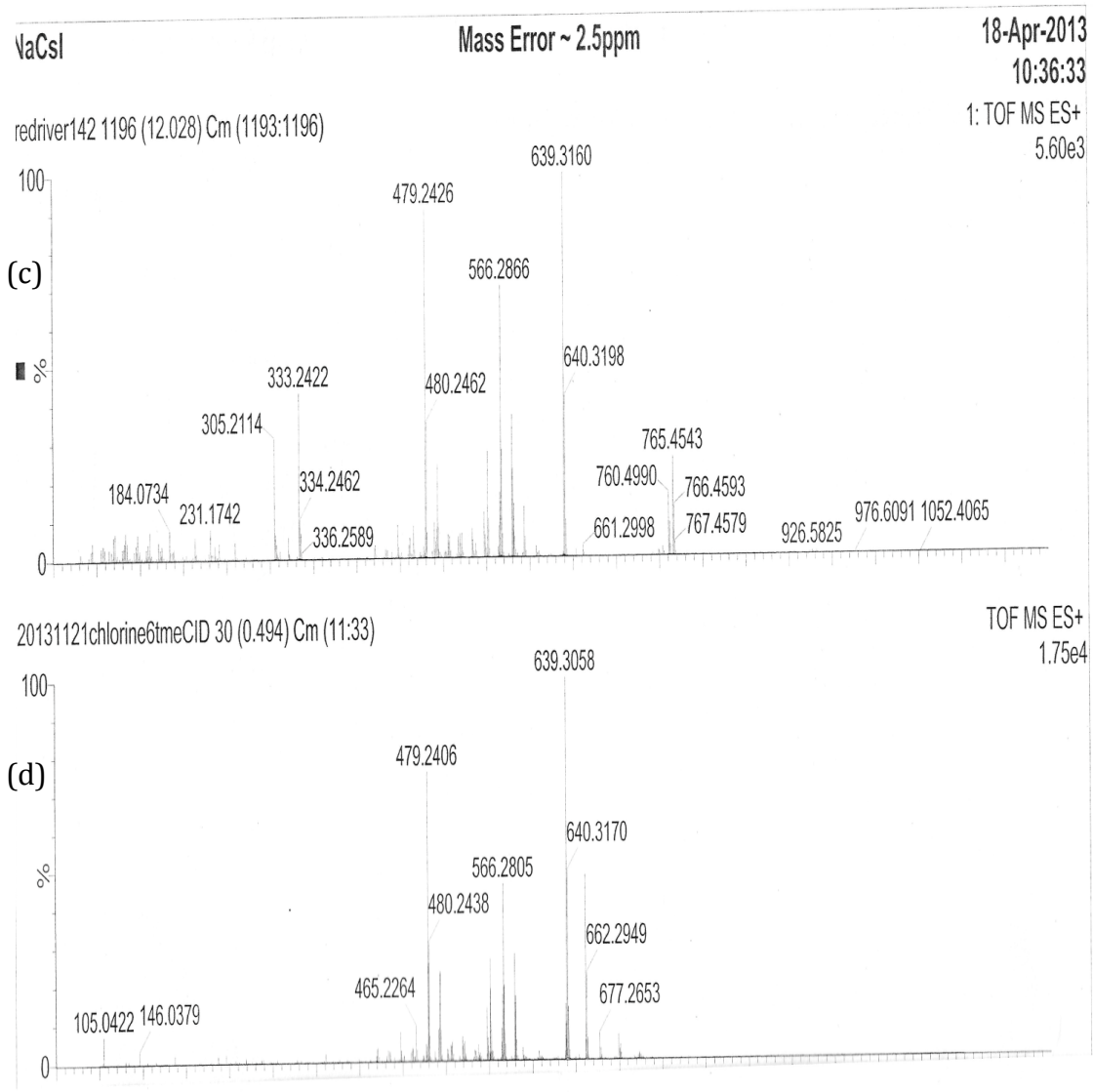

Figure 3. Pathways of production formation from chlorine6 trimethylester ion $(M+H)^+$.

