THE DEVELOPMENT OF A NEW APPROACH TO EVALUATE ENVIRONMENTALLY INDUCED GENETIC DAMAGE IN HUDSON RIVER BIOTA

A Final Report of the Tibor T. Polgar Fellowship Program

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ABSTRACT

The Hudson River has one of the highest levels of polycyclic aromatic hydrocarbon (PAHs) contamination levels of any estuary in the United States (7,100-34,000 ng PAHs/g dry weight). These PAHs are believed to cause damage to bottom dwelling organisms. The Atlantic tomcod, *Microgadus tomcod*, was used as a sentinel species of environmental quality to test the extent of DNA damage caused by PAHs. DNA damage was quantified in juvenile, one-year-old, and two-year-old age classes of Hudson River tomcod using a new extra-long PCR-based technique. DNA from all three age groups of Hudson River fish was amplified and compared. It was hypothesized that the older age classes of fish would have increased levels of DNA damage due to longer exposure to Hudson River sediments. Results suggest that there was a lower yield of amplification product in one-year-old fish compared to juvenile tomcod, possibly indicating a bioaccumulation of DNA damage over time. Two-year-old Hudson fish had higher levels of amplification product than expected, greater than the one-year-olds, and only slightly less than the juveniles. DNA from two-year-old tomcod from the Margaree, a pristine Canadian river, was also amplified and compared to the Hudson two-year-olds. The Margaree samples appeared to have higher yields of extra-long-PCR product compared to the Hudson samples. These results offer promise for further use of this PCR-based technique to quantitatively and reproducibly assess overall DNA damage in Hudson River biota.

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INTRODUCTION

The Hudson River estuary is polluted with a variety of both halogenated aromatic hydrocarbons (e.g., PCBs and dioxins) and polycyclic aromatic hydrocarbons (PAHs) (O'Connor et al. 1982). The organic contaminants found in estuaries generally have low water solubility and thus accumulate in the sediment. Hudson River sediments have one of the highest levels of PAHs (7,100-34,000 ng PAHs/g dry weight) of any estuary in the United States (NOAA Technical Memorandum 1987). PAHs are formed by the incomplete combustion of fossil fuels and the action of internal combustion engines. They enter the water systems through runoff from roadways, atmospheric fallout and precipitation, as well as through petroleum spillage and seepage (Neff 1985; Baek et al. 1991).

Once in the cell, PAHs are metabolized to highly mutagenic and carcinogenic forms. Previous studies have shown that metabolites of benzo[a]pyrene (a PAH commonly found in Hudson River sediments) rapidly and preferentially accumulate in mitochondria (Ballinger et al. 1996; Backer and Wernstein, 1980; Allen and Coombs, 1980). Once in the mitochondria, PAH metabolites adduct with DNA and cause mutations. DNA adducts interfere with the organelle's ability to make critical enzymes needed for cellular metabolism.

An organism which has considerable contact with the PAH-bound sediment is the Atlantic tomcod (*Microgadus tomcod*). The tomcod is an anadromous, bottom dwelling fish species of the northeast coast of North America whose distribution extends from Labrador to Virginia (Bigelow and Schroeder 1953). The Hudson River supports the tomcod's southern most spawning location. Therefore, the fish may be thermally stressed

during the warmer summer months (Dew and Hecht 1976; Grabe 1978). Tomcod also have extremely high hepatic lipid levels. Elevated hepatic lipid levels increase the bioaccumulation of lipophilic organic contaminants in the Hudson River tomcod's liver (Wirgin et al. 1994). Tomcod are confined to estuaries and undergo annual winter spawning migrations. Since Atlantic tomcod are benthic, they are exposed to sediment-bound lipophilic environmental agents throughout the lower Hudson River estuary by direct dermal contact and their benthic diet.

The age structure of Hudson River tomcod is different from Canadian tomcod. The Hudson River tomcod population is truncated, with few two-year-old fish and hardly any older fish. Canadian fish, on the other hand, attain an age of 3-7 years. The Hudson tomcod population also has a history of a high prevalence of hepatocellular carcinomas. Previous studies have indicated that the prevalence for carcinomas exceeds 50% in one-year-old tomcod and is as high as 90% in two-year-old fish (Dey et al. 1993). A large percentage of Hudson River tomcod also exhibit a high frequency of DNA mutations, perhaps promoting formation of liver tumors. An elevated level of hepatic DNA adducts, caused by PAHs, was also found in the Hudson population (Wirgin et al. 1994). Therefore, they are an excellent sentinel species of environmental quality (Klauda et al.1988).

