Final Report

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

(Second Year Grant Number SR03-038)

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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.

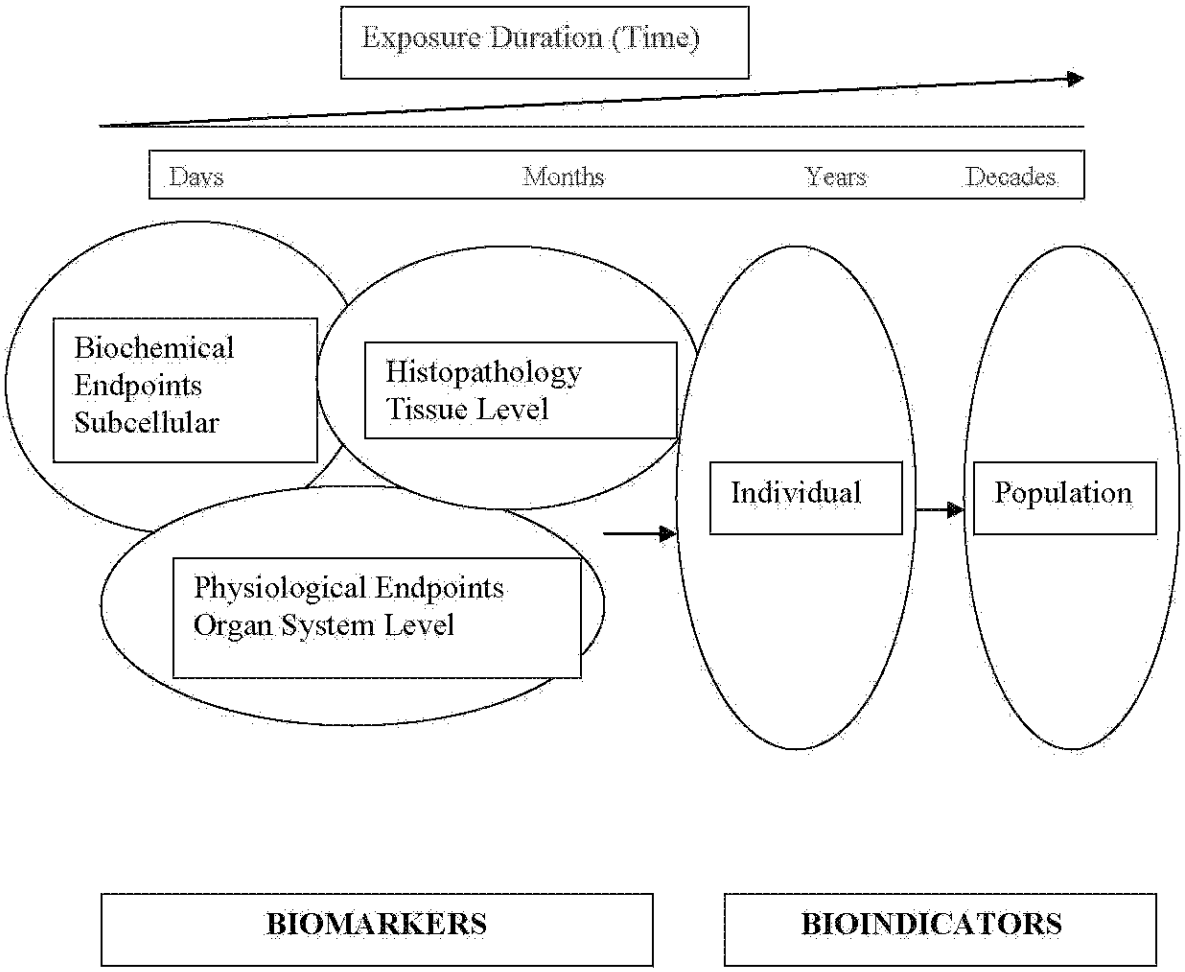


Figure 1. Relationship between biomarkers and bioindicators as they relate to exposure Duration.

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.*

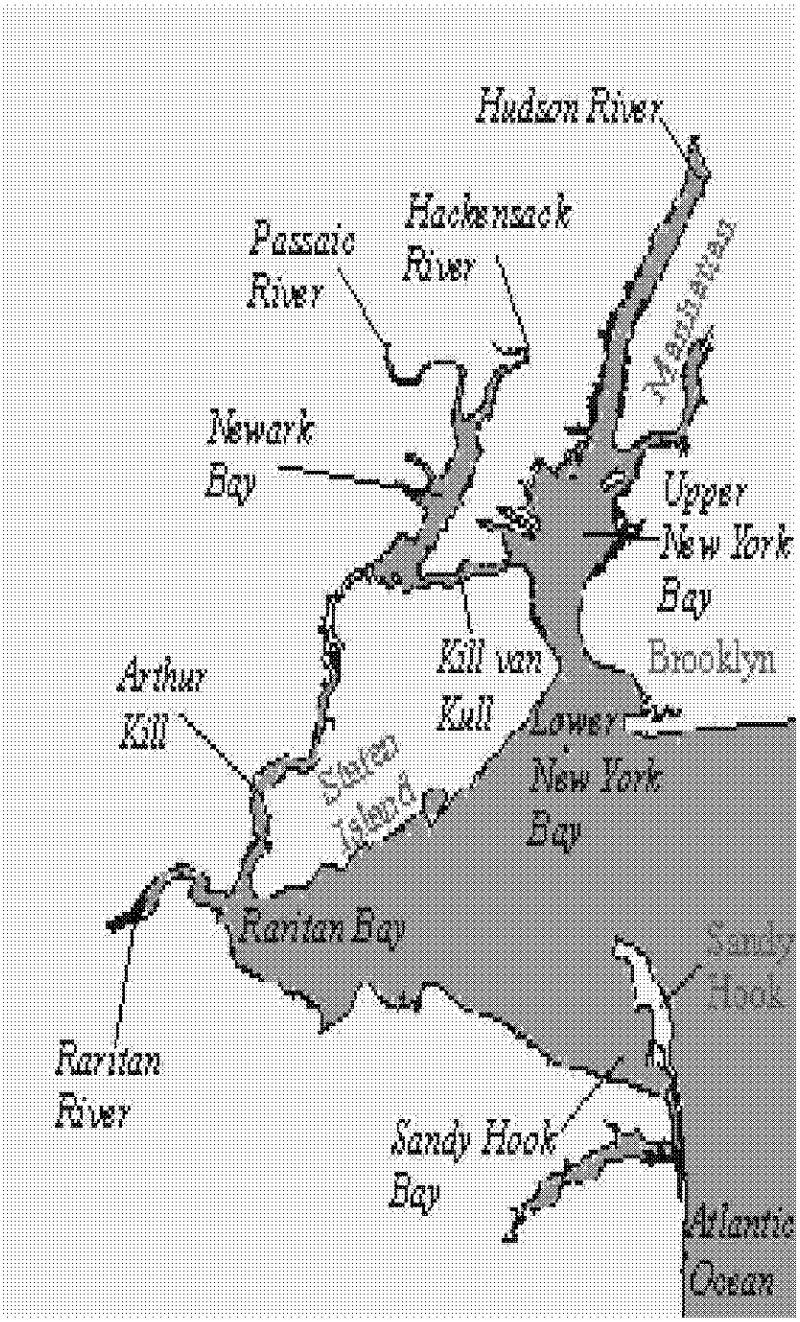
Problem Statement: The New York-New Jersey Harbor Estuary is heavily impacted by anthropogenic sources of contamination and it also has a large biotic community that is living within these waters. There are currently no established biological based indicators of ecosystem health for evaluating management 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

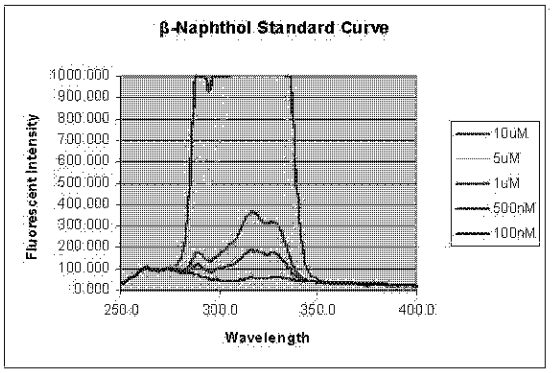
Date	Site	Number of Fish	Biomarkers Run
Oct 29, 2004	Delaware River	10 White Perch	all
Nov 19, 2004	Hackensack River1	18 White Perch	all
Apr 27, 2005	Hackensack River2	17 White Perch	all
Apr 17, 2006	Passaic River	13 White Perch	all
May 18, 2006	Passaic River	2 White Perch	all
June 7/22, 2006	Tuckerton	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.

The detection limits for naphthalene, pyrene and benzo(a)pyrene were 50 ng/µl, 0.5 ng/µl and 50 pg/µl, respectively. These values were used when calculating statistical values for any non-detect values. This technique allows for estimation as to the extent of PAH exposure for the fish that were examined in this study.



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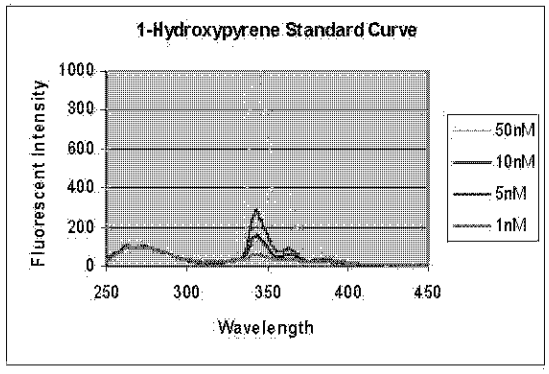

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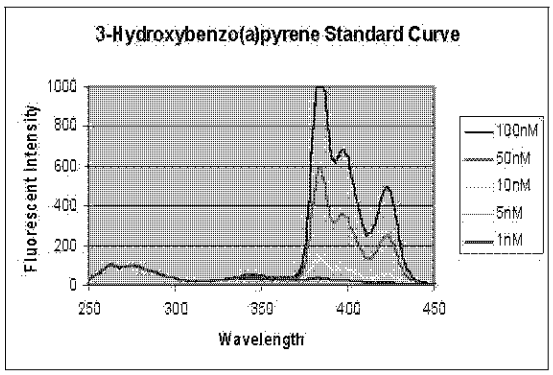


C.

Figure 2. Synchronized fluorescence scans of pure β-naphthol, 1-hydroxypyrene and 3-hydroxybenzo(a)pyrene.

Statistical Analysis:

The statistical analysis carried out to date on the data in this study has examined single biomarker parameters between different fish collected at different locations. The analysis has used Sigma Stat, which allows for a number of statistical comparisons using relatively straightforward descriptive statistics and more traditional statistical tests (t-test, one-way ANOVA, two-way ANOVA). When the data failed normality or equal variance an ANOVA on Ranks was used (Dunn's Method and Kruskal-Wallis). In all other cases the Student-Newman-Keuls method was used. Significant differences were set at $p \leq 0.05$. Therefore any

time a parameter is stated in the text as statistically different, means that the p value was less than or equal to 0.05 unless otherwise noted.

Results and Discussion

All of the raw data is provided in a spreadsheet format in Excel as a separate file. What is presented below is a summary of the data in graphic form along with discussion. Table 3 below is a master table of descriptive statistics for all organismal level biomarker (organ weights, gender ratio, hematocrit). Table 4 below is a master table of descriptive statistics for all biochemical and chemical level biomarkers. Refer to these tables when looking at all statistical comparisons in this section for averages and standard deviations of each biomarker.

Table 3. Master sheet of descriptive statistics for all organismal level biomarkers

	# M/F	Hematocrit (%)	LSI	Male GSI	Female GSI	Male SSI	Female SSI
FUNDULUS							
May 2004 Newark Bay	7/23	35.7±6.5	0.05±0.01	0.04±0.01	0.09±0.04	0.004±0.001	0.003±0.002
May Piles Creek	6/9	39.1±4.4	0.05±0.07	0.04±0.01	0.09±0.02	0.004±0.002	0.002±0.001
May Union Beach	12/18	31.9±9.7	0.03±0.07	0.02±0.01	0.05±0.02	0.003±0.001	0.002±0.001
May Sandy Hook	7/17	40.1±10.7	0.03±0.04	0.04±0.01	0.09±0.05	0.003±0.001	0.002±0.001
May Tuckerton	6/23	38.3±9.7	0.03±0.07	0.02±0.01	0.03±0.02	0.002±0.001	0.002±0.001
July 2004 Newark Bay	13/16	42.5±9.4	0.03±0.01	0.02±0.02	0.02±0.01	0.004±0.002	0.003±0.001
July 2004 Piles Creek	12/18	43.2±7.8	0.05±0.06	0.01±0.01	0.01±0.01	0.004±0.002	0.003±0.002
July 2004 Union Beach	8/10	34.2±5.6	0.04±0.05	0.01±0.01	0.01±0.01	0.005±0.001	0.004±0.001
July 2004 Sandy Hook	9/24	35.0±10.3	0.03±0.01	0.03±0.01	0.04±0.02	0.004±0.002	0.004±0.002
July 2004 Tuckerton	4/26	40.9±12.0	0.03±0.01	0.01±0.01	0.01±0.01	0.002±0.001	0.003±0.001
July 2006 Newark Bay	6/8	33.6±6.9	0.03±0.01	0.03±0.01	0.03±0.01	0.002±0.001	0.002±0.001
July 2006 Piles Creek	7/5	34.2±5.1	0.04±0.06	0.04±0.04	0.02±0.01	0.003±0.001	0.003±0.001
July 2006 Union Beach	4/5	28.7±8.0	0.03±0.01	0.02±0.01	0.04±0.03	0.003±0.001	0.002±0.001
July 2006 Sandy Hook	1/10	43.1±11.2	0.04±0.01	0.03	0.11±0.06	0.003	0.002±0.002
July 2006 Tuckerton	4/9	46.2±8.9	0.07±0.09	0.03±0.01	0.08±0.05	0.004±0.002	0.002±0.001
WHITE PERCH							
Passaic River	11/4	37.2±11.4	0.02±0.01	0.06±0.02	0.06±0.03	0.002±0.001	0.002
Hackensack River 2004	5/12	47.2±7.7	0.02	0.01±0.02	0.01±0.03	0.012±0.020	0.01±0.01
Hackensack River 2005	8/7	40.5±3.4	0.02	0.05±0.04	0.13±0.03	0.003±0.003	0.002
Delaware River	2/8	34.2±9.1	0.02	0.01	0.01±0.01	0.002	0.002
Tuckerton	8/11	45.4±9.8	0.01	0.01±0.01	0.02±0.01	0.002±0.001	0.001

Table 4. Master sheet of descriptive statistics for all chemical level biomarkers

	Naphthalene (ng/uL)	Pyrene (ng/uL)	B(a)P (pg/uL)	CYP1A1 (ng/uL)	MT (ng/uL)	Male VT (ng/uL)	Female Vt (ng/uL)
FUNDULUS							
May Newark Bay	761±304	0.59±0.25	175±158	6.79E-06	8.22E-05	5.51E-07	5.13E-03
May Piles Creek	nd	nd	Nd	1.24E-05	5.03E-06	1.50E-07	4.80E-04
May Union Beach	783±232	0.57±0.20	178±130	1.99E-06	5.09E-06	1.27E-06	2.51E-04
May Sandy Hook	533±321	0.69±0.47	146±187	1.21E-06	1.22E-05	1.01E-06	2.80E-04
May Tuckerton	nd	nd	nd	2.67E-06	2.85E-05	3.45E-07	3.24E-04
July 2004 Newark Bay	758±556	1.21±0.99	579±744	3.38E-06	1.45E-05	1.53E-07	2.13E-05
July 2004 Piles Creek	904±354	0.66±0.45	247±226	1.04E-06	9.46E-06	1.50E-07	1.74E-07
July 2004 Union Beach	1280±409	0.76±0.61	426±417	4.11E-06	7.92E-06	2.00E-07	5.58E-06
July 2004 Sandy Hook	1026±360	0.57±0.19	268±180	5.34E-06	4.16E-05	8.97E-06	5.67E-05
July 2004 Tuckerton	1167±409	0.77±0.49	491±377	1.47E-05	4.19E-05	1.50E-07	1.41E-05
July 2006 Newark Bay	2104±886	5.87±3.84	2158±1234	nd	nd	nd	nd
July 2006 Piles Creek	1635±287	2.58±2.37	1095±649	nd	nd	nd	nd
July 2006 Union Beach	1621±436	1.36±0.39	969±461	nd	nd	nd	nd
July 2006 Sandy Hook	1546±276	0.51±0.02	814±391	nd	nd	nd	nd
July 2006 Tuckerton	992±655	1.35±1.38	564±450	nd	nd	nd	nd
WHITE PERCH							
Passaic River	1358 ± 530	3.54±2.70	1266±956	2.46E-07	4.79E-06	2.29E-07	4.28E-07
Hackensack River 2004	1212±382	3.61±2.22	1126±872	3.60E-06	8.04E-06	5.37E-06	1.04E-06
Hackensack River 2005	1030±344	3.94±1.76	1039±853	8.17E-07	1.27E-05	3.81E-07	7.47E-07
Delaware River	841 ± 295	1.11±0.58	341±198	1.15E-06	8.49E-06	2.56E-06	7.03E-07
Tuckerton	580 ± 321	0.76±1.01	243±258	3.45E-08	9.54E-06	3.28E-07	5.18E-06

nd = no data, samples either lost or degraded

Fundulus heteroclitus Results:

Collection Demographics:

Refer to Table 2 to see how many fundulus were collected at each site. However it should be noted here that in all collections and at every site females outnumbered males and at best the F/M ratio was 1, but generally varied from 2-6. Whether this represents a true population demographic is unknown, but may be due to collection methods. While gender ratio in fish may not always be 1, it is surprising to see such a skewed gender ratio.

