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Genotoxicity in Atlantic killifish (*Fundulus heteroclitus*) from a PAH-contaminated Superfund site on the Elizabeth River, Virginia

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Abstract

The Atlantic Wood Industries Superfund site (AWI) on the Elizabeth River in Portsmouth, VA is heavily contaminated with polycyclic aromatic hydrocarbons (PAHs) from a wood treatment facility. Atlantic killifish, or mummichog (*Fundulus heteroclitus*), at this Superfund site are exposed to very high concentrations of several carcinogens. In this study, we measured PAH concentrations in both fish tissues and sediments. Concurrently, we assessed different aspects of genotoxicity in the killifish exposed *in situ*. Both sediment and tissue PAH levels were significantly higher in AWI samples, relative to a reference site, but the chemistry profile was different between sediments and tissues. Killifish at AWI exhibited higher levels of DNA damage compared to reference fish, as measured via the flow cytometric method (FCM), and the damage was consistent with sediment PAH concentrations. Covalent binding of benzo[*a*]pyrene (BaP) metabolites to DNA, as measured via LC-MS/MS adduct detection methods, were also elevated and could be partially responsible for the DNA damage. Using similar LC-MS/MS methods, we found no evidence that oxidative DNA adducts had a role in observed genotoxicity.

Keywords

Fundulus heteroclitus; DNA adduct; chromosomal damage; Elizabeth River; Atlantic Wood Industries Superfund site; polycyclic aromatic hydrocarbon; biomarker

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic carbon compounds composed of fused aromatic ring structures. These compounds are released into the environment primarily through the incomplete combustion of organic matter (Walker et al. 2005). Unlike other compounds of concern, concentrations of PAHs in the environment have been rising

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due to increases in industrial activities as well as rapid urbanization in recent years (Van Metre and Mahler 2005; Chang et al. 2006). There is also continued release of PAHs via oil spills, as evidenced by the 2010 Deep Horizon Gulf of Mexico oil spill. Several of these chemicals are considered highly carcinogenic or mutagenic (Srogi 2007) and are listed on the US EPA Priority Pollutant List (Appendix A of 40 CFR Part 423). Moreover, recent studies have shown that PAHs are also indicated in other deleterious effects such as immunotoxicity (Carlson et al. 2004; Reynaud and Deschaux 2006) and impaired cardiovascular and neurological development in organisms (Sanyal and Li 2007; Perera et al. 2009). In the environment, PAHs are always present as mixtures of several individual compounds. Importantly, PAH mixtures can have synergistic carcinogenic effects (Hermann 1981; Schneider et al. 2002), as well as teratogenic effects on developing fish (Wassenberg and Di Giulio 2004a; Billiard et al. 2008). Therefore, assessment of exposure and effects of environmentally prevalent PAHs in mixtures to the biota is gaining recognition as an important issue (USEPA 2010).

The Atlantic Wood Industries Superfund Site (AWI) along the Elizabeth River in Portsmouth, VA provides a unique opportunity to study the effects of chronic exposure to PAHs on the biota, and allows for the evaluation of different types of biomarkers of PAH exposure and effect. This site housed a wood treatment facility between 1926 and 1992 and is heavily contaminated with creosote, wood preservatives that mostly consist of PAHs, from the facility. This site has been designated as a National Priority List Superfund site since February 1990 (USEPA 2007). This site is polluted with a complex mixture of contaminants including PAHs, metals, and pentachlorophenol. Concentrations of total PAHs in the sediment of this site have been reported to average ~ 200–400 g/g, and include carcinogenic PAHs, such as benzo[*a*]pyrene (BaP), chrysene, and dibenzo[*a,h*]anthracene (Hartwell and Hameedi 2007; Vogelbein et al. 2008). In short, the PAH concentrations in the sediments at AWI are considered some of the highest in the world (Walker et al. 2004). As a result, the Atlantic killifish (*Fundulus heteroclitus*) found at AWI are exposed to very high concentrations of a complex mixture of PAHs. This history of chronic exposure is reflected in the killifish population in two distinct ways. On one hand, the deleterious effects of the exposure are manifested as high occurrences of hepatic lesions (Vogelbein et al. 1990; Van Veld and Nacci 2008) and elevated levels of DNA adducts (Rose et al. 2000). On the other hand, the fish show adaptation to the PAH-rich environment by alteration of the aryl hydrocarbon receptor pathway which is involved in the metabolism of PAHs (Meyer et al. 2003; Wills et al. 2009; Wills et al. 2010) and in oxidative stress (Meyer et al. 2003; Bacanskas et al. 2004), two pathways through which PAHs commonly exert toxicity in vertebrates. Considering the fact that organisms chronically exposed to chemicals can have complex biological responses to such chemicals, it is important to choose appropriate endpoints, particularly those that can be correlated to environmental pollutant levels.

