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Evaluation of Quantitative-Extra Long PCR to Assess Levels of DNA Damage in an Estuarine Fish Population

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Abstract—Metabolites of PAHs cause DNA damage by the generation of bulky DNA adducts and persistent halogenated aromatic hydrocarbons generate reactive oxygen species which may also modify the structure of DNA. We evaluated the applicability of Quantitative-Extra Long PCR (Q-XLPCR) to monitor and compare DNA damage in mitochondrial DNA (mtDNA) and at the aryl hydrocarbon receptor 2 (AHR2) gene in nuclear DNA in Atlantic tomcod *Microgadus tomcod*, an estuarine indicator species along the Atlantic coast of North America. Initially, mtDNA and AHR2 sequences were obtained to develop tomcod-specific primers and amplification parameters were optimized to allow for reproducible Q-XLPCR amplification of genomic DNA. We found no significant differences in yield of hepatic Q-XLPCR mtDNA or nDNA products between chemically (B[a]P, PCB77, TCDD) and vehicle treated juvenile tomcod, between environmentally exposed adult tomcod from the highly polluted Hudson River and the cleaner Miramichi River, or among three size classes of environmentally exposed tomcod from the Hudson River. We also found no significant differences in Q-XLPCR mtDNA yield between chemically (B[a]P, PCB77) and vehicle treated tomcod larvae. Our results indicate that Q-XLPCR may not be a sensitive approach to quantify DNA damage in fishes from chemically contaminated waterways.

Keywords: DNA damage, mitochondrial DNA, Atlantic tomcod, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, polychlorinated dibenzo-*p*-dioxins

INTRODUCTION

Aquatic ecosystems are often contaminated with aromatic hydrocarbons that are potentially genotoxic. For example, sediments in the Hudson River (HR) estuary, New York, contain some of the highest levels of PCDD/Fs, PCBs and PAHs worldwide (Wirgin and Waldman, 1998). The bioavailability of these contaminants is evidenced by the elevated hepatic levels of these xenobiotics (Fernandez *et al.*, 2004) and their metabolites (Wirgin *et al.*, 1994) in resident benthic fishes including Atlantic tomcod (*Microgadus tomcod*). Exposure of HR tomcod to these contaminants probably has had adverse consequences at the organismic, population, and community levels. In the early 1980s, tomcod from the HR

exhibited one of the highest prevalences of hepatic neoplasms ever observed and concurrently the population exhibited a truncated age class structure (Dey *et al*., 1993). Further studies demonstrated that tomcod from the HR have developed heritable resistance to PCBs and TCDD toxicities at the molecular (Courtenay *et al*., 1999; Yuan *et al*., 2006; Wirgin *et al*., 2011) and organismic levels (Wirgin and Chambers, 2006).

Elevated levels of DNA damage in fishes have been correlated with higher-level toxicities including apoptosis, neoplasia, and early life stage toxicities (Wirgin and Theodorakis, 2002). Genetic damage may be particularly pernicious because of its potential long-term evolutionary consequences. PAHs can be metabolized to electrophilic reactants that covalently bind DNA causing bulky DNA adducts that may result in mutations and initiation of chemical carcinogenesis. Associations between exposure to PCDD/F and PCBs and DNA damage are less clear, although these chemicals may generate DNA-damaging reactive oxygen species (ROS) such as superoxide anions and hydroxyl radicals in fishes (Schlezinger *et al*., 1999) that can damage DNA through single-and double-stranded breaks, abasic sites, and damaged bases.

Quantitative-Extra Long PCR (Q-XLPCR) allows for the detection of damage at specific gene loci which may be important in quantifying overall DNA damage and in mechanistically linking levels of lesions to impaired function. The assumption behind this approach is that such lesions along a DNA template will "block" DNA polymerases and halt the extension step in PCR. Thus, increased levels of DNA damage will result in reduced yields of Q-XLPCR product. The longer the stretch of template DNA that is screened, the greater the opportunity to detect differences in DNA integrity. It is imperative that multiple controls be introduced in the analysis to ensure that levels of Q-XLPCR product reflect the extent of template DNA damage rather than vagaries of the technique, differences in starting copy number, or overt quality of starting template DNA. Thus, absolute copy number among samples of template DNA must be standardized, quality of template DNA evaluated, and the presence of possible inhibitors of amplification evaluated.