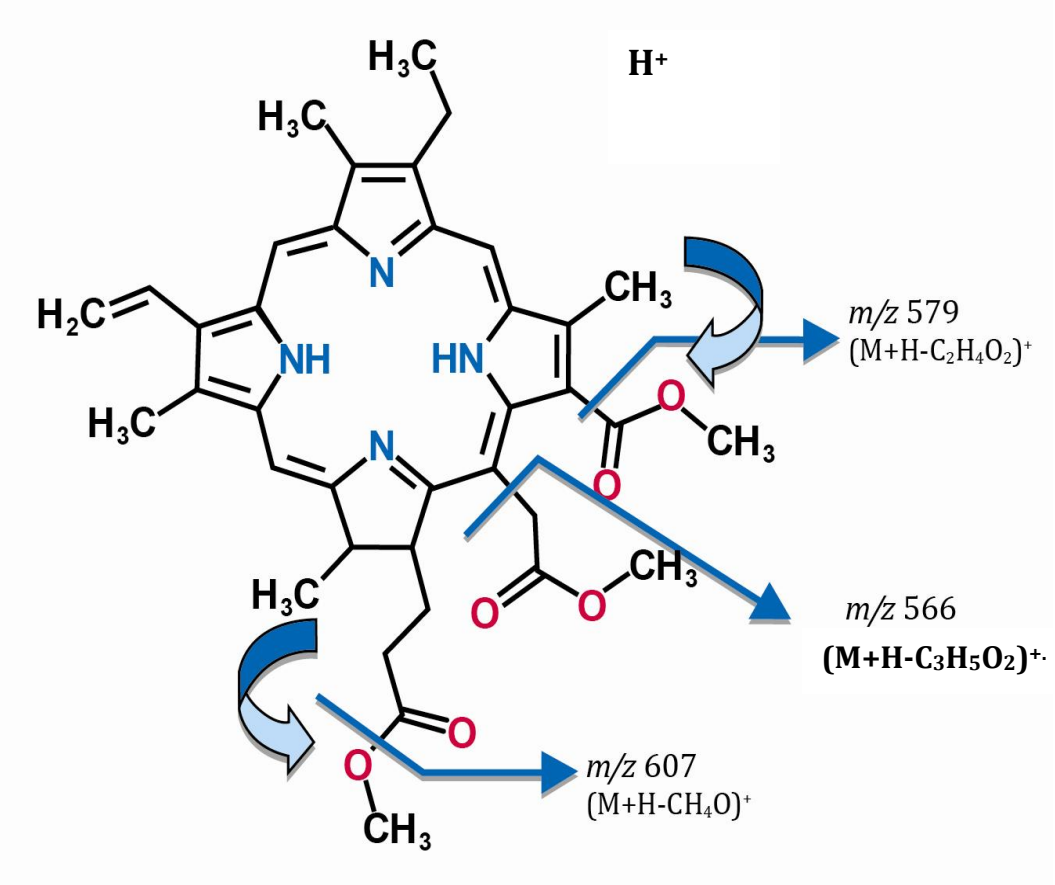

Figure 4. Likely ammonolysis transformation product of chlroine6 trimetylester, yielding *m/z* 624 Da, $(M+H)^+$

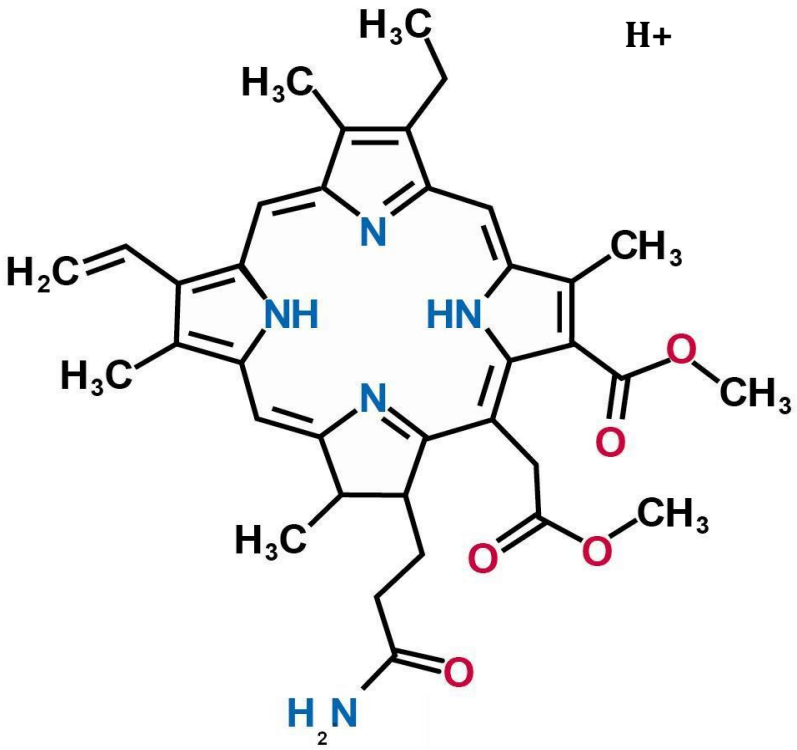

Figure 5. Ion chromatograms of (a) unreacted chlorine6 trimethylester and (b) reacted chlorin e6 trimethylester with ammonium hydroxide solution.

