APPLYING AN EFFECT-DIRECTED STRATEGY TO INDTIFY PREVIOULSY UNRECOGNIZED TOXIC CHEMICALS IN HUDSON RIVER SEDIMENTS

A Final Report of the Tibor T. Polgar Fellowship Program

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Abstract

Hudson River sediments highly contaminated with PCBs, PCDDs, and PCDFs are a concern for the watershed and humans that live in the surrounding area. Potential health risks to humans from these compounds are due to fish consumption and contaminated water. For this reason, recent research has focused on the behavior of mixtures of toxic chemicals rather than individual chemicals to get a better assessment for potential toxicity. Previous studies have shown that the biological responses from fractions with known concentrations of estrogenic compounds or dioxin-like compounds do not correspond. This indicates that there are either unrecognized toxic chemicals or mixtures of chemicals that behave differently from individual chemicals. An effect-directed strategy was developed to distinguish between these two hypotheses. Four sediment samples from the upper Hudson River were analyzed using the effect-directed strategy. We performed a sequential extraction using ASE to initially separate non-polar, semi-polar, and highly polar compounds found in the sediment. All extracts inhibited *Vibrio fischeri* metabolism, indicating the presence of toxic compounds in the extracts. MeOH extracts for River miles 152.7 (36-40 cm) and 188.6 (28-32 cm) exhibited high dioxin or dioxin-like activity and high estrogenicity, respectively, leading to the conclusion there are highly polar compounds present that exhibit toxicity. A di-chlorinated compound and tri-chlorinated compound were found in the MeOH extracts of River mile 152.7 (36-40 cm) and River mile 188.6 (28-32 cm) using liquid chromatography mass spectrometry (LC-MS). Further investigation of these compounds is needed.

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Introduction

Over a span of 30 years, ceasing in the 1970's, the General Electric Company (GE) released as much as 1.3 million pounds of polychlorinated biphenyls (PCBs) into the upper Hudson River from its two capacitor manufacturing plants located in Hudson Falls and Fort Edward, New York. High concentrations of PCBs are still present in the sediments of the 40 miles of the upper Hudson River. In addition to the PCB contamination, the Hudson River receives runoff from both agricultural waste and human sewage sources that contribute a variety of other organic contaminants such as pharmaceuticals, detergent metabolites, fire retardants, and other compounds that may cause endocrine disrupting effects on fish species and other wildlife. High concentrations of these compounds can persist in sediment. In addition to the known compounds above, metabolites and degradation products from weathering, photolysis, and hydrolysis may also contribute to the ecotoxicity of commonly known organic compounds. As a result, there is concern for the effects of fish consumption and contaminated water supplies on human health and for the health of the river and its surrounding watershed (McArdle et al. 2004).

A previous study of Hudson River sediment conducted by Dr. Richard Bopp of Rensselaer Polytechnic Institute (RPI) indicates a range of PCB, PCDD and PCDFs in sediment samples taken from the upper Hudson River at River miles 152.7, 163.6 and 188.6. In fact all sediments had part per million (ppm) levels of PCBs and high part per trillion (ppt) levels of polychlorinated dibenzo dioxins (PCDDs) and polychlorinated dibenzo furans (PCDFs) (Table 1) (Personal correspondence with Dr. Bopp, Bopp et al. 1982, Bopp et al. 1998).

Sediment Sample	Total PCB (ppm)	Total PCDD (ppt)	Total PCDF (ppt)
163.6 (2-4 cm)	7.7	388	1,268
163.6 (36-40 cm)	64	3,600	32,322
152.7 (36-40 cm)	40	33,610	928,863
188.6 (28-32 cm)	460	9,510	74,647

Table 1. Previously collected PCB, PCDD, and PCDF concentrations for all sediment samples.

Considering these previous data collected (Personal correspondence with Dr. Bopp, Bopp et al. 1982, Bopp et al. 1998), the focus in this study was on analyzing the polar extracts from the same sediment samples for degradants and metabolites of PCBs, PCDDs and PCDFs, because these compounds tend to be more polar and more toxic than their precursors. Studies have shown that concentrations of known toxic chemicals in sediment extracts fail to correspond to biological response of fractions from these extracts (Suzuki et al. 2004, Schlenk et al. 2005). This indicates that there are either unrecognized toxic chemicals remaining undetected by current analytical techniques or mixtures of chemicals that behave differently from individual chemicals. An effect-directed strategy was developed to distinguish between these two hypotheses.

Methods

Suzuki et al. (2004) showed non-additive increase in toxic effects from extracts and fractions of compost extracts containing polycyclic aromatic hydrocarbons (PAHs). As a result, conventional methods of determining toxicity of mixtures, such as taking toxic equivalency factors (TEF) of individual compounds to calculate the toxic equivalent

quantity (TEQ) for a mixture, were found to be ineffective for assessing the risk associated with environmental samples (Burgess et al. 1999). Brack et al. (1999), Brack and Schirmer (2003), Suzuki et al. (2004), Sundberg et al. (2005), and Schlenck et al. (2005) determined that different assays measuring different toxic effects need to be utilized to determine the full extent of toxicity within a sample. Based on these studies we developed a strategy incorporating assays to measure dioxin or dioxin-like activity, estrogenicity, and energy metabolism inhibition in environmental samples.

Our effect-directed strategy was selected to facilitate the identification of toxic compounds present in Hudson River sediments. This strategy has several key steps: sequential extraction, biological assay and, finally, separation/mass spectrometric analysis (Figure 1.). A combination of these three analysis techniques not only allows for identification of contaminants but also provides toxicity data on the sediment extracts before and after separation. This strategy, illustrated in the schematic diagram shown in Fig. 1, first starts with three sequential extractions of the sediment sample using solvents with increasing polarities for initial sub-fractionation of contaminants based on their polarity. After concentration and cleanup, extracts are tested for toxicity using several different bioassays. Extracts that indicate any degree of toxicity are analyzed by liquid chromatography ion trap mass spectrometry (LC-ITMS) and gas chromatography mass spectrometry (GC-MS). Fractions are also collected from the LC and subjected to bioassays to determine individual peaks that exhibit toxicity. Fractions that are toxic are analyzed further by LC-ITMS and GC-MS to determine if more fractionation and bioassay analysis is necessary. Once individual components have been isolated, MS^2 data are collected as necessary for structural elucidation and identification.