Mitochondrial DNA (mtDNA) is circular, 16–20 kb, and is found in multiple cellular copies. Damage to mtDNA has been implicated in loss of mitochondrial function, cancer, and aging (Salazar and Van Houten, 1997). Evidence suggests that mtDNA is significantly more susceptible to PAH and ROS-induced damage than is nuclear DNA (nDNA) (Mandavilli *et al*., 2002) because of preferential accumulation of PAHs in mitochondria, absence of protective histones and nucleosome structure, higher concentrations of ROS, and relatively poor DNA repair. Although nDNA is not as sensitive as mtDNA to damage, previous studies in mammalian models have demonstrated that xenobiotics can induce significant nDNA damage (Yakes and Van Houten, 1997; Ayala-Torres *et al*., 2000).

We evaluated the utility of Q-XLPCR in assessing *in vivo* mtDNA damage and nDNA damage at the aryl hydrocarbon receptor2 (AHR2) in a chemically impacted fish population. We targeted AHR2 because of its pivotal role in mediating toxic effects of aromatic hydrocarbons and available tomcod sequence

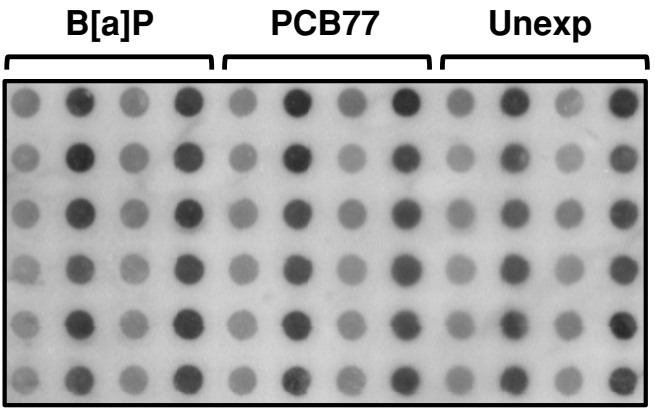

Fig. 1. Slot blot analysis of 1 μg and 250 ng of tomcod genomic DNA hybridized with ^{32}P radiolabeled tomcod mtDNA probe. Tomcod were treated with B[a]P, PCB77, or vehicle control.

(Roy and Wirgin, 1997). We hypothesized that levels of DNA damage detected by Q-XLPCR would be dose responsive to levels of PAHs, PCBs, and PCDD/Fs, significantly higher in environmentally exposed adult tomcod from the HR than those from a cleaner estuary, and greater in older than younger environmentally exposed tomcod.

MATERIALS AND METHODS

Q-XLPCR and Q-PCR mtDNA and AHR2 primers

TCCRD(5′-GTCCATCCTAATATCTTCAGTA-3′) and TCCRA(5′-TCCACCTCTAACTCCCAAAGC-3′) amplified a 1 kb mtDNA control region product that was used in Q-PCR. TC12SXL(5′-CCCTAAGACCTCTGATTCCACGAAAGCCAT-3′) and TCCYTBXLNEW(5′-TACGTCACGGCAGATGTGTACGACAGACGA-3′) amplified a 16.5 kb mtDNA product and were used in Q-XLPCR reactions. Y5(5′-CCTCTGCGATTTCCTTA-3′) and T18(5′-GACTGTTTTCCGACTG-3′) amplified 1040 bp of AHR2 that was used in Q-PCR reactions. AHRXL791(5′-CCTCTTCCACAATCCAAGACTACCTGGGC-3′), and AHRXL5746(5′-TTGGCGTCCAGGTAATGCGGCATGGCAGCC-3′ yielded an 8.0 kb tomcod AHR2 product that was used in Q-XLPCR.