For all these reasons, it is expected that Hudson tomcod populations should have a greater extent of DNA damage compared with Canadian populations, which are not subjected to large quantities of sediment-bound lipophilic environmental agents. Therefore, the Atlantic tomcod is an excellent species to test a new PCR-based approach to quantitatively and reproducibly assess overall DNA damage in animal cells. The extra

long PCR (XL-PCR) technique can amplify a 16 Kb fragment of DNA, in contrast to regular PCR techniques which amplify 0.1 to 5 Kb fragments. The technique is based on the assumption that the DNA polymerase used in the PCR will stop when it encounters damaged DNA, such as a DNA adduct caused by PAHs. Therefore, increased DNA damage will result in decreased yields of PCR products. The longer the length of DNA amplified, the more likely PAH-induced damage to DNA will be encountered, and the more likely the yield in PCR products between damaged and undamaged DNA will differ.

This PCR-based approach was used in a study which evaluated the levels of mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) damage in lung cells from smokers and non-smokers (Ballinger et al. 1996). Ballinger's results did find that smokers exhibited approximately six-fold higher levels of damage than did non-smokers. This is an extremely high induction for a human biomarker study and exemplifies the potential sensitivity of this approach to assessing DNA damage. In addition, this study found that mtDNA was far more susceptible to damage than nDNA. This is possibly due to the less efficient DNA repair process of mtDNA compared to nDNA. Or it could be due to the fact that PAHs, which are also found in smoke, accumulate preferentially in mitochondria (Ballinger et al. 1996; Backer and Wernstein, 1980; Allen and Coombs, 1980).

For several reasons, we hypothesize that this technique should find an even greater level of DNA damage in Hudson River tomcod compared with human smokers. First, lung alveolar macrophages are replaced approximately every 30 days, whereas liver cells of a tomcod are not replaced, and thus, should show accumulated DNA damage over

time. Secondly, the levels of PAHs bound in the sediment which tomcod are continually exposed to are much greater than that found in smoke. Therefore, Hudson River tomcod should exhibit a much greater level of DNA damage than the human smokers did.

METHODS

Samples Collected

A total of 41 specimens of Atlantic tomcod were collected from three different river systems, 18 from the Hudson, 12 from the Miramichi, 6 from the St. Lawrence, and 5 from the Margaree (Figure 1). The Hudson River tomcod were composed of three age groups: six juveniles (< 6 months), six one-year-old fish, and six two-year-old fish. The twelve tomcod from the Miramichi were composed of six two-year-olds and six one-year-old fish. All of the St. Lawrence and Margaree fish were two years of age. The fish's ages were estimated by their total lengths.

Tomcod DNA was extracted from frozen liver tissue using the ammonium hydroxide triton X-100 protocol and standard phenol chloroform extractions (Downs and Wilfinger 1983). The DNA was then precipitated in ethanol and high molecular weight DNA was spooled on a glass pipette and transferred to another tube. The DNA was then resuspended with TE Buffer (Sambrook et al. 1989). All samples produced high molecular weight DNA with the exception of the St. Lawrence River fish. These liver samples were freezer burned. As a substitute, DNA previously extracted from the St. Lawrence fish was used.