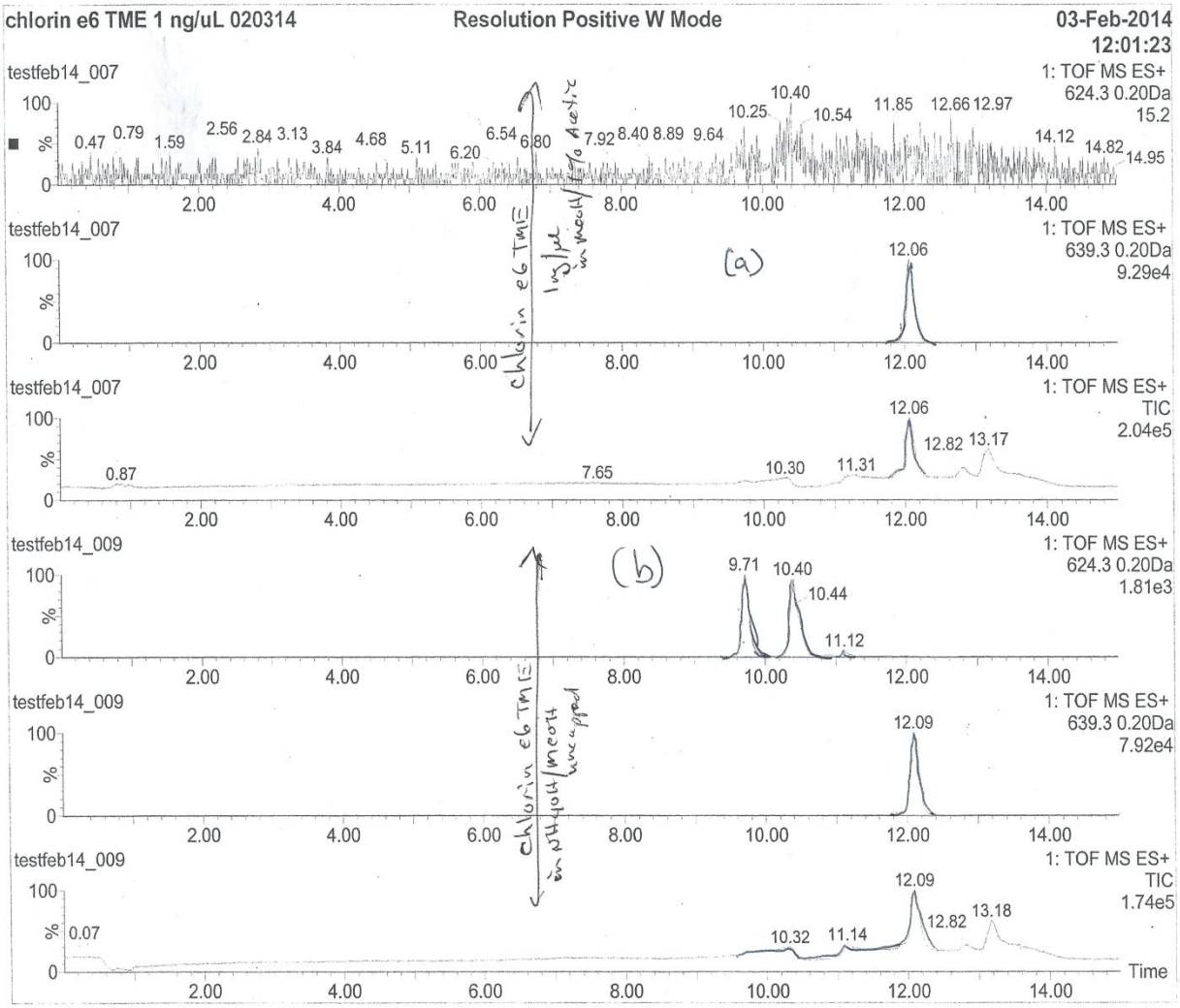

Figure 6. Fish kill graph and mass spectra of unknown m/z 639.3

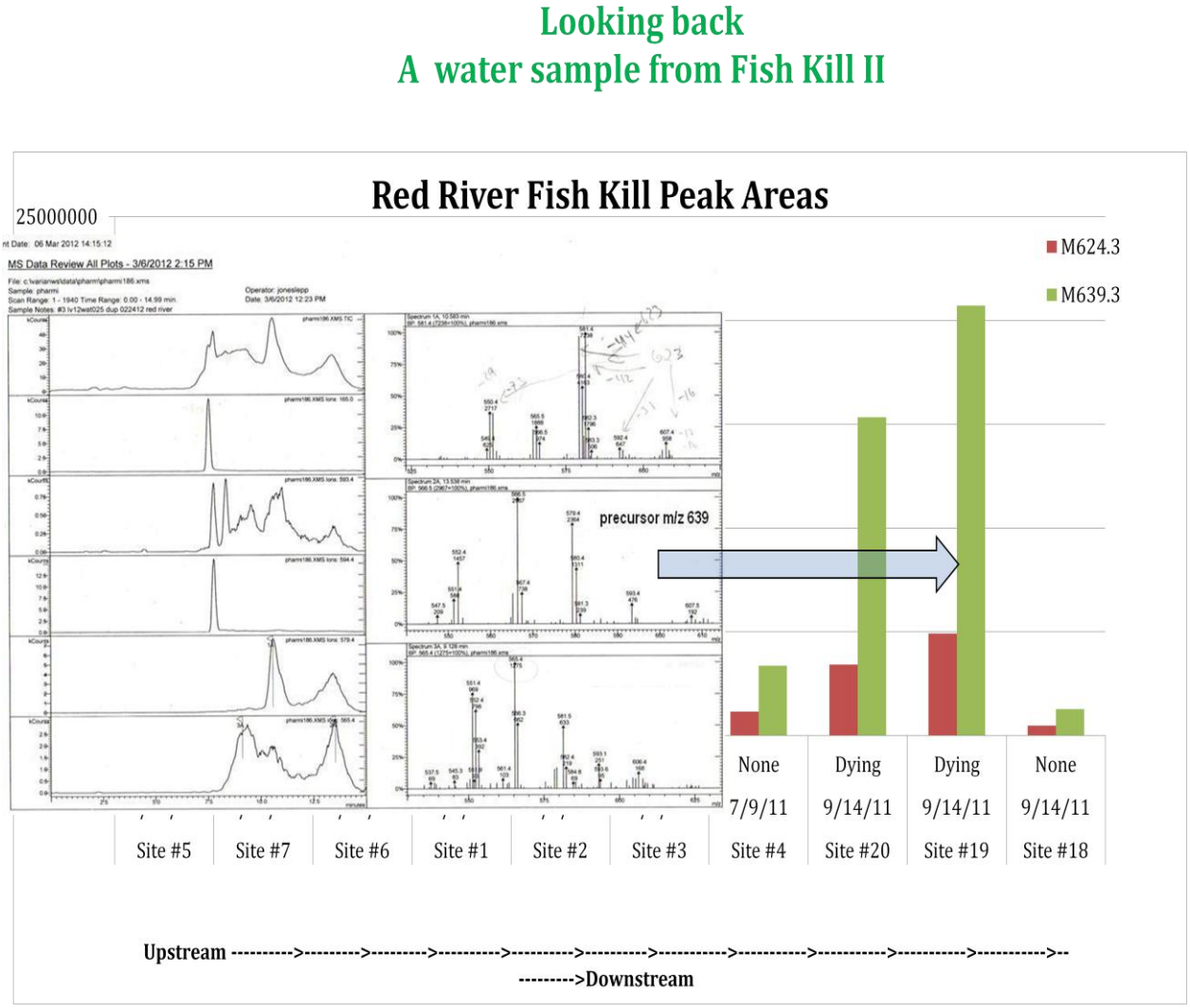

Table 1. ITMS or TOFMS screen and confirmation of Chlorine6 TME by CID ITMS or TOFMS

