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Wildlife toxicology: biomarkers of genotoxic exposures at a hazardous waste site

Cole W. Matson^{1,2}, Annika M. Gillespie³, Chris McCarthy⁴, Thomas J. McDonald⁵, John W. Bickham^{2,6}, Robert Sullivan⁷, and K.C. Donnelly^{3,5,*}

¹Nicholas School of the Environment, Duke University, Durham, NC, 27708-0328, USA

²Department of Wildlife and Fisheries Sciences, Texas A&M University, College Station, TX, 77843-2258, USA

³Department of Veterinary Anatomy and Public Health, Texas A&M University, College Station, TX, 77843-4458, USA

⁴CH2M Hill, Houston, TX, 77040-5719, USA

⁵Department of Environmental and Occupational Health, School of Rural Public Health, Texas A&M University Health Science Center, College Station, TX, 77843-1266, USA

⁶Center for the Environment, Purdue University, West Lafayette, IN, 47907-2966, USA

⁷U.S. Environmental Protection Agency, Dallas, TX, 75202-2750, USA

Abstract

A large number of hazardous waste sites in the United States have undergone the initial stages of remediation or containment. At many of the remaining sites, the potential for exposure to ecological receptors is a primary concern. This manuscript reports on studies to investigate the impact on ecological receptors exposed to complex mixtures at a former creosote facility. Currently there are isolated areas on-site that were not addressed in the initial removal action that appear to be releasing polycyclic aromatic hydrocarbons (PAHs) to the surrounding environment. The U.S. EPA collected environmental samples and performed *ex situ* sediment bioassays to measure chronic toxicity; whereas, this study describes an *in situ* study to measure biomarkers of effect in two ecological receptors. Mosquitofish (*Gambusia affinis*) and cricket frogs (*Acris crepitans*) were collected from a small intermittent creek adjacent to the site, and reference stations. A weight-of-evidence ecological risk assessment was completed for the amphibian and fish communities. The ecological risk assessment was developed using analysis of media chemistry, body burden of specific PAHs, bioassay results, community surveys, and cellular genome size variation as a biomarker of genotoxicity. Flow cytometric estimates of chromosomal damage were significantly elevated for both mosquitofish and cricket frogs inhabiting the contaminated site, relative to at least one reference site. Surface water screening values for fish and amphibians exceeded screening values for PAHs by more than one order of magnitude in the on-site creek, and sediment PAH concentrations were extremely high (up to 1,549 µg/dry g). Tissue concentrations of PAHs were below screening values. Media chemistry, bioassay and genotoxicity data all support the same conclusion that on-site PAHs continue to impact aquatic receptors. The genotoxicity findings are consistent with and contribute to results of the weight-of-evidence ecological risk assessment. The results support continuing efforts to incorporate biomarkers as valuable lines of evidence within ecological risk assessment.

*To whom correspondence should be addressed: Department of Environmental and Occupational Health School of Rural Public Health Texas A&M University Health Science Center College Station, TX 77843-1266 kdonnelly@cvm.tamu.edu, telephone: (979) 847-8981.

Keywords

mosquitofish; *Gambusia affinis*; cricket frogs; *Acris crepitans*; flow cytometry; chromosomal damage; genotoxicity; biomarker; polycyclic aromatic hydrocarbons; DNA damage

Introduction

The release to the environment of chemicals capable of causing genetic damage represents a threat to human and ecological health. Genetic ecotoxicology is the study of pollutant-induced genetic changes in organisms (Depledge 1994). A broad range of potential biochemical and molecular effects result from exposure to certain xenobiotics including alterations to DNA (Landis and Yu 1995). One of these alterations, chromosomal breakage, can be readily measured in exposed organisms. Unlike the formation of DNA adducts and other subtle molecular changes, chromosome breaks cannot be repaired and are indicative of permanent genetic damage. These types of breaks have the potential to cause cancer and reduced fitness in the affected organism, and can be a useful tool for assessing ecosystem health at the individual and population levels (Bickham et al. 2000; Shugart et al. 2003). Exposure to certain environmental contaminants such as complex mixtures of polycyclic aromatic hydrocarbons (PAHs) have been shown to be genotoxic to animals in controlled laboratory experiments (Bickham et al. 1998b; Maria et al. 2003; Incardona et al. 2004; Peterson and Bain 2004; Winter et al. 2004) and through *in situ* field studies (Wirgin and Waldman 1998; Gauthier et al. 2004; Maria et al. 2004; Winter et al. 2004; Matson et al. 2005b; Barbee et al. 2008).

