Final Report

Integrated Biomarkers for Assessing the Exposure and Effects of Endocrine Disruptors and Other Contaminants on Marine/Estuarine Fish

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Submitted By: Keith R. Cooper, Ph.D.
Rutgers University Cook College
88 Lipman Drive
New Brunswick, New Jersey 08824

Project Manager: Gary A. Buchanan, Ph.D.
The Department of Environmental Protection
PO Box 409
Trenton, New Jersey 08625

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Executive Summary:

The New York-New Jersey Harbor Estuary is heavily impacted by anthropogenic sources of contamination and it also has a large biotic community that is living within these waters. Within the scientific literature there are numerous reports of using single biological endpoints to determine the extent of pollution within a body of water. The work carried out in the second year of funding included sampling the original (year one) sites consisting of Tuckerton, NJ; Sandy Hook, NJ; Union Beach, NJ; and Newark Bay, and one additional site (Piles Creek) for Mummichog, *Fundulus heteroclitus*. These sites were selected to represent a contaminant gradient with elevated concentrations in Newark Bay to the reference location at Tuckerton. In addition, preliminary sampling was carried out examining White Perch (*Morone americana*) from the Delaware River, Hackensack River, Passaic River and near Tuckerton, NJ. The studies were designed to examine a suite of biomarkers in these fish and determine which if any correlated with the anthropogenic inputs at those locations. It is implicit that the greater the extent of the pollution, the greater the impact on the biomarker, and as the pollution is decreased so should the effect on the biomarker. This assumption may however be altered by the ability of the in-bred population to reset normal values or develop transport systems to eliminate higher chemical contamination and therefore adjust to chronic chemical exposure. There are a number of reports that have established that chronic exposure to xenobiotics can result in biochemical and or physiological pathways that allow the population to survive in contaminated environments (Nacci et al. 2000, Wirgin and Waldman 2004, Weis 2000).

It is important to use a battery of endpoints to measure fish health since there is a large mixture of compounds present in the NY-NJ Harbor Estuary that may or may not cause pathoneumonic (specific) lesions observed in the fish. This second year study also examined vitellogenin as a biomarker of endocrine disruption. There are currently no established biological based finfish indicators of ecosystem health for evaluating management decisions concerning toxics in the estuary. This report discusses the findings from approximately 400 Mummichog and 80 White Perch samples. The findings support the need to conduct both classical toxicological evaluations (histopathology, organ to body weight ratios) along with biochemical endpoints (CYP1A1, metallothionein, vitellogenin). The use of micronuclei was not found to be reliable as a biomarker, due in part to interference from cytosolic parasites and very low occurrence. An alternative method may be more useful in examining

DNA damage i.e. Comet Assay or DNA adduct identification. Because of the detection of neoplasms (i.e., tumors) in the livers from several locations believed to have reduced contaminant input, additional sampling is warranted. There is also a need to include chemical analysis to better determine the chemicals present in these different populations. There is some chemical analysis information on White Perch, but due to limited funding analysis was not carried out on the majority of the fish sampled. The detection of PAHs in the bile at similar levels in all of the samples examined, points to the chronic exposure of these compounds to all fish inhabiting estuaries in heavily populated localities.

Based on the findings from these preliminary studies the following recommendations are made:

1. The biomarkers that were useful in evaluating the health of the organisms included grossly visible lesions (external and internal), standard hematology (hematocrit, blood smears), standard body morphometric (length and weight), histopathology, biochemical endpoints (CYP1A1, vitellogenin) and bile fluorescence (specific PAHs).
2. Neither the micronuclei nor the hepatic metallothionein appeared to be useful biomarkers in differentiating between various populations.
3. Future studies could include, if available, species specific gene activation based on gene chips, circulating hormones and proteins and alternative methods for evaluating DNA adducts.
4. A more extensive sampling campaign should be conducted to better characterize the extent of wild fish having hepatic neoplasms.
5. Conduct chemical analysis concomitantly with biomarker analysis in order to have a better handle on the relationship of tissue dose and lesion occurrence.
6. Fund studies to better understand how *Fundulus heteroclitus* has adapted to multi-chemical exposure.
7. Evaluate if chemical challenge experiments looking at the organism's response can be used to identify resistant populations.
8. Expand the survey to include other fish species such as eel, bluefish and flounder.
9. In order to establish a metric to evaluate the health of the organisms inhabiting the NY/NJ Estuary there is a requirement that adequate funding be made available for research into establishing a baseline and for prospective environmental based epidemiological studies. Without such an approach it will be impossible to establish whether policy decisions have had an impact on restoring the health of the ecosystem.