External Evaluation:

In all of the fish examined there were no ulcerations, fin erosion or major external lesions. Upon dissection there were grossly visible parasites in the gastrointestinal track and alteration in liver color, but they did not appear to be specific for any one site (see Excel data set). This would be in agreement with the histological findings of widespread parasitism by Digenea (trematodes), and Eimeria sp. (protozoan) (Hawkins, W.E., Fournie, J.W., and Overstreet, R.M. 1983a, 1983b).

Blood Evaluation:

From the previous years work it was determined that the examination of micronuclei in RBCs was not a useful biomarker. A recent review of the technique discusses the difficulties with the detection of micronuclei in wild populations of teleosts compared to laboratory exposed fish (Udroiu 2006). It is recommended that an alternative method be used in evaluating mutational events in these fish. This could include running the assays for large DNA adducts, hemoglobin adducts or the Comet Assay (Cotelle and Ferard 1999, Lee and Steinert 2003, Nehls and Segner 2005).

The hematocrit determinations were reproducible and did give an indication of the RBC levels circulating in the fish. No significant differences were found in hematocrit between sites in the May collection while the range was 30-40%. There was however significant differences found in summer months (summary in Table 5). In July 2004 Newark Bay (42.5%) and Piles Creek (43.2%) were both higher than Union Beach (34.2%) and Sandy Hook (35.0%). In the July 2006 collection Tuckerton (46.2%) and Sandy Hook (43.1%) were both significantly higher than Union Beach (28.7%), Newark Bay (33.6%) and Piles Creek (34.2%). Hematocrit is an indication of the percentage of RBCs circulating in the blood of the fish. Unlike humans, fish control values can range from 25 to 50% depending on the species and time of year. In the majority of the studies we have carried out the hematocrit is normally between 40 and 50% for mummichog collected at Tuckerton. In both summer collections Union Beach was consistently lower than other sites. Union Beach and Tuckerton also had the least variation with no statistical differences between collections, suggesting that while hematocrit at other sites fluctuates between collections. Further studies would have to examine what is the cause of the significantly decreased hematocrits. It is worthy to note that hematocrit results between July 2004 and July 2006 collections also

conflict with each other. In July 2004 the more contaminated sites have higher hematocrits and in July 2006 the less contaminated sites have higher hematocrits.

Table 5. Summary of Fundulus hematocrit significant differences following ANOVA[1]

Date	Comparison	P<0.05
July 2004	Piles Creek vs Union Beach	Yes
July 2004	Piles Creek vs Sandy Hook	Yes
July 2004	Newark Bay vs Union Beach	Yes
July 2004	Newark Bay vs Sandy Hook	Yes
July 2006	Tuckerton vs Union Beach	Yes
July 2006	Tuckerton vs Newark Bay	Yes
July 2006	Tuckerton vs Piles Creek	Yes
July 2006	Sandy Hook vs Union Beach	Yes
July 2006	Sandy Hook vs Newark Bay	Yes
July 2006	Sandy Hook vs Piles Creek	Yes

[1]May data and all other comparisons were non-significant

Organ to body Weight Ratios:

Organ to body weight ratios are an easy biomarker that can indicate enlarged or atrophied organs either due to chemical exposure or in some cases histopathological lesions. Tables 6, 7 and 8 show significant differences in liver to body weight ratios (LSI), gonad to body weight ratios (GSI) and spleen to body weight ratios (SSI), respectively

Table 6 shows the liver somatic index comparisons. For the May 2004 collection the Newark Bay LSI (0.049) was significant higher than Tuckerton (0.020), Sandy Hook (0.030) and Union Beach (0.030). In July 2004 Piles Creek (0.045) has significantly higher liver to body weight ratios than Sandy Hook (0.030), Tuckerton (0.030) and Newark Bay (0.030). No significant differences for liver to body weight ratios were found in July 2006 and the range in values was 0.020-0.040. For the Newark Bay fish these are consistent with work carried out in the mid 1990s from the same locations (Prince and Cooper 1995a,b).

Table 6. Summary of Fundulus LSI significant differences following ANOVA

Date	Comparison	P<0.05
May	Newark Bay vs Tuckerton	Yes
May	Newark Bay vs Sandy Hook	Yes
May	Newark Bay vs Union Beach	Yes
May	Piles Creek vs Tuckerton	Yes
July 2004	Piles Creek vs Sandy Hook	Yes
July 2004	Piles Creek vs Tuckerton	Yes
July 2004	Piles Creek vs Newark Bay	Yes

[1]July 2006 data and all other comparisons were non-significant

When analyzing GSI the data was separated by gender because results were expected to differ depending on sex. Statistical differences for GSI are shown in Table 7. The May results show no significant differences in male GSI between sites. Females however showed that fish in Piles Creek (0.09), Sandy Hook (0.08) and Newark Bay (0.09) had significantly higher GSI than Union Beach (0.04) and Tuckerton (0.03). Unlike May data, July data is the same disregarding sex. In both males and females Sandy Hook (0.03 in both) had statistically higher GSI than all other sites (0.01 in all sites and sex). In July 2006 no differences were found between male populations. In Females however, Sandy Hook (0.10) stood out as significantly higher than Piles Creek (0.02), Newark Bay (0.035), Union Beach (0.04) and Tuckerton (0.08). Tuckerton was also significantly higher than Piles Creek. Several trends are seen in this analysis. Males tend to have fewer significant differences between populations. There is generally little to no variation amongst male GSI at any particular site. Females on the other hand show greater differences between sites and amongst individuals. There is generally greater variation in the data, and many more significant differences between sites. Sandy Hook is of notable interest because it was consistently higher than all sites in July 2004 and July 2006 and higher than 2 sites in May 2004. In the two July collections Sandy Hook had a ratio up to five times higher than all other sites which suggests that these fish were still gravid. Therefore interpreting GSI in some cases may be complicated depending on the time of year because of this phenomenon.

Table 7. Summary of Fundulus GSI significant differences following ANOVA[1]

Date	Comparison	P<0.05
May Females	Piles Creek vs Tuckerton	Yes
May Females	Piles Creek vs Union Beach	Yes
May Females	Sandy Hook vs Tuckerton	Yes
May Females	Sandy Hook vs Union Beach	Yes
May Females	Newark Bay vs Tuckerton	Yes
May Females	Newark Bay vs Union Beach	Yes
July 2004 Males	Sandy Hook vs Union Beach	Yes
July 2004 Males	Sandy Hook vs Tuckerton	Yes
July 2004 Males	Sandy Hook vs Piles Creek	Yes
July 2004 Males	Sandy Hook vs Newark Bay	Yes
July 2004 Females	Sandy Hook vs Tuckerton	Yes
July 2004 Females	Sandy Hook vs Piles Creek	Yes
July 2004 Females	Sandy Hook vs Union Beach	Yes
July 2004 Females	Sandy Hook vs Newark Bay	Yes
July 2006 Females	Sandy Hook vs Piles Creek	Yes
July 2006 Females	Sandy Hook vs Newark Bay	Yes
July 2006 Females	Sandy Hook vs Union Beach	Yes
July 2006 Females	Sandy Hook vs Tuckerton	Yes
July 2006 Females	Tuckerton vs Piles Creek	Yes

[1]All other comparisons were non-significant

There were few significant differences between sites and genders with SSI (Table 8). In the case of the spleen somatic index there was a significant difference observed between males and females at Sandy Hook and Union Beach, while at Newark Bay, Piles Creek and Tuckerton there was not a significant difference. The male SSI is generally smaller than the female and may reflect the generally smaller body size of the male ($p=0.01$). Therefore any comparison across sites must separate out the female from the male. Lack of SSI differences between male and female may indicate an enlarged spleen in females or possibly an effect on growth.

The data was divided into genders and compared across sites. There were few statistical differences between sites in all collections. The only two differences were found between Newark Bay females and Union Beach females in the May collection.

Table 8. Summary of Fundulus SSI significant differences following ANOVA[1]

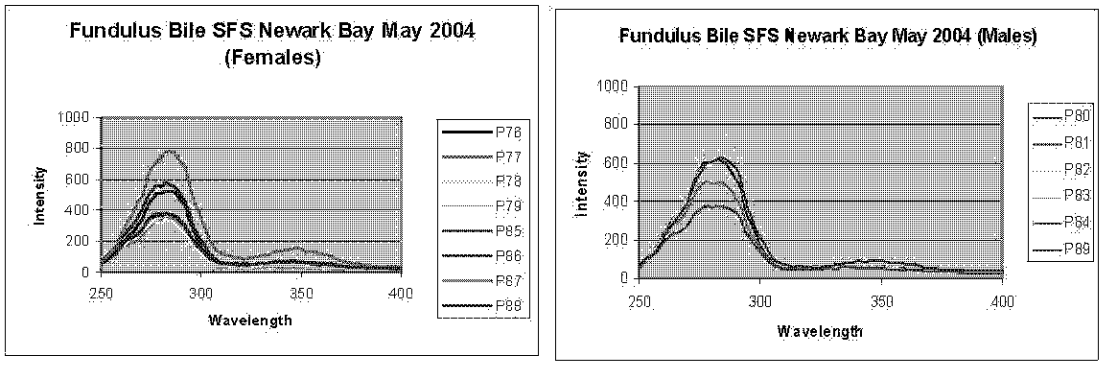
Date	Comparison	P<0.05
May	Union Beach males vs females	Yes
May	Sandy Hook males vs females	Yes
May	Newark Bay vs Union Beach	Yes
July 2004	Tuckerton vs Union Beach	Yes

[1]All other comparisons were non-significant

Organ to body weight ratios should be evaluated in any sampling effort, especially because it is a simple, quick and easy measure. Total gonadal weight should also be determined but differences in spawning patterns between sites should be considered. In the case of fish that have begun to spawn the gonadal weight may reflect loss of egg or sperm. In the case of Fundulus we recommend that collections be consistently carried out at the same cycle of the moon. For example all collections should be carried out within one or two days of a new or full moon.

Bile Concentrations:

The source of these compounds may be from any number of point and non-point sources. It was expected that there would be less of these compounds in Tuckerton and Sandy Hook. There was no qualitative difference observed between the male and female fish spectrum collected from the same location (Figure 3). Statistically there was no difference between male and female fish bile levels for naphthalene, pyrene or benzo(a)pyrene.



A. B.

Figure 3. Comparison of fluorescence scans between male and female bile samples collected from Newark Bay *Fundulus heteroclitus*.

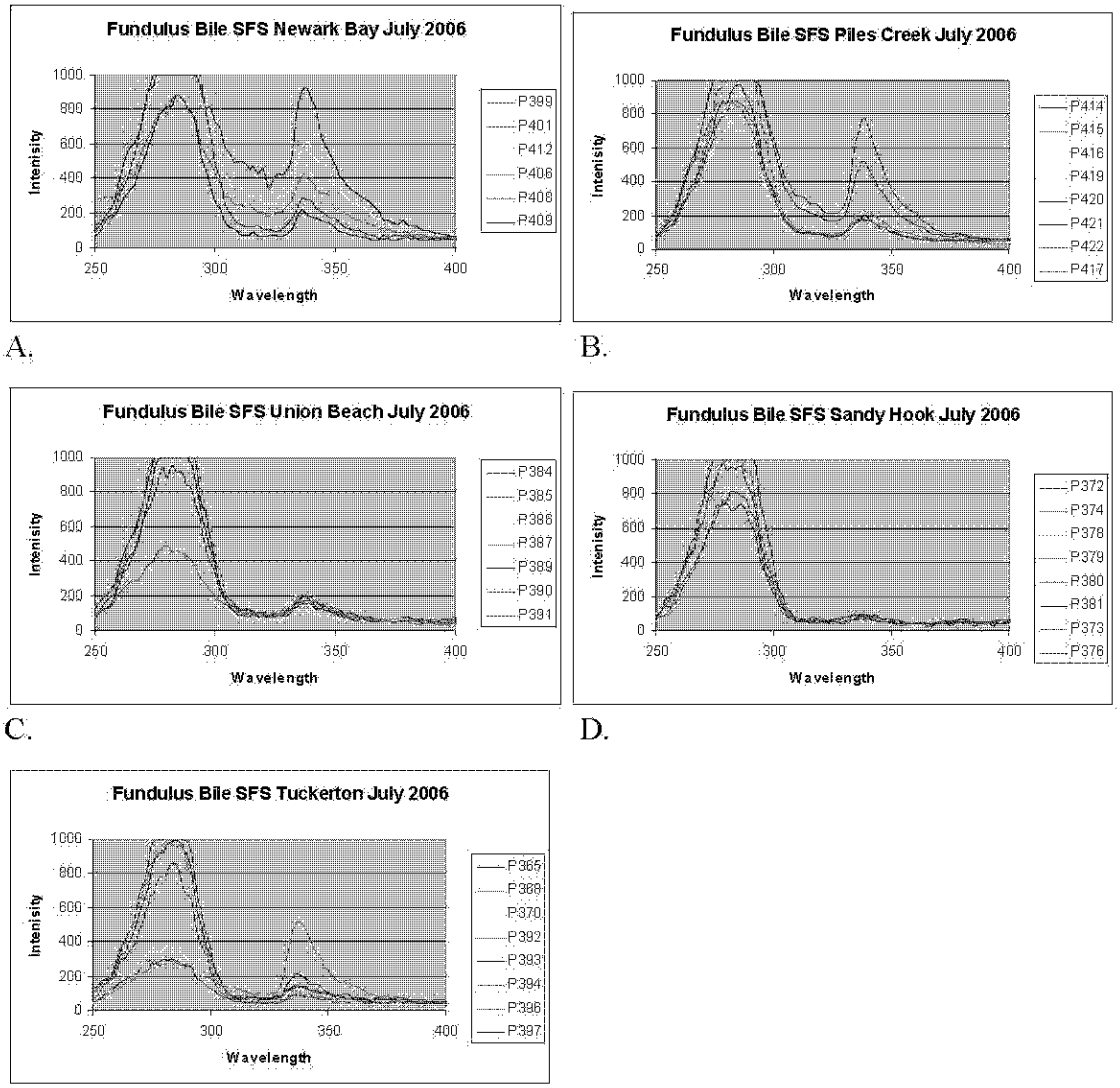


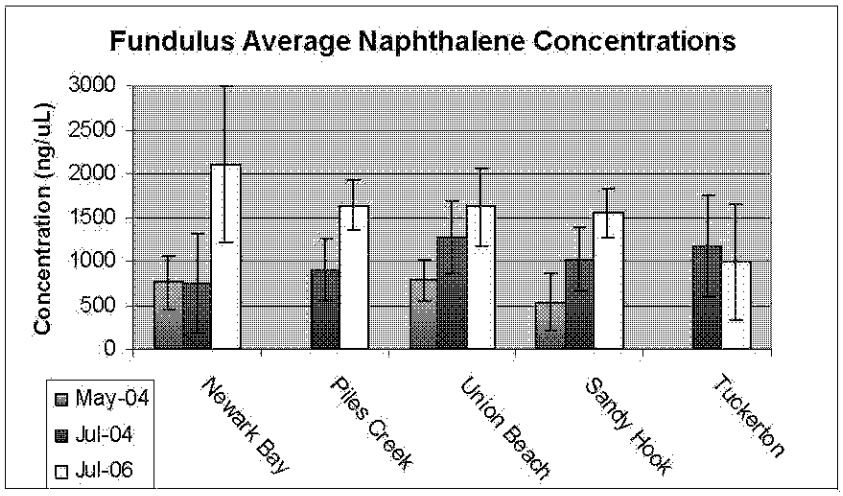
Figure 4. Comparison of different synchronous fluorescence scans for *Fundulus heteroclitus* in July 2006 bile samples. A. Newark Bay. B. Piles Creek. C. Union Beach. D. Sandy Hook and E. Tuckerton.