sample ID	Date collected	639.3	Confirmed by CID ITMS and/or TOFMS
Site 1 lv12wat002	07/09/11	x	No sample left
Site 2 lv12wat007	07/09/11	x	Yes (02/13/12)
Site 2 lv12wat004	07/09/11	x	Yes (09/18/13)
Site 3 lv12wat011	07/09/11	x	No sample left
Site 3 lv12wat008	07/09/11	x	Yes (09/18/13)
Site 4 lv12wat013	07/09/11	x	No sample left
Site 5 lv12wat014	07/13/11	x	No sample left
Site 6 lv12wat017	07/13/11	x	No sample left
Site 7 lv12wat020	07/13/11	x	No sample left
Site 18 lv12wat023	09/14/11	x	Not confirmed
Site 19 lv12wat025	09/14/11	x	Yes
Site 20 lv12wat027	09/14/11	x	Yes
sample ID	Date collected	639.3	
lv12wat110A	06/13/12	nd	
lv12wat110B	06/13/12	nd	
lv12wat111A*	06/13/12	nd	masses *m/z* 562.3 and *m/z* 564.3 present
lv12wat112A	06/13/12	nd	
lv12wat112B	06/13/12	nd	
lv12wat113A*	06/13/12	nd	masses *m/z* 562.3 and *m/z* 564.3 present
lv12wat114 (TB)	06/13/12	nd	
lv12wat115A*	06/12/12	nd	masses *m/z* 562.3 and *m/z* 564.3 present
lv12wat116A	06/12/12	nd	
lv12wat116B	06/12/12	nd	
lv12wat117 (TB)	06/12/12	nd	
lv12wat118a	06/14/12	nd	
lv12wat119A	06/15/12	nd	

lv12wat120A	06/21/12	nd	
lv12wat121	06/21/12	nd	
lv12wat122	06/20/12	nd	
lv12wat123A	06/20/12	nd	
lv12wat124A	06/21/12	nd	
lv12wat125A	06/21/12	nd	
lv12wat126A	06/21/12	nd	
sample ID	Date collected	639.3	
lv12wat135	02/14/12	nd	
lv12wat136	02/14/12	nd	
lv12wat137	02/14/12	nd	
lv12wat139	02/14/12	nd	
lv12wat140	02/14/12	nd	
lv12wat145	02/14/12	nd	
lv12wat146	02/14/12	nd	
sample ID	Date collected	639.3	
lv13wat006	01/31/13	nd	
lv13wat007	01/31/13	x	Yes
lv13wat008‡	01/31/13	x	Yes
lv13wat009	01/31/13	x	Yes
sample ID	Date collected	639.3	
lv13wat018	09/04/13	nd	
lv13wat019	09/04/13	x	Yes
lv13wat020	09/03/13	x	Yes
lv13wat021	09/04/13	x	Yes
lv13wat022	09/03/13	x	Yes
lv13wat023	09/03/13	x	Yes
sample ID	Date collected	639.3	

* These three samples have possibly different geoporphyrins present at masses *m/z* 562.3 $(M+H)^+$ and *m/z* 564.3 $(M+H)^+$

‡ Large mass detected at *m/z* 826.7 Da ($M^{+\cdot}$); nd = not detected; x = detected during screening analysis

Table 2. Sediment data – CID ITMS screening for *m/z* 639.3, chlorine6 trimethylester.

Site identification	OK ID	EPA ID	MS/MS confirmation
Site #2/Approx. 5.72 mi. US of I-35	506356 B	lv12sed002	nd
Site #3/Approx. 3.56 mi. US of I-35	506357 A	lv12sed003	nd
Site #5/Ketchum Bluff	506648 A	lv12sed004	nd
Site #6/Approx. 2.24 mi. DS of BR	506649A	lv12sed005	nd
Site #7/Primitive BR @ Oscar	506650 A	lv12sed006	nd
Hwy 89 near Courtney	521445A	lv12sed007	Yes
Hwy 81/Ryan, Ok (Barn stockpile)	521452A	lv12sed009	nd
Ketchum Bluff	521446A	lv12sed011	Yes
Co. Rd. 2940	521447A	lv12sed013	Yes
Union Valley Rd. near Oscar	521448A	lv12sed015	< trace
Bub Wilcoxin (lower)	521449A	lv12sed017	nd
Bub Wilcoxin (upper)	521454A	lv12sed019	Yes
Hwy 32	521450A	lv12sed021	nd
S11RC East Tribute		lv13sed001	Yes
S12RC N2900		lv13sed002	Yes

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APPENDIX A

STANDARD OPERATING PROCEDURE FOR EXTRACTION AND DETECTION OF EMERGING CONTAMINANTS USING SOLID-PHASE EXTRACTION AND LIQUID CHROMATOGRAPHY-ION TRAP MASS SPECTROMETRY

January 2014

United States Environmental Protection Agency
Environmental Sciences Division
Environmental Chemistry Branch

_____ _____
Brian Schumacher, Branch Chief Date

_____ _____
Tammy Jones-Lepp, Principal Investigator Date

_____ _____
Ed Heithmar, Branch Quality Assurance Representative Date

_____ _____
ESD Quality Assurance Manager Date

STANDARD OPERATING PROCEDURE FOR:
EXTRACTION and ANALYSIS OF EMERGING CONTAMINANTS in AQUEOUS SAMPLES USING SPE and LC-IONTRAP MS

1.0 Disclaimer

This standard operating procedure (SOP) has been prepared for use of the Environmental Sciences Division, Environmental Chemistry Branch, National Exposure Research Laboratory, Office of Research and Development, of the U.S. Environmental Protection Agency and may not be specifically applicable to the activities of other organizations. **THIS IS NOT AN OFFICIAL EPA APPROVED METHOD.** This document has not been through the Agency's peer review process or ORD clearance process. Additionally, this SOP is equipment and/or instrument-specific.

2.0 Purpose (Scope and Application)

This document describes the procedure for the determination of emerging contaminants (ECs), in aqueous samples using a Thermo Fisher (formerly Dionex) Autotrace for an automated solid-phase extraction (SPE) procedure, and an Agilent (formerly Varian) liquid chromatography-ion trap mass spectrometer (LC-ITMS) for detection.