DNA isolations and evaluation of DNA quality

DNAs were isolated by incubation of tissues in 1 N NH_4OH, 0.2% Triton X-100 solution (Wirgin *et al*., 1990), phenol chloroform extractions, and ethanol precipitations. Undigested DNAs were electrophoresed in 1.0% agarose gels and

Table 1. Q-XLPCR analysis of hepatic mtDNA damage in juvenile HR tomcod that were injected with PCB77, TCDD, or B[a]P and sacrificed after 10 d or 20 d.

Treatment	No. of samples	Mean yield of Q-XLPCR mtDNA product (ng)
Vehicle control	9	17.18 ± 5.03
100 ppm B[a]P	12	17.28 ±12.16
1 ppm TCDD	7	15.20 ± 3.53
10 ppm PCB 77	6	16.19 ± 5.89

those that appeared fragmented or whose 260/280 spectrophotometric ratios did not fall between 1.8 and 2.0 were omitted from subsequent analysis. Undigested DNAs were also subjected to Southern hybridizations with ^{32}P radiolabelled tomcod mtDNA probes to visualize the extent of DNA fragmentation. Degraded DNA samples were omitted from subsequent analysis.

Normalization of mtDNA and nDNA copy number

Although spectrophotometric readings at 260 nm provide an estimate of total DNA concentrations, it was important that the actual amounts of template mtDNAs or nDNAs were standardized among samples. For this, slot blot analysis was used to quantify the total amount of mtDNA and nDNA in each sample. One μg and 250 ng of DNA (based on spectrophotometric readings) from each specimen were applied to membranes in a dot blot manifold. The membranes were hybridized to ^{32}P radiolabeled tomcod mtDNA and AHR2 probes and phosphorimaging was used to quantify hybridizeable mtDNA and AHR2 levels (Fig. 1). This allowed us to use equivalent amounts (500 ng) of mtDNA and nDNA as template in subsequent Q-XLPCR and Q-PCR reactions.

Juvenile tomcod treatments

Juvenile HR tomcod were i.p. injected three times (0, 3, 6 d) with B[a]P (100 ppm) and sacrificed after 20 d or a single dose of 10 ppm B[a]P and sacrificed after 7 days. Livers were excised and frozen at –80°C. Another group of juvenile HR tomcod was injected with 100 ppm B[a]P and sacrificed after 4 hr and 24 hr based on the possibility that DNA repair may have been extensive during the 20 d between treatment and sacrifice in the first B[a]P experiment. Another group of juvenile HR tomcod was injected with 10 ppm PCB77 and 1 ppm TCDD and sacrificed after 20 days, because of the possibility that DNA damage may occur by the generation of ROS. A final group of juvenile HR tomcod was injected with methyl methansulfonate (MMS) (10 ppm and 100 ppm) and sacrificed at 4 hr and 24 hr after treatment. MMS has been shown to alkylate DNA at multiple sites on all nucleobases and produces single strand breaks in mammalian cells. Negative controls for all treatment groups were solvent vehicle injected juvenile HR tomcod.

Table 2. Q-XLPCR analysis of hepatic mtDNA damage in three size classes of environmentally exposed adult tomcod from the HR and the Miramichi River (n = 11–12 fish/group).

	Mean Q-XLPCR yield (ng) in three size classes of tomcod		
River	15.1–17.5 (cm)	18.4–23.9 (cm)	26.3–31.2 (cm)
Hudson	30.1 ± 38.6	28.2 ± 15.2	19.7 ± 11.8
Miramichi	16.7 ± 8.5	56.4 ± 68.1	29.6 ± 28.0

Hepatic DNA damage in environmentally exposed adult tomcod from two rivers

Three different-sized/aged groups of environmentally exposed adult tomcod were collected from the Miramichi River, New Brunswick, and from the HR. Livers were excised and immediately frozen at –80°C.

Exposure of tomcod embryos to PCB77 and B[a]P

Ten day-old embryos of HR and Miramichi River descent were statically waterborne exposed to 1 ppm of PCB77 for 34 d and two doses of B[a]P (0.1 ppm and 1.0 ppm) for 14 d. Contaminant solutions were renewed every 48 hr. Specimens were sacrificed as larvae.