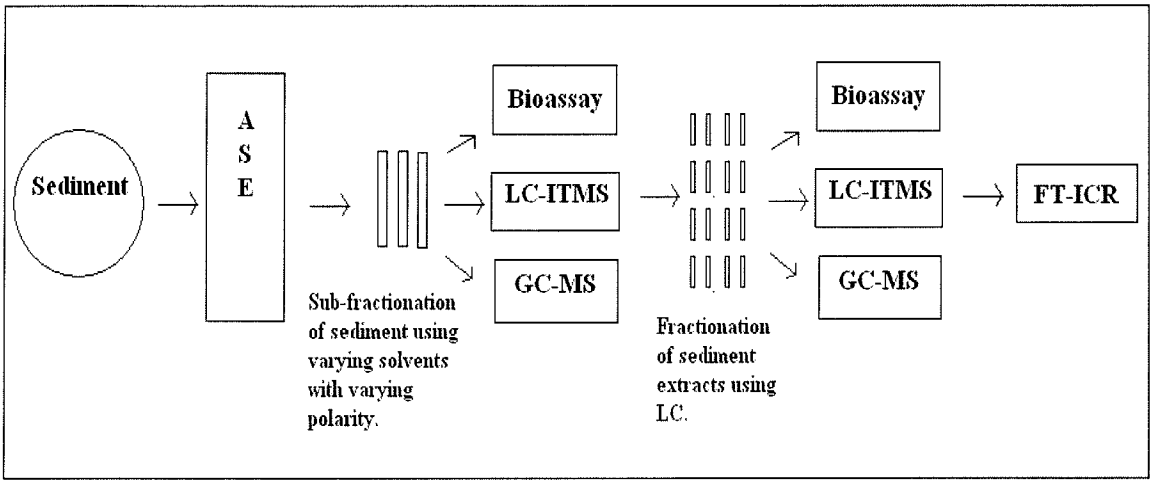

Figure 1. Detailed schematic of effect-directed strategy.

Sediment Extraction & Cleanup

Accelerated Solvent Extraction (ASE) was utilized for sediment extraction (Houtman et al. 2006). A Dionex ASE-200 System was used for extraction. Two grams of sediment and 10 grams of anhydrous sodium sulfate were placed in the extraction chamber. Three extraction solvents were used sequentially. First, hexane extracted nonpolar compounds. Second, a 50:50 mixture of hexane: isopropranol (IPA) extracted semi-polar compounds. Finally, methanol (MeOH) extracted highly polar compounds. The other operating conditions were as follows: pressure of 70 bar, temperature of 100°C and time of 5 minutes. After extraction, extracts were concentrated to ~3mL and passed through an activated alumina column to remove humic acids and other interfering compounds.

Bioassay Analysis

Several bioassays were used to assess the toxicity of sub-fractions from the ASE and fractions collected from the High-Performance Liquid Chromatograph (HPLC): the luciferase reporter gene bioassay, yeast estrogen screen (YES) assay and a rapid toxicity

screening assay. The luciferase reporter gene bioassay is able to detect 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD) levels as low as 1 picoMolar (Gasiewicz et al. 1996, Kostyniak et al. 2005). The analysis was performed by Dr. James Olson and Barbara McGarrigle from the Toxicology Department at the State University of New York at Buffalo. A yeast estrogen screen assay (YES) was used to determine estrogenic activity for all of the extracts from River mile 188.6 (28-32 cm) sediment sample. This colorimetric estrogen reporter gene assay uses *Saccharomyces cerevisiae* (yeast) transfected with human estrogen receptor to measure estrogenicity of samples compared to 17β-estradiol (Routledge and Sumpter 1996, Holbrook et al. 2002). Approximately 2mL of sediment extract was evaporated to dryness and shipped to Dr. Katharine Knowlton at Virginia Tech University for analysis.

The Abratox^{TM} assay is a rapid toxicity screening assay. This assay, manufactured by Abraxis Kits LLC, is a luminescence assay which utilizes *Vibrio fischeri* for the detection of toxic compounds in samples (Brack et al. 1999). Toxic compounds inhibit the bacteria from luminescing by interrupting energy metabolism. Dilutions of sediment extracts were made at 2.5% of extract in 1.5mL of nanopure water. For extracts, a 2.5% solution of acetonitrile (ACN) or MeOH in nanopure water was used for the negative control. The positive controls used were a solution of zinc chloride provided in the assay kit and a 400 parts per billion (ppb) solution of triclosan. Triclosan was selected as an additional positive control for its structural similarities to common organic contaminants found in Hudson River sediments.

Liquid Chromatography-Ion Trap Mass Spectrometry (LC-ITMS)

LC-ITMS was performed on a Thermo (San Jose, CA) LCQ Advantage system. Approximately 1-1.5mL of extract was evaporated to dryness and reconstituted into 0.5mL of ACN for LC-ITMS analysis. Separation was performed on a reversed-phase column with an injection volume of 15μL, with a gradient starting at 30:70 CAN: water (with 0.3% formic acid), ramping up to 100% ACN over 15 minutes. Ionization for mass spectral detection was done by electrospray ionization (ESI) in positive and negative mode (Maziarz et al. 2005, Kemmochi et al. 2002). Positive mode had a capillary temperature of 205°C, sheath gas flow of 20, spray voltage of 4.5 kV, and a capillary voltage of 4.00 V. In negative mode capillary temperature, sheath gas flow and spray voltage were the same but the capillary voltage was -10.00 V. A full scan from 100-1000 *m/z* was acquired. All extracts were initially analyzed and fractionated by LC-ITMS, however only certain extracts with peaks of interest were analyzed using MS^2 and MS^3. Fractions were collected at 2 minute intervals from all extracts and these fractions were subjected to the Abratox™ assay.

Gas Chromatography Mass Spectrometry (GC-MS)

GC-MS is a common environmental analytical technique used for determination of organic contaminants in environmental samples, especially those that are relatively nonpolar and difficult to detect by LC-MS (Ryan et al. 2005, Focant et al. 2001). An Agilent (Palo Alto, CA) single quadrupole GC-MS (model 5973) was used for sediment analysis. Approximately 1mL of the extract was evaporated to dryness and reconstituted with 0.3mL hexane for GC/MS analysis. The GC injection port was at 250°C. A Restek (Bellefonte, PA) RTX-5MS column (40.0m x 250μm x 25μm) with stationary phase of

5% diphenyl/95% dimethyl polysiloxane was used for separation. The method used helium as a carrier gas and the oven started at 50°C held for 1 min, then ramped to 250°C at 20°C/min held for 4 min, and finally ramped to 325°C at 10°C/min held for 4 min with a total run time of 26.50 mins.

Results

The Abratox™ assay showed that all extracts were inhibiting the energy metabolism pathways of *Vibrio fischeri* (Fig. 2).