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Introduction:

Analysis of contaminant concentrations in water, sediment or biotic (e.g., fish) tissue have been used as surrogate measurements to determine aquatic ecosystem or aquatic organism health. For example, these measurements are typically collected during the investigation of contaminated sites and often used in ecological risk assessments to estimate adverse impacts to the ecosystem. However, these measures do not directly measure organism health and may not be accurate representations of ecosystem health, whereas full contaminant analysis can be very expensive and not indicate whether or not there is a biological impact. Biomonitoring is the centerpiece of ecological assessment and is essential for assessing the well-being of any ecosystem (Burger 2006).

The purpose of this study was to evaluate several different biomarkers for their usefulness in evaluating fish health, which is one measurable response for evaluating the current health of an estuary. Because of the large number of contaminants entering the watershed it is also unlikely that a single biomarker would be sensitive to multiple contaminants, therefore using a battery approach would appear to be more prudent. The question therefore is which biomarkers are appropriate and which ones are not? It is assumed that these baseline measures if consistent enough and associated with a pollutant gradient can be used to monitor future improvements in the general water and sediment quality of the system. The study area was selected because a number of previous studies have reported a wide variety of contaminants present in the aquatic life living in these waters (Longwell et al. 1996, Steinberg et al., 2004). There is no question that the levels of these contaminants are high due to anthropogenic activity, which has impacted the area during the post-Industrial Revolution era. Persistent contaminant inputs into the system from point sources (e.g. sewage treatment facilities, industrial dischargers) and non-point sources (e.g. atmospheric deposition, combined sewer outflows) ultimately end up in the sediments. The sediments in this region act as sinks for the persistent metals (Hg, Cu, Pb, Zn and Cr) and organic compounds (polyaromatic hydrocarbons-PAHs, polychlorinated biphenyl-PCBs, polychlorinated dibenzo-p-dioxins-PCDD). The major rivers continue to deposit contaminated sediments into estuarine waters. The tidal nature of the system also contributes to the extended retention time in these bays for sediments and their continual suspension in the water column (Kim et al. 2006).

In addition to these historically documented contaminants there is a new class of compounds (tributlytin, surfactants, phthalates, pesticides and synthetic steroids), endocrine disruptors that over the past decade have begun to be recognized as important contaminants in rivers and estuaries (Denslow and Larkin 2006, Gimeno et al. 1996, 1997, Houtman et al. 2004, Sumpter and Jobling 1995). Unlike the PCBs or dioxins there is not a structural similarity between these compounds, but in the biological affect they have on the organism. These compounds result in alteration of normal hormonally controlled systems or tissues within an organism (e.g. cancer, embryonic development, reproduction, and neurodevelopment). The evidence that endocrine-disrupting chemicals are widespread due to anthropogenic sources and are having effects on invertebrates and fish reproduction is growing (Arcand-Hoy and Benson 1998, Nash et al. 2004). Specific endocrine disruptors that are likely impacting fin and shellfish in the NY/NJ Hudson Estuaries include the following: tributlytin from shipping, the large metropolitan human populations contribute 17-*B* estradiol (E_2) and ethinylestradiol (EE_2) (Desbrow et al. 1996), nonionic surfactants such as nonylphenol and octylphenol (Gronen et al. 1999, Jobling et al. 1996), phthalate esters (Patyna et al. 1999, Patyna et al. 2005) and bisphenol A to secondary water treatment facilities, and various pesticides (pyrethroids, DDT/DDE, endosulfan, methoxychlor). There is not only concern for aquatic and terrestrial wildlife (Tyler et al. 1998), but also the exposure to humans through drinking water (USGS 2002, USGS 2006) and consuming contaminated food stuffs (NAS 2003).

As indicated above there are many sources for these compounds and one important characteristic is that at the concentrations of concern they do not result in acute toxicity but alter normal pathways that can impact an organisms ability to reproduce, grow or respond to environmental factors. The effects can be at the molecular level which may be reversible if the system is not permanently altered (Figure 1). Effects can be manifested at the tissue level where gross or histological changes are evident. If the xenobiotic is removed the organism may be able to revert back to pre-exposure biochemical or histological conditions if permanent changes are not caused (e.g. fibrosis of the liver). In some instances population level effects can be manifested that generally requires longer periods of time to be recognized. The classic example of this is with DDT/DDE and PCBs/dioxins where the impact was on the survival of the offspring that resulted in the decline of bird populations around the world (Fry 1995, Hoffman et al. 1996). The inability of successful reproduction of

Lake Trout in the Great Lakes is a similar situation where chemical levels prohibited successful recruitment (Walker and Peterson 1991).