Q-PCR and Q-XLPCR of tomcod DNA

Fifty μl Q-PCR reactions included 500 ng of mtDNA or nDNA template (determined by dot blot analysis), 5 μl of 10× reaction buffer, 0.2 mM of each dNTP, 1.0 μM of each primer, 0. 5 U of Taq DNA polymerase, and $_{dd}H_20$ to volume. Cycling parameters were denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1.5 min, and a final extension at 72°C for 7 min.

Fifty μl Q-XLPCR reactions included 500 ng of mtDNA or nDNA template, 15 μl of XL Buffer II, 0.2 mM of each of the four dNTPs, 0.6 μM of each primer, 2.2 mM of magnesium acetate, 2 units of r*Tth* DNA polymerase XL enzyme (PE Applied Biosystems), and $_{dd}H_20$ to volume. Cycling parameters were denaturation at 94°C for 1 min, incubation at 80°C for 10 min during which 2 U of r*Tth* DNA polymerase was added, denaturation at 94°C for 30 sec followed by 23 cycles (mtDNA) or 26 cycles (AHR2) of 94°C for 30 sec, extension at 70°C for 15 min initially followed by 15 sec increments in each subsequent cycle, followed by a final extension at 70°C for 15 min. The number of Q-XLPCR cycles was empirically optimized for tomcod mtDNA and AHR2.

Gel electrophoresis and data analysis

Q-PCR and Q-XLPCR products were electrophoresed in 1.2% and 0.8% agarose gels, respectively along with known amounts of molecular weight marker. AlphaEase FC software was used to quantify products using an

Table 3. Mean yield of Q-XLPCR mtDNA product in tomcod larvae exposed as embryos to PCB77 (1 ppm) for 34 d or B[a]P (1 ppm) for 14 d.

Treatment	No. of samples	Mean Q-XLPCR yield (ng)
Vehicle control	12	160.3 ± 68.6
B[a]P	12	107.2 ± 69.9
PCB77	12	168.1 ± 74.0

AlphaImager system or Kodak 1D 3.5.2 USB software was used to quantitate amounts of PCR product in gels imaged with a Kodak camera. Data was expressed as mean yield of Q-XLPCR product. Statistical comparisons of treatment groups to their matched vehicle control groups were performed using unpaired Student's *t* tests and were considered significant at $P < 0.05$.

RESULTS

Q-XLPCR of hepatic DNA from B[a]P-treated juvenile tomcod

Initially, we used Q-XLPCR to quantify hepatic DNA damage in hatchery-bred and reared juvenile HR tomcod that were injected with single doses of 10 ppm B[a]P and sacrificed after 7 d or three times with B[a]P (100 ppm) and sacrificed after 20 d. For all three replicates of the 10 ppm B[a]P-treated group, mean yield of both mtDNA and AHR2 in B[a]P-treated fish was approximately half that of untreated controls. For example, in replicate #1, mean yield of mtDNA was 152 ng in negative controls compared to 76 ng in B[a]P-treated fish. Similarly, in replicate 1, mean yield of AHR2 was 50 ng in negative controls and 23 ng in B[a]P-treated fish (data not shown). Thus, B[a]P-induced DNA damage was evident through use of this assay. However, variability was high both among samples within and among replicates and differences in Q-XLPCR yield between B[a]P treated and control groups were not statistically significant.

As a control, Q-PCR of approximately 1.0 kb of mtDNA and AHR2 products was used to ensure that inhibitors to PCR were not present in the sample preparations. Mean yield of Q-PCR products was similar between control and B[a]P-treated fish. For example, mean yield of mtDNA Q-PCR products was 121 ng for B[a]P-treated and 149 ng for control fish. Similarly, mean yield of AHR2 Q-PCR products was 84 ng for B[a]P-treated and 78 ng for control fish. These results suggested that differences in Q-XLPCR yield between B[a]P-treated and control fish was likely due to increased DNA damage in chemically-treated fish.