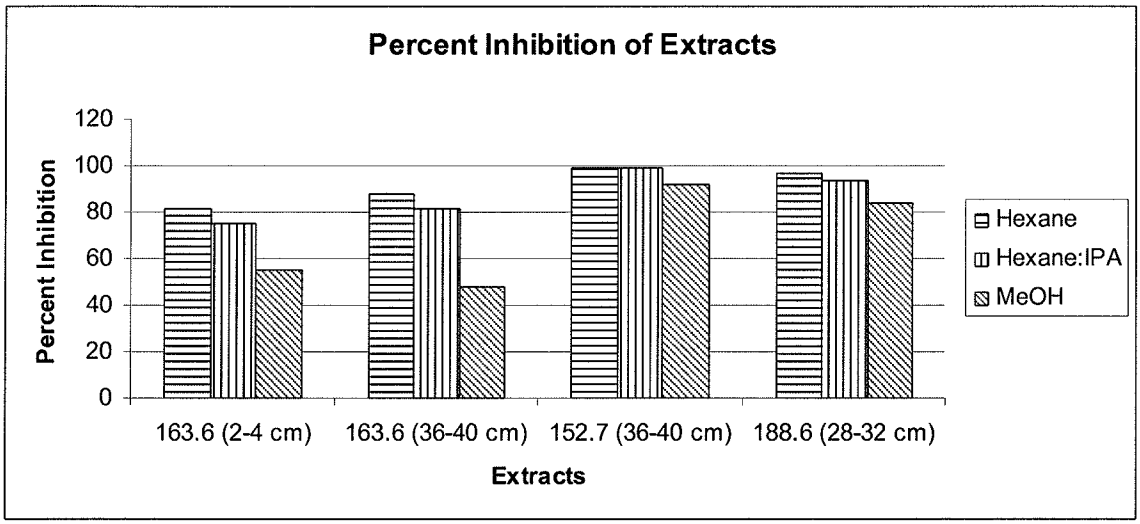

Figure 2. Percent inhibition of luminescence from *Vibrio fischeri* for all sediment extracts.

The MeOH extracts were least toxic of all the extracts within the Abratox™ system, second were the hexane: IPA extracts and, finally, the hexane extracts were most toxic to *Vibrio fischeri*. However, not all extracts showed dioxin or dioxin-like activity in the luciferase reporter gene assay as shown in Fig. 3. Three of the four hexane extracts were below the limit of detection for toxicity; however the hexane extract for River mile 152.7 (36-40 cm) showed dioxin or dioxin-like activity. All hexane: IPA extracts showed dioxin or dioxin-like activity. River mile 152.7 (36-40 cm) showed the highest toxicity,

then River mile 188.6 (28-32 cm) was the next highest, River mile 163.6 (36-40 cm) was third highest, and River mile 163.6 (2-4 cm) was the lowest for hexane: IPA extracts. The MeOH extract for River mile 152.7 (36-40 cm) had the highest toxicity of all extracts. The MeOH extract the River mile 188.6 (28-32 cm) was the second highest toxicity, third was River mile 163.6 (36-40 cm) and, finally, River mile 163.6 (2-4 cm) MeOH extract had the lowest toxicity of the MeOH extracts.

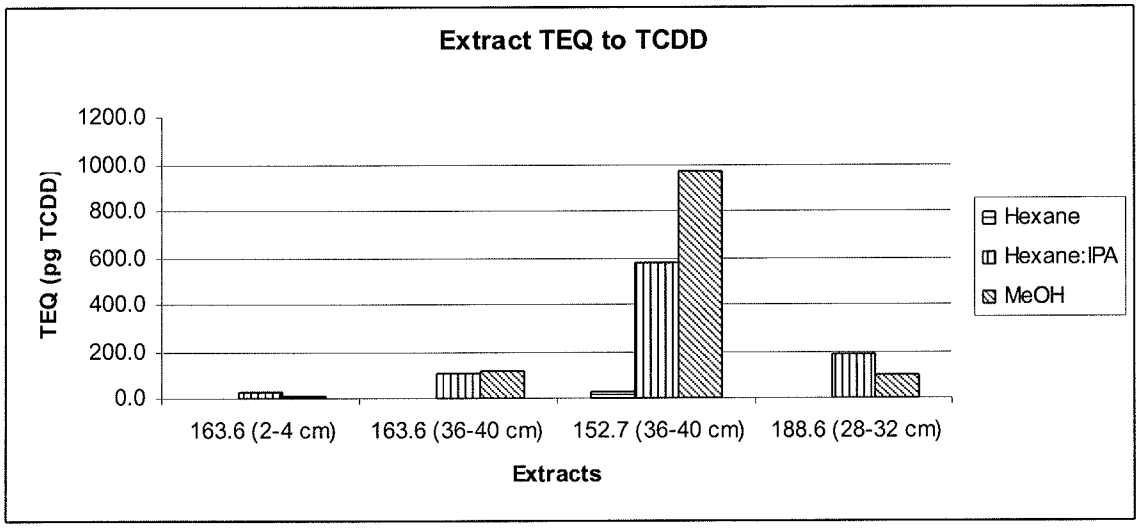

Figure 3. Toxicity equivalents for all extracts compared to pg TCDD.

The hexane, hexane: IPA, and MeOH extracts for River mile 188.6 (28-32 cm) were also run with the YES assay. The MeOH extract had the most estrogenic activity, the hexane extract had the second highest activity, and the hexane: IPA extract had the lowest estrogenic activity seen in Figure 4.

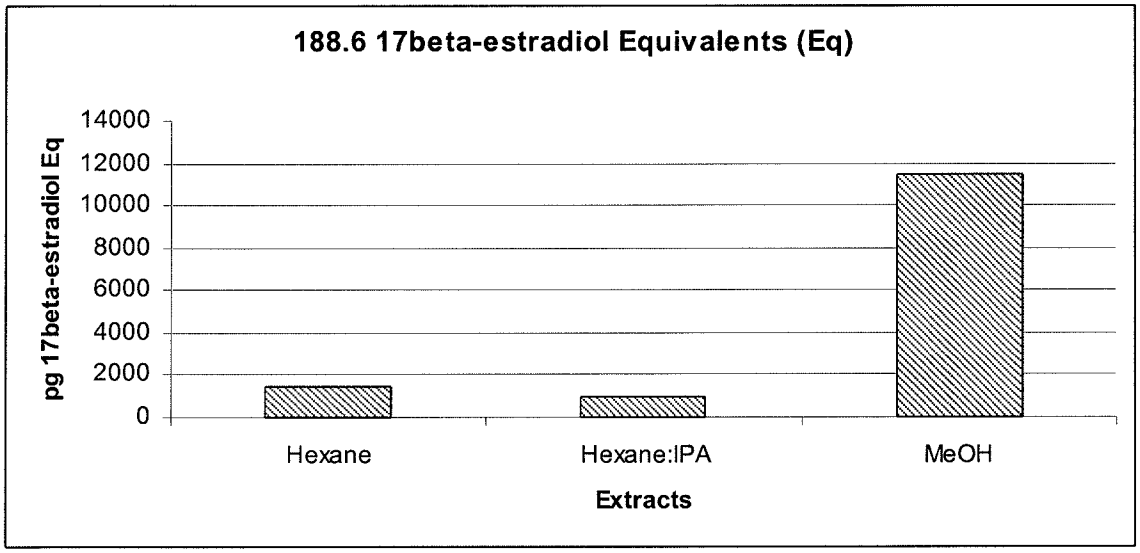

Figure 4. Estrogenicity of River mile 188.6 (28-32 cm) extracts compared to pg of 17β-estradiol.