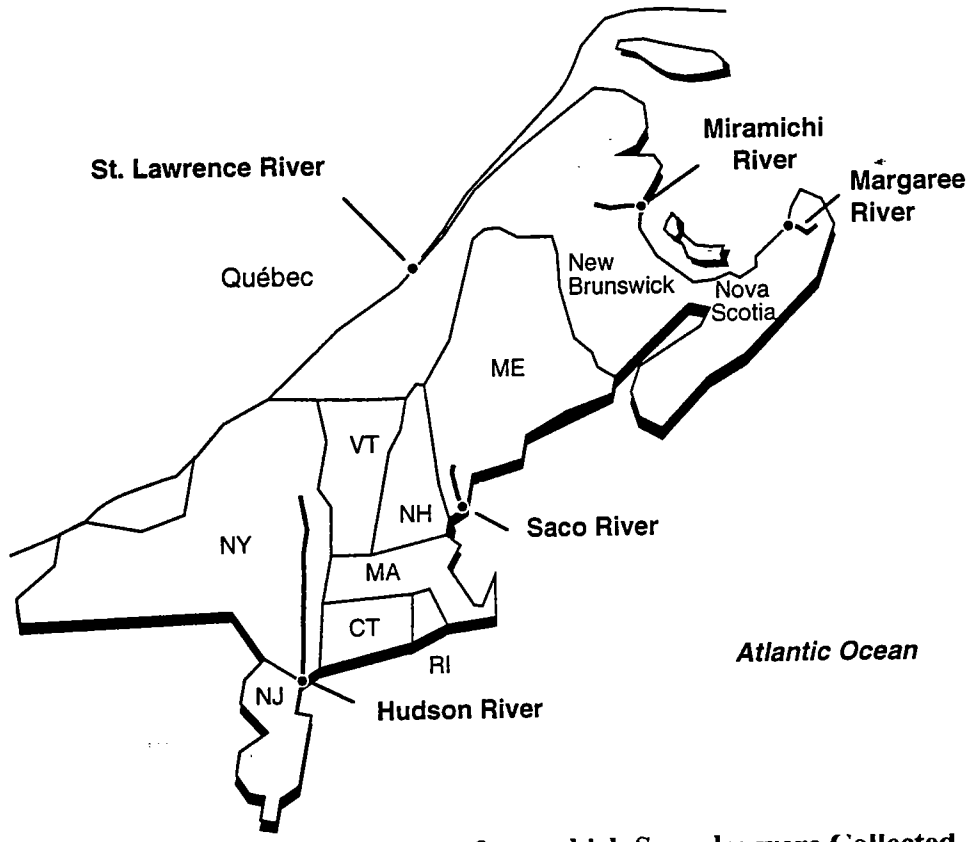

Figure 1: Map of Rivers from which Samples were Collected

Development of Atlantic tomcod-specific PCR primers

Originally, Atlantic tomcod-specific PCR primers were designed from published DNA sequence data of the Atlantic tomcod control region (Woo-Jai Lee et al. 1995). Two regular PCR primers, TCCRD and TCCRA, and two XL-PCR primers, TCCR 84-60 and TCCR 641-664, were ordered from Gibco-BRL. The position of the primers is shown in Figure 2.

No product was initially obtained with the original XL-PCR primers. An additional set of XL-PCR primers was developed by sequencing the mtDNA control region and the 12S ribosomal DNA (rDNA) gene of an Atlantic tomcod from the Hudson River. The PCR product was generated with TCCA and a universal 12S rDNA primer

and run on a low melting point agarose gel. The band of amplified DNA was cut out and both ends were directly sequenced using TCCA and the 12S rDNA primers (Kretz and O'Brien 1993). From the sequence data obtained, two new XL-PCR primers were ordered, TC12SXL and TCCYTBXLNEW. (Table 1)

Table 1: Primer Sequences

Primer Name	Primer Sequence	Source
TCCRD	5' GTCCATCCTAATATCTTCAGTA 3'	1
TCCRA	5' TCCACCTCTAACTCCCAAAGC 3'	1
TCCR 84-60	5' ATATCTAGGACATCTGTACATGGTA 3'	1
TCCR 641-664	5' TACTCCTCCTCGATGAGTTCCTAA 3'	1
TC12SXL	5' CCCTAAGACCTCTGATTCCACGAAAGCCAT 3'	2
TCCYTBXLNEW	5' TACGTCACGGCAGATGTGTACGACAGACGA 3'	2

1. Woo Jai et al.
2. Zimmermann et al. in prep

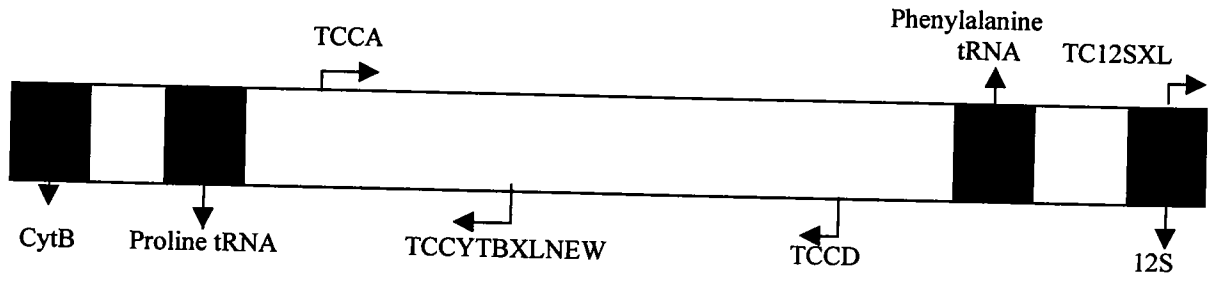

Figure 2: Map of Atlantic Tomcod mtDNA Control Region.