Figure 4 is an example of the individual differences in the intensity of the three PAH peaks between sites in July 2006. More specifically regarding May naphthalene concentrations, Newark Bay (761.0ng/μL) and Union Beach (782.8ng/μL) were both higher than Sandy Hook (532.6ng/μL). There were no significant differences in May between sites for pyrene and benzo(a)pyrene concentrations. In July 2004 there were no significant differences between sites for pyrene and benzo(a)pyrene. There were however several differences for naphthalene. Union Beach (1280.3ng/μL) was higher than Newark Bay (757.7ng/μL) and Piles Creek (904.4ng/μL). In July 2006 there were no differences for naphthalene between sites, although there were differences for the other compounds. Pyrene concentrations in

Newark Bay (4.52ng/µL), Piles Creek (1.45ng/µL) and Union Beach (1.50ng/µL) were statistically higher than both Sandy Hook (0.50ng/µL) and Tuckerton (0.93ng/µL). The only differences for benzo(a)pyrene in July 2006 was between Newark Bay (1782.6pg/µL) and Tuckerton (519.8pg/µL).

There is also an indication that the samples collected in July had higher levels of pyrene compared to the May levels It is interesting that one of the Tuckerton fish bile samples seen in Figure 4E show much higher levels than the majority of the samples. This does demonstrate the individual variation at a site, as well as difference in concentrations between the sites. Both Newark and Piles Creek have higher total levels of bile fluorescence than the other sites.

Shown in Figure 5 below are summarized the concentrations for naphthalene, pyrene and benzo(a)pyrene by date of collection and by site. In the majority of the sites there was a significant increase in the concentration of the compound in the later collection times. The two exceptions to this were the naphthalene values at Tuckerton Figure 4A and Sandy Hook Figure 4B. In both these cases there was a decrease in the summer value. For naphthalene the naphthalene equivalents in the bile appear to increase as you move from May into late summer (Figure 5). A similar pattern is observed with pyrene with the exception of Sandy Hook fish where the pyrene levels appear to be decreasing. B(a)P also showed an increase in concentration as the summer progressed. What these results show is that these PAHs occur in all of the sites where these fish were collected



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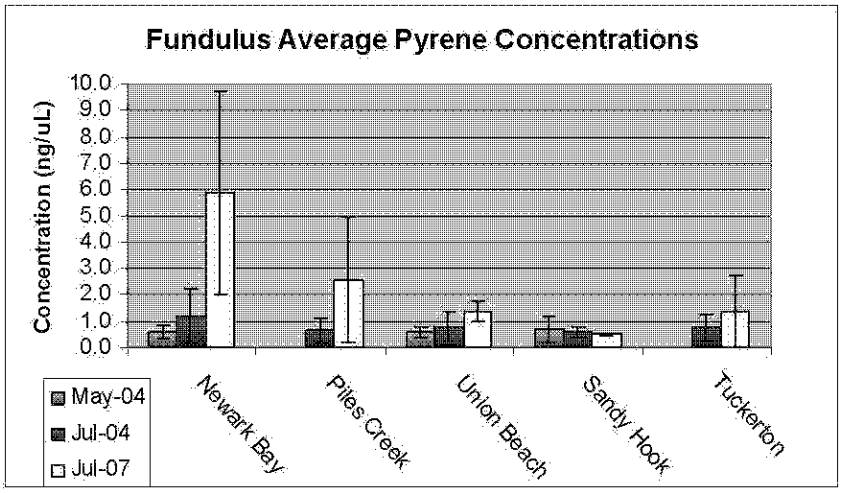

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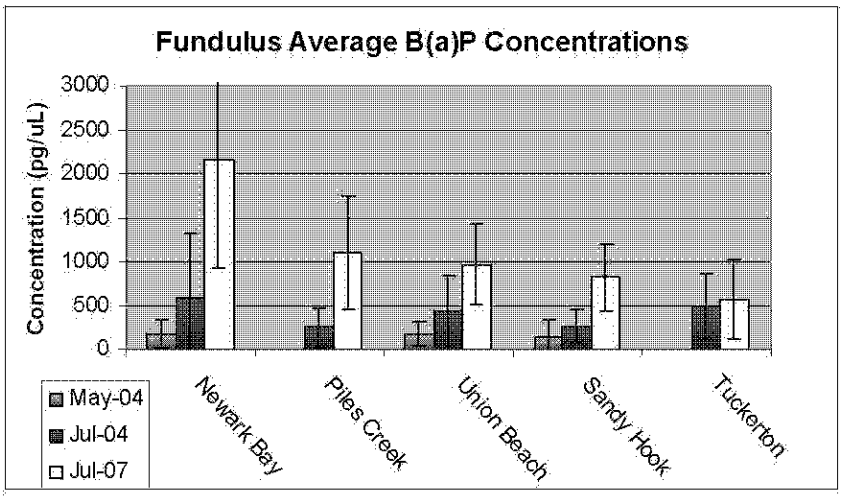


C.

Figure 5. Comparison in *Fundulus heteroclitus* bile of naphthalene, pyrene and benzo(a)pyrene by site and collection date.

The bile fluorescence findings suggest that higher concentrations of contaminants are present in the bile in the latter summer months. This is not surprising since the fish would have been eating locally contaminated prey during this time period. In addition, while in many cases there were few significant differences between sites it is always the more contaminated sites that are shown to be significantly higher than the other sites.

This is a very good technique for qualitative determination of PAHs that the fish are being exposed to at the time of collection. This also gives some indication of the PAHs that the liver might be being exposed to and could induce enzymes such as CYP1A1 or result in associated lesions.

Histopathology:

What are summarized below are the results from the examination of the Fundulus livers from the various locations. In all sites there were both large encysted trematodes (granulomas) as well as a small encysted protozoan tentatively identified as *Eimeria sp.* It is apparent that the number of lesions were fewer in the more heavily impacted (i.e., contaminated) sites such as Newark and Piles Creek than in the less impacted locations (i.e., Sandy Hook and Tuckerton). The presence of hemosiderin (black deposits), lipofuschin (yellow/brown deposits) and fibrosis at several sites indicated chronic exposure to parasites and possible chemical damage and subsequent tissue repair.

Table 9. Summary of histopathology from *Fundulus heteroclitus* in 2006.

SITE	DATE (M/Yr)	NSL[1]	LESION	DESCRIPTION
Newark Bay	4/04	22	8 (27%)	Encysted trematodes, fibrosis, sm. Protista
	6/06 & 7/06	10	3 (23%)	Hemosiderin, sm. Protista
Piles Creek	5/04	25	4 (14%)	Altered staining, lg # trematodes, fungal hyphae
	7/06	9	0 (0%)	
Union Beach	5/04	17	10 (37%)	Hemosiderin, necrosis, uneven staining, ext. sm. Protista
	6/06	2	8 (80%)	Mod. sm. Protista
Sandy Hook	5/04	11	11 (50%)	Hepatic neoplasia, necrotic areas In pancreatic and hepatic tissue
	7/04	19	11 (37%)	Hepatic neoplasia, fatty liver, macrophage centers, Inflammatory cells in liver
	6/06	1	9 (90%)	Mod. sm. Protista
Tuckerton	4/04	22	8 (27%)	Trematodes pancreas, fibrosis, and sm. Protista
	6/06 & 7/06	10	3 (23%)	Hemosiderin, mod. sm. Protista

[1] NSL = No Significant Lesions

In the Newark Bay fish encysted parasites, fibrosis and the occurrence of *Eimeria sp.* were the primary lesions observed. In Piles Creek there were altered hepatocytes staining that could be considered pre-neoplastic lesions. There were several fish with what appeared to be fungal infections. Uneven hepatocyte staining was also observed in Union Beach mummichog. In the June 2006 Union Beach sample the primary lesion was extensive *Eimeria sp.* encysted in the liver. In the Sandy Hook fish neoplasms were observed in liver samples collected in May 2004 and June 2006 samples. Similar to Union Beach the liver was heavily parasitized with *Eimeria sp.* Similar parasitic lesions were observed in Tuckerton fish, but no altered staining or neoplasms were observed. In all of the fish examined there was extensive damage to the pancreatic tissue. It is likely that such damage would impact the pancreatic tissues ability to regulate glucose and could result in altered metabolic activity. Monitoring of glucose levels in the fish blood could be an additional biomarker that would likely correlate with pancreatic function.

Histopathological evaluation is a good biomarker for establishing the health of an individual organism and the prevalence of lesions in a specific population. Information on background prevalence of these lesions is necessary to establish how much of the lesion prevalence is attributed to site specific reasons. A reference site can estimate background prevalence assuming there is no exposure to any contaminants, but Tuckerton may not be a good estimate for background prevalence in this case. In any case these histopathological lesions measured are chronic lesions and contribute to deteriorating health conditions. The presence alone of any preneoplastic and neoplastic lesion indicates poor health of the fish.

Cytochrome P450 mRNA (CYP1A1):

Cytochrome P450 is a membrane bound oxidative enzyme primarily found in the mitochondria and endoplasmic reticulum which is involved in phase I metabolism. It is responsible for metabolizing drugs and xenobiotics to help facilitate excretion and detoxification. Polyaromatic hydrocarbons are known to induce hepatic mRNA expression which can be used as a measure of exposure to a wide variety of drugs and endogenous compounds (Hahn, 1998b). While expression patterns are often useful for indicating exposure to contaminant loads, interpretation is often complicated by locally resistant and sensitive populations (Wirgin 2004). Nevertheless, relative mRNA induction levels between sites and variation amongst each site gives valuable information regarding exposure (Coutrenay et al., 1999).

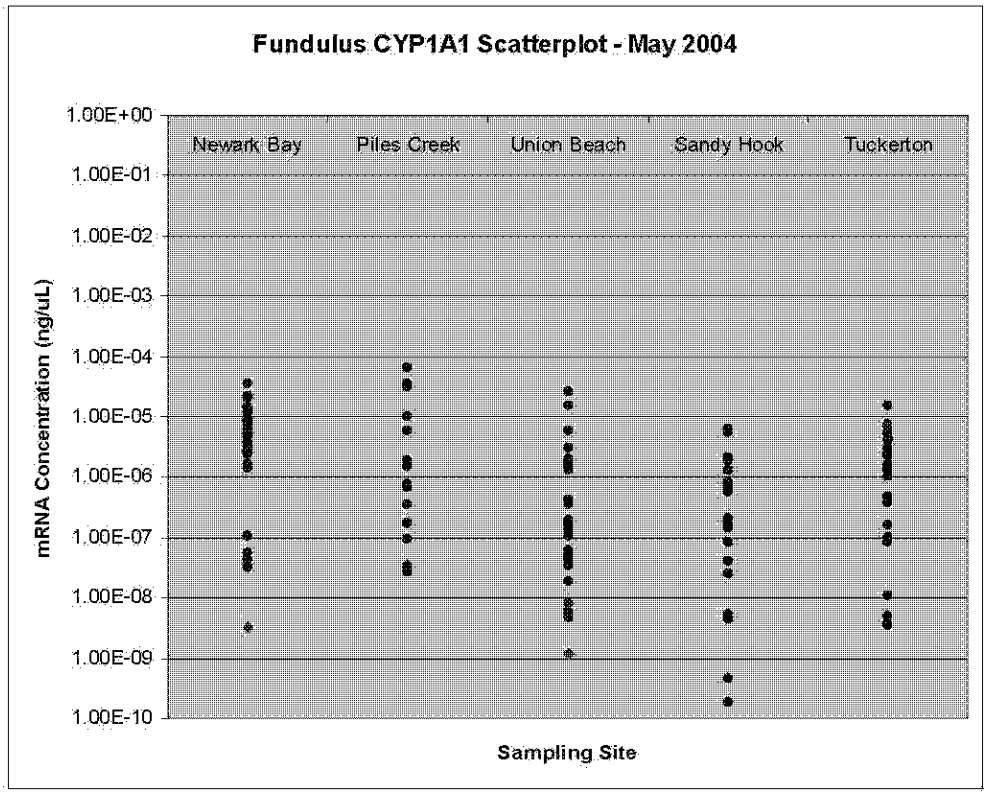
The data presented in Figure 6 and Figure 7 below illustrates the range of mRNA detected in the various fish livers collected from the same location and time of year. It should be noted that the values range over three orders of activity. This indicated the heterogeneity even in these in-bred populations. Significant statistical comparisons are summarized in Table 10. In May 2004 CYP1A1 levels in Newark Bay fish were significantly higher ($p=0.000142$) than the Union Beach and Sandy Hook levels and not significantly different from Piles Creek or Tuckerton. The difference between Newark Bay induction levels and Union Beach induction levels was approximately 30 fold which demonstrates the wide range of induction levels. Comparing the CYP1A1 in July resulted in different groups being significantly different than observed in May. Piles Creek was significantly lower from Tuckerton, Sandy Hook, Newark Bay and Union Beach. Tuckerton had the highest induction which was twice that of Sandy Hook, four times that of Union Beach, five times that of Newark Bay and

approximately 330 times that of Piles Creek. Tuckerton was statistically higher than Newark Bay and Piles Creek, but none of the other sites. These findings are counter-intuitive considering the level of contamination in Newark Bay and Piles Creek. There is a possibility that chemical stressors at these sites are effecting the mRNA signaling pathway or some other biochemical feedback mechanism.

CYP1A1 induction levels differed significantly between all sites from May and July 2004. Interestingly, the more contaminated sites (Newark Bay and Piles Creek) decreased induction levels from May to July while the less contaminated sites (Tuckerton, Sandy Hook and Union Beach) increased induction levels during the May to July transition.