Once toxicity of extracts was determined they were analyzed by LC-ITMS in positive and negative mode. Fractionation was performed to determine which peaks to focus on. Below in Fig. 5 is an example of a positive mode chromatogram and Fig. 6 is negative mode for the MeOH extract of River mile 188.6 (28-32 cm).

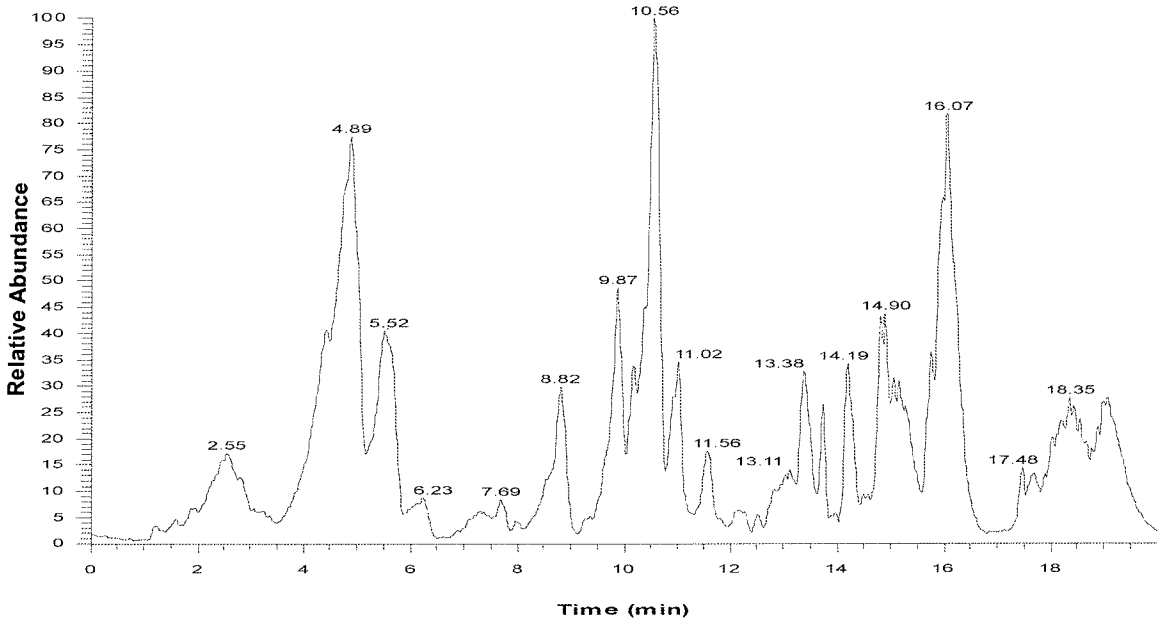

Figure 5. Positive mode LC chromatogram from River mile 188.6 (28-32 cm) MeOH extract.

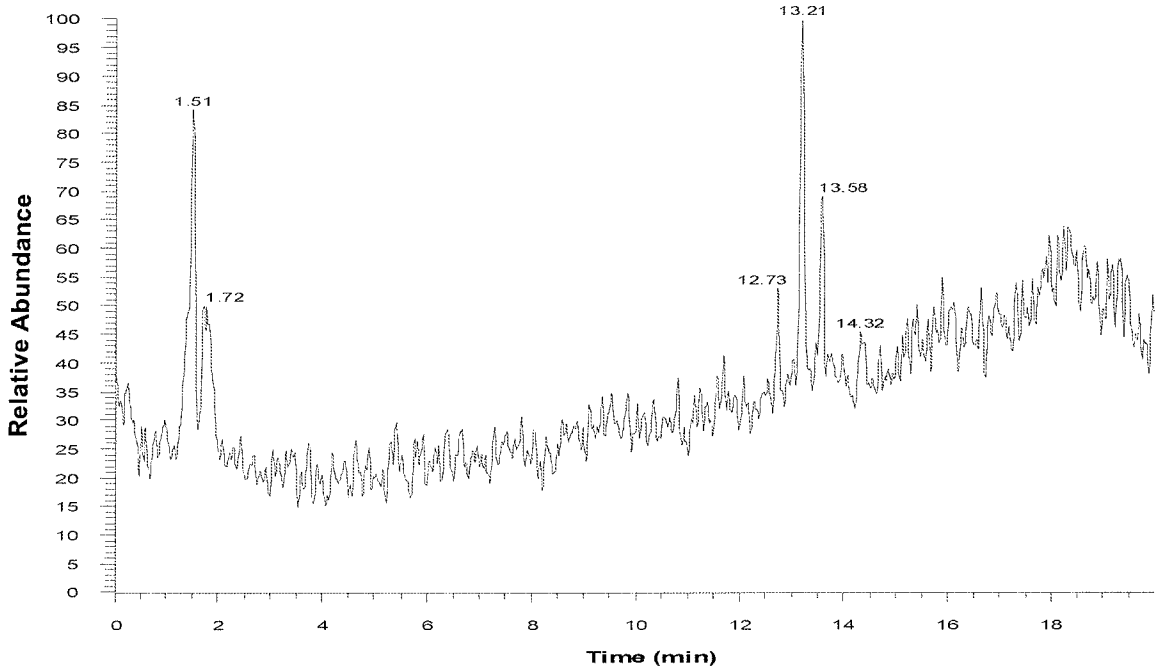

Figure 6. Negative mode LC chromatogram from River mile 188.6 (28-32 cm) MeOH extract.

Although positive mode had more ionizable compounds, negative mode showed two compounds of interest, tri- and di- chlorinated compounds at approximately 13.1 and 13.5 minutes from several of the extracts, specifically the hexane:IPA and MeOH extracts of River miles 152.7 (36-40 cm) and 188.6 (28-32 cm). Shown below in figs. 7, 8, and 9 is a chromatogram and mass spectra of the chlorinated compounds from the MeOH extract of River mile 152.7 (36-40 cm). The two peaks in River mile 152.7 (36-40 cm) MeOH extract correspond to the toxic effect of the fraction from this extract at 12-14 min shown in Fig. 10.