PAHs are primarily produced from the incomplete combustion of organic compounds such as coal, crude oil and natural gas. The PAH mixture investigated for this research is wood preserving waste (WPW) which is composed of creosote and diesel hydrocarbons. Creosote is obtained from the distillation of coal tar, which contains hundreds of organic compounds, at high temperatures. More than 100 chemicals have been identified in creosote (USEPA 2005a), of which greater than 85% are PAHs (Mueller et al. 1991). In the United States, creosote has been found in at least 33 of the 1,430 national priorities list sites identified by the United States Environmental Protection Agency (U.S. EPA) (ATSDR 2004). There are 17 priority pollutant PAHs detected in WPW that are considered a hazard to ecological receptors (Table 1), seven of which, including benzo[a]pyrene (BaP), have been classified by the U.S. EPA as probable human carcinogens (USEPA 2005b). Exposure to WPW has the potential to adversely affect wildlife that come in contact with contaminated environmental media. Due to environmental conditions, PAHs are generally persistent in contaminated sediments. Species that are particularly sensitive to pollution in aquatic environments are fish and amphibians. Studies suggest that fish and amphibians exhibit a variety of adverse effects (including carcinogenesis, teratogenesis, genetic damage, and mortality) from environmentally-relevant PAH exposures (Eisler 1987; Malins et al. 1988; Gagne et al. 1995; Bickham et al. 1998b; Wirgin and Waldman 1998; Monson et al. 1999; Wassenberg and Di Giulio 2004a, 2004b; Matson et al. 2005b; Timme-Laragy et al. 2007; Barbee et al. 2008). As a result, they may be used as sentinel species when investigating ecological risk at WPW contaminated sites. Due to the interspecies variability in contaminant toxicokinetics, and in food web dynamics it is important to have a multi-species approach to discern the genetic effects of contaminants across taxa (Bihari and Fafandel 2004). Cricket frogs (*Acris crepitans*) and mosquitofish (*Gambusia affinis*) were chosen for this research because of their abundance at the contaminated site and at associated reference areas. Additionally, the short generation times (< 1 yr) and relative site fidelity for these two species make them ideal for *in situ* biomarker studies.

One of the potential endpoints that can be measured in exposed species is chromosome damage using the flow cytometric method. Flow cytometry is useful for detecting sublethal, genetic damage from PAHs, which can be both mutagenic and clastogenic (Custer et al. 2000). This method has been shown to detect chromosome damage in a number of species exposed to complex mixtures of environmental contaminants (Bickham et al. 1988; McBee and Bickham 1988; George et al. 1991; Lamb et al. 1991; Bickham et al. 1992, 1994; Theodorakis et al. 2001; Matson et al. 2004, 2005a, 2005b; Barbee et al. 2008). Moreover, flow cytometry data tend to correlate well with petroleum product concentrations in animal tissues and specifically with PAHs (Bickham et al. 1998a; Custer et al. 2000). A recent paper by Matson et al. (2005a) used flow cytometry to demonstrate a correlation between chromosome damage in turtles and concentrations of three-ring PAHs.