Up regulation of CYP1A1 mRNA is complicated since it can be induced by a number of PAHs, dioxins, and PCBs (Karchner et al., 1999, Waxman 1999). There is also the situation that populations that have been chronically exposed to these compounds have been reported to have elevated basal levels of this protein and are less able to be induced (Nacci et al 2000, Prince and Cooper 1995 a,b). There was no significant correlation between CYP1A1 levels as they related to liver weight, LSI, and bile concentrations of individual compounds. However, the dependent variable CYP1A1 for Newark Bay fish can be predicted from a linear correlation of the independent variables liver wt, bile B(a)P concentration and bile naphthalene. This would support the idea that multiple factors influence the CYP1A1 mRNA level.

CYP1A1 mRNA is a good biomarker for examining the effect from compounds that induce aryl hydrocarbon receptor (AHR) activity. A number of these compounds are known hepatotoxic and carcinogenic compounds. The difficulty arises when fish have developed a tolerance to these compounds. It is recommended that not only mRNA be evaluated but also enzymatic activity and CYP1A1 protein levels (Western Blot Analysis) (Ostrander 1996).



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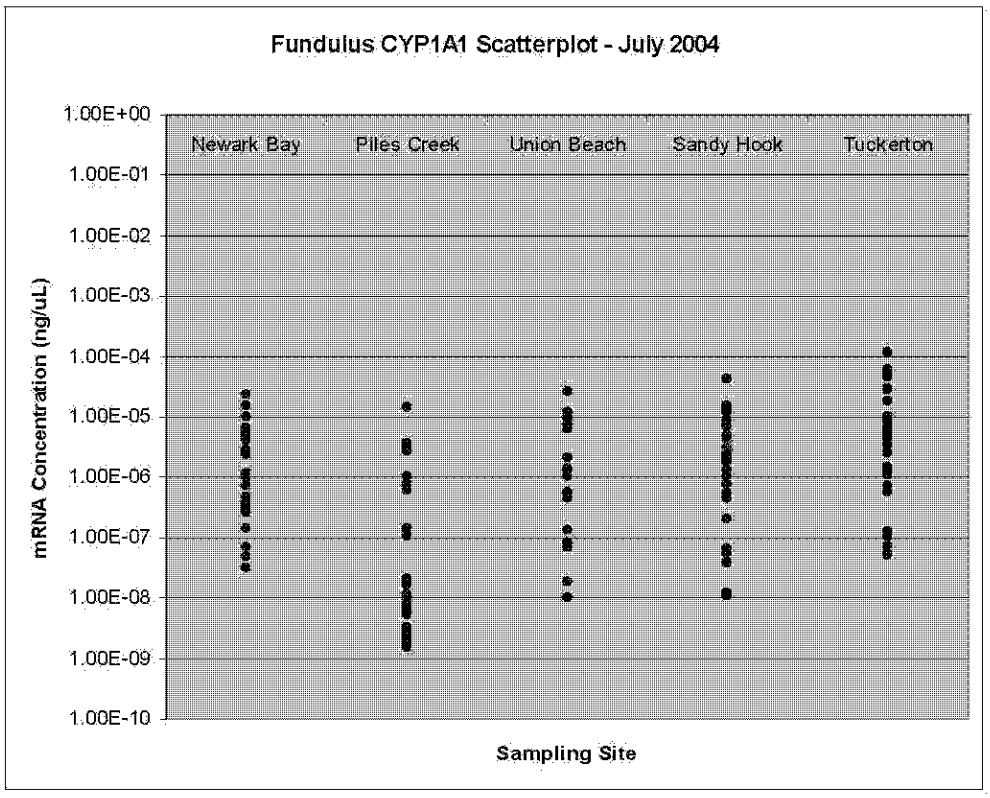


B.

Figure 6. Scatter plots of hepatic CYP1A1 mRNA in *Fundulus heteroclitus*. Each point represents the average value for one individual run in triplicates.

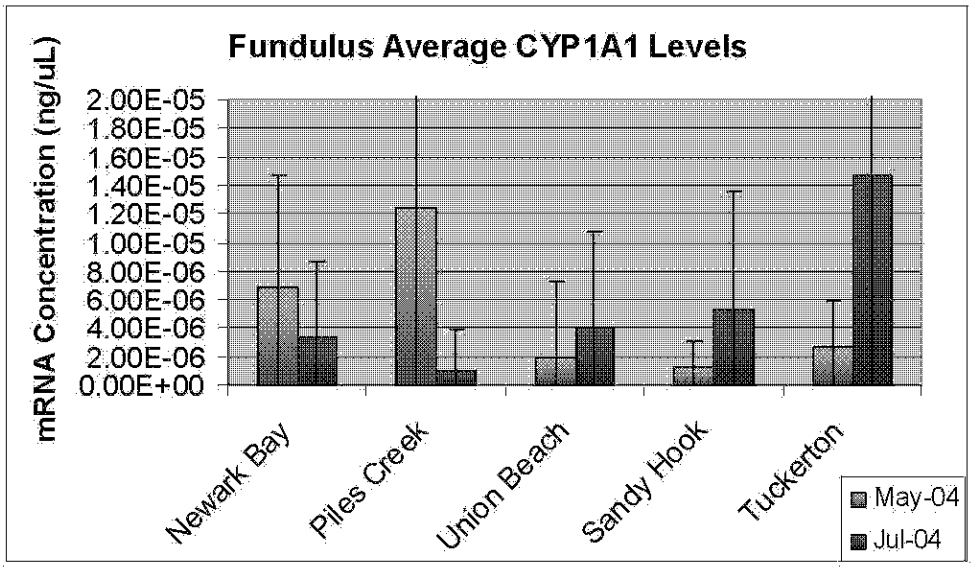


Figure 7. Average CYP1A1 values for *Fundulus heteroclitus* in May and July, 2004.

Table 10. Summary of Fundulus CYP1A1 significant differences following ANOVA[1]

Date	Comparison	P<0.05
May	Newark Bay vs Union Bay	Yes
May	Newark Bay vs Sandy Hook	Yes
July 2004	Tuckerton vs Piles Creek	Yes
July 2004	Sandy Hook vs Piles Creek	Yes
July 2004	Newark Bay vs Piles Creek	Yes
July 2004	Union Beach vs Piles Creek	Yes

[1]July 2006 samples missing, all other comparisons were non-significant

Metallothionein (MT) Hepatic mRNA:

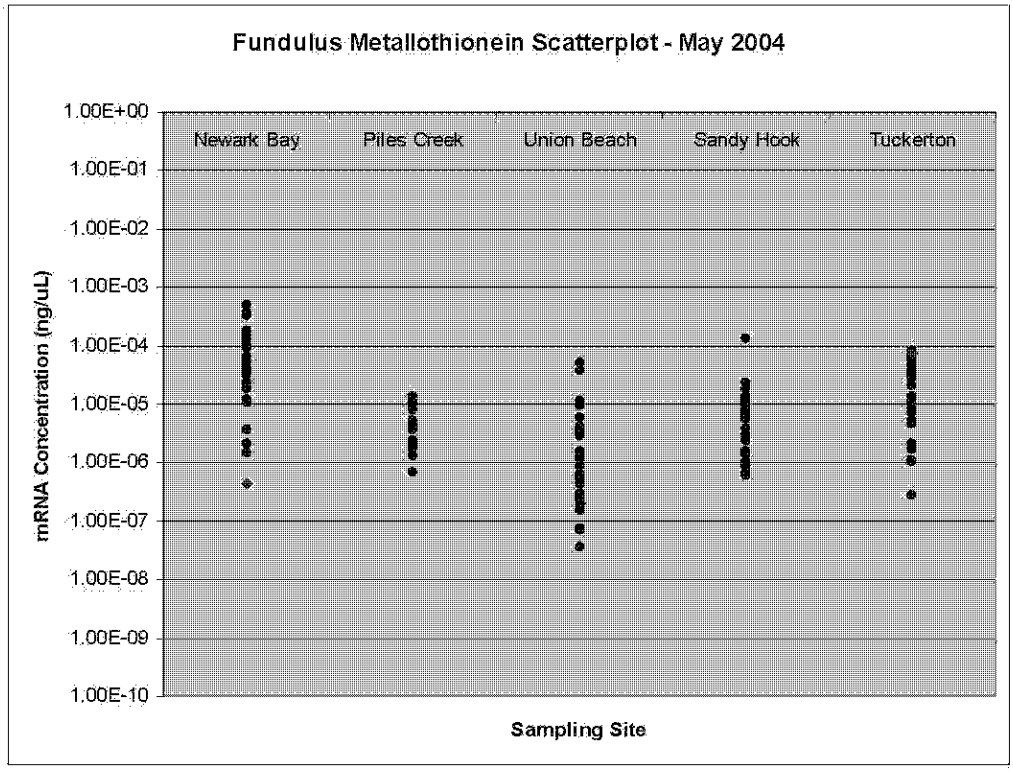
Metallothionein (MT) is a low-molecular-weight (MW 6000-7000) metal binding protein. MT consists of a multigene family that has a high capacity to bind metallic cations due to its high content of cysteine. In some forms of MT 20 of the approximately 61 amino acids are cysteines. The cysteine sulfhydryl groups are capable of binding Zn^{2+}, Cd^{2+}, Hg^{2+}, methyl-Hg^{+} and other cations that bind to sulfhydryl groups (Hodgoson and Smart 2001). It is believed that MT acts as a storage protein for Zn^{2+} and Cu^{2+} as well as an antioxidant.

Shown in Figure 6 are the scatter plots for liver metallothionein mRNA. In the May sample Newark Bay levels were significantly elevated above Union Beach, Piles Creek and Sandy Hook fish (Table 11). Newark Bay MT levels were not significantly different from Tuckerton levels. Tuckerton levels were significantly higher than Union Beach. In the July sample Newark Bay was significantly different from Piles Creek. Union Beach and Piles

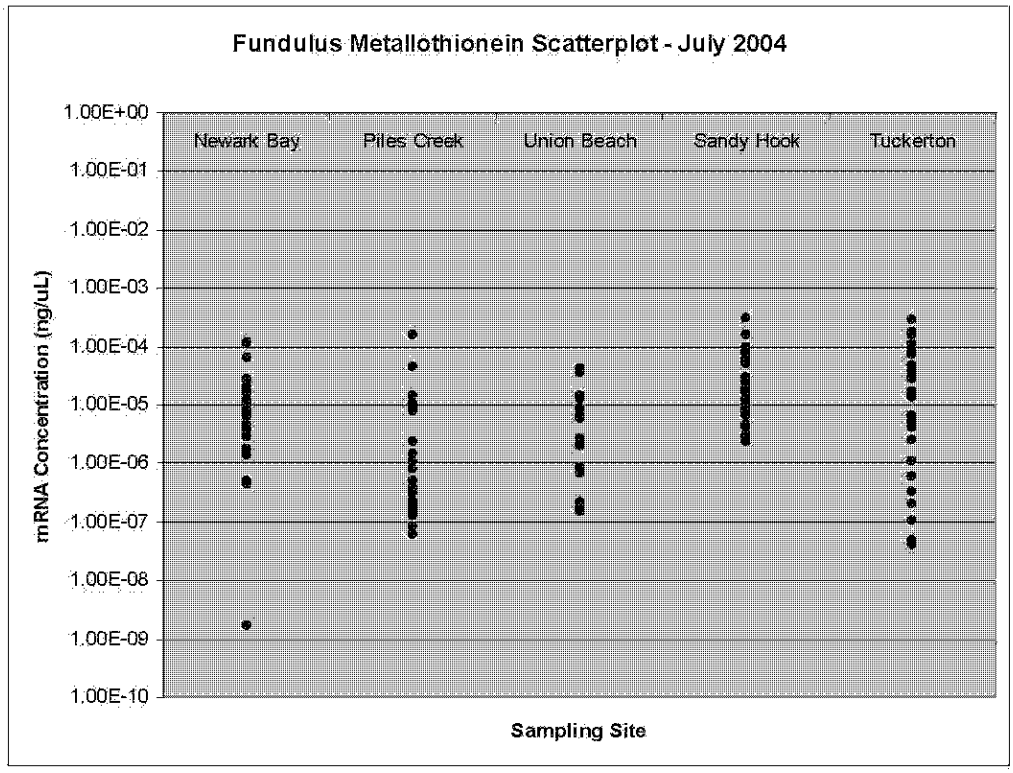
Creek induction levels were significantly higher from Sandy Hook. Sandy Hook and Piles Creek were significantly different, Sandy Hook of which was 5 times that of Piles Creek. The liver mRNA from the July 2006 collection was not prepared properly and was unable to be used for determining MT levels.

Several observations are seen with the metallothionein expression patterns. In May, Newark Bay is significantly higher than all sites except Tuckerton. In July Piles Creek is significantly lower than all other sites except Union Beach. What is not known is the effects of chronic chemical exposure on this pathway or the impacts from other chemicals present at Piles Creek. Another observation is that induction between May and July decreases significantly in Newark Bay and Piles Creek, increases at Sandy Hook, and remains the same in Union Beach and Tuckerton. Metal concentrations in the liver would allow for a better interpretation of this data.

There was no clear pattern for induction of hepatic MT mRNA, and without chemical quantification of metals I would not recommend evaluating this as a biomarker. It might also be useful to evaluate MT from the kidney which is known to accumulate heavy metals. What is not known is the ability of these local populations to be induced and what effect chronic exposure has had on the regulation of MT.



A.



B.

Figure 8. Scatter plots of *Fundulus heteroclitus* hepatic metallothionein. Each point represents the average value for one individual run in triplicates.

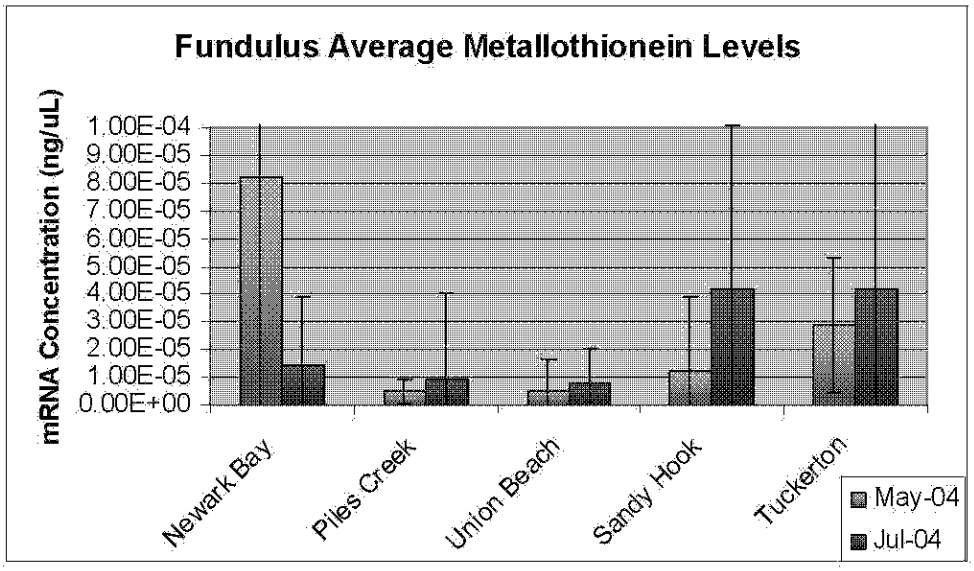


Figure 9. Average hepatic metallothionein levels in *Fundulus heteroclitus* for May and July, 2004.

Table 11. Summary of Fundulus metallothionein significant differences following ANOVA

Date	Comparison	P<0.05
May	Newark Bay vs Union Beach	Yes
May	Newark Bay vs Piles Creek	Yes
May	Newark Bay vs Sandy Hook	Yes
May	Tuckerton vs Union Beach	Yes
July 2004	Sandy Hook vs Piles Creek	Yes
July 2004	Sandy Hook vs Union Beach	Yes
July 2004	Tuckerton vs Piles Creek	Yes
July 2004	Newark Bay vs Piles Creek	Yes

[1]July 2006 samples missing, all other comparisons were non-significant

Vitellogenin (VT) mRNA Levels:

Vitellogenin data was separated by gender because of the difference in gender use of this protein. Site-site differences of the same gender were investigated as well as the differences between genders at the same site.