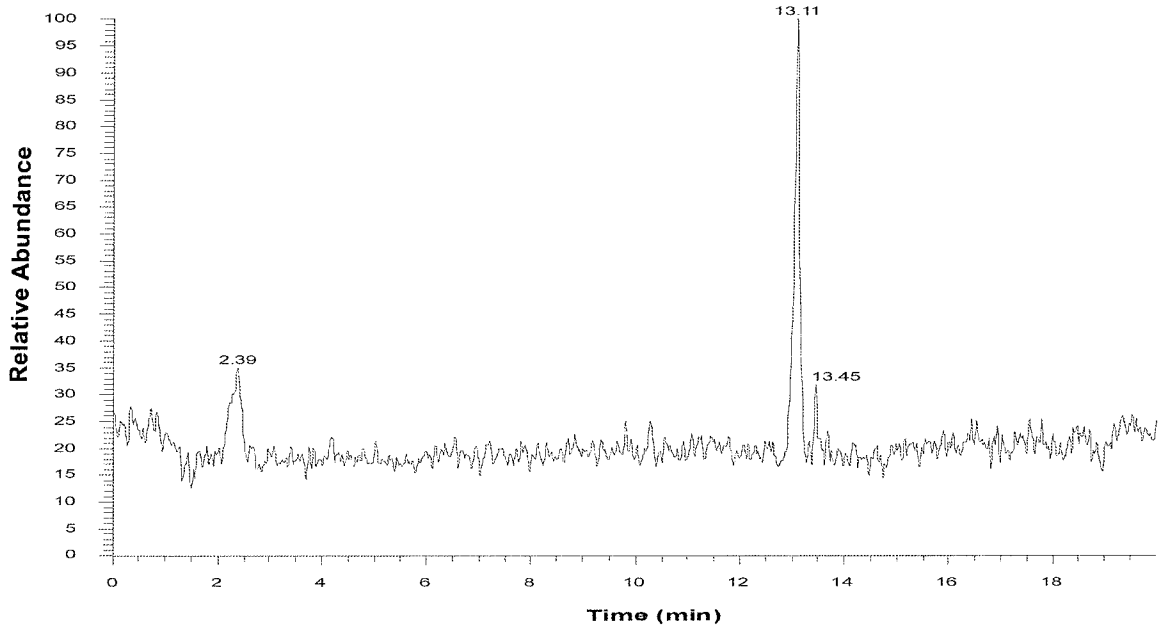

Figure 7. Negative mode LC chromatogram of River mile 152.7 (36-40 cm) MeOH extract.

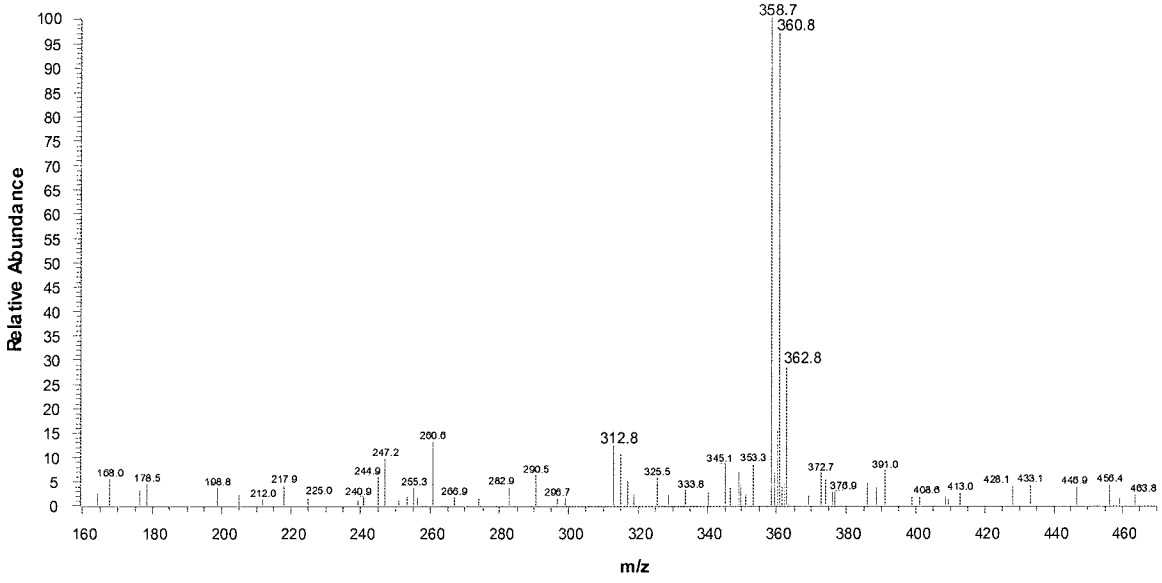

Figure 8. Mass spectra of peak at 13.11 min in Fig. 9 (trichlorinated compound)

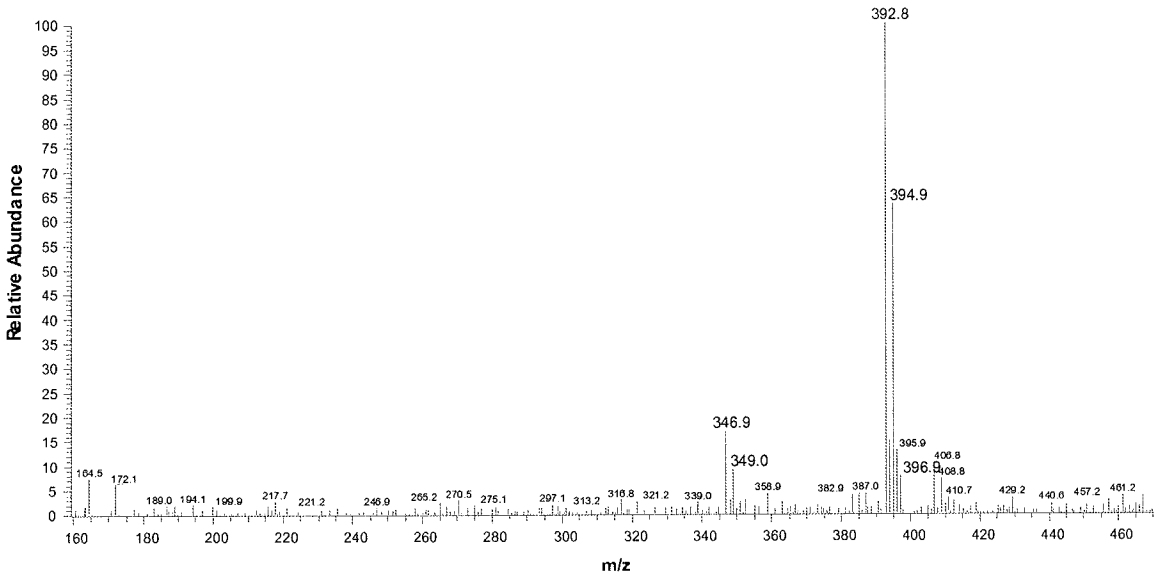

Figure 9. Mass spectra of peak at 13.45 min in Fig. 9 (dichlorinated compound).