Currently in ecological risk assessment, chemical analysis is often combined with *ex situ* toxicity tests and species surveys to determine environmental risk. *Ex situ* toxicity testing is valuable for determining the sensitivity of species or comparing chemical toxicity, but there is a lack of ecological realism in controlled laboratory environments (Preston and Shackelford 2002). Laboratory tests where the data generated are extrapolated to ecosystems often involve considerable uncertainty (Preston and Shackelford 2002). One of the major sources of uncertainty results from the use of laboratory organisms that may not be representative of indigenous species (La Point and Waller 2000). In addition, laboratory tests are typically conducted with single compounds, whereas ecological receptors are most often exposed to complex mixtures. Laboratory testing usually overlooks the physical and chemical variation that is characteristic of environmental settings (Preston et al. 2001). Although standard methods for ecological risk assessment usually assume additive effects from multiple stressors, synergistic or antagonistic interactions are certainly possible, and may not be uncommon (Folt et al. 1999; Wassenberg and Di Giulio 2004a; Timme-Laragy et al. 2007). Moreover, biomarker methods such as genotoxicity testing are rarely employed in the risk assessment process primarily because the results cannot easily be translated into remedial goals for the contaminated site.

Under field conditions, ecological receptors must cope with multiple anthropogenic and natural stressors that may affect ecosystem health. For example, toxic anthropogenic chemicals and natural variability in total suspended solids or dissolved oxygen can stress aquatic environments (Preston and Shackelford 2002). With multiple stressors, it is a challenge in field studies to create a link between stressor and effect (Preston and Shackelford 2002). Despite these challenges, *in situ* studies are valuable because effects seen in the field are a result of the net effects of stressors and can be directly observed (Preston and Shackelford 2002).

This manuscript describes a study to incorporate genotoxicity testing into ecological risk assessment in a weight-of-evidence approach. The primary goal was to generate data regarding biomarkers of exposure in multiple ecological receptors. For those areas where elevated levels of contamination have been observed in environmental media or biota samples, the flow cytometry data provides an alternative metric to determine if exposed receptors are experiencing increased levels of genetic damage. A combined testing protocol that integrates *in situ* and *ex situ* data can be used as a supplement to chemical analysis and may help to increase the accuracy of the risk assessment process.

Materials and methods

Site History

Samples were collected from a former wood preserving facility in the southern United States that was active until 1993 (USEPA 2004). The facility used coal-tar creosote for wood

treatment operations and disposed of waste on-site into four unlined ponds. During the initial removal action these ponds were drained and the water treated. The remaining sludge from the ponds was excavated and stored on-site in a fenced waste cell. This temporary waste cell was completed in 1995, capped and the area seeded (USEPA 2004). However, there are some areas on site that were not targeted during the removal action and still contain visible product. One of these areas is on the west side of the property along the bank of a creek (Figure 1). This unnamed creek feeds into a larger creek which is used for sport fishing (USEPA 2004).

Sample Collection

Mosquitofish and frogs were collected under a scientific collecting permit from Texas Parks and Wildlife Department and an approved animal use protocol on file at Texas A&M University. Samples were collected in May 2004, August 2004, February 2005, and May 2005 from the on-site creek (Figure 1). Reference sites were a private rural pond (Ref.-1), and a state park less than 15 miles from the creosote site (Ref.-2). During these sampling events, the level of rainfall and amount of water in the intermittent on-site creek was highly variable. In May 2004 approximately 7 inches of total rain was recorded over the days that sampling took place plus one week prior to sample collection. In August 2004 and February 2005 rainfall was roughly 1 and 3 inches, respectively, over the same time period. In May 2005, no rainfall registered during the sampling event or one week prior to collection.

Cricket frogs were collected along the banks of the intermittent on-site creek as well as from the top of the capped waste cell on-site (Figure 1). Reference animals were collected from the state park (Ref.-2), and from the banks of a private pond (Ref.-3).