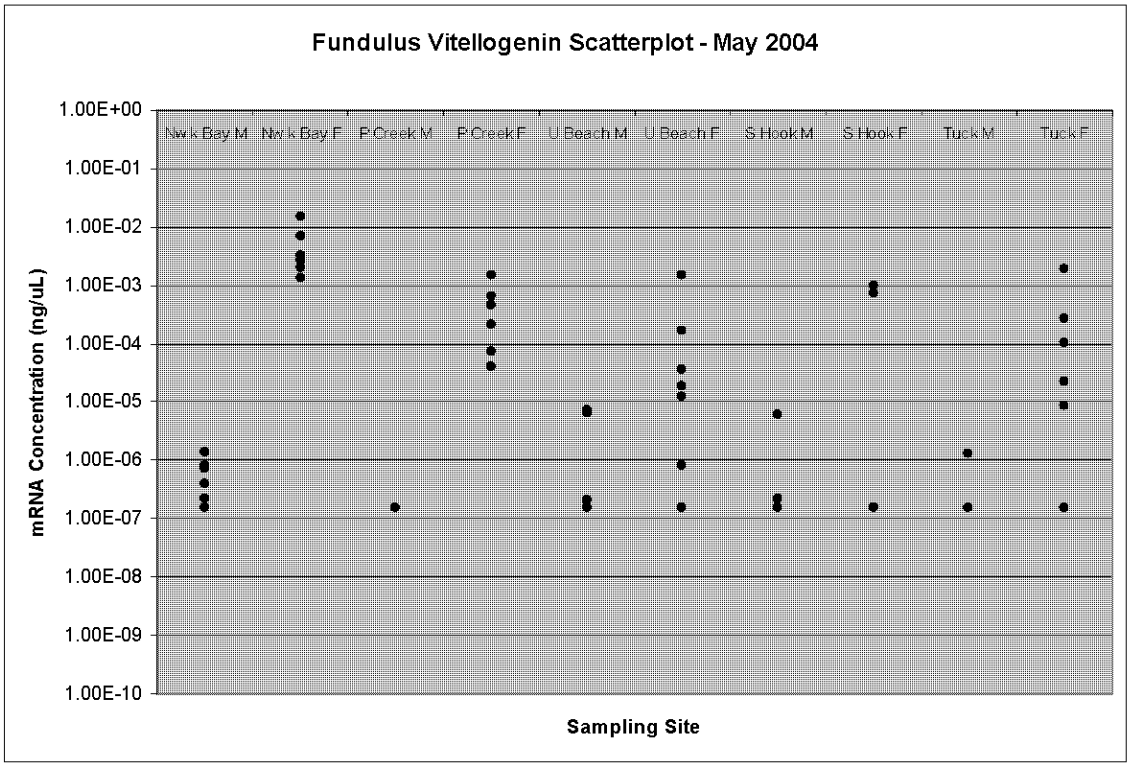
Male VT levels were generally one or two orders of magnitude lower than the female fish collected at the same time and location (Figure 10). There were several exceptions however. Females at Sandy Hook in May had vitellogenin levels no different than the male population. Although females at Sandy Hook in July 2004 were statistically higher than males the difference in relative induction was not at the same level of induction at all other sites which

tended to be much higher between genders. This data is interesting because of the regularly high GSI found at Sandy Hook which might suggest Sandy Hook fish have irregular gonadal development and reproduction.

Female VT expression was not significantly different than the male population at Piles Creek and Union Beach. This was however for the July 2004 collection which is expected and consistent with the end of spawning which is when vitellogenin should be low.

When comparing sites, females at Newark Bay were statistically higher than all other sites. Sandy Hook having the lowest level of expression, Newark Bay had a relative induction of 20 fold over Sandy Hook. There were no site-site significant differences between male populations found in May and July 2004 collections.

Based on these data there does not appear to be a shift in the male population VT levels toward female levels. This would agree with the lack of feminization of the male testis in the fish examined histologically. Based on the values obtained across the groups VT levels in a male that would be higher than $1.00x10^{-05}$ng/µL would be suspect of feminization. In a similar fashion a female that has VT levels lower than $1.00x10^{-05}$ng/µL would indicate very low levels of VT and could indicate a pre-spawning female or a female with low VT possibly due to androgenic compounds. In order to determine masculinization of the female aromatase or circulating hormone levels would need to be determined.



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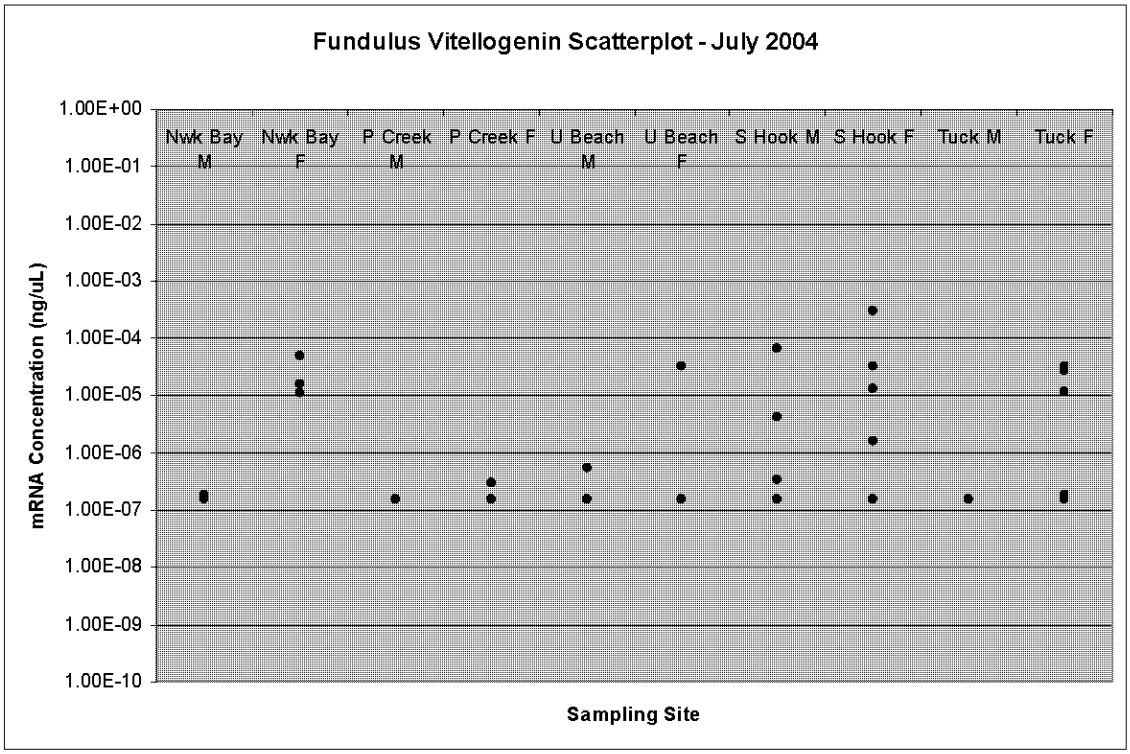


B.

Figure 10. Vitellogenin mRNA scatterplot levels from *Fundulus heteroclitus* collected in May and July 2004.

*All values falling on threshold (10^{-7}ng/µL) will converge on 1 point

White Perch Results:

White perch were collected from the Hackensack River, Mullica River (Lower Bank) near Tuckerton, Delaware River and the Passaic River. They were collected by NJDEP, Philadelphia Academy of Science and Rutgers personnel. They were collected by boat trawl, hoop nets and gill net. Collection dates were not uniform and this fact needs to be taken into consideration when comparing different populations. These results will offer preliminary insight into which biomarkers are useful in White Perch.

Collection Demographics:

Refer to Table 17 (pg 46) to see how many white perch were collected at each site and the male to female ratios. It should be noted that in the majority of collections females outnumbered males with the exception of Passaic River in which males outnumbered females almost 3:1. Until population based studies are carried out shifts in sex ratios can not be interpreted.

External Evaluation:

In all of the fish examined there were no ulcerations, fin erosion or major external lesions except for some abrasions which were likely due to the method of fish collection. Upon dissection there were grossly visible parasites in the gastrointestinal track and alteration in liver color, but like the Fundulus they did not appear to be associated with any particular location.

Blood Evaluation:

There were significant differences (Table 12) between the hematocrit values between Hackensack (median = 45.5%), Delaware fish (35.9%) and Passaic River fish (36.5%). The Tuckerton HMT (44.2%) values were also significantly different from Delaware and Passaic River fish. There is a need to collect a larger number of White Perch to better assess the role of parasite infestation as it relates to hematocrit values. Since there does appear to be an effect on hematocrit values it would be important to section through the head kidney area where RBCs are produced to determine if the "bone marrow" cells appear normal. Because of the extensive parasite loads that these fish have and the damage to the pancreatic tissue (see Histopathology below). An evaluation of the circulating glucose levels would be

another parameter that I would recommend including in any future studies. Decreased glucose could indicate effects on the pancreatic ability to release insulin and maintain appropriate glucose levels.

Table 12. Summary of White Perch hematocrit significant differences following ANOVA[1]

Comparison	P<0.05
Hackensack 04 vs Delaware	Yes
Hackensack 04 vs Passaic	Yes
Tuckerton vs Delaware	Yes
Tuckerton vs Passaic	Yes

[1]All other comparisons were non-significant

Organ to body Weight Ratios:

There were significant differences for the liver somatic index (LSI) between the different groups (Table 13). The LSI (mean $\pm$ SD) for Hackensack (0.0235 $\pm$ 0.004) in 2004, Hackensack (0.0245 + 0.003) in 2005 and Passaic River (0.0175 $\pm$ 0.006) fish were larger than either the Tuckerton (0.0103$\pm$ 0.002) fish. The Hackensack River fish LSI were significantly greater than the Tuckerton and Delaware River fish. There was no significance difference from the Hackensack and Passaic fish. The Passaic River fish were significantly different from the Tuckerton fish but not the Delaware fish. The Delaware and Tuckerton fish were not significantly different one from the other. This would indicate that LSI is a good biomarker for correlating with increased chemical contamination.

Table 13. Summary of White Perch LSI significant differences following ANOVA[1]

Comparison	P<0.05
Hackensack 05 vs Tuckerton	Yes
Hackensack 04 vs Tuckerton	Yes
Passaic River vs Tuckerton	Yes

[1]All other comparisons were non-significant

Altered spleen somatic index (SSI) can be an indication of chronic infection or the response of the fish to parasite loads. Following the pattern seen in fundulus where few differences were found, there was no difference in SSI between genders at any site. There were however differences between sites Hackensack (2004) and Tuckerton fish, both male and female populations. There were no other differences between sites.

Females in the Hackensack River (2004) had a GSI (mean = 0.008) that was significantly less from the Passaic River fish (0.070) and the 2005 Hackensack River collection (0.13). The GSIs for Tuckerton (0.00148), Passaic (0.002) and Delaware River (0.00191) were not significantly different. Following the trend seen in fundulus in which few significant differences were found between males, there were no significant differences between male white perch.

Bile Concentrations:

As can be seen in Figure 11 below the Passaic River (1358 ± 530) and Delaware (841 ± 295) were significantly higher than the reference site Tuckerton (580 ± 321) fish for naphthalene (Table 14). Hackensack River (1205 ± 371) fish were also significantly different. While no other significant differences were found, Figure 11 shows a decreasing trend of naphthalene concentration when sites are put in order of levels of contamination. This indicates white perch at all sites are being exposed to naphthalene.

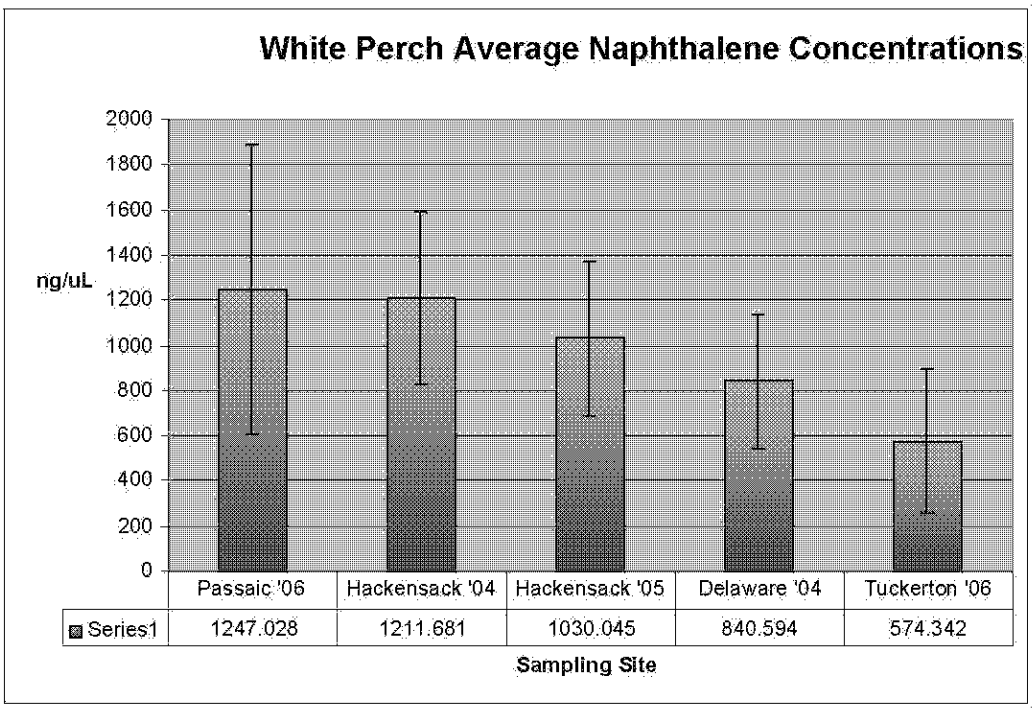


Figure 11. Comparison of White Perch bile naphthalene concentrations (mean ± SD).

Table 14. Summary of White Perch Naphthalene significant differences following ANOVA[1]

Comparison	P<0.05
Passaic vs Tuckerton	Yes
Hackensack 04 vs Tuckerton	Yes
Hackensack 05 vs Tuckerton	Yes

[1]All other comparisons were non-significant

In the case of pyrene (Figure 12 and Table 15 below) the Hackensack River fish (both collections) and Passaic River fish were significantly higher from the Delaware River fish and Tuckerton fish. These significant findings correlate with the naphthalene data which suggest these three sites have higher contaminant loads than Delaware River and the reference site in Tuckerton.