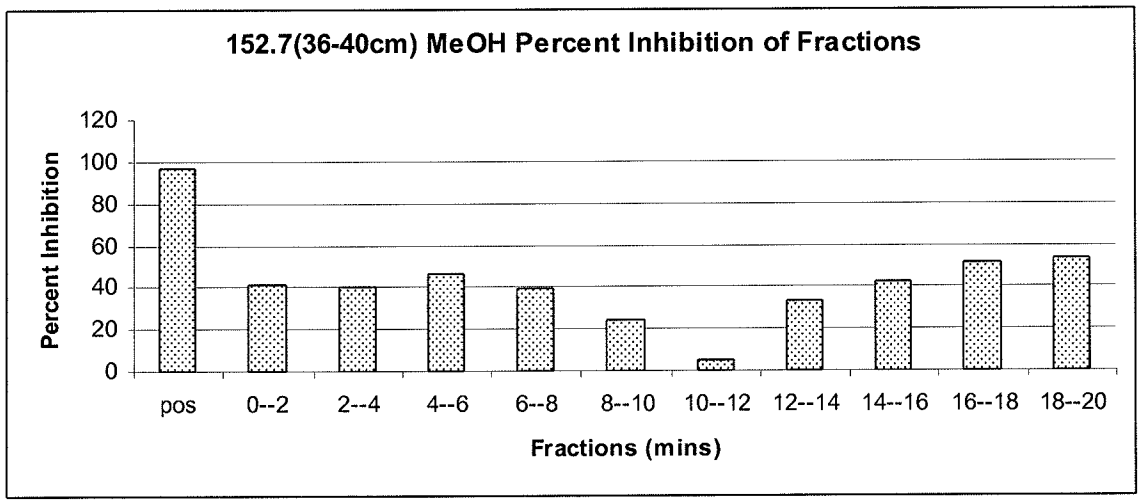

Figure 10. Percent inhibition of *Vibrio fischeri* by fractions from MeOH extract of River mile 152.7 (36-40 cm).

In Fig. 6 the tri-chlorinated compound is also observed at 12.73 minutes, which is confirmed by the mass spectra in Fig. 11.

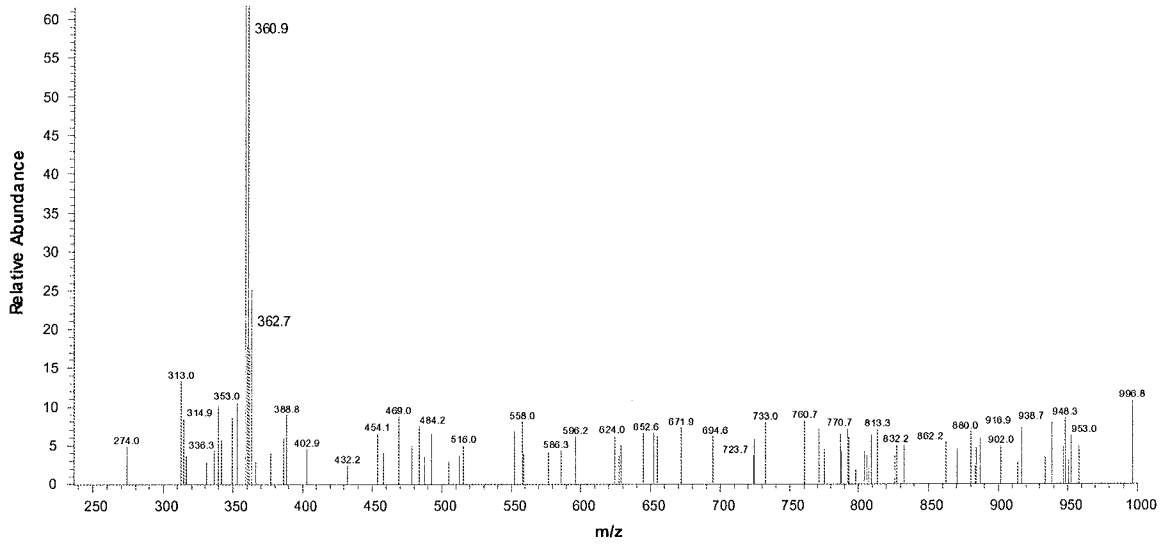

Figure 11. Mass spectra of peak at 12.73 min in the MeOH extract at River mile 188.6 (28-32 cm)

There is also a toxic response seen for the fraction 12-14 min from the MeOH extract of River mile 188.6 (28-32 cm) (Fig. 12).

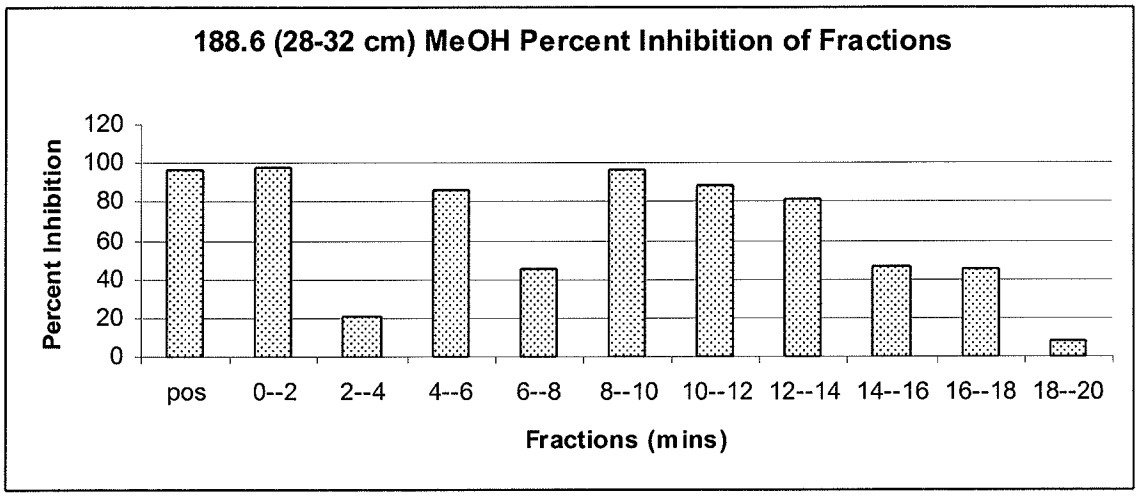

Figure 12. Percent inhibition of *Vibrio fisheri* by fractions from MeOH extract of River mile 188.6 (28-32 cm)

GC-MS analysis was also performed on all sediment extracts. Several peaks of interest were found. A library search provided possible matches for these peaks. Below

is Fig. 13 showing a GC chromatogram from the hexane extract of River mile 163.6 (2-4 cm). One peak of interest at 3.5 min is a 94% library match for 1-chloro-4-(trifluoromethyl)-benzene (Fig. 14).