Normalization of mtDNA copy number from all tomcod DNA samples

The DNA concentrations of all samples were normalized prior to performing the PCR reactions. For each sample, DNA concentration was read using a spectrophotometer at 260 nm. This reading gave a general idea of the total DNA concentration, which could

include nDNA, mtDNA, and high and low molecular weight DNA. Next, three micrograms of total DNA from each sample was run on low percent gel (0.7%) to verify the concentration of high molecular weight DNA. This gel was then Southern blotted to a nylon membrane and hybridized to a ^{32}P-labeled mtDNA probe as previously described (Wahl et al. 1979). The membrane was then washed and exposed to x-ray film.

A slot blot analysis was then performed on the DNA samples to quantify the total amount of mtDNA in each sample. Six micrograms of DNA were filtered on to a nylon membrane using the manufacturer's protocol (Schleicher & Schuell). The membrane was hybridized to a mtDNA probe and exposed to x-ray film. The integrated optical densities of the visualized fragments were quantified using a Millipore-Bioimage densitometer and the DNA concentrations of each sample were adjusted accordingly. The slot blot procedure was repeated two more times as described in order to confirm that the mtDNA concentrations of all the samples were equivalent.

DNA sample quality test for PCR (Q-PCR)

QPCR primers TCCRA and TCCRD were used to amplify an approximate 1,000 base pair fragment of the mtDNA control region. Amplification occurred in 25 µl volumes containing 2.5 µl of 10x buffer (Gibco), 0.5 µl of each dNTP, 1.0 µl of each QPCR primer, 0.3 µl of Taq polymerase (Gibco), 100 ng of template DNA, and ddH_2O to volume. Amplification parameters were as follows: 94 C for 5 min, 35 cycles of denaturing at 94 C for 1 min, annealing at 58 C for 1 min, and extension at 72 C for 1 min, followed by a final 7-min extension at 72 C.

After amplification, the products were electrophoresed in an ethidium bromide stained agarose gel (0.8%) and the PCR fragments were visualized on an UV transilluminator.

Long Template PCR – XLPCR and quantification of the products

XL-PCR primers, TC12SXL and TCCYTBXLNEW, were used to amplify an approximate 15 kbp fragment of the mtDNA genome in tomcod. Amplification occurred in 50 µl volumes containing 15.0 µl Buffer (Perkin Elmer), 4.0 µl dNTPs, 1.0 µl of each XL-PCR primer, 1.0 µl of enzyme (Perkin Elmer), 2.2 µl of Mg, 100 ng of template DNA, and ddH_2O to volume. Amplification parameters were as follows: 94 C for 30 sec, hot start at 80 C for 4 min, 94 C for 1 min, 26 cycles of denaturing at 94 C for 20 sec, and annealing and elongation at 70 C for 12 min, followed by a final 10-min extension for 10 minutes.

The amplification products were electrophoresed in an agarose gel (0.7%) stained with ethidium bromide and visualized on an UV transilluminator. The intensity of the stained bands was quantified using the Millipore Bioimage densitometer.

RESULTS

Determination of High Molecular Weight DNA

Southern blot analysis of the gel confirmed the presence of high molecular weight mtDNA in all samples. The DNA hybridized to a tomcod mtDNA probe, confirming the DNA's mitochondrial origin. Extracted DNA from all tomcod samples ran above the 23.1 Kb molecular weight marker indicating its quality. (Figure 3)

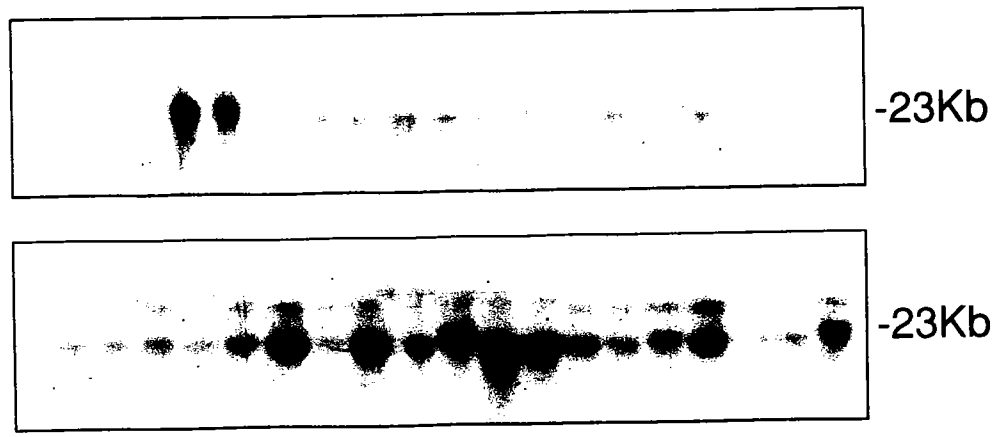

Figure 3: Southern blot of high molecular weight mtDNA : Ethidium bromide stained agarose gel (0.7%) southern blotted to a mtDNA probe confirming the high molecular weight of the DNA (>30 Kb).