3.0 Method Summary

3.1 The method employs high-performance liquid chromatography (HPLC) coupled with positive (or negative) electrospray ionization (ESI-) ion trap collision induced (CID) mass spectrometry (MS/MS) for the determination of emerging contaminants in aqueous matrices.

3.2 Aqueous samples are extracted through Oasis MCX SPE cartridges to extract the ECs from solution before concentrating the eluants to 0.5 mL.

3.2 Unknown ECs are tentatively identified by using LC-ITMS and searching known mass spectral databases and operator knowledge of mass spectrometry. Known ECs can be quantified using select internal standards.

4.0 Interferences

4.1 All glassware must be washed with detergents free from alkylphenol ethoxylates. Powdered Alconox does not contain ethoxylated alcohols, but any comparable detergent free from these interferences may also be used. This is then followed by acid washing, rewashing in DI water, rinsing with methanol and heated in an oven. See section 11.5.

4.2 Method interferences can be caused by contaminants in glassware, solvents, and other apparatus producing discrete artifacts or elevated baselines. These materials are routinely demonstrated to be free from interferences by analyzing laboratory reagent blanks and method blanks under the same conditions as the samples.

4.3 Matrix interferences may be caused by contaminants that are co-extracted from the sample.

4.4 It must also be demonstrated that the AutoTrace is free of contamination. It has been shown that certain recalcitrant ECs can remain in the Teflon lines of the Autotrace. After suspected EC contamination the whole system of the AutoTrace must be cleaned with a mixture of water and methanol (50:50) until all traces of the contaminants are purged.

4.5 Instrumentation blanks must be analyzed before, during and subsequent to mass spectral analyses to ensure contamination free analyses. Certain ECs are recalcitrant and can remain in various parts of the LC-ITMS, causing "ghost" peaks, and interfering with subsequent analyses. It is incumbent upon the mass spectrometer operator to ensure contamination free analyses, and demonstrate this.

5.0 Safety

5.1 All of the samples and chemicals used in this procedure should be handled only while using proper personal protective equipment such as gloves, lab coats, safety glasses and fume hoods. The analyst should review the Material Safety Data Sheet for each chemical in this procedure so that safe working conditions can be achieved.

5.2 The toxicity of each sample received, and the reagents used in this method may not be fully established. Each sample and chemical should be regarded as a potential health hazard, and exposure should be kept as low as reasonably achievable.

5.3 Waste must be disposed of in appropriate waste containers. Contact the onsite SHEM Program Manager to dispose of full waste containers.

5.4 Exhaust fumes from the LC-MS must be properly vented.

5.5 All applicable safety and compliance guidelines set forth by the EPA and by federal, state, and local regulations must be followed during the performance of this SOP. Stop all work in the event of a known or potential compromise to the health and safety of any person and

immediately notify the SHEM Program Manager and other appropriate personnel.

5.6 Analysts must be cognizant of all instrumental hazards (i.e., dangers from electrical shock, heat, or explosion).

6.0 Reagents/Chemicals/Gases

6.1 HPLC-grade methanol

6.2 HPLC-grade water

6.3 HPLC-grade acetonitrile

6.4 Deionized (DI) water: in-house 18 MΩ-cm DI water

6.5 ACS research grade methyl t-butyl ether (MTBE)

6.6 ACS reagent grade sodium chloride (NaCl)

6.7 ACS reagent grade ammonium hydroxide (NH_4OH), 28% - 30%

6.8 ACS reagent grade hydrochloric acid (HCl), 12 N

6.6 Deuterated internal standards, to be chosen by the mass spectral analyst to be consistent with possible ECs present. For example, if the target ECs are pharmaceuticals, then use a 10 ng/µL mixture of d_3-azithromycin, d_3-clarithromycin, and 20 ng/µL d_5-MDMA, in methanol. If target ECs are aromatase inhibitors, then use 10 ng/µL of d_{12}-anastrozole, d_3-exemestane, and d_5-tamoxifen, and 20 ng/µL of d_4-letrozole, in acetonitrile. The labeled pharmaceuticals cover a wide mass range and are suitable for use with unknown ECs.

6.7 LC/MS tuning solutions available from a variety of instrument manufacturers. Must contain compounds that are in the instrument manufacturers tuning and mass calibration procedures. Analyst needs to follow instrument manufacturer's protocol for mass spectrometric tuning and mass calibration procedures.

7.0 Equipment and Supplies

7.1 HPLC-Ion Trap MS system: (Agilent (formerly Varian) 500MS coupled with Varian/ASI HPLC and autosampler).

7.2 HPLC 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}). Other columns may be used if they provide sufficient retention and separation of analytes.

7.3 Variable volume standard pipettors (0.5 -10 µL, 20-200 µL, 100-1000 µL)

7.4 Disposable pipet tips

7.5 Glass beakers, volumetric flasks, sized as appropriate

7.6 Disposable borosilicate Pasteur pipets

7.7 Ultra-high-purity grade compressed nitrogen

7.8 1.8 mL autosampler vials with PTFE/silicone septa

7.11 TurboVap concentrator, and 50 mL nipple tubes, 0.5 mL endpoints, for concentrating samples

7.12 AutoTrace 6-station SPE Workstation

7.13 Oasis MCX SPE cartridges (200 mg, 6 cc size)

8.0 Sample Collection, Preservation, and Storage

8.1 This SOP does not describe sample collection procedures; however, the following guidelines are followed once samples are received in the laboratory.

8.2 Samples must be stored at 4°C in a designated sample refrigerator.

8.3 Holding time studies have not been performed on these analytes; however, samples should be analyzed as soon as possible, and within 28 days.

9.0 Quality Control

9.1 The following are relevant QC criteria for this method.

Table 1. Data Quality Indicators of Measurement Data.