In the event that DNA damage induced by B[a]P was repaired in the 14 d between the last exposure and the time of sacrifice, we injected two new groups of juvenile HR tomcod with 100 ppm B[a]P and sacrificed them after 4 and 24 hr. Results indicate that the amount of product obtained from Q-XLPCR amplifications of mtDNA was almost identical between negative control and B[a]P-treated fish. As expected, no difference in yield of Q-PCR products was observed.

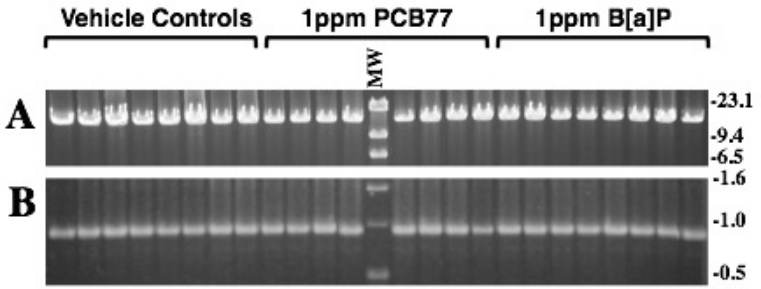

Fig. 2. A. Q-XLPCR analysis of 16.5 Kb mtDNA in tomcod larvae exposed as embryos to 1 ppm PCB77, 1 ppm B[a]P, or vehicle control for 14 d. MW is molecular weight marker. B. Q-PCR amplification of the same larval DNAs as in Panel A.

Q-XLPCR of hepatic DNA from PCB77 and TCDD treated juvenile HR tomcod

Juvenile HR tomcod were treated for 20 d with halogenated aromatic hydrocarbons, 10 ppm PCB77 and 1 ppm TCDD, that may generate elevated levels of ROS and damage DNA by base modification. Yields of Q-XLPCR products were very similar between chemically-treated, TCDD or PCB77, and vehicle-treated fish (Table 1). Although there was a slight reduction in yield in the TCDD and PCB77-treated fish, this was not statistically significant. Q-PCR yields were almost identical among treatment groups.

Q-XLPCR of hepatic mtDNA from MMS-treated juvenile HR tomcod

We projected that MMS could serve as a positive control to test the efficacy of the Q-XLPCR assay in detecting *in vivo* DNA damage in tomcod. Two groups of tomcod were i.p. injected with either 10 ppm or 100 ppm of MMS and sacrificed after 4 or 24 hr. Surprisingly, we saw no significant reduction in Q-XLPCR yield in the tomcod treated with 10 ppm MMS compared to the control group (data not shown). Tomcod that were treated with 100 ppm MMS and sacrificed at 24 hr exhibited an 18%, but not significant, reduction in Q-XLPCR mtDNA yield.

Q-XLPCR of hepatic mtDNA from three size classes of environmentally exposed tomcod from two rivers

Three distinct size classes of environmentally-exposed adult tomcod were collected from the HR and the Miramichi River and their livers immediately flash frozen. We hypothesized that within the HR collection, Q-XLPCR yield would be highest for the smallest and lowest for the largest size group. Within the Miramichi collection, we expected no difference in yield among size groups. We also hypothesized that Q-XLPCR yield would be lower in HR tomcod compared to those from the Miramichi River across all size ranges. As depicted in Table 2,

while Q-XLPCR yields were lower in larger than smaller size classes of HR tomcod, these differences were not statistically significant probably because of large inter-individual variation in yield. Also, while Q-XLPCR yields were lower for larger HR tomcod compared to those from the Miramichi River, this difference was not observed for the smallest size class of fish.

Q-XLPCR of mtDNA damage in B[a]P or PCB77 exposed embryos

Because young life-stages of fishes are most sensitive to AHR2 mediated toxicities we hypothesized that DNA damage would be extensive in embryos chronically exposed to 1 ppm PCB77 for 34 d or 0.1 and 1.0 ppm B[a]P for 14 d. However, we saw little evidence of significantly increased damage in the mtDNA of larvae exposed to either of these agents. Although, yield of Q-XLPCR was reduced by 34% in 1 ppm B[a]P treated larvae, this reduction was not statistically significant. There was no evidence of elevated mtDNA damage in the PCB77 exposed group (Table 3, Fig. 2).