McCarty and Munkittrick (1996) broadly define biomarkers as biochemical, physiological or ecological structures or processes that are linked to or correlated to biological effects measured at one or more levels of biological organization. The World Health organization defines a biomarker as "any substance, structure or process that can be measured in the body…and influence or predict the incidence or outcome of disease" (Bartell 2006, WHO 2001). The levels of organization go from sub-cellular to organ specific endpoints to individual to population to community. Bartell (2006) and Burger (2006) have made the suggestion that biomarkers be limited to sub-organism levels of biological organization and that bioindicators include structures and processes at the higher levels of organization (e.g. organism, population, community and ecosystem). It is important to asses any biomarker as it relates to the control of a physiological response in the organism that may or may not be translated into higher level effects. Most biomarkers have been developed based on perturbation of a system from control organisms that are exposed to a toxicant over relatively short periods of time (e.g. induction of P450 enzymes, metallothionein). This paradigm allows for an assessment of the up or down regulation of a specific biomarker, but does not in most cases examine what the response will be to organisms exposed for longer periods of time and the effect on the population and ecosystem community structure (Figure 1). Biochemical or physiological responses have both positive and negative feed back mechanisms that regulate a system and prevent an organism from expending too much of its energy resources. Homeostasis of an organism is required in order to be able to successfully reproduce and have adequate resources to grow. Chemical selective pressures can result in loss of fitness and the loss of organisms that are unable to adapt. This results in the selection of species able to tolerate the presence of chronic chemical exposure and will eliminate the less tolerant species from the ecosystem. Burger and Gochfeld (1992) discuss the importance of temporal scale exposure and impact on conducting ecological risk assessments. What is now apparent is that in any ecological risk assessment there needs to be an estimating or predicting exposure to critical life stages of both economically important species as well as important prey species in an ecosystem and establishing appropriate temporal scales for predicting impacts and recovery. Therefore, the species diversity present in an ecosystem will

depend on the extent and duration of the perturbation and the tolerance of the tolerant species to successfully reproduce and grow.