Mosquitofish (*Gambusia affinis*) were collected using hand-held dip nets from the on-site creek (Creek-1-4) and associated references during May 2004 ($n = 1$ composite sample for both the on-site creek (Creek-1-4) and Ref.-2), August 2004 (Creek-1-4 $n = 10$, Ref.-1 $n = 22$), February 2005 (Creek-1-4 $n = 18$, Ref.-1 $n = 23$, Ref.-2 $n = 14$), and May 2005 (Creek-1-2 $n = 26$, Ref.-2 $n = 38$). Approximately 1-4 μL of caudal vein blood was collected from individual fish using a heparinized microhematocrit capillary tube (Fisher Scientific, 22-362566) and stored in 25 μL of citrate buffer (Vindelov and Christensen 1990) in cryovials (no blood was collected from fish in May 2004). All blood samples and fish carcasses were frozen on liquid nitrogen until they could be stored at -80°C .

Cricket frogs (*Acris crepitans*) were collected in August 2004 using hand-held dip nets from along the creek bank, on top of the waste cell, and from associated references (Creosote Site $n = 18$, Ref.-3 $n = 8$). Animals were sacrificed, placed in cryovials, frozen whole on liquid nitrogen and then stored at -80°C before dissection.

Environmental media including co-located surface water samples were collected in August 2004, and May 2005. In August 2004 surface water samples were collected from the on-site creek, Ref.-1, Ref.-2, and Ref.-3. In May 2005 surface water and sediment samples were collected from the on-site creek and Ref.-2. Additionally, sediment samples were also collected from Ref.-3. Surface waters were collected from points along the on-site creek by hand using 1-L amber glass I-CHEM certified sampling bottles (VWR Scientific). Each sample represents water from a single point within a shallow narrow creek. Surface sediments were also collected at the same sites by hand using pre-cleaned stainless steel trowels and stored in 16-oz. glass I-CHEM certified sampling jars (VWR Scientific). After collection, the water and sediments were transported on ice and stored at 4°C prior to extraction.

Chemical Extraction

For tissue extractions, cricket frogs from August 2004 were grouped for extraction according to high and low HPCV values. Composite samples were also made for mosquitofish. These groupings were dependent on the number of specimens collected and the amount of tissue that was available for extraction. Composite tissues were ground in a blender and dried with hydromatrix (Varian Inc., Palo Alto, CA, part #198003). The samples were extracted using a Dionex Model 200 Accelerated Solvent Extractor (ASE) (USEPA, 1996b) using pesticide-grade methylene chloride (VWR, BJ300-4). Tissue sample extracts were then run through silica gel columns (Resprep, Bellefonte, PA, part #24038) and eluted with 1:1 methylene chloride:pentane. The extracts were concentrated to 3 mL in a water bath (60°C), re-suspended in methylene chloride, and processed through HPLC to minimize matrix interference. All samples were then analyzed using Gas Chromatography/Mass Spectrometry (GC/MS) for PAHs and semi-volatile organics (SVOCs) (USEPA 1996c).

Separatory funnel liquid-liquid extraction using pesticide-grade methylene chloride (VWR, BJ300-4) was performed on surface waters following the methods outlined in U.S. EPA method 3510C (USEPA 1996a).

Sediment extractions were performed using a Dionex (Dionex Corp., Sunnyvale, CA) Model 200 Accelerated Solvent Extractor (ASE) using a 1:1 ratio of hexane and acetone (U.S. EPA Method 3545, U.S. EPA 1996). Approximately 30 g of sediment from each sample was oven dried at 60°C for 16 hours. Ten grams (± 1 g) of the dried sediment was weighed for extraction. Following extraction, samples were transferred to pre-weighed sterile culture tubes with Teflon-lined caps, dried under a stream of nitrogen and stored at 4°C until chemical analysis.

Chemical Analysis

Samples were analyzed for PAHs, PCP and other semivolatile organic compounds (SVOCs) using USEPA method 8270C (USEPA 1997). Analyses were conducted on a Hewlett-Packard 5890 Series II gas chromatograph with a 5972 mass selective detector in selected ion monitoring mode. A 60 m $\times$ 0.25 mm ID $\times$ 0.25 mm film thickness column (Agilent Technologies, Palo Alto, CA) was used. The injection port was maintained at 300°C and the transfer line at 280°C. The temperature program was as follows: 60°C for 6 min, increased at 12°C/min to 180°C and then increased at 6°C/min to 310°C and held for 11 min for a total run time of 47 min.