Normalizing DNA Concentrations

The high molecular weight DNA was filtered on to the slot blot membrane and hybridized to the tomcod mtDNA probe. The integrated optical densities (IOD) of the visualized fragments were quantified using the Millipore-Bioimage densitometer. The DNA concentrations were adjusted and this procedure was repeated two more times in an effort to normalize all DNA samples. After the third slot blot, DNA concentrations appeared equivalent, but IODs ranged from 2.854 to 1.650. The two-year-old Hudson fish tended to have higher concentrations of mtDNA compared to the juveniles and one-year-olds. (Figure 4)

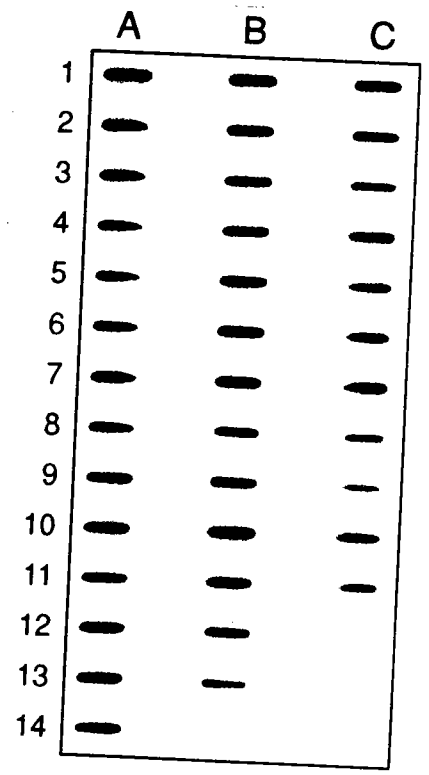

Figure 4: Third Slot Blot Analysis: Slot blot of dilutions used in the PCR reactions. The Hudson River juveniles samples included B10-12&C1,C3,C4; Hudson River 1-yr.-olds samples included A1,A4,A5,A8,A11,B9; Hudson River 2-yr.-olds samples included A2,A3,A6,A7,A12,A13; Miramichi River fish were A9,A10,A14, B1-8,B13; and St. Lawrence River fish were C2, C5-10

QPCR

All samples were amplified using PCR according to the QPCR analysis. The visualized fragments also appeared to be equivalent in size, therefore leading us to believe the mtDNA concentrations in each sample were close to equivalent. (Figure 5)

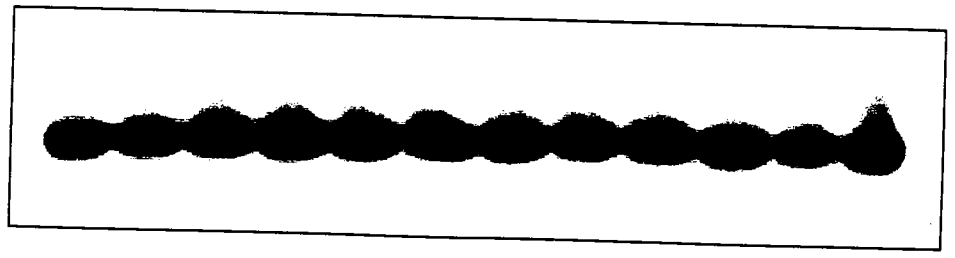

Figure 5: QPCR Ethidium Bromide Stained Gel: One of three QPCR gels illustrating the visualized 1000 bp products.

XL-PCR

Due to time constraints and initial difficulty in getting the XL-PCR technique to work, only Hudson River samples were amplified and quantified. A difference in the yield of XL-PCR product was observed among the three age groups of Hudson River tomcod. (Figure 6). The average IOD for juveniles was greater than that of the one-year-old samples. The two-year-old samples had a greater yield than expected. Their average IOD was greater than the one-year-old fish and equivalent to the juvenile group. (Table 2).