Many studies correlate environmental levels of pollutants to actual biological effects on organisms by measuring a variety of different endpoints or biomarkers (Varanasi et al. 1989; Bickham 1990; Varanasi and Stein 1991; Lyons et al. 2000; Siu et al. 2004; Machella et al. 2005; Matson et al. 2005). These studies show the importance of choosing appropriate endpoints when measuring the effects of chemical mixtures that can potentially have several mechanisms of action, such as PAH mixtures. Choosing appropriate biomarkers, in terms of efficiency and accuracy, will give us insight into the overall physiological effects of complex mixture exposures.

From an ecotoxicological point of view, the Atlantic Wood Industries Superfund site and the killifish in the area, which are resistant to the acute toxic effects of the chemicals present, provide us with a unique and useful situation in which we can utilize various methods to assess exposure, effect, and response in organisms chronically exposed to extremely high

levels of a PAH mixture. At the same time, by taking advantage of this well-characterized site and fish population, we can compare various methods that assess the effects of PAHs. Therefore, in this study, we examined environmental and tissue PAH levels, and assessed the effects of this exposure by evaluating genotoxicity in killifish using two different methods; (1) measurement of chromosomal damage using flow cytometry, and (2) measurement of covalent and oxidative DNA adduct measurement using liquid chromatography-mass spectrometry (LC-MS/MS).

Materials and Methods

1. Sample collection

Killifish were captured at the Atlantic Wood Superfund site on the Elizabeth River (Portsmouth, VA; 36°48'27.4"N, 76°17'36.1"W) and the King's Creek reference site (Gloucester County, VA; 37°17'52.4"N, 76°25'31.4"W) using baited minnow traps. Within 24 h of transfer back to the laboratory, blood and liver were harvested from adult males (5 – 13 g) from each population. Blood samples for chromosomal damage analysis were collected (n = 20 per population) via caudal vein puncture into heparinized collection tubes (Fisher Scientific, Pittsburgh, PA, USA), flash frozen, and kept at –80°C. Liver samples for DNA adduct analysis (n = 3) were also flash frozen and kept at –80°C. In addition, whole individuals from each site (n = 5) were stored at –20°C for tissue chemistry analysis. Sediment samples were also collected at the sites at the time of fish collection, moved to the laboratory, and kept at 4°C until sediment chemistry analysis.

2. Sediment total organic carbon measurement

To measure the total organic carbon content in each site, we used a loss-on-ignition method (Dean 1974). Briefly, roughly 20 g of sediment was dried at 100°C in pre-weighed tins for 24 h, weighed, then heated to 500°C in a muffle furnace for 24 h, then reweighed. Percent organic matter was calculated from mass lost on ignition.