Flow Cytometry

Flow cytometry was used to detect chromosome damage and DNA changes in the collected specimens following the methods of Vindelov and Christensen (1990, 1994). All samples were randomized before processing to avoid experimental bias. For the *Actris* samples, a small portion of the livers was excised for analysis while the carcasses were still frozen. The remainders of the carcasses were stored again at -80°C until contaminant screening. Due to the small amount (1-4 μ L) of blood collected from the mosquitofish, the entire 25 μ L blood +citrate buffer sample from each fish was used in the analysis. Samples were quickly thawed and added to a trypsin/detergent solution for digestion. The trypsin inhibitor solution and RNase were added after 10 min. to stop the reaction and to degrade the RNA (which can also be stained by the propidium iodide). The solution was then filtered through a 30 μ m nylon mesh and 375 μ L of propidium iodide (PI) was added. The samples were kept on ice for 15 min. and then analyzed on a Coulter Epics Elite flow cytometer (Beckman Coulter Inc., Fullerton, CA). Cells were illuminated with a laser (Coherent, Santa Clara, CA, USA) at 514 nm and 500 mW of power to excite the PI, and fluorescent emission was measured. Cells were gated on forward scatter, side scatter, and the ratio of peak to integrated

fluorescence. From each sample, 10,000 nuclei which satisfied all gating parameters were measured and intercellular variation in DNA content was reported as either full or half peak coefficient of variation (FPCV, HPCV). Previous flow cytometry studies in fish suggest that FPCVs are generally a better estimator of DNA damage (Barbee et al. 2008); however, amphibian studies have performed better with HPCVs (Matson et al. 2005b). Samples that did not yield 10,000 nuclei within a 4-min run were excluded from the statistical analyses.

Statistics

All flow cytometry data were compared using SPSS ver. 15.0 software (SPSS Inc., Chicago, IL). Reference and experimental samples were compared by testing the samples for normality (Shapiro-Wilk test for normality) and equal variance (Levene's test for equal variances). To allow data from multiple experiments to be combined and because of non-normally distributed data, the mosquitofish FPCVs were rank transformed by experiment. Additionally, mosquitofish data were analyzed in two separate analyses to test for differential biomarker responses in flood versus drought conditions, and because of a combinability issue with experiments that did not contain all of the sites. Therefore, all samples from the Aug. 2004 and Feb. 2005 collections were combined for the first comparison. Whereas, the May 2005 samples were analyzed independently for the second comparison. As mosquitofish were only collected from the on-site creek and Ref.-2 in May 2005, the second comparison excluded Ref.-1. An ANOVA with Fisher's LSD *post hoc* multiple comparisons was used on the rank transformed data to test for differences among the two references and the on-site creek in the first comparison. For the second comparison, a Welch's *t*-test was used to look for differences between the on-site creek and Ref.-2. For the cricket frog data, which were normally distributed and which came from a single experiment, an ANOVA with Fisher's LSD *post hoc* multiple comparisons was used to evaluate differences between the on-site creek and reference cricket frogs. For all tests, *p* 0.05 was considered significant.