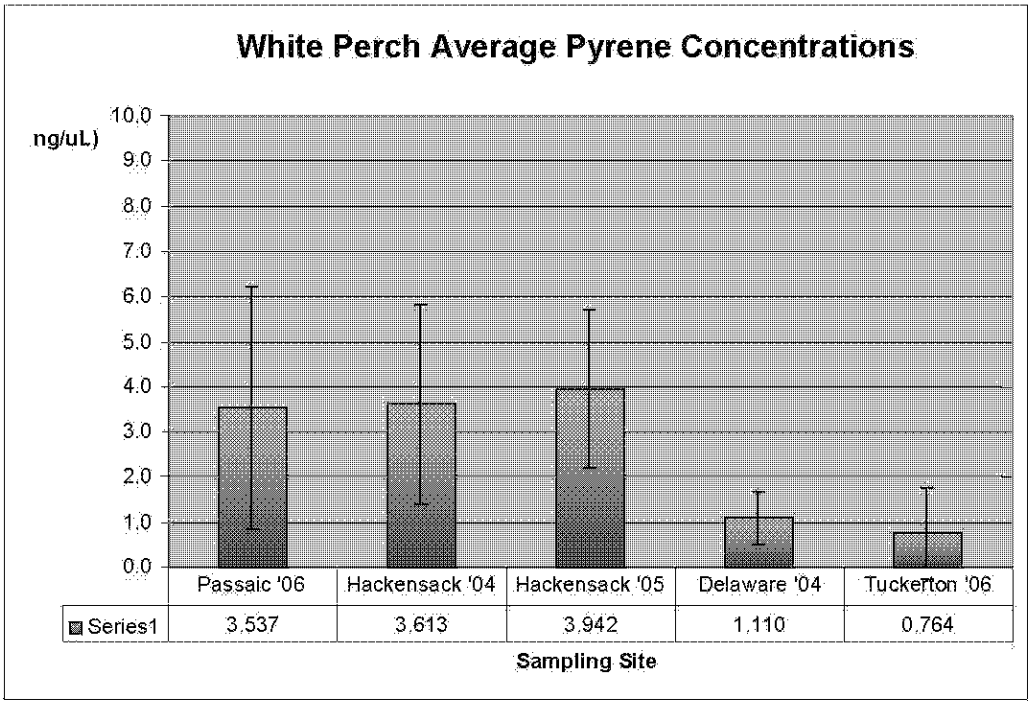


Figure 12. Comparison of White Perch bile fluorescence for pyrene (mean ± SD).

Table 15. Summary of White Perch Pyrene significant differences following ANOVA[1]

Comparison	P<0.05
Passaic vs Tuckerton	Yes
Hackensack 04 vs Tuckerton	Yes
Hackensack 05 vs Tuckerton	Yes

[1]All other comparisons were non-significant

In the case of B(a)P the Passaic River and Hackensack River fish (2004) were significantly higher from the Tuckerton fish on average by an order of magnitude. White Perch B(a)P bile concentrations from Hackensack River in 2005 were also significantly higher than Tuckerton ($p = 0.056$). They were not however significantly different from the Delaware River fish values (Figure 13 and Table 16).

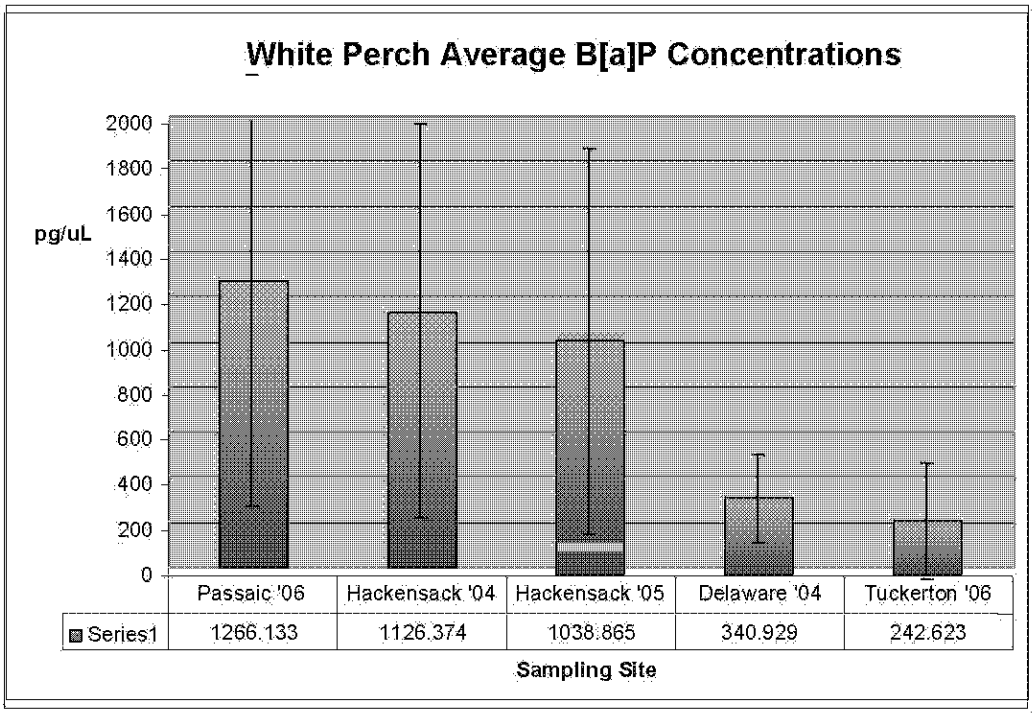


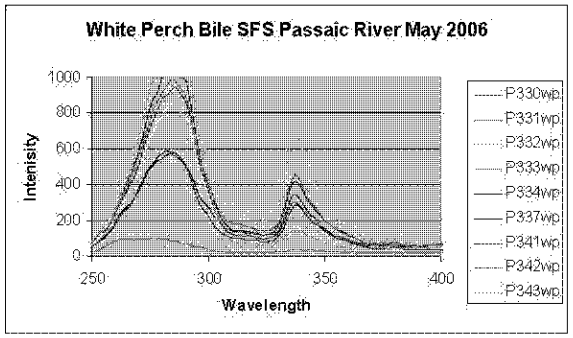
Figure 13. Comparison of White Perch bile fluorescence for B(a)P (mean ± SD).

Table 16. Summary of White Perch B(a)P significant differences following ANOVA[1]

Comparison	P<0.05
Hackensack 04 vs Tuckerton	Yes
Hackensack 05 vs Tuckerton	P=0.056
Passaic vs Tuckerton	Yes

[1]All other comparisons were non-significant

Within Figure 14 are comparative scans from White Perch from the different locations. The levels and extent of pyrene is strikingly different. This is a very useful biomarker to evaluate the level of PAH equivalents that the fish are being exposed to through their water or food sources and is consistent with other researchers (Britvic et al. 1993). Results of all three compounds agree with one another and show that fish in the Passaic River and Hackensack River contain higher loads of PAH contaminants.



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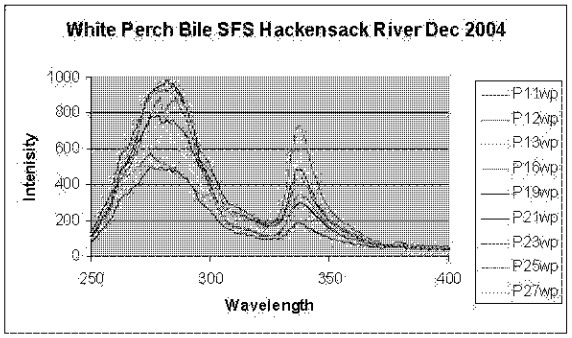

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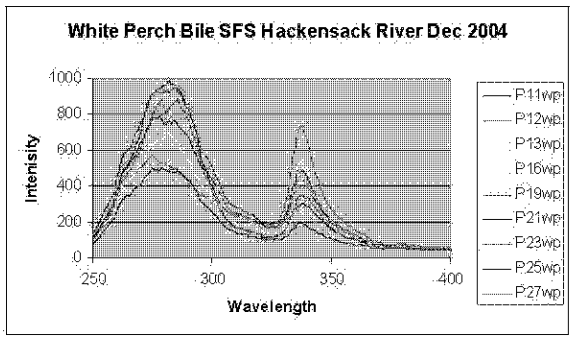

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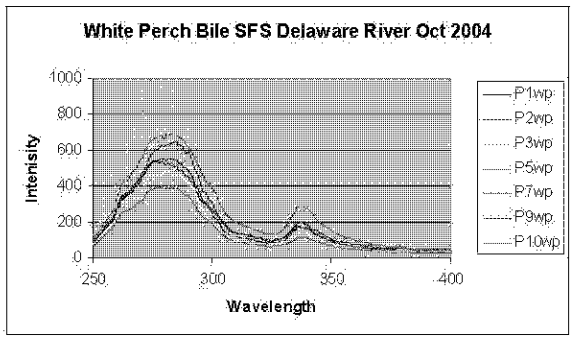

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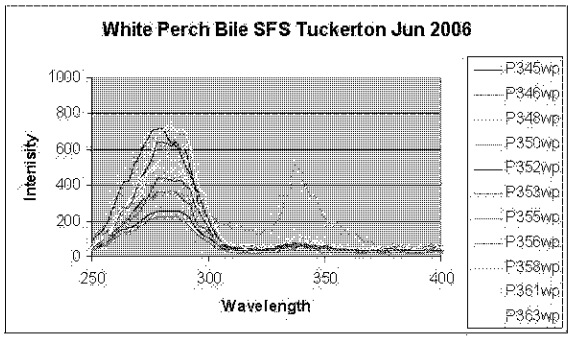

E.

Figure 14. Comparison of different synchronous fluorescence scans from individual White Perch bile samples at different sampling locations.

Histopathology:

Summarized in Table 17 below are the results from the livers examined from White Perch collected from several river systems. At all locations there was evidence of parasites present in both the pancreatic tissue and the hepatic tissue. After reviewing the morphometric data and the occurrence of lesions it became apparent that in the smaller fish parasites were present, but the hepatocytes appeared normal with few if any lesions. In the larger fish there was an increase in altered basophilic or eosinophilic staining and in a number of fish there was neoplasia. There were only a few very large fish but those had parasites but no

neoplastic tissue (Table 17). The alteration in hepatic staining and then progression to neoplasia is consistent with that reported from other fish species (Dawe and Harshbarger 1975, Hawkins et al. 1990, Sinnhuber et al. 1977, Vogelbein et al. 1990, Vogelbein and Fournie 1994). Size related occurrence of hepatic neoplasms have been reported in tomcod inhabiting the Hudson River (Cormier 1989; Dey et al. 1993).

These observations are being made on a relatively small number of fish, and there is a need to expand the number of fish being examined. In addition, I would recommend looking at least 20 fish per year class or size range <100g, between 100 and 200g and > 200g. If possible using year class or based on age would be the ideal approach. It would also be important to see if other organ systems may have neoplasms or other lesions as well.

White perch would appear to be sensitive to the development of hepatic pre- and cancerous lesions. Histopathological evaluation is important in evaluating the health of a fish population inhabiting contaminated and reference waters.

Table 17. Summary of histopathological evaluation of White Perch.

Location	Date	Wt. Range (g)	F/M[1]	NSL[2]	Lesion	Description
Passaic River	April 2006	47.5-489	3/10	12	1 (8%)	Focal area alter staining
	May 2006	83.9-185	1/1	1	1 (50%)	Neoplasm & parasites
Hackensack River	Nov 2004	5.1-167.8	12/5 *	11	7 (39%)	Parasites & focal area altered staining
	April 2005	184-340#	7/8 **	2	10 (83%)	4 neoplasm, 5 focal area altered staining, & parasites
Delaware River	Oct 2004	19.1-27.6	8/2	6	4 (40%)	Parasites
Tuckerton	June 2006	44.7-200	11/8	19	7 (28%)	2 neoplasm, 2 focal area altered staining, & parasites

[1] F/M = female to male ratio
[2] NSL = No significant lesions
* One fish was undifferentiated sexually.
** Two fish were undifferentiated sexually.
# Range only from four fish.

Cytochrome P450 mRNA (CYP 1A1).

Shown in Figure 15 below are the CYP1A1 data from the White perch collected at the different locations. Fish collected at Tuckerton had significantly lower CYP1A1 induction than all other sites. However, there were no other significant differences between sites. As with the Fundulus, CYP1A1 as a dependent variable could be predicted from a linear correlation of the bile concentrations of three bile PAHs.

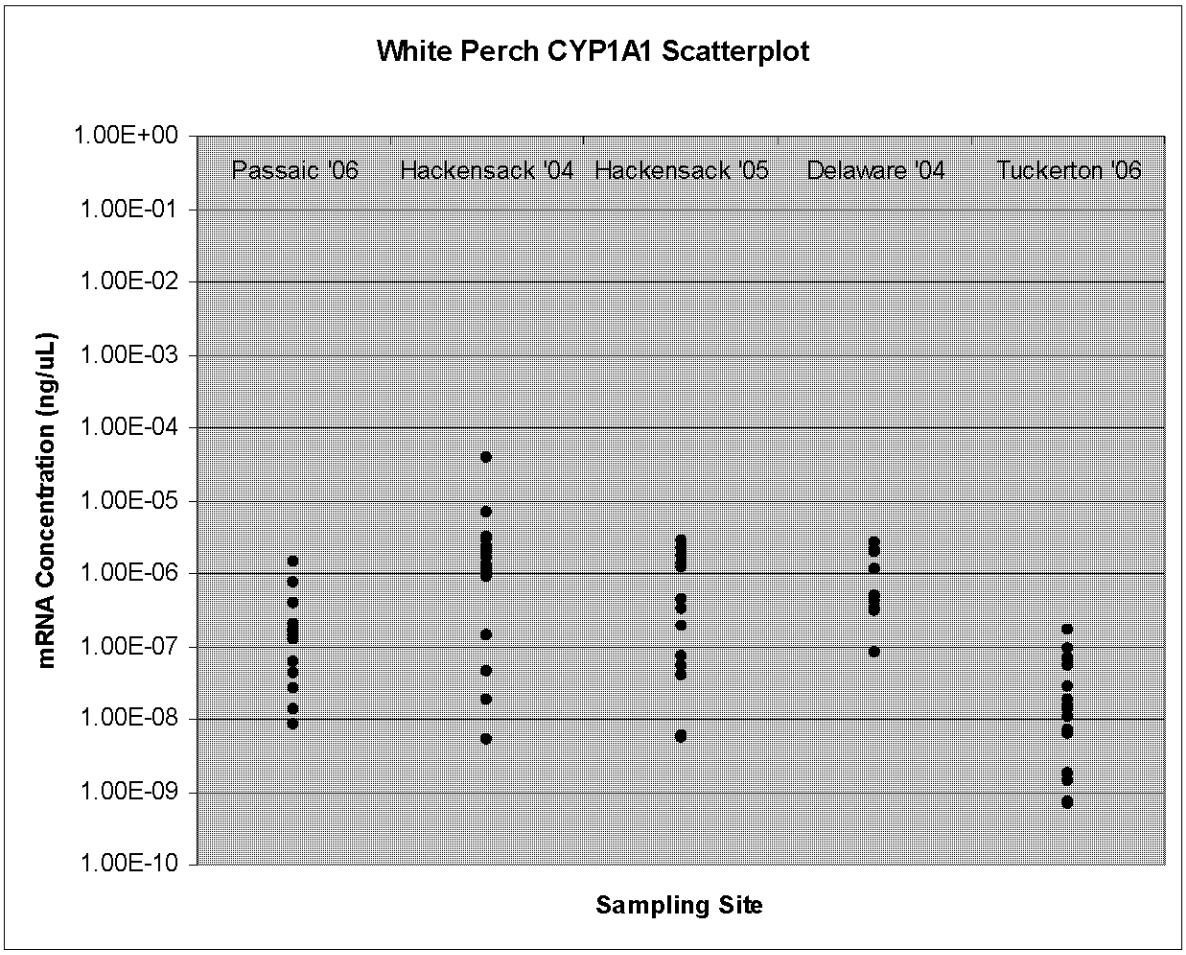

Figure 15. CYP1A1 values determined from white perch collected at various locations. Each point represents the average value for one individual run in triplicates.

Metallothionein (MT) Hepatic mRNA:

Shown in Figure 16 are the scatter plots for liver metallothionein mRNA. From the scatterplot it can be seen that there is a greater variability in the Tuckerton fish which may reflect a greater ability to respond in a broader range. There was not a significant difference between the different groups that could not be attributed to random sampling variability. It

would appear that hepatic mRNA MT is not a very sensitive biomarker endpoint for white perch. This may be due to the fact that the liver is not being exposed to high levels of metals in these groups of fish. A more sensitive organ to metal toxicity is the kidney and this might be more appropriate for that organ in future studies.