QC Check	Frequency	Completeness	Precision	Accuracy	Corrective Action
Initial known standard 3-pt calibration	Prior to sample analysis	100%	RSD≤30%	$R^2 > 0.98$	Review data, re-analyze.

Laboratory blank	One per batch of samples[a]	100%	N/A	< PQL[b]	Inspect the system and reanalyze the blank. Samples must be bracketed by acceptable QC or they will be invalidated.
Instrument blank	One at beginning of each analysis of analytical 8-hr day, and between samples if high level contaminants	100%	N/A	< PQL[b]	Inspect the system and reanalyze the blank. Samples must be bracketed by acceptable QC or they will be invalidated.
Laboratory spiked sample (LSS)	One per batch of samples[a]	100%	RPD≤30%[c]	± 30% of known value	Check the system and reanalyze the standard. Re-prepare the standard if necessary. Recalibrate the instrument if the criteria cannot be met. Samples must be bracketed by acceptable QC or they will be invalidated.
Laboratory replicates	One per batch of samples[a]	100%	RPD≤30%[c]	TBD	Inspect the system, narrate discrepancy. Samples must be bracketed by acceptable QC or they will be invalidated.
Minimum detection limit	Each chemical	100%	TBD for each EC chemical	TBD for each EC chemical	TBD for each EC chemical

[a]Batch of samples not to exceed 12; [b]PQL=practical quantitation limit, 5 times the MDL; [c]Precision among replicates if more that 1 batch of samples are analyzed. RSD may be applicable if more than 2 replicates are analyzed.

10.0 Calibration and Standardization

10.1 Tune and calibrate MS according to manufacturer's directions using the manufacturer's recommended tuning solution.

10.3 Tuning to determine the correct system settings (e.g., curtain gas, temperature, ion spray voltage, declustering potential, etc.) for particular analytes is performed as needed and according to the manufacturer's directions. This is done according to the manufacturer's instructions.

10.4 Record all instrument maintenance in the instrument maintenance log book.

10.5 Calibration by isotope dilution: isotope dilution is used for calibration of each native compound for which a labeled analog is available. Please refer to EPA SW-846 Method 1694, Section 10.

10.6 Calibration by internal standard: internal standard calibration is applied to the determination of the native compounds for which a labeled compound is not available. Please refer to EPA SW-846 Method 1694, Section 10.

11.0 Procedure

11.1 Sample preparation

11.1.1 Transfer 500 mL of the environmental water sample (DI water if it's a blank) into numerically labeled volumetric flasks.

11.1.2 Use pH paper to test pH of each water sample and record the initial pH of sample in notebook.

11.1.3 Spike internal standard (50 µL internal standard mix, see section 6.6) directly into water sample, and indicate spiked samples in notebook.

11.1.3.1 Spike known QC spiking compound(s) directly into one water sample per extraction batch. This data will be used to evaluate extraction efficiencies.

11.1.4 Cap and shake volumetric flask.

11.1.5 Add 100 µL of NH_4OH (or 700 µL of HCl for acidic pH) to each 500 mL water sample.

11.1.6 Cap and shake volumetric flask.

11.1.7 Test pH of water samples.

11.1.7.1 If basic pH is desired, ensure that each water sample has a pH > 9. If the pH is not > 9, then add more NH_4OH (50 µL at a time) until a pH of > 9 is attained. Record the final pH and the amount of NH_4OH added in notebook.

11.1.7.2 If acidic pH is desired, ensure that each water sample has a pH of < 3. If the pH is not < 3, then add more HCl (100 µL at a time) until a pH of < 3 is attained. Record the final pH and the amount of HCl added in notebook.

11.1.8 Add 3g of NaCl to each sample.

11.1.9 Cap and shake volumetric flask.

11.2 AutoTrace solid-phase extraction

This method was developed as an automated SPE method. If need be it could be converted to a manual method using the appropriate SPE cartridges and a vacuum SPE manifold, and following the steps as outlined below.

11.2.1 Place water samples in sample holder. Rinse the outside of the sample lines with methanol, then DI water, and place into respective prepared water samples.

11.2.2 Program the computer to input the following conditions as outlined in steps 11.2.3 through 11.2.6. Including a "pause" step, as noted in step 11.2.4. Be sure to take note of the time that the machine will "pause" and be ready to add 50 mL of DI water to each sample.

11.2.3 Load cartridges into AutoTrace SPE Workstation and precondition the Oasis MCX cartridges with 5 mL methanol, 5 mL de-ionized (DI) water, and 95% water/5%methanol at a flow rate of 1 mL min^{-1} . Divert eluant to waste stream.

11.2.4 Load 500 mL aqueous sample through the SPE cartridges at a flow rate of 7 mL min^{-1}. After the 500 mL have loaded through the cartridges, pause system, rinse the sample volumetric flasks with 50 mL DI water (leave the rinsate in the flask), and continue to load the rinsate (left in the flask) through the SPE cartridges.

11.2.5 Dry the cartridges with N_2 for 40 min.

11.2.6 Elute 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}.

11.2.6.1 After all samples have loaded, and elution has begun, remove lines from empty volumetric flasks. Rinse the outside of the lines with methanol, then DI water, then place lines in container of DI water.

11.2.6.2 Once method is complete, machine will make a beeping noise. Press "Cont" to purge lines with a 50:50 mixture of methanol:DI water. If emerging contaminant levels were high it may be necessary to repeat his step 2 or 3 times.

11.2.7 Qualitatively transfer the eluate from the AutoTrace collection tube to a TurboVap 0.5 mL (or 1 mL) endpoint nipple tubes. This involves gently pouring and subsequent rinses of the AutoTrace elution collection vials with final solvent to be used for mass spectrometric analysis, i.e., methanol/1% acetic acid, or acetonitrile/1% acetic acid.