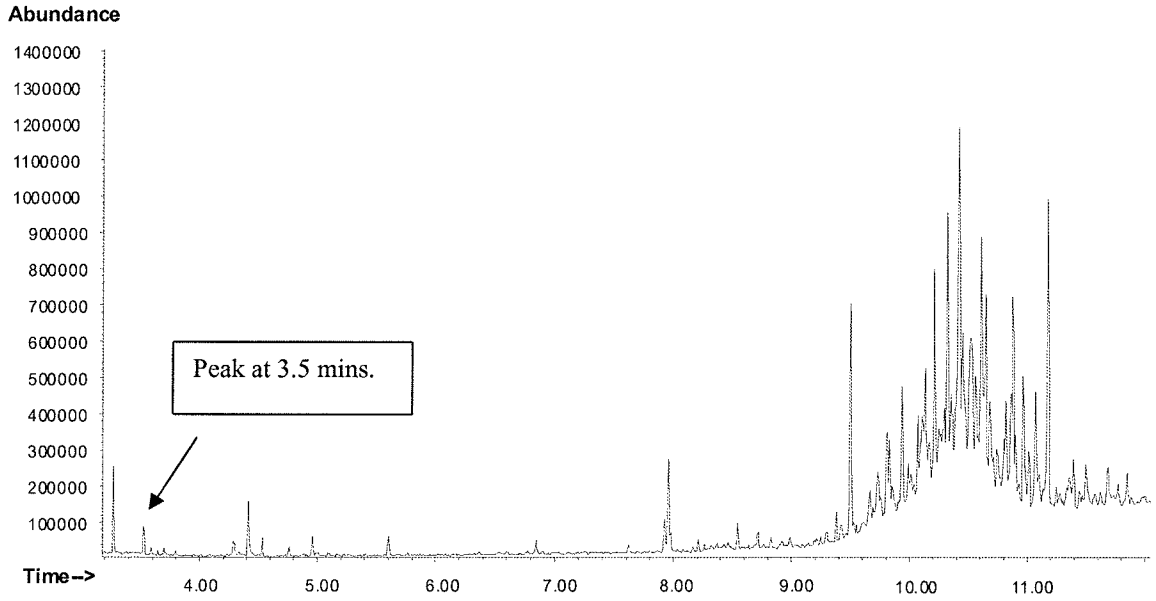

Figure 13. GC chromatogram from hexane extract of River mile 163.6 (2-4 cm).

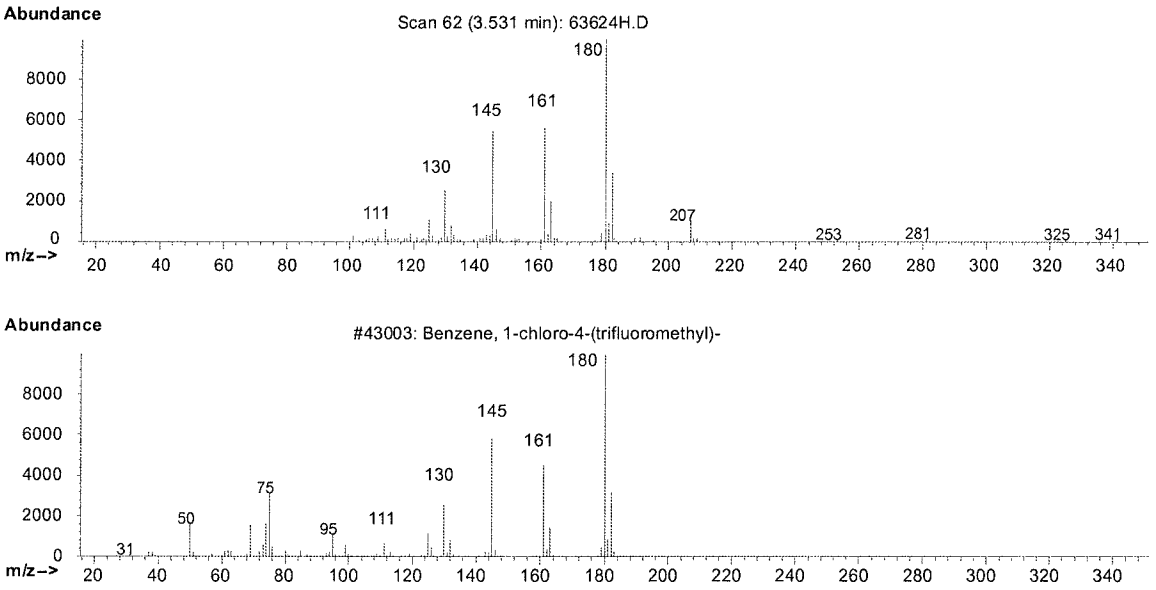

Figure 14. Mass spectra and library match for peak at 3.5 min in Fig. 15 (hexane extract of River mile 163.6 (2-4 cm)).

During GC-MS analysis PCBs were also found in the extracts. Mono- to penta-chloro biphenyls were detected. Below in Fig. 15 is a chromatogram of the hexane: IPA extract from mile 163.6 (36-40 cm). The peak at 8.9 min corresponds to a dichloro biphenyl which is confirmed by the mass spectra in Fig. 16.

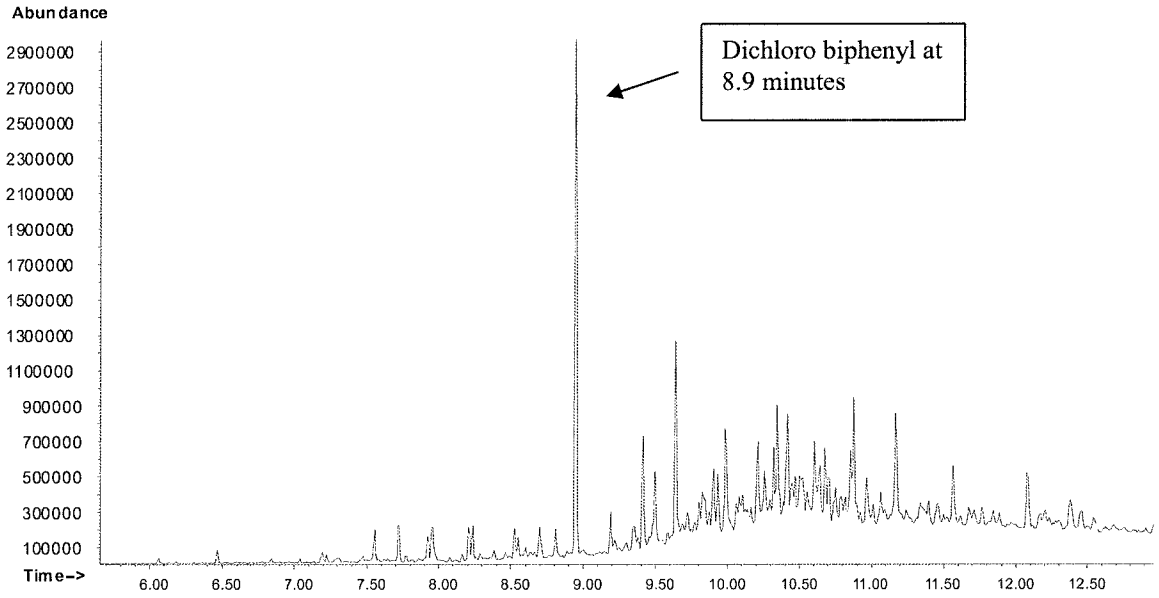

Figure 15. GC chromatogram of the hexane:IPA extract from River mile 163.6 (36-40 cm). Peak at 8.9 min. corresponds to a dichloro biphenyl.