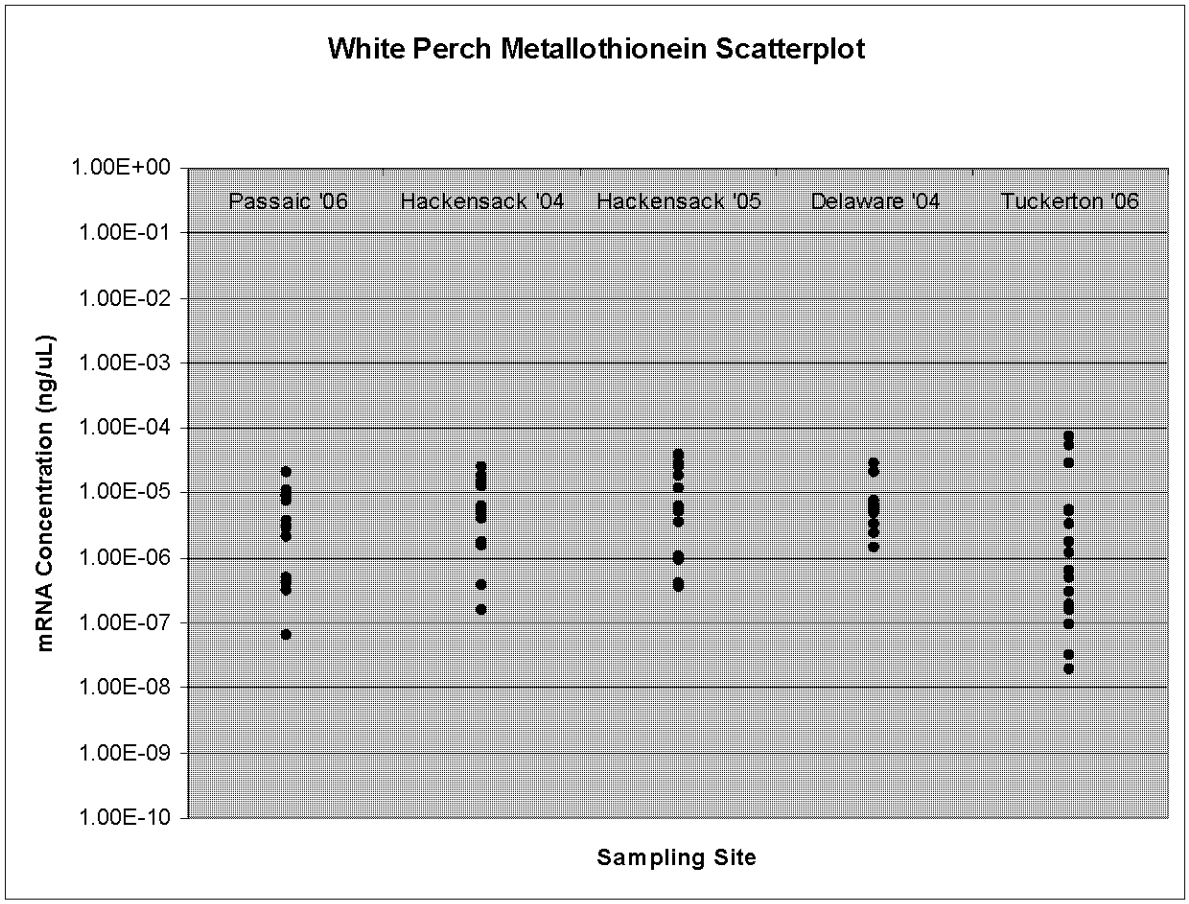

Figure 16. Metallothionein determined from White Perch livers at different locations. Each point represents the average value for one individual run in triplicates.

Vitellogenin (VT) mRNA Levels:

The vitellogenin levels for white perch are shown in Figure 17 below. Care must be exercised because of the small number of samples collected and the size differences that were present in these fish.

In all comparisons between male and female levels there was no statistical difference. This is in contrast to what has been observed in other fish species. The reason could be related to the small size of the fish and the lack of sexual development which would result in both

females and male liver levels of VT as being similar. This does point out that larger fish that are sexually mature should be collected if this biomarker is to be meaningful. It does appear as though male fish collected in Hackensack (2005) may be higher than females, although there was no statistical difference (P = 0.08) and there are too few male fish to have confident statistical analysis. Not having sufficient numbers of both male and female fish is a problem for all of these sites. Even with the small number it would appear that there is not a major difference between the male and female liver VT levels.

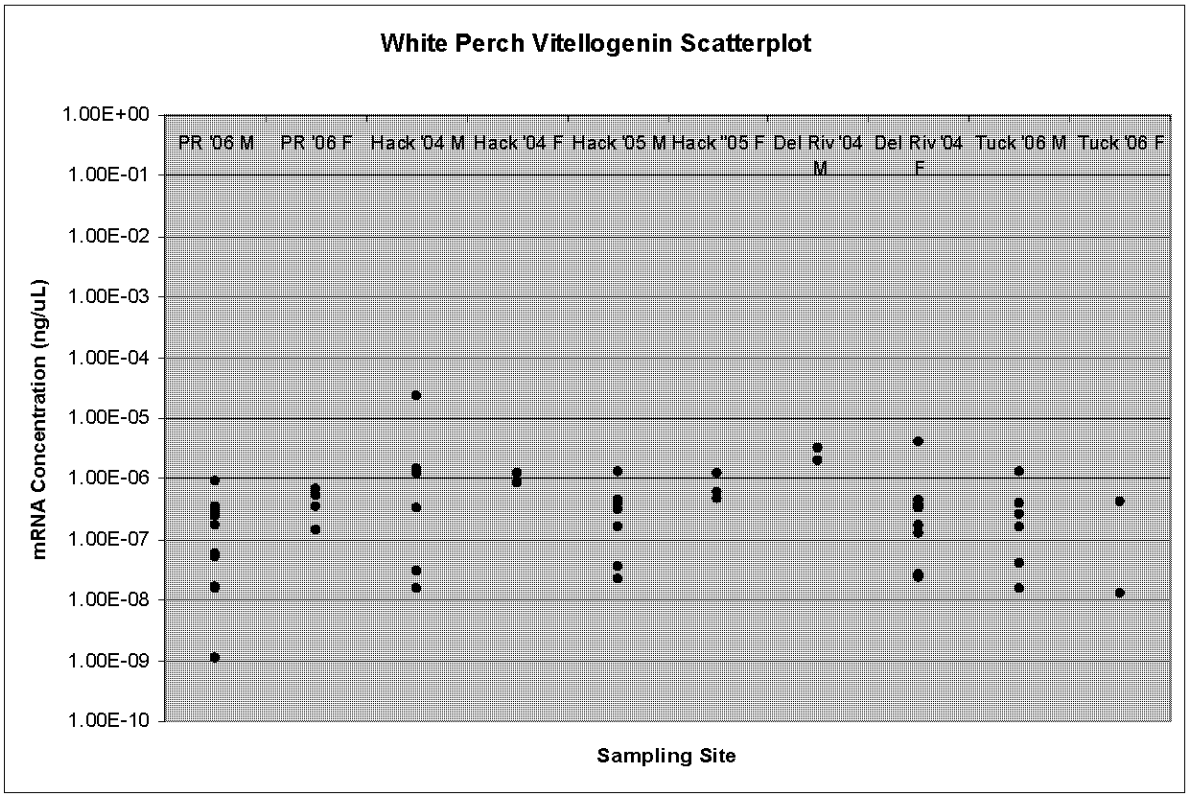

Figure 17. White Perch vitellogenin levels determined from hepatic tissue collected at various locations.

Conclusions and Recommendations for Future Research:

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 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, develop transport systems to eliminate higher chemical contamination, modify feedback loops to not respond to chemical stimulus, 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 (Sternberg 2004). 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. In order to evaluate the health of the fish inhabiting the estuary there is a need to adequately fund the research so that a total picture can be obtained. The under funding and piece meal approach currently being carried out will not meet the short or long term goal of assessing the overall health of the NY/NJ Harbor Estuary. 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. The neoplasms

observed in the White Perch appeared to be in fish of greater than 100 grams and may reflect a similar situation that was observed in Tomcod where specific year classes had high tumor occurrence (Wirgin 2004). The White Perch would appear to be a very good fish to examine throughout New Jersey's estuaries.

Summarized in Table 18 below are the endpoints that were evaluated in the past year and based on the results how useful they were in assessing the animal's health as a biomarker. Based on my familiarity with biomarkers in fish I have included several additional biomarkers that would be considered as potential additions.

Table 18. Summary of biomarker endpoint effectiveness.

Biomarker	Purpose	Endpoint	Useful Biomarker
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	**Yes**
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	**No** Micronuclei **Yes** RBC & WBC
Hematocrit	Determine packed cell volume	• Anemia or altered RBC production	**Yes**
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	**Yes** Liver and spleen. **Yes** Gonad at specific spawning cycles
Histopathology	Evaluate liver cellular structure at light microscopic level.	• Evaluation of normal vs. altered structures	**Yes** Expand to other organs: gill kidney etc.
Biochemical Endpoints	Evaluate if populations inhabiting different locations have different levels of endogenous enzymes.	• Real time PCR for quantification of mRNA enzyme levels for P450 1A1, metallothionein (MT)and hepatic vitellogenin (VT)	**Yes** CYP1A1 & metabolism **No** Hepatic MT **Yes** Kidney MT **Yes** Hepatic VT **Yes** Circulating Hormones **Yes** DNA adducts or protein adducts, or Comet assay
Fluorescent Activity	Fluorescent activity in bile has been correlated with PAH activity	▪ Increased basic fluorescence indicates increased aromatic contamination	**Yes** PAH fluorescence **Yes** Metal & organic compounds

Recommendations and Application and use by NJDEP:

Based on the findings from these preliminary studies the following recommendations are being 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 (CYP 1A1, 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 and potential other organs and initially focusing on White Perch.
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 (adults and embryos).
8. Expand the survey to include other fish species such as eel, bluefish and flounder.
9. Establish set locations to be sampled every 3 to 5 years to evaluate the health of target estuarine fish within the specific watershed.

Because of the importance of fisheries within New Jersey from a commercial, recreational and ecological standpoint, it is critical that there be a better understanding of the health of the fish within our waters and the chemical body burdens they are carrying. It is also critical to have this information because of the higher trophic level organisms (avian species, turtles, aquatic mammals) that rely on these organisms for food. From a human health perspective it is also essential to know the body burdens of the organisms being consumed. This approach will also allow for evaluating if remediation actions being taken are removing chemicals of concern and if the affects on the organism's health are improving. The use of a battery of biomarkers in fish will allow for the aquatic environmental quality to be assessed over time. Because of the large number of chemicals within our estuaries it is impossible to select which ones are to be examined. The use of fish species or biological based endpoints allows for the overall impact of the total mixture on a biological organism to be economically assessed (i.e.,

as compared to expensive analytical testing). The biological systems will also react to new chemicals of concern that may be only now reaching concentrations high enough to affect an organism's health.

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Quality Assurance and Quality Control Document for Biomarker Studies.

Purpose:

The purpose of these studies are to develop a series of biomarkers that can be examined to assess the health of feral fish populations living in various polluted and less polluted sites within New Jersey Waters. This multiyear study will attempt to examine a battery of endpoints (Table 1) to determine which have potential to be used for long-term monitoring programs and which seem to be unaffected by historical pollution exposures. In the first and second year of this study *Fundulus heteroclitus* will be collected from the sites described below. In the second year we expanded collections to include white perch. Initially Tom cod were to be collected but due anticipated difficulty in obtaining Tom cod other species have been proposed.

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 (Steinberg et al., 2004). 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 surrogate measure of an ecosystem's health.

Table 1 Endpoints: The endpoints to be evaluated are listed in the table below.[1]

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

1. Not all endpoints will be evaluated for all fish. Since some of this is protocol development "proof of principle" will be done for several of the biochemical endpoints

Sampling:

Atlantic mummichogs (*Fundulus heteroclitus*) will be collected from 5 estuary sites along the Atlantic coast in New Jersey using baited minnow traps and or beach seines. Additional sites may be added or deleted depending on approval from the program officer. There will be an attempt to collect fish of the second and third year class. Specifically, 20-30 fish or as many fish as can be captured will be collected from each of the following sites: Newark Bay, Piles Creek, Union Beach, Sandy Hook, and Tuckerton. The first 4 sites represent a contamination gradient from high to low while the last site is assumed to be pristine and fish from this site will be considered reference organisms.

The sampling of the other agreed upon fish species (White Perch will likely be collected by trawl net or fish trap. These fish will be transported back to the laboratory in the least stressful manner possible.

Among fish from each site, 30 euthanized (for detail, see below) fish will be processed immediately for blood work and tissue sampling. Appropriate portions of each liver will be used for histological examination, a portion frozen at -80C and RNA extraction. These studies are aimed at examining several commonly used biomarkers to determine how reproducible they are and if there is a seasonal variation. The chosen biomarkers examine different endpoints (see Table 1 above). In any evaluation of biomarkers it is essential that the overall health of the animals be evaluated. This is important because health endpoints can dramatically affect the toxicokinetics and toxicodynamics of contaminates and expression of proteins and directly impact the biomarker expression. Determine if hepatic mRNA expression for potential biomarkers in wild killifish is impacted by other contaminants, such as metals and/or PAHs, by comparing the gene expression in wild fish from contaminated sites, and reference sites. Although not proposed in these studies challenging chronically exposed fish with known contaminants with an ecosystem has resulted in shifts in dosage response curves that can indicate a selection pressure for either resistance or increased sensitivity (Nacci et al., 2000, Prince Hahn et al., 2004 , Meyer et al., 2003, Wirgin and Waldman 2004, Yuan et al. 2006).

Fish husbandry:

Standards of laboratory animals handling at Rutgers will be adhered to (see approved Rutgers IRB appendix). At a minimum the following procedures and conditions will be maintained.

Adult Fish per Tank: 40 L tanks – 30 fish

Feeding: Fish are fed Wardley Goldfish Floating Pellets twice daily.

Water Quality Control: Aquaria are placed in a recirculation system for containment of adult fish. Salt water originating in a heated head tank is first filtered through a biofilter and then passed through a second filter containing gravel and activated carbon. Following filtration, water runs through the individual tanks and is then pumped back up to the biofilter using a centrifugal pump. Water is recycled in this manner for

approximately two weeks, after which system water change in conducted. Specifically, approximately one quarter of the water being recycled is removed and replaced with fresh salt water from the head tank. Fresh water is added as needed to maintain the correct salinity. The following conditions are maintained: (1) Temperature – 18-20°C; (2) pH - ~7; (3) Ammonia – 0 ppm; (4) Nitrite – 0 ppm; (5) Salinity – 20 ppm. These levels are monitored once a month and recorded.

Water Change Rate: Maintained at ~1mL/min in each tank.

Light/Dark Cycle: 12 hours light/12 hours' dark via automatic timers.

Tank Maintenance: Tanks are cleaned twice weekly. Cleaning involves siphoning and disposing of fecal debris. Tanks are scrubbed as needed to remove any algae accumulation.

Visible External and Internal Examination:

The fish will be weighed and measured (total and/or standard length) and then examined for visible lesions of the external skin, eyes (cataracts), and fins. Any parasites present will be noted. The gills will be examined for color (bright red), lamellar tip erosion (pale or irregular edge), parasites or other lesions.