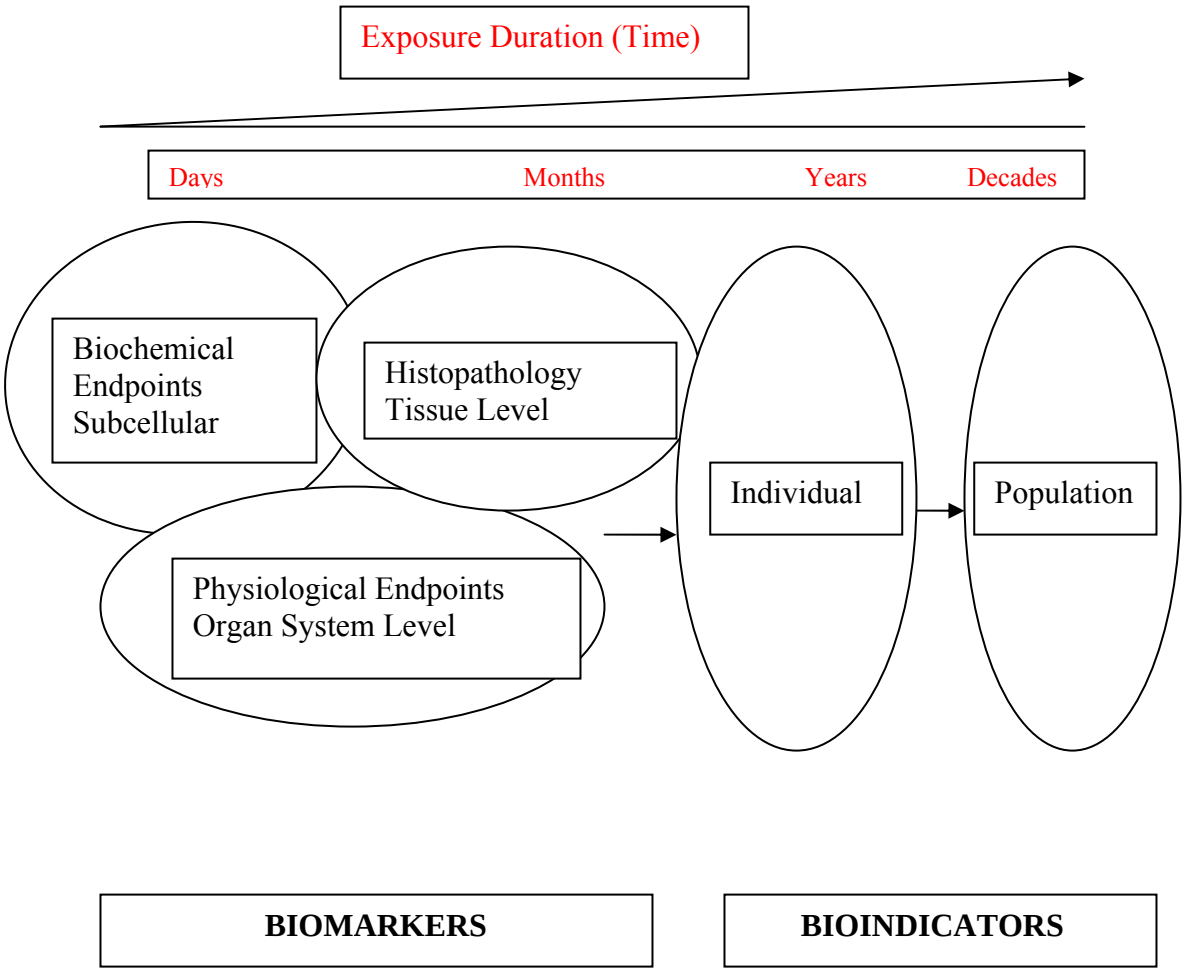

Figure 1. Relationship between biomarkers and bioindicators as they relate to exposure Duration.

The specific sites selected for sampling were determined in consultation with Dr. Gary Buchanan at NJDEP. It was assumed that the sites would represent a gradient of contamination with the highest being in Newark Bay followed by sites in Piles Creek, Union Beach, Sandy Hook and Tuckerton (reference location). This is a very general assumption since there could be local contamination that may impact the resident populations. Due to funding constraints, one drawback to this study is the lack of analytical evaluations to compare to the biomarker analysis. However, if the biomarkers are too variable then having

additional analytical data are unlikely to shed any additional light on a cause and effect relationship.

Because these sediments act as a sink for these contaminants, benthic organisms are at greatest risk of coming in contact with these contaminants. Contaminants can move into higher trophic organisms through their food web or through direct exposure from ingestion of sediment, contaminated prey and or across epithelial tissues. Biological based tests can be used to ascertain if the contaminants present in the sediments, prey species or water can reach high enough concentrations to significantly change a biomarker in the fish. The presence of epithelial and/or hepatic tumors are visible biomarkers of exposure to carcinogenic compounds that are either direct acting or require metabolic activation (Vogelbein and Fournie 1994). Other studies have also shown hepatic tumors in flounder from heavily contaminated sites such as Boston Harbor and Black Rock Harbor. The species that was examined is the common mummichog, *Fundulus heteroclitus*. The mummichog is an important prey species and has a limited home range that can be used to pinpoint local contaminated environments (Elkus et al., 1999; Smith and Weis 1997; Weis et al., 1982). The White Perch (*Morone americana*) was also selected because of its occurrence throughout many estuaries in New Jersey and along the East Coast and its ability to accumulate high levels of organic compounds.

The main objectives of this study are the following:

1. To develop a battery of biomarkers that can be used to evaluate fish health, which will correlate with levels and classes of toxic compounds in the estuary.
2. To begin to establish a baseline data set concerning these biomarkers for comparison with fish collected in latter years following remediation measures carried out in the harbor.
3. Make recommendations as to the suitability of the specific biomarker.

These fish biomarkers will provide an ***integrated*** measure of contaminant exposure from multiple contaminants, and provide an *indicator of estuarine/marine water quality and overall ecosystem health.*

Problem Statement: The New York-New Jersey Harbor Estuary is heavily impacted by anthropogenic sources of contamination and it also has a large biotic community that is living within these waters. There are currently no established biological based indicators of ecosystem health for evaluating management decisions concerning toxics in the estuary. Therefore, the use of biomarkers in finfish can serve as a biological-based measure of an ecosystem's health including endocrine disruption.