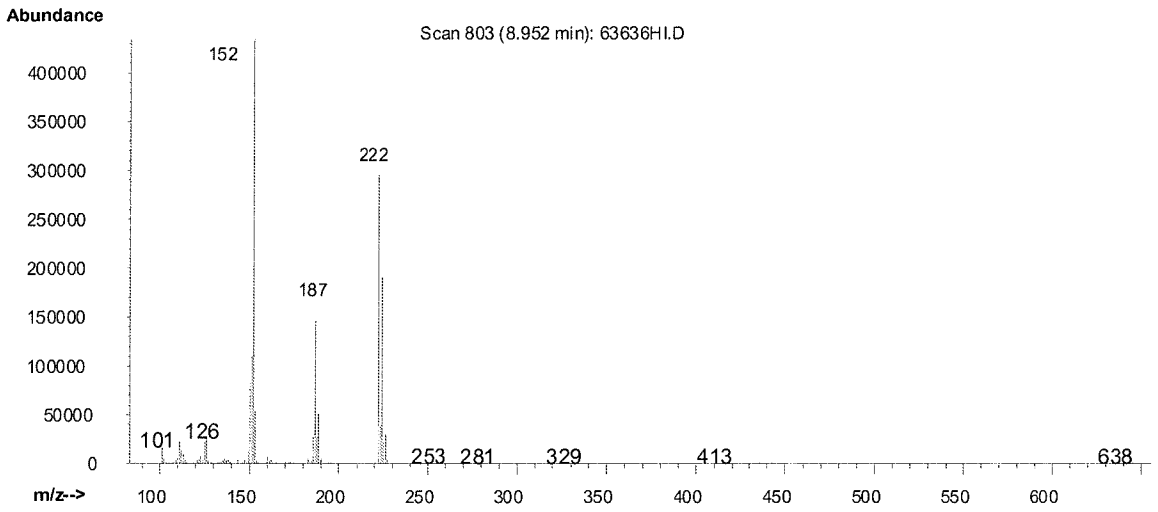

Figure 16. Mass spectra of dichloro biphenyl peak at 8.9 min in Fig. 17 from the hexane: IPA extract at mile 163.6 (36-40 cm).

Discussion

Our results indicate that extracts from Hudson River sediments are toxic in varying ways. Some extracts are highly estrogenic such as the MeOH extract of River mile 188.6 (28-32 cm), while others show dioxin or dioxin-like activity like the MeOH extract of River mile 152.7 (36-40 cm). However, all extracts inhibited the energy metabolism of *Vibrio fischeri* (Fig. 2). The Abratox^{TM} assay is mainly for water soluble compounds and the hexane extracts contain non-polar compounds that are unlikely to be water soluble. Some of the inhibition could be due to the presence of sulfur in the extracts (Brack et al. 1999), particularly in the hexane or hexane: IPA extract. As a result, all hexane and hexane: IPA extracts showed higher inhibition compared to the MeOH extracts due to the possible presence of sulfur. Sulfur can be removed by running extracts through a copper powder column.

The highly estrogenic extract (Fig. 4) from mile River 188.6 (28-32 cm) does not exhibit high dioxin or dioxin-like activity (Fig. 3); however it does exhibit high inhibition

to *Vibrio fischeri*. This indicates that it is likely the MeOH extract for River mile 188.6 (28-32 cm), does not have a large concentration of dioxins or similar compounds but has a high concentration of highly polar, water soluble compounds that are exhibiting high estrogenicity. Further analysis for estrogenicity needs to be conducted on fractions from the MeOH extract to identify the estrogenic compounds.

The fractions from River mile 188.6 (28-32 cm) extract exhibit increased inhibition for *Vibrio fischeri* (Fig. 12) indicating that there is more than one component in the extract that is toxic and that the toxic effects are non-additive, similar to what was seen by Suzuki et al. (2004), Sundberg et al. (2005) and Schlenk et al. (2005). Further analysis of fractions by combining them to determine fraction and mixture toxicity needs to be completed.

Schlenk et al. (2005) found that high concentrations of estrogens did not correspond to biological response. It was thought that there was co-extraction of anti-estrogenic and androgenic compounds. Also, they found that fractions did not yield any identification of sex-steroids as potential toxic agents but the occurrence of polar compounds in extracts did produce a biological response. Taking this into account, it is possible that the toxicity seen in the more polar fractions from the MeOH extract of River mile 188.6 (28-32 cm) could be due to degradation products.

Brack et al. (1999) determined that photodegradation products are possibly more phytotoxic and produce photo-induced ecotoxicity where water depths allow for photolysis of contaminants. This occurrence results in the possibility of these sediment samples having more toxic and polar degradation products due to photodegradation.

However, we found that extracts contained other toxics but we could not identify those compounds.

The tri-chlorinated compound found in the MeOH extract from River mile 188.6 (28-32 cm) at 12.7 minutes (Fig.6 and Fig. 11) corresponds to a toxic effect for fraction 12-14 minutes from the HPLC (Fig. 12). However, further analysis needs to be completed before this compound is identified.

A di-chlorinated compound was found in the extract from River mile 152.7 (36-40 cm) at 13.5 minutes (Fig. 9) which could contribute to the toxicity exhibited in the corresponding fraction from this extract (Fig. 10). More MS^2 and MS^3 data need to be collected for these peaks and also for peaks in positive mode before identification can be made.

GC-MS revealed more possible compounds through library matches. For example, the peak in the GC chromatogram for the hexane extract from River mile 163.6 (2-4 cm) (Fig. 13) at 3.5 minutes provides a library match of 1-chloro-4-(trifluoromethyl) benzene (Fig. 14) at 94%, indicating that some non-polar compounds are not detected by LC-ITMS.

In addition, PCBs were detected in the extracts by GC-MS analysis. Confirmation was seen in the mass spectra for a di-chloro biphenyl from the hexane: IPA extract for mile 163.6 (36-40 cm) at 8.9 minutes in the chromatogram in Fig 15 and Fig. 16. Further investigation needs to be done for the GC-MS data to determine which compounds are present.

Future work for this study involves not only more fractionation of extracts until single components are isolated for identification by Fourier transform Ion Cyclotron mass

spectrometry (FT-ICR) but also determination of toxicity relative to that of the mixture. Also, further inquiry into the role of pH and adsorption, or *in-situ* conditions, in toxicity needs to be conducted. Adsorption and pH could affect bioavailability for aquatic organisms and humans that use the river. Once bioavailability is determined further exposure studies can be conducted to determine the real risk to humans and wildlife from these contaminants.

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