3. Sediment PAH concentration measurements

Sediment PAH levels were measured by gas chromatography mass spectrometry (GC/MS), operated in electron impact mode (GC/EI-MS), using previously reported methods (Schneider et al. 2001; Stapleton et al. 2008). All standards were purchased from AccuStandard, Inc. (New Haven, CT, USA). Sediments were first mixed with pre-cleaned sodium sulfate to remove water residues and then spiked with four labeled surrogate standards (d¹⁰-2-methylnaphthalene, d¹⁰-fluorene, d¹⁰-fluoranthene, d¹²-perylene). Extraction was performed using accelerated solvent extraction (ASE 300, Dionex Corp) with 50:50 hexane:dichloromethane. After extraction, samples were concentrated to 1 mL and purified via elution through a column packed with alumina (4.0 grams 6% deactivated) with petroleum ether. The solvent was exchanged to hexane, concentrated to 1 mL, and spiked with four labeled internal standards (d⁸-naphthalene, d¹⁰-phenanthrene, d¹⁰-pyrene, d¹⁰-benzo[a]pyrene). Samples were analyzed by GC/MS (Agilent 5890N GC, 5975 MS).

4. Tissue PAH concentration measurements

Tissue PAHs were analyzed via GC/MS using the same method for sediments as outlined above with one additional step included to remove lipid residues. Thawed tissues were homogenized in pre-cleaned sodium sulfate, and spiked with surrogate standards (as described above). Samples were extracted with 50:50 hexane:dichloromethane on Soxhlets for approximately 16 h. The samples were purified via elution through deactivated alumina with petroleum ether and reduced in volume to 1 mL in DCM. To remove any lipid interferences, the remaining extracts were injected into an HPLC system for size exclusion

chromatography (SEC) using a divinylbenzene-polystyrene column (10 μ m particle size, 100 Å pore size, 2.5 cm i.d. $\times$ 60 cm, PL-Gel, Polymer Labs, Inc., Amherst, MA). Samples were eluted through the SEC using a mobile phase of 100% dichloromethane at a flow rate of 5.0 mL/min. The collected extract was solvent exchanged to hexane, concentrated to 1 mL, spiked with internal standards (as described above), and analyzed by GC/MS using the conditions described above.

5. Quality assurance

Recovery rate for surrogate standards were 32.5 ± 6.2 %, 42.0 ± 3.0 %, 77.1 ± 5.6 %, and 90.3 ± 3.4 % for d¹⁰-2-methylnaphthalene, d¹⁰-fluorene, d¹⁰-fluoranthene, d¹²-perylene respectively for sediment PAH measurement. Recovery rate for surrogate standards were 47.5 ± 4.3 %, 66.3 ± 2.7 %, 87.6 ± 2.2 %, and 89.2 ± 1.3 % for d¹⁰-2-methylnaphthalene, d¹⁰-fluorene, d¹⁰-fluoranthene, d¹²-perylene, respectively, for tissue analysis. Both sediment and tissue PAH measurements were corrected for recovery. A few PAHs were detected in the laboratory blanks. Therefore, sample values were blank-corrected by subtracting average blank values. Method detection level (MDL) was calculated as three times the standard deviation of laboratory blanks divided by the mass extracted.

6. Chromosomal damage estimation

Chromosomal damage in blood samples was estimated using a flow cytometric method (FCM) to measure intercellular variability in DNA content as described by Vindelov and Christiansen (1994). Briefly, 15 μ L of heparinized blood was added to 50 μ L of citrate buffer and 280 μ L of trypsin/detergent solution for digestion. Following a 10 min digestion, 280 μ L of trypsin inhibitor/RNase was added to stop the reaction and to degrade RNA. The solution was then filtered with 30 μ m nylon mesh, followed by the addition of 325 μ L of propidium iodide. Samples were placed on ice for 15 min and then analyzed on a Beckman Coulter (Fullerton, CA) Quanta SC flow cytometer. Fluorescent emission was measured for cells that were illuminated with a 488 nm laser diode to excite propidium iodide. Cells were gated on side scatter, EV, and fluorescence. A total of 20,000 cells were collected in the G0/G1 peak for each sample and the intercellular variation in DNA content (genome size) was reported as the half-peak coefficient of variation. Genome size variation was used as a measure of chromosomal damage. Variability in genome size is likely the result of the unequal distribution of chromosomes or chromosome fragments during cell division. This can result from clastogenicity or aneugenicity. Thus FCM provides a generalized estimate of large-scale DNA damage (i.e. chromosomal).