Quality Assurance:

All animal protocols were approved through Rutgers University Animal Care Committee. All methods for collection, handling and sampling of tissues from the fish were approved by the Rutgers University Animal Rights Committee in accordance with AALAC accreditation and NIH guidelines (Protocol # 03-014 and 04-013). An expanded QA document is attached to this document (Appendix 2) and contains extensive descriptions of protocols that have been developed during the first and second year study.

Project Design and Methods:

Site selection: The sites were selected based on historical information, meetings and discussions with a number of researchers who had carried out research in the Raritan/Newark Bay complex. These included academic as well as government researchers. The specific sites to be sampled for *Fundulus heteroclitus* were agreed upon by NJDEP. The reference site is in the Great Bay-Little Egg Harbor Inlet estuary near Tuckerton, NJ (not shown on map below). The four additional sites in the NY/NJ Harbor estuary included Sandy Hook Bay, Union Beach (southern shore of Raritan Bay), Piles Creek (tributary to the Arthur Kill) and Newark Bay (near Newark Airport). The collections of the white perch were carried out from the following locations: Delaware River, Passaic River, Hackensack River and Mullica River (Lower Bank) near Tuckerton, NJ.

Fish collection: The *Fundulus heteroclitus* were collected using baited minnow traps and or seine nets. The animals were transported back to the laboratory in aerated coolers. If necessary, they were maintained for a short period in the re-circulating system in the Rutgers University Marine Science building. The fish were housed in glass aquaria.

White perch were collected by either gill net, trawls or fish traps. In the case of gill netting the nets were checked frequently to minimize stress and damage to the fish. The preferred method is using fish traps or trawls.

Table 1. Endpoints evaluated in these studies

Biomarker	Purpose	Endpoint
External Examination	▪ Examine the fish for external lesions involving skin, fins, gills or eyes and internal color and shape of internal organs.	▪ Incidence of external lesions and grossly visible lesions
Blood Smear	▪ Morphological evaluation of RBC and WBCs following staining with Wright/Giemsa stain and/or a DNA specific stain.	▪ Micronuclei in RBC ▪ WBC shift infection ▪ Morphology of RBCs
Hematocrit	▪ Determine packed cell density	▪ Anemia or altered RBC production
Total & Organ Weights	▪ Size of organs are correlated with size of the organism (liver, spleen, gonads) ▪ Gross morphological evaluation (color & shape) is an indicator of disease	▪ Stressors or diseased organs will have altered organ to body weight ratios
Histopathology	▪ Evaluate liver cellular structure at light microscopic level.	▪ Evaluation of normal vs. altered structures
Biochemical Endpoints inc. Endocrine Disruption	▪ Evaluate if populations inhabiting different locations have different levels of endogenous enzymes.	▪ Real time PCR for quantification of mRNA enzyme levels for P450 Cyp1A1, metallothionein (MT) and hepatic vitellogenin (VT) (Endocrine Disruption)
Fluorescent Activity	▪ Fluorescent activity in bile has been correlated with PAH activity	▪ Increased basic fluorescence indicates increased aromatic contamination

Based on this battery of tests the overall health of the fish can be determined. As shown in the table above different levels of biomarkers ranging from external examination to enzyme induction can be considered as a biomarker. Some of these are similar endpoints examined in humans (e.g., visiting a doctor for a routine checkup). Many of these techniques are discussed in great detail in Gary Ostrander's book 'Techniques in Aquatic Toxicology". When diagnosing the health of an individual it is essential to examine gross appearance and external appearance as well as blood work and specific organ function. The biochemical endpoints can be used to ascertain whether there are compounds that have resulted in altered levels of enzymes and or proteins (Courtenay et al. 1999; Munkittrick and McCarty 1995; Nelson et al., 1991; Wirgin, 1994). It is realized that when dealing with biomarkers that in some instances a U-shaped dose response curve (hormesis) may occur (Calabrese and

Baldwin 2001). Hormesis is defined as a dose-response relationship which there is a stimulatory response at low doses, but an inhibitory response at high doses, resulting in a U-shaped or inverted U-shaped dose response. Hormesis is often observed at concentrations below the NOAEL. The histopathological evaluation will indicate if there are any acute or chronic disease and overall health of the fish from these various sites. These biomarkers would cover exposure from PAHs, chlorinated PAHs, estrogenic compounds (i.e., endocrine disruptors), certain heavy metals, PCBs, dioxins/furans, and parasitic infections (NRCC 1985; Prince and Cooper, 1995a; USEPA 1998).