11.2.8 Initially set the TurboVap to a gentle nitrogen stream, approximately 3 or 4 psi. As the solvent in the nipple tubes decrease the flow can be increased to 13 psi. During the evaporation process rinse the sides of

the TurboVap tubes at least 4 times, with the final LC-MS compatible solvent. Concentrate eluants to 0.5 mL.

11.2.9 Transfer the concentrated sample with Pasteur pipette to an appropriate sized LC-MS autosampler vial, capped with a PTFE/silicone septa.

11.2.10 Filter the samples, if necessary, with a syringe filter prior to MS analysis.

11.3 **LC-MS analysis**

11.3.1 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.

11.3.2 The following LC gradient is used to analyze ECs (column temperature approx room temp 23°C)

Table 1. LC gradient conditions.

Time (min)	Flow rate (mL/min)	%A	%B
Initial	0.30	100	0
2	0.30	100	0
5	0.30	30	70
10	0.30	30	70
13	0.30	100	0
15	0.30	100	0

11.3.3 Starting MS analysis conditions: Source conditions: Electrospray needle voltage: 5000 to 5800kV, Ion Source temperature: 350^{o} C, Housing chamber 50^{o} C; drying gas, 20 psi; nebulizer gas, 40 psi; spray shield, 600 V. Capillary voltage and percent radio frequency (%RF, on the hexapoles) are set dependent upon the optimized response of the precursor and product ions of interest. See table 2 for suggested precursor and product ions as produced by the Varian LC-ITMS. Other LC-MSMS instruments may produce different product ions, at varying intensities.

11.3.3.1 Operator should turn instrument gases, source and LC on 1/2 hr to 1 hr before operation. Ensure LC flow starts and is in the inject mode into the MS. This will allow the trap to warmup and have ions flowing into the trap and towards the detector.

11.3.4 Load samples into the autosampler. Program autosampler method file with correct sample id's, contents, volume injected, vial position. Refer to manufacturer manual.

11.3.5 Select browse function in sample table and ensure proper method is loaded.

11.3.5.1 For unknown screening, initially a full-scan method should be utilized such that the %RF is set to 50% and the capillary voltage to 40 eV to 60 eV. There will be ions missed at these voltages and RF, so the operator may want to run a second pass at different %RF and capillary voltages, if enough sample extract permits.

11.3.5.2 Once an unknown of sufficient intensity is discovered (the operator will need to ensure that the unknown is NOT a background ion by analyzing a sufficient number of instrument and method blanks) the operator will set up a MS/MS method file, such that the CID energies are sufficient to produce product ions from the selected precursor ion(s). A single MS/MS method can be set up such that multiple precursor ions detected during the screening phase can be analyzed during a single analytical run. See table 2 for several known ECs and their precursor and product ions as produced by the Varian LC-ITMS under optimized conditions. This types of data can be produced by other mass spectrometers that are capable of isolating and producing product ions. As each instrument is unique, the optimized settings will need to be set by a skilled analyst trained in the art of mass spectrometry.

Table 2. MS/MS ions for several known ECs

Compound	Precursor ion	Major Product ion(s)
Urobilin hydrochloride	591.3 $(M + H - HCl)^+$	343.3 $[M+H- HCl - 2(C_7H_{10}NO)]^+$
Azithromycin	749.5 $(M+H)^+$	591.4 $(M+H-C_8H_{16}O_2N)^+$
d_3-Azithromycin (ISTD)	752.5 (M+H)+	594.4 $(M+H-C_8H_{16}O_2N)^+$
Clarithromycin	748.4 $(M+H)^+$	590.1 $(M+H-C_8H_{16}O_2N)^+$
d_3-Clarithromycin (ISTD)	751.4 $(M+H)^+$	593.4 $(M+H-C_8H_{16}O_2N)^+$
Clindamycin	425.2 $(M+H)^+$	377.2 $(M+H-SH-CH_3)^+$
Methamphetamine	150 $(M+H)^+$	119 $(M+H-CH_3NH_2)^+$
MDMA(Ecstasy)	194 $(M+H)^+$	163.0 $(M-CH_3NH_2+H)^+$
d_5-MDMA (ISTD)	199 $(M+H)^+$	165.0 $(M-CD_3NH_2+H)^+$
Pseudoephedrine	166 $(M+H)^+$	148.2 $(M+H-H_2O)^+$
Hydrocodone	300 $(M+H)^+$	199 $(M+H-C_5H_{11}NO)^+$
Chlorin e6 trimethyl ester	639 $(M+H)^+$	579 $(M+H-C_2H_4O_2)^+$, 566 $(M+H-C_3H_5O_2)^{+\cdot}$

11.3.6 Ensure that LC solvent levels are adequate and that there is enough N_2 gas to complete the analyses. Once the instrument is ready, begin the sample acquisition process.

11.3.7 At the end of each analytical day the operator should open the source door, gently spray methanol onto the spray shield and wipe the source surfaces with a clean kimwipe (or similar material). **Hazard**: Spray shield is very hot, and there may be toxic contaminants on the shield, use appropriate personal protective gear.

11.4 Data Analysis

11.4.1 Inspect each prominent chromatographic peak for a Gaussian appearance. The peaks may not be Gaussian in appearance due to the presence of multiple isomers, or interferences. The operator can try changing the LC conditions to try for better separations on subsequent analyses.

11.4.3 Identify and confirm the presence of unknown ECs in the samples by reviewing the total ion chromatograms (TICs) for large, > 20% intensity above background signal, for masses that are not common background contaminants, i.e., surfactants (unless looking specifically for surfactants).

11.4.4 Once an intense unknown EC precursor ion has been selected the operator will set up a MS/MS method file, such that the CID energies are sufficient to produce product ions from the selected precursor ion(s).

11.4.4.1 The operator will then review the CID fragment ions produced and try to determine a structural assignment to the fragment ions. Also, using the precursor ion the analyst should be able to assign a molecular weight to the unknown.