The fish will be bled either from the severed tail or from the cutting of the aorta or by cardiac puncture. The blood sample will be prepared as described below. The fish will then be dissected. An incision will be made from the anal pore to below the gill arches. The peritoneal cavity will be examined for any hemorrhages or abnormal discoloration. The organs in the cavity will be examined for any grossly visible lesions or abnormal appearance. If the gallbladder is present then a sample or the entire gall bladder will be frozen for possible fluorescence analysis. In some specimens the gall bladder is not dilated and is difficult to locate on the liver. The liver, gonads, spleen will be removed and weighed. Peritoneal fat will also be removed along with a small piece of muscle for possible chemical analysis. They will be either used for biochemical evaluation or histological preparations (see below). The rest of the organs will be examined and the carcass will be fixed in 10% buffered formalin if needed for latter analysis.

Blood and Hematocrit Evaluation:

Blood will be collected in heparin zed capillary tubes. The capillary tubes will be sealed and then spun on a Hematocrit centrifuge. Following centrifugation the percentage of packed cell volume will be determined. Any hemolysis will be noted in the plasma layer. This can indicate fragility of the RBCs or improper handling of the blood. In either case it should be noted. From a separate Hematocrit tube several drops of blood will be placed on a clean glass slide, and then streaked and allowed to air dry. Several slides will be made in case one is lost in processing. The slide will then be stained with a Giemsa stain or H&E which are standard stains. These slides will be used to evaluate if there are any micronuclei present in the cytoplasm of the RBCs and also a white cell differential count if adequate cells are present. Multiple fields (four in different areas) will be read for the micronuclei until approximately 400 cells have been examined. Number of cells with micronuclei per 100 cells will be calculated. In addition, the staining characteristics of the RBCs will be noted and any alteration in the membrane crenulations noted.

Histological biomarkers:

Killifish and other species will be maintained as described above until used in toxicological studies. Prior to necropsy, fish will be euthanized in a solution prepared by dissolving 100 – 500 mg of methane sulfonate (MS-222) in 1 L of filtered system water. This euthanization procedure is widely utilized and accepted by the scientific community (Saidel et al., 2001; Cerda-Reverter et al., 2003; Ma, 2003; Webber and Haines, 2003). Tissue obtained from fish euthanized by MS-222 will be used for histological, pathological, and immunohistochemical analysis when appropriate.

Some fish (especially large fish) will not be euthanized using MS222. These fish will be euthanized via ice/cold treatment (Roex et al., 2003). This has been reported to be an alternative method which results in less stress to the fish. Fish will be taken from tanks and placed immediately into a crystallization bowl filled with ice and a small amount of water. Fish metabolism will immediately decrease, as the fish ceases all activity. The spinal cord will then be cut and the necropsy performed. The use of ice as a euthanization method allows for greater enzyme stability, and the maintenance of tissue architecture.

Following euthanization, fish will be bled and necropsied (see attached approved Necropsy Procedure). Tissues that could potentially be collected include brain, heart, liver, intestines, kidneys, spleen, gonads, gills, and skin. Tissues used for histological, pathological, and immunohistochemical evaluation will be fixed in 10% phosphate buffered formalin for at least 24 hours then transferred to 70% ethanol until tissue processing. The tissues will then be transferred into cassettes and placed on an Autotechnicon for dehydration, and paraffin infiltration. The paraffin blocks will be sectioned at 6 microns and stained with H&E. The stained tissue sections will be examined under a light microscope and any lesions recorded. The person examining the slide will not know the specific site being examined. Tissues used for biochemical endpoints will be frozen in liquid nitrogen and stored at –80 °C until analysis.

Molecular biomarkers:

Total RNA Extraction:

Total RNA was extracted as described by Kreamer et al (1991), a modified phenol/chloroform extraction as described by Chomczynski & Sacchi, 1987. Approximately 10mg of each frozen liver tissue was directly homogenized in 200µL Trizol® Reagent in 1.5mL microcentrifuge tubes to which 800µL Trizol® was added and left to sit at room temperature for 5min. To each tube, 200µL of chloroform was added, vortexed, and incubated at room temperature for 3min. Homogenates were centrifuged at 14,000x g for 15min at 4°C. The aqueous phase was then transferred to new 1.5mL microcentrifuge tubes (RNase, DNase, pyrogen and DNA free) and

mixed with 100µL of cold 70% ethanol. The precipitating RNA was centrifuged at 14,000x g for 10min at 4°C. The supernatant was discarded and RNA pellets were left to air dry before being resuspended in 60µL of Diethyl pyrocarbonate treated water. Concentrations of each total RNA sample was determined on a UV spectrophotometer (Beckman, Germany) and stored at -80°C.

Representative RNA samples were analyzed with gel electrophoresis to verify mRNA integrity. Integrity was determined by loading 6µg of total RNA on a 1% denaturing agarose gel cast in 1X MOPS containing 1% (v/v) formaldehyde to be electrophoresed and stained in ethidium bromide. The gel was photographed under UV illumination to assess quality of the 28S and 18S ribosomal RNA bands.

RNA analysis using quantitative RT-PCR (Real Time-Polymerase Chain Reaction):

A portion of each RNA sample (10µg) was treated with DNA-free (Ambion, Austin, TX) to remove residual genomic DNA. Reverse transcription was then performed on 1µg DNase treated RNA to create cDNA template necessary for RT-PCR using iScript™ cDNA Synthesis Kit (BIO RAD, Hercules, CA).

Messenger RNA targeted for quantification with RT-PCR included *B-actin*, Cytochrome P4501A1 (*cyp1a1*), Metallothionein (*mt*), and Vitellogenin (*vit*). *B-actin* and *CYP1a1* primers were designed using gene sequences found in PubMed for killifish (Locus AY735154), which were also homologous to white perch. Metallothionein primers were designed for killifish using winter flounder sequences (*Pseudopleuronectes americanus*, Locus T23119). Killifish vitellogenin primers were obtained from Meyer *et al* (2005). *Mt* and *cyp1a1* primers for white perch were designed from sequence information found on PubMed for striped bass (*Morone saxatilis*, Locus AF091100) and european seabass (*Dicentrarchus labrax*, Locus AJ251913), respectively. Vitellogenin primers were obtained from Hiramatsu *et al* (2004). Forward and reverse primers used for each species and mRNA target are:

Killifish *B-actin* forward: 5'-GCT CTG TGC AGA ACA ACC ACA CAT-3'
Killifish *B-actin* reverse: 5'-TAA CGC CTC CTT CAT CGT TCC AGT-3'
Killifish *cyp1a1* forward: 5'-TGT TGC CAA TGT GAT CTG TG-3'
Killifish *cyp1a1* reverse: 5'-CGG ATG TTG TCC TTG TCA AA-3'
Killifish *MT* forward: 5'-ATG GAT CCC TGC GAT TGC-3'
Killifish *MT* reverse: 5'-GCA CAC GCA GCC AGA GG-3'
Killifish *VT* forward: 5'-AGG ATT CGT CCG AAC AAC AC-3'
Killifish *VT* reverse: 5'-TTT CAG ACG GCA CTC AGA TG-3'

White Perch *B-actin* forward: (same as killifish sequence)
White Perch *B-actin* reverse: (same as killifish sequence)
White Perch *cyp1a1* forward: 5'-TGC AAT GAG GGA TAG TGA AGG GCA-3'
White Perch *cyp1a1* reverse: 5'-GTT CAT GGA CAT CAA TGC CCG CTT-3'
White Perch *MT* forward: 5'-GTA CCC AGG TGG TTG TAA TTG GCT-3'
White Perch *MT* reverse: 5'-TGG TTA AGG GTG TGT AGC CTG GAA-3'
White Perch *VT* forward: 5'-GCT CCG ACG AGA GCG TAG AA-3'
White Perch *VT* reverse: 5'-CCG CTC CGC AGC TAA ACT C-3'

PCR products for each fish species and primer pair were confirmed by verifying the correct anticipated amplicon sizes with electrophoresis on a 1.4% agarose gel made in 0.5X tris-borate EDTA. Melt curves were also used to show there were no secondary products for all genes in all samples. *B-actin* products were expected to be 136bp for both killifish and white perch. *Cyp1a1* products were expected to be 258bp for killifish and 419bp for white perch. Metallothionein products were expected to be 147bp for killifish and 185bp for white perch. Vitellogenin products were expected to be 416bp for killifish and 69bp for white perch.

RT-PCR was carried out using 100ng total cDNA template using iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA) for amplification and Bio-Rad iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA) for fluorescent detection. Each gene was run in triplicates to use average threshold values for quantification.

RNA Quantification:

Standard curves were generated by using serial dilutions (0.1ng template-0.00001ng template) of each gene. Threshold values (C_t) were used for quantification. For each group of samples amplified B-actin was included for each sample to standardize all samples to the lowest B-actin C_t of the group. This method normalizes all genes to *B-actin*, a reference housekeeping gene by using the formula below where NE is normalized gene expression, E is the efficiency of amplification for a particular gene, C_t is the threshold cycle, ref is the housekeeping gene, and the target is the gene of interest (Muller *et al*, 2002). Normalized Ct values were used to quantify each template and were expressed as nanograms of mRNA per microliter of template.

Threshold concentrations for *VT* were set at detection limits of $1.5x10^{-7}$ ng/μL in fundulus and $1.5x10^{-8}$ng/μL in white perch. Values below this were scored as these detection limits for data analyses.

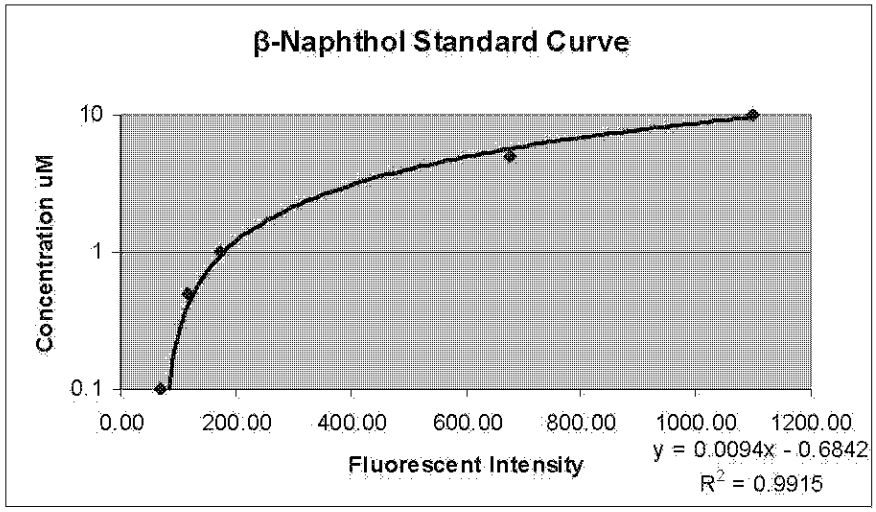
$$NE = \frac{\{E_{ref}\}^{C_t\text{-ref}}}{\{E_{target}\}^{C_t\text{ target}}}$$

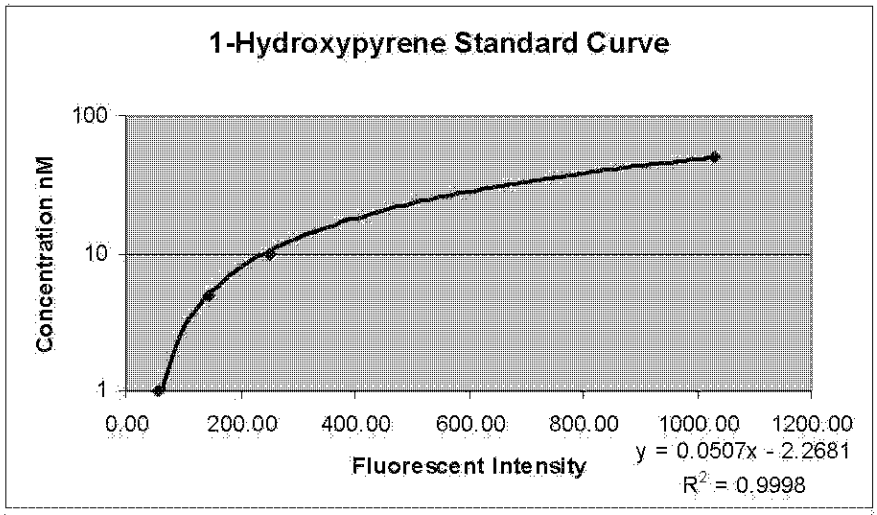
b

Bile Fluorescence:

The gall bladder was removed and placed into a 1.5mL centrifuge frozen at -80°C. Sample preparation and Synchronous Fluorescence Spectrophotomety (SFS) methods used were modified from Eickhoff *et al* (2002). Upon removal from the freezer prior to fluorescence the bile was diluted 1:1500 in 50% spectral grade ethanol and mixed thoroughly. A 3mL sample was then placed in a Cary Eclipse Fluorescent Spectrophotometer and the total fluorescence was measured using SFS with an excitation/emission wavelength offset of 44nm, an excitation/emission slit width of 5nm/5nm and maximum detector voltage (800v). The spectrum between 250nm-450nm was obtained for quantification of peaks for naphthalene (290nm), pyrene (346nm) and benzo(a)pyrene (380nm). Intensities of these peaks were used to semi-quantify concentrations of these compounds.

Standard curves of β-naphthol (Sigma-Aldrich, St. Louis, MO), 1-hydroxypyrene (Sigma-Aldrich, Milwaukee, WI) and 3-hydroxybenzo(a)pyrene (Midwest Research Institute, Kansas City, MO) were generated using detectable concentration ranges of 0.1-10μM, 1-50nM and 1-100nM respectively. Concentration estimates were converted and recorded as grams of standard equivalent per microliter of bile. Threshold values were set slightly above the lowest concentration of the standard curves, 5ng/μL for naphthalene, 0.5ng/μL for pyrene and 50pg/μL for benzo(a)pyrene. Any sample below these set detection limits were scored as threshold values for all analyses. An arbitrary sample was also chosen to be scanned daily as an internal standard to ensure spectrophotometer variation from day to day was not an issue.





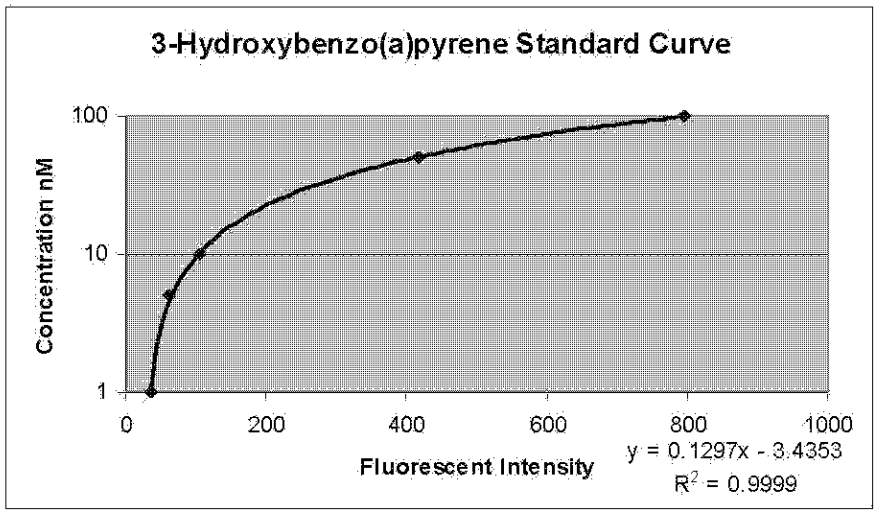


Figure 1. Standard curves for β-naphthol, 1-hydroxypyrene and 3-hydroxybenzo(a)pyrene.

Statistics:

All statistical tests will be performed with SigmaStat. When three different treatments are compared, ANOVA will be performed followed by the appropriate multiple comparison test as the post-hoc statistical test. When comparing two different treatments, unpaired *t*-tests will be performed. A *P*-value of ≤ 0.05 is regarded as significantly different from control. For data which is appropriate a one way or two-way ANOVA will be carried out to examine significance with such variables as time. Discrete data such as that obtained from histopathology will be run using a Chi Square analysis.

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