7. DNA adduct measurement

For measuring oxidative (8-OH deoxyguanosine, or 8-oxo-dG) and covalent DNA adducts formed by the BaP-metabolite (benzo[a]pyrene diol-epoxide deoxyguanosine, or BPDE-dG), DNA from killifish livers harvested as mentioned above were isolated and enzymatically hydrolyzed as described in Jung et al. (2009a). The final volume of each sample containing all reagents and internal standard was 300 μ L. The [¹⁵N₅]8-Oxo-dG internal standard for all samples was purchased from Cambridge Isotope Laboratories (Andover, MA, USA) and 8-Oxo-dG analyte standard was purchased from Sigma-Aldrich. BPDE-dG standards ([¹⁵N]BPDE-dG internal standard for all samples and N²-BPDE-dG analyte standard for positive controls) were generous gifts from Dr. Natalia Tretyakova (University of Minnesota).

Samples of hydrolyzed DNA were chromatographed on an Agilent 1200 HPLC system with automated fraction collector. Separation was performed on an Ultrasphere ODS C18 4.6 $\times$ 250 mm 5 μ m column (Beckman, Fullerton, CA) using a gradient of 10 mM ammonium formate in water (adjusted to pH 4.3 with formic acid) and methanol. Methanol composition

was held at 7% from 0 to 22 min, then increased linearly to 80% in 1 min, was held at 80% for 6 min to elute TEMPO, decreased to 7% in 1 min, and held at 7% for 6 min for column re-equilibration. A 275 μ L aliquot of sample was injected, and the flow rate was 1 mL per min. The column oven, autosampler tray and fraction collector chamber temperatures were maintained at 30°C, 4°C and 4°C, respectively. The retention time was determined by using 2'-deoxyguanosine (dG) as a retention time marker and multiplying its retention time by 1.5 (~18 min for 8-oxo-dG and ~23.5 min for N^2 -BPDE-dG). Targeted fractions were automatically collected from 1.5 min before until 1.5 min after their predicted retention time. The fraction collection tubes were placed in a SpeedVac concentrator (ThermoFinnigan, San Jose, CA) and evaporated to dryness. Sample residue was transferred to autosampler vials via 2×130 μ L washings with 50:50::water:methanol, evaporated to dryness in a SpeedVac concentrator, and finally redissolved in 20 μ L HPLC grade water for subsequent analysis by LC-MS/MS. The 2'-deoxyguanosine (dG) amount in each sample was determined during fraction collection by comparison with dG calibration standards using UV detection at 264 nm.