11.4.4.2 If the analyst has available enough sample extract and access to an accurate mass, mass spectrometer, for example, a Time-of-flight mass spectrometer (TOFMS), then the extract should be analyzed by TOFMS for accurate mass of the unknown(s). Accurate mass can help eliminate many chemical formulas that are generated by less accurate mass measurements. Ideally, the ability to generate accurate mass product ions would

help greatly in the identification of unknown emerging contaminants.

11.4.5 To ensure good quality of the sample process on occasion samples will be spiked with a known compound. These can be quantitated by using isotope dilution or internal standard techniques. By adding a known amount of a labeled compound to every sample prior to extraction, correction for recovery of the native analog of that compound can be made because the native compound and its labeled analog exhibit similar chemical properties upon extraction, concentration, and chromatography.
See Section 10 and Section 17 of the US EPA Method 1694. Note: During calculations, take into account the concentration factor from the 500 mL sample down to 0.5 mL following extraction/concentration.

11.5 Glassware cleaning

11.5.1 Prepare soapy bath with hot water and approximately 1 tsp Alconox detergent. Scrub glassware with bottle brushes and/or pipe cleaners until visibly clean (do not scratch glassware with metal from brushes).

11.5.2 Rinse glassware first with non-DI water, and then with DI water.

11.5.3 Soak glassware in acid bath (3 mL HCl, 3 mL HNO_3, 4 L water, pH 1-2) overnight.

11.5.4 Remove glassware and rinse with DI water; rinse glassware with methanol and air dry.

111.5.5 Place glassware in oven at 100°C for 6 hours. Let cool in oven. Remove cooled glassware and put away in appropriate areas.

12.0 Method Performance

12.1 Method performance can be evaluated based on the criteria in Table 1.

12.2 MDLs have not been determined yet because this is a method for screening and identification of unknown emerging contaminants.

13.0 References

EPA Method 1694. "Pharmaceuticals and Personal Care Products in Water, Soil, Sediment, and Biosolids by HPLC/MS/MS", 2007.

APPENDIX B

GCMS ANALYSIS ON WATER EXTRACTS DATED 2.22.2013

Analysis done by Charlita Rosal and Wayne Sovocool
Extraction was performed by Trevor Nance Jr. and Matt Ward

The following Agilent data file numbers with corresponding sample IDs are provided below:

#1	13022806	LV13WAT006 (blank)
	13022808	DUPLICATE RUN OF LV13WAT006
#2	13022810	LV13WAT007
	13022812	DUPLICATE RUN OF LV13WAT007
#3	13022814	LV13WAT007 DUP
	13022816	DUPLICATE RUN OF LV13WAT007 DUP
#4	13022818	LV13WAT008
	13022820	DUPLICATE RUN OF LV13WAT008
#5	13022822	LV13WAT008 spike
	13022824	DUPLICATE RUN OF LV13WAT008 spike
#6	13022826	LV13WAT009
	13022828	DUPLICATE RUN OF LV13WAT009

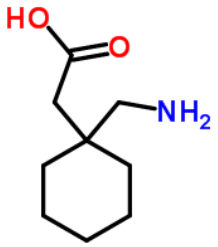
Water extracts were provided in 0.5-mL volume of methanol/1% acetic acid. These extracts were solvent exchanged and brought up to 1 mL with ethyl acetate prior to gas chromatography-mass spectrometry (GC-MS) analysis. All samples above were analyzed using an Agilent GC-MS in pulsed-splitless injection, electron impact (EI) scan mode.

Results and discussion

Except for sample #1(blank sample), all three environmental water samples contained gabapentin at unknown concentrations.

Gabapentin (common name Neurontin) is used primarily to treat seizures, neuropathic pain, including concussions, and hot flashes (http://en.wikipedia.org/wiki/Gabapentin). Gabapentin (1-(aminomethyl) cyclohexaneacetic acid), has a molecular formula of $C_9H_{17}NO_2$, and mw171.24 Da. Gabapentin is a white to off-white crystalline solid with a pK_{a1} of 3.7 and a pK_{a2} of 10.7. It is freely soluble in water and both basic and acidic aqueous solutions.

The structural formula of gabapentin is:



In order to confirm the presence of gabapentin in the water samples, we acquired prescription gabapentin in 100-mg capsules, as well as a neat standard from Sigma-Aldrich. We dissolved these materials individually in DI/methanol and then analyzed by GC-MS using the same method as the water samples. Retention time and spectra of both the prescription drug and the neat standard confirmed that what was detected in the environmental water samples was gabapentin. Using the same approach as was used with the environmental water samples an extraction of gabapentin was repeated in DI water. Two 100-mg portions of prescription gabapentin were each dissolved in 500-mL DI water (200 mg/L), extracted, and analyzed by GC-MS. Preliminary studies suggest that high levels of gabapentin could be present in the water samples. However, the extraction procedure used was not optimized for this compound, therefore further method optimization would be necessary. Also, this compound is not ideal for GC-MS analysis and confirmation by liquid chromatography-mass spectrometry (LC-MS) would be a better analytical technique.

Another interesting peak was detected in all of the water extracts (except the blank). This included those extracts from DI water spiked with neat standard and the prescription gabapentin. The spectra resemble that of gabapentin's, but with a base peak of *m/z* 195 Da. This unknown did not show in the neat standard and prescription gabapentin dissolved in solvent and directly injected into the GC-MS. No further identification was done on this unknown.

Additionally, low levels of several alkylorganophosphate fire retardants (CAS#'s 13674-84-5, 115-96-8, and 137909-40-1) were found in the samples. However, a literature search did not find much evidence for fish toxicity for these compounds. Low levels of the pesticide terbutylazine (CAS# 5915-41-3) were also found in the samples. This compound does have some fish toxicity, but at much higher levels than what was extrapolated as found. The largest peaks detected in the ion chromatograms, that were not present in the blanks or that were not common contaminants, e.g. plasticizers; were unsaturated lipids.

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