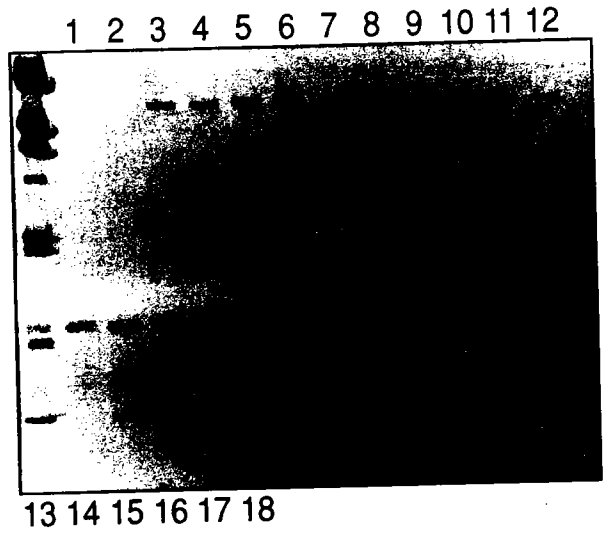

Figure 6: XL-PCR Ethidium Bromide Stained Gel: Ethidium bromide stained agarose gel (0.7%) containing XL-PCR products of three different age classes of Hudson River tomcod. Numbers 1-6 are juvenile Hudson fish, 7-12 are one-year-old Hudson fish, and 13-18 are two-year-old Hudson fish.

Table 2: Quantification of XL-PCR Products (IOD readings)

Juvenile Hudson Fish	One-Year-Old Hudson Fish	Two-Year–Old Hudson Fish
0.186 (3)	0.118 (7)	0.219 (13)
0.199 (4)	0.113 (8)	0.196 (15)
0.225 (5)	0.111 (9)	0.127 (16)
0.138 (6)	0.166 (10)	0.181 (18)
	0.138 (11)	
	0.111 (12)	
Avg.=0.187	**Avg.= 0.126**	**Avg.=0.181**

DISCUSSION

Results suggest that the XL-PCR technique shows promise in assessing DNA damage in animal cells. Amplification products were obtained for all tomcod DNA samples using the Q-PCR technique, indicating that there were no contaminants in any tomcod DNA sample that would inhibit amplification. In addition, all tomcod DNA samples, regardless of their geographic origin, had equivalent yields of the Q-PCR product. Thus, the results of the X-LPCR should indicate the extent of DNA damage present in the original sample.

Differences in the yield of XL-PCR products were observed among the three age classes of Hudson River tomcod. As expected, the average IOD of the juvenile age class was greater than that of the one-year old group. This general trend of less yield in the older fish was hypothesized to be the result of a lengthy exposure time to PAHs in Hudson River sediments. However, two-year-old Hudson River tomcod had high yields of XL-PCR products. There are a number of possible factors why two-year old Hudson River fish did not exhibit the level of DNA damage hypothesized. First, there were only a small number of two-year old fish examined in this study. It is possible that only

tomcod which avoid or are somehow adapted to high levels of PAHs are able to survive to the age of two. Secondly, there is a great deal of inter-individual variability in how Hudson River tomcod react to organic pollutants. This is evidenced in Hudson River tomcod's inducibility of the cytochrome P450 gene (CYP1A1). Upon exposure to an organic pollutant, the CYP1A1 gene is expressed. The enzyme encoded by this gene is responsible for detoxifying the pollutant. Previous studies have shown that individual Hudson River tomcod have different levels of expression of this gene, probably reflecting genetic variability in the population (Courtenay, et. al. 1994) Therefore, it is possible that the small number of two-year-olds that survive may be better able to cope with high levels of Hudson River PAHs. Finally, the average concentration of initial template DNA used in the XL-PCR reaction was greater in two-year-old fish than the other age groups. This would lead to an overestimate of the final XL-PCR product, because of the greater number of mtDNA copies in two-year-old samples.

Technical difficulty in getting the XL-PCR technique to work used up a great deal of the research time allotted for this study. There was considerable difficulty in getting the technique to work consistently. To improve the study, a better normalization of the DNA concentrations is necessary. The initial template DNA concentrations were not close enough to allow for an accurate interpretation of the results. Further, the sample size must also be increased for both the Hudson and Margaree Rivers.

DNA from four Margaree River fish was also amplified and compared to the Hudson River samples. The Margaree samples did appear to have higher yields of XL-PCR products but were not formally added to this study because the concentration of their initial template DNA was not normalized to that of the Hudson River fish. The