The quantitative analysis of 8-oxo-dG was performed with an Acquity UPLC (Waters, Milford, MA, USA) coupled to a TSQ-Quantum Ultra triple-quadrupole mass analyzer (ThermoFinnigan) according to the protocol described in Jung et al. (2009a). The quantitative analysis of N^2 -BPDE-dG was performed with a nanoAcquity UPLC (Waters) coupled to a TSQ-Quantum Ultra triple-quadrupole mass analyzer (ThermoFinnigan) using the nanospray source in positive mode. The trap column was a Waters 180 μ m $\times$ 20 mm Symmetry C18 5 μ m, and separation was performed on a 100 μ m $\times$ 100 mm BEH300 C18 1.7 μ m column (Waters). The mobile phase consisted of 10 mM ammonium acetate, 0.1% acetic acid in water and methanol. The sample was first injected onto the trap column at a flow rate of 10 μ L per min for 1.5 min at an initial methanol concentration of 5%, then flow was directed in-line with the analytical column. Gradient elution was performed at a flow rate of 600 nL per min. The methanol composition started at 5% and increased linearly to 80% in 10 min, was held at 80% for 2 min, decreased to 5% in 2 min, then held at 5% for 6 min for column re-equilibration. The retention time of N^2 -BPDE-dG was 12 min, and the total run time was 20 min. The analyte and internal standard were detected in selected reaction monitoring mode (SRM), monitoring the transitions of m/z 570 to 257 (collision energy 15 eV) and m/z 570 to 454 (collision energy 35 eV) for N^2 -BPDE-dG and m/z 575 to 257 (collision energy 15 eV) and m/z 575 to 459 (collision energy 35 eV) for [$^{15}\text{N}_5$] N^2 -BPDE-dG. Other nano-electrospray conditions were as follows: positive mode, spray voltage of 2000 V, capillary temperature of 280°C. The detection limit was 10 fmol for 8-oxo-dG and 2.5 fmol for BPDE-dG.

8. Statistics

For all statistical analyses, Prism (version 5.0a, GraphPad Software Inc., La Jolla CA, USA) was used. For DNA adduct analysis and chromosomal damage estimation, Mann-Whitney U tests were conducted. Two-way ANOVA with Bonferroni post-hoc analysis was used to compare the tissue and sediment PAH concentrations for the two sites. We used $p = 0.05$ as our cut-off for significance.

Results

1. Sediment PAH concentrations

Sediment concentrations of EPA Priority Pollutant PAHs are summarized in Figure 1. Sediment from the AWI Superfund site had significantly higher concentrations of PAHs than sediments from the King's Creek reference site ($p < 0.01$). In addition, composition of PAHs was statistically significantly different between the sites ($p < 0.01$). Generally, there

were much higher concentrations of high molecular weight PAHs (HMW, chemicals with four or more ring structures) in the sediment from the AWI Superfund site compared to the reference site. Complete sediment chemistry data can be found in Supplement Table 1. Concentrations of PAHs in sediments are strongly dependent on organic matter (Viguri et al. 2002). Therefore, we also measured sediment total organic carbon (TOC) content to verify whether the organic content of sediments from King's Creek was similar to the sediments from the AWI Superfund site. Loss on ignition experiment (24 h at 500°C) showed that the AWI sediment contained 13% organic matter, whereas King's Creek sediment contained 4% organic matter.

2. Tissue PAH concentrations

As seen in Figure 1, tissue PAH concentrations from whole fish homogenates were different between the two populations ($p < 0.01$). As with the sediment PAH profile, the chemical composition of tissue PAHs differed between the two populations ($p < 0.01$). However, contrary to the sediment data, AWI killifish had significantly higher levels of three-ring PAHs (acenaphthene and acenaphthylene) than King's Creek fish. Higher molecular weight-PAHs were not significantly different between the two populations. Complete tissue chemistry results are available in Supplement Table 2.

3. Chromosomal damage

We measured chromosomal damage in blood samples of fish from both populations using flow cytometry ($n = 20$ per population). There were higher levels of chromosomal damage, as measured by intercellular variability in DNA content, in the AWI killifish compared to the King's Creek killifish ($p = 0.006$, Fig 2). Mean half-peak coefficients of variation (HPCV) in DNA content were 3.03 ± 0.14 and 3.39 ± 0.12 , for King's Creek and AWI fish, respectively. Increased HPCVs represent greater variability in DNA content, resulting from a number of processes, including both clastogenic (inducing breakage of chromosomes) and aneugenic (causing abnormal homologous pair) events.