Sample collection and analysis: Within the QA section are more extensive descriptions of the methods used in these studies. An abbreviated sample collection and analysis methods are described in this section of the report. Collection dates, locations, numbers of fish and biomarkers run are summarized in Table 2. Each fish was assigned a unique accession number that was used for all tissues collected from that animal. Fish were anesthetized using a small container with MS222 added to the water. When the animal lost the righting ability it was removed and their weight and length recorded. The animal was examined for any external abnormalities involving the skin, gills and fins. The caudal portion of the fish just anterior to the caudal fin was severed and blood was collected into a heparinized micro-capillary tube. A drop of blood was placed on a glass slide and a blood smear was made. In most cases two hematocrit tubes were collected. Because of the non-consistent findings examining blood cells for micronuclei in the first year preliminary study this biomarker was excluded from the second year testing. An incision was then made along the ventral peritoneal area from the anus to the pericardial cavity. The endometrial lining of the peritoneal cavity was observed for any hemorrhagic areas or other visible lesions. At the same time any grossly visible abnormalities on the liver, spleen, GI track, and gonadal tissues were observed. The liver was then removed, weighed and divided into two portions, one for histopathology and the second for biochemical parameters. The liver was snap frozen and maintained at -80 C until processed for Real Time Polymerase Chain Reaction (RT-PCR) for CYP1A1, vitellogenin and metallothionein. The gallbladder containing the bile was collected into a plastic microcentrifuge tube and frozen at -20 C. The spleen and gonadal tissue (if sufficient mass was present) was also removed and weighed. The liver and gonad tissue were fixed in formalin, and embedded in paraffin. The tissues were then cut into six-micron sections and stained with Hematoxylin and Eosin. The tissues were examined for

lesions and other abnormalities. All histological slides were evaluated without knowing their site or time of collection. The liver somatic index (LSI), gonad somatic index (GSI) and spleen somatic index (SSI) were calculated. In order to determine the hematocrit, blood was extracted into capillary tubes and spun in a microcentrifuge for six minutes. All of the raw data for each animal is presented in the Excel spread sheet that is provided in a separate file.

Table 2. Dates, locations and fish counts for all fish collections

Date	Site	Number of Fish	Biomarkers Run
Oct 29, 2004	Delaware River	10 White Perch	all
Nov 19, 2004	Hackensack River1	18 White Perch	all
Apr 27, 2005	Hackensack River2	17 White Perch	all
Apr 17, 2006	Passaic River	13 White Perch	all
May 18, 2006	Passaic River	2 White Perch	all
June 7/22, 2006	Tuckerton	20 White Perch	all
May 20, 2004	Tuckerton	29 Fundulus	all but bile fluorescence*
May 29, 2004	Sandy Hook	24 Fundulus	all
May 20, 2004	Union Beach	30 Fundulus	all
May 20, 2004	Piles Creek	15 Fundulus	all but bile fluorescence*
May 21, 2004	Newark Bay	30 Fundulus	all
July 1, 2004	Tuckerton	30 Fundulus	all
July 1, 2004	Sandy Hook	33 Fundulus	all
July 1, 2004	Union Beach	18 Fundulus	all
July 1, 2004	Piles Creek	30 Fundulus	all
July 1, 2004	Newark Bay	30 Fundulus	all
June 22/July 13, 2006	Tuckerton	13 Fundulus	all
July 11, 2006	Sandy Hook	11 Fundulus	all but RT-PCR**
July 11, 2006	Union Beach	10 Fundulus	all but RT-PCR**
July 14, 2006	Piles Creek	10 Fundulus	all but RT-PCR**
July 14, 2006	Newark Bay	14 Fundulus	all but RT-PCR**

*Samples lost
**RNA degraded and quality too low for RT-PCR

Analysis for Naphthalene, Pyrene and Benzo(a)pyrene from Fish Bile:

The figures shown below are the synchronous fluorescent scans for each of the compounds analyzed for in the bile of these fish. This method was a slight modification of previous reported methods examining polyaromatic hydrocarbons in fish bile (Aas et al., 1998, Aas et al. 2000, Vuontisjarvi et al., 2005). These curves were then used to generate standard curves of metabolites that were used to calculate the equivalent amount of each parent compound.