DISCUSSION

It has been proposed that DNA damage in chemically impacted natural populations can provide a quantitative and ecologically important measure of environmental health (Wirgin and Theodorakis, 2002; van der Oost *et al*., 2003). PAHs and halogenated PCDD/Fs and PCBs have been demonstrated to elicit significant DNA damage in vertebrates by at least two different mechanisms. For example, we previously demonstrated that HR tomcod exhibited significantly higher levels of bulky DNA adducts as quantified by ^{32}P-postlabeling, presumably from PAH exposure, than tomcod from elsewhere (Wirgin *et al*., 1994). However, the gold-standard approach, ^{32}P-postlabeling, is difficult, costly, time consuming, and only measures exposure from DNA-adducting PAH metabolites. Therefore, Q-XLPCR has been proposed as an alternative approach to sensitively quantify DNA damage in populations from their exposure to a broader suite of contaminants and at specific gene loci (Meyer, 2010). However, in our study we found that Q-XLPCR did not provide a sensitive, dose responsive approach to quantify *in vivo* mtDNA or nDNA damage in juvenile or larval tomcod treated with B[a]P, PCB77, or TCDD under controlled laboratory conditions or in environmentally exposed adult tomcod from the contaminated HR.

Most previous studies that have successfully applied Q-XLPCR to quantify DNA damage have been *in vitro* in mammalian cells or *in vivo* in laboratory rodent models (Hunter *et al*., 2010). However, recent *in vivo* studies have reported successful use of Q-XLPCR to demonstrate elevated DNA damage in two environmentally relevant models. For example, Neher and Sturzenbaum (2006) reported elevated levels of nDNA damage in whole nematode worms *Caenorhabditis elegans* treated with B[a]P, fluoranthene, and their mixtures. Similarly, use of Q-XLPCR demonstrated elevated levels of mtDNA and nDNA damage in brain, liver, and muscle of Atlantic killifish *Fundulus heteroclitus* that were treated with B[a]P and in liver and muscle of an environmentally exposed

killifish population from a creosote contaminated site (Jung *et al*., 2009).

Why did we fail to detect significantly elevated levels of DNA damage in tomcod that were chemically treated or which are known to bioaccumulate high levels of genotoxicants in natural populations? In part, our results reflect the high levels of inter-individual variability in our Q-XLPCR yields that compromised the statistical sensitivity of our analyses. High levels of inter-individual variation in Q-XLPCR yields was also reported among individual *C. elegans* treated with 5 ppm B[a]P (Neher and Sturzenbaum, 2006), but these workers failed to statistically compare aggregate DNA yields between treated and control groups. Previously, we reported 40 fold higher levels of hepatic DNA adducts, presumably PAH metabolites, in environmentally exposed HR tomcod compared to those in cleaner estuaries (Wirgin *et al*., 1994). However, in the current Q-XLPCR study we failed to see significant DNA damage in tomcod by PAHs, either by experimental treatment with B[a]P or by environmental exposure. It might be argued that because HR tomcod are resistant to PCBs and TCDD induced CYP1A expression they are also resistant to the generation of DNA damage by ROS. However, tomcod from the HR are not resistant to PAH-induced CYP1A and therefore should suffer detectable DNA damage from their exposure (Courtenay *et al*., 1999; Yuan *et al*., 2006). We suggest that this discrepancy in results between the two experimental approaches may be due to reduced sensitivity of Q-XLPCR compared to ^{32}P-postlabeling in detecting DNA damage. It has been suggested that ^{32}P post-labeling has a sensitivity limit of 1 adduct in 10^7–10^{10} nucleotides (Reddy and Randerath, 1986; Phillips and Arlt, 2007) compared to less than 1 lesion in 10^5 nucleotides using Q-XLPCR (Meyer, 2010). Our results indicate that Q-XLPCR may not be a sensitive and quantitative approach to detect DNA damage in environmentally exposed natural populations.

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