4. DNA adduct formation

The results from both liver 8-oxo-measurements and liver BPDE-dG measurements are shown in Figure 3. Levels of 8-oxo-dG, one of the most commonly measured oxidative DNA adducts, was not different between the two populations ($p = 0.20$). Moreover, the levels detected (1.1 to 1.5 adducts per 10^6 dG in the King's Creek population and 1.4 to 2.6 adducts per 10^6 dG in the AWI killifish) were barely above detection limits. We also measured the levels of BPDE-dG, formed by the covalent binding of BPDE (a common metabolite of BaP) to DNA. AWI killifish had elevated levels of the BPDE-dG adduct compared to the King's Creek killifish ($p = 0.038$). The adduct levels ranged from 10.7 to 54.4 adducts per 10^6 dG in the AWI population (average = 27.49) and below detection to 1.9 adducts per 10^6 dG in the King's Creek population (average = 0.63).

Discussion

In this study, we compared several biomarkers for genotoxicity in killifish collected from a well-characterized Superfund site. In addition, we measured sediment and tissue PAH concentrations so that we could determine the relationships among our markers for genotoxicity and the actual environmental levels of the pollutants. Interestingly, we saw a greater prevalence of low molecular weight (LMW) PAHs in the tissue samples of AWI fish. Moreover, the amount of HMW PAHs in the tissue samples was not significantly different between the two populations. In addition, the differences in tissue PAH concentration was not as noticeable in the two populations as was the case in the sediment samples. Such contradictory tissue and sediment chemistry profiles are not entirely

unexpected (Varanasi and Stein 1991; van der Oost et al. 1994b; Soclo et al. 2008). This could be due to greater bioavailability of LMW compounds (Nye and Witt 1995), or due to the lower efficiency of teleosts in metabolizing LMW PAHs than HMW PAHs (Schnell et al. 1980; Varanasi and Stein 1991). One important consideration here would be the health implications of LMW PAHs (Park et al. 2002). For example, acenaphthylene and acenaphthene, two LMW PAHs that are present in significantly higher levels in the AWI killifish tissues, are known to be strong aryl hydrocarbon receptor (AhR)-independent CYP1A2 inducers in mammals (Chaloupka et al. 1994; Ryu et al. 1996). In addition, we have previously shown that synergistic effects of different PAHs are important factors in developmental toxicology in fish (Wassenberg and Di Giulio 2004a, b; Billiard et al. 2006). Given the high accumulation of LMW PAHs in the resident fish populations, it may be important to take these compounds into consideration when evaluating the toxicity of PAH mixtures.

A previous study with larval killifish in our laboratory indicated that the AWI killifish population might be coping with chronic exposures to high levels of PAHs by reducing the rate of PAH metabolism or shifting metabolism to less toxic metabolites (Wills et al. 2009). The tissue concentrations of HMW PAHs were not different between the two populations in the samples that we examined (Fig 1, Supplement Table 2). This suggests that if the fish are being exposed to comparable amounts of PAHs as measured in the sediment, the metabolism of PAHs in the AWI killifish population is still extremely rapid, and that the AWI killifish are more efficiently shifting metabolism to yield less toxic compounds. Characterizing the PAH-metabolites of adults would provide more information regarding this issue.

Although teleosts metabolize PAHs quite rapidly, their DNA repair capacity, especially nucleotide excision repair, is low relative to mammals. (Varanasi et al. 1989; Meador et al. 1995; Mitchell et al. 2004; Groff et al. 2010). In this sense, it may be questionable whether tissue PAH concentrations correctly represent what the organisms were actually exposed to, and whether tissue PAH concentrations are a good indicator of the exposure to and effects of pollutants. Our results also show that tissue PAH levels cannot explain the extensive liver DNA damage levels measured. In fact, sediment PAH levels were more reflective of the DNA damage levels in the liver. Others have documented this discrepancy in studies of liver DNA damage and tissue chemistry (van der Oost et al. 1994a; van der Oost et al. 1994b) and concluded that adduct measurement is a better marker of exposure than tissue PAH concentrations (Meador et al. 1995; French et al. 1996). This result stresses the importance of obtaining environmental data to infer the actual exposure levels of organisms in field studies.

The FCM measures general chromosomal damage and loss. This method has been used widely as a biomarker of genotoxicity, including in areas contaminated with PAHs (Bickham 1990; Theodorakis 2001; Custer et al. 2005; Matson et al. 2005; Goanvec et al. 2008; Matson et al. 2009). Generally, this method measures severe damage in the organism's DNA. In this study, AWI killifish had significantly higher levels of chromosomal damage than King's Creek killifish. This result suggests that this population suffers from substantial chromosomal damage-most likely due to high levels of several genotoxic compounds. In a highly polluted area, such as the AWI Superfund site, genotoxicity of the chemicals in the environment can be picked up using biomarkers of large-scale damage. However, we were hopeful that measuring more specific markers would provide us with more striking differences between the two populations-indicating that such methods might be better candidates for assessing sites that do not have particularly high pollution levels.

Killifish at the AWI Superfund site are exposed to several genotoxic PAHs that can be metabolized and then bind covalently to DNA. Among these, we chose to measure the frequency of BPDE-dG adducts, the most commonly measured form of covalent DNA adduct derived from BaP. Previous surveys have shown that BaP is one of the most abundant PAHs at the AWI Superfund site (~11% of total PAHs by mass) (Vogelbein et al. 2008). In addition, BaP is thought to contribute significantly to the carcinogenicity caused by PAH mixtures and is frequently used as marker for total PAH exposure in human exposure studies (Petry et al. 1996; Lin et al. 2002). Therefore, we hypothesized that a large proportion of DNA damage at AWI would be induced by specific adducts such as BPDE-DNA adducts and that what we see in terms of BPDE-adducts could be a representation of bulky adducts formed by PAH metabolites. Although there was broad variation among the samples, the adduct levels in the AWI killifish were quite high compared to King's Creek killifish. The BPDE-adduct results are in agreement with previous biomarker surveys (Rose et al. 2000; Jung et al. 2009b), and support our hypothesis that BPDE-DNA adducts can be a representation of bulky adducts formed by PAH metabolites. These results also suggest that for sites containing PAH mixtures, this BaP-specific adduct can be a useful biomarker.

Production of reactive oxygen species during PAH metabolism is one of the modes of action of PAH carcinogenicity (Bolton et al. 2000; Park et al. 2006). Oxidative DNA damage can be caused by a number of PAHs found in the sediments of the AWI Superfund site. In fact, Malins et al. (1990) found elevated levels of 2,6-diamino-4-hydroxy-5-formamidopyrimidine, another marker of oxidative DNA damage, in English sole (*Parophrys vetulus*) from a site with documented PAH contamination. Therefore, we hypothesized that the AWI killifish population would have higher levels of oxidative DNA adducts compared to King's Creek killifish. In addition, since 8-oxo-dG frequency can reflect DNA damage from all chemicals that produce oxidative DNA damage, we expected the differences in adduct frequency to be higher than for the BPDE-adduct. However, we did not find any evidence that oxidative DNA damage was playing a major role in observed DNA damage in the AWI population. This result was in agreement with previous research on killifish exposed to BaP in the laboratory (Jung et al. 2009a). It is possible that the 8-oxo-dG measurement method is not as sensitive for measuring general oxidative DNA damage in wild populations. More studies that involve measuring different types of oxidative adducts, such as 3-(2'-deoxy- β -D-erythro-pentofuranosyl)-pyrimido[1,2-a]-purin-10(3H)-one, may shed more light on this issue (Jeong et al. 2008). Another possibility regarding the lack of oxidative DNA adducts, is that the AWI killifish are extremely efficient in countering oxidative stress. This agrees with our previous studies which indicated that AWI killifish have higher tolerance and better defense mechanisms against oxidative stress (Meyer et al. 2003; Bacanskas et al. 2004).

In a previous study, we measured the extent of general DNA damage in the mitochondria and nuclei of killifish collected from King's Creek and AWI using the long amplicon-quantitative PCR (LA-QPCR) method (Jung et al. 2009b). In that study, significantly higher levels of mitochondrial and nuclear DNA damage were observed in the livers of AWI killifish relative to King's Creek reference fish. In the present study, similar results were seen with FCM. However, when we measured specific DNA adducts (8-oxo-dG and BPDE-dG), the results varied widely depending on which specific adduct we were looking at. Statistically, more significant differences were seen in LA-QPCR compared to either of the two adduct measurements. This increased discrimination observed between the populations by LA-QPCR method compared to the current study is most likely due to the fact that the LC-MS/MS method measures very specific types of DNA adducts, whereas the LA-QPCR method measures any type of polymerase-inhibiting DNA damage (Hunter et al. 2010). In an environment where organisms are exposed to a complex mixture of genotoxic chemicals, such as AWI, DNA damage is likely caused by more than one type of chemical. Therefore,

this type of general assay would likely be more sensitive to the damage in the genome caused by multiple agents, and be more indicative of the actual *in situ* situation as is the case with the AWI killifish (Meyer 2010). This is also true for FCM, as it is not specific with regard to the damage-initiating event, only that damage has occurred. Field studies of this kind are particularly suited for methods that cover a wide range of impacts; however, more specific endpoints are also quite valuable for sorting out which contaminants/mechanisms might be driving observed biomarker responses. In the case of the AWI population, measuring different types of adducts with LC-MS/MS allowed us to further hypothesize that covalent binding of the PAH metabolites may be a more dominant mechanism behind DNA damage in the killifish than DNA damage generated by reactive oxygen species.

In summary, we have demonstrated the importance of selecting an appropriate method when assessing genotoxicity of pollutants in the environment. We have shown that methods that identify general damage, as well as those for specific types of damage, are both necessary to accurately assess the extent and type of genotoxicity occurring in an area contaminated with multiple contaminants. Specifically, the killifish at AWI exhibit higher levels of DNA damage. The damage is severe enough to cause significant damage in the chromosomal structure. Metabolites of BaP seem to be partially responsible for the DNA damage, but we did not see evidence of genotoxicity arising from oxidative DNA adducts. In addition, PAH concentrations in sediment samples were a better predictor of the biological effects of the compounds than PAH concentrations from tissue samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Concentrations of PAHs on EPA Priority Pollutant list from the sediment (3 separate samples from each site) and whole fish extracts ($n=5$ from each site) collected from the Superfund (square) and reference site (circle). Both tissue (open symbols) and sediment (filled symbols) samples from the Atlantic Wood Industries Superfund site had significantly higher levels of PAHs overall ($p < 0.001$). KC: King's Creek, AWI: Atlantic Wood Industries Superfund site. Inset shows total amount of priority pollutant list PAHs (ppPAHs) in the sample (ng/g wet weight). * indicates significant difference between KC and AWI for each chemical ($p \leq 0.05$).

Fig. 2. Atlantic killifish from the Atlantic Wood Industries Superfund site (black bar) show significantly higher levels of chromosomal damage in blood samples ($n = 20$ from each site), as indicated by half-peak coefficient of variation measured with flow cytometry, compared to killifish from King's Creek reference site (white bar). KC: King's Creek, AWI: Atlantic Wood Industries Superfund site. Graph represents mean value SEM. * indicates significant difference between the two groups ($p \leq 0.05$).

Fig. 3.

Oxidative and covalent DNA adducts in the liver of three individuals each from the Superfund site (AWI, black bar) and reference site (KC, white bar) killifish populations were measured by LC-MS/MS. Oxidative DNA damage (8-oxo-dG) is minimal and not different between the two populations. However, BaP-derived bulky adduct (BPDE-dG) was significantly higher in the Atlantic Woods Superfund site population. Bar represents mean value SEM. * indicates significant difference between the two groups ($p \leq 0.05$).