Results

Mosquitofish (*Gambusia affinis*)

Composite samples of mosquitofish from the on-site creek, Ref.-1, and Ref.-2 were analyzed for PAHs. Data from the chemical analyses revealed elevated levels of total PAHs in mosquitofish from the on-site creek compared to the references (Figure 2). The maximum tissue concentrations of priority pollutant PAHs were seen in the composite sample collected in May 2004 during a flooding event. At this time the fish were evenly distributed throughout the sampled creek area. Looking at the maximum surface water concentrations taken from the on-site creek in May 2005 (a year later), eight of the priority pollutant PAHs were above the screening level criteria for fish in surface waters (Table 1). During the period in which the surface water sample in Table 1 was taken (May 2005), the creek water level was low and there were free standing pools of water. The pool from which this surface water was sampled yielded no aquatic organisms. Adjacent pools of water of similar size and depth did yield live fish suggesting that contamination was playing a role in fish survival. The data also suggest that seasonal variability in the creek water levels affect fish exposure. Exposure appears to be highest during flooding events when the increased water flux increases migration of contaminants through the water column. During dry periods, fish isolated to the water pools with highest contaminant concentrations are unable to survive. Whereas, fish isolated in cleaner pools of water (such as those fish we collected in May 2005) would have increased survivability and decreased contaminant body burden. Table 2 shows the variability in the co-located water samples collected from the reference sites and the on-site creek in August 2004 and May 2005. In May 2005, with no observable water flow, there was a definite contamination gradient seen as samples were collected from areas

with the highest concentration (Creek-3 & 4) where there was no life, to pools with lower concentrations (Creek-1 & 2) from which fish were taken (Table 2).

The flow cytometry data for mosquitofish collected in August 2004 from the on-site creek and Ref.-1 were not normally distributed. Using a non-parametric test there was not a significant difference in full-peak coefficients of variation (FPCVs) between the sites (Mann-Whitney U, $p = 0.084$). It was decided to repeat sampling at the same locations, increase the sample numbers and add another reference for comparison. After the February 2005 collections, combined FPCV data from the on-site creek, Ref.-1, and Ref.-2 were not normally distributed. Data were fractionally rank transformed by experiment and analyzed using a one-way ANOVA, there was a significant difference between the sampling sites ($p = 0.028$). A Fisher's LSD *post hoc* comparison was used to compare the 3 different sites. There were significant differences between the rank transformed FPCV values from the on-site creek and both references (Figure 3A; Ref.-1, $p = 0.048$; Ref.-2, $p = 0.012$). The contaminated site showed significantly elevated levels of genetic damage compared to both references.

The final sampling event was conducted in May 2005. Ref.-1 was deleted from the sample design in favor of using Ref.-2 located within the state park. This decision was based on the close geographic proximity of Ref.-2 to the experimental site, and the fact that the previous experiments suggested that levels of genetic damage were higher at Ref.-1. Using FPCV values, the data were not normally distributed (Shapiro-Wilk test; $p < 0.05$). Following a fractional rank transformation of the data, a non-parametric Welch's *t*-test was used to compare the sites. There was not a significant difference between the sampling sites (Figure 3B; $p = 0.288$). It is important to again note that during this collection period mosquitofish were not collected from the most contaminated areas as this section of the on-site creek was devoid of aquatic life.

Cricket Frogs (*Acris crepitans*)

Initially, the specimens collected in August 2004 along the banks of the on-site creek and from the top of the waste cell near the on-site pond were grouped separately during flow cytometry analysis. However, statistically there was no difference between these groups (Student's *t*-test, $p = 0.95$). Thus, all on-site specimens were combined as one site in the final analysis. HPCV values were normally distributed. Using an ANOVA, there was a significant difference between contaminated and reference sites ($p = 0.036$). A Fisher's LSD *post hoc* comparison was used to evaluate pairwise differences among sites. There was a significant difference in HPCV values between the contaminated site and Ref.-3 ($p = 0.017$) with the contaminated site showing elevated levels of genetic damage compared to the reference (Figure 4). However, there was not a significant difference between the contaminated site and the reference station at the state park (Ref.-2) ($p = 0.908$).

Following flow cytometry analysis, individual frogs were equally grouped according to low and high HPCVs for chemical analysis. While a correlation analysis of body burden and HPCVs would have been preferable, pooling of individuals was necessary for the chemical analysis due to body mass limitations. Levels of genetic damage corresponded well with total PAH concentrations in the pooled tissues (Figure 5).

Co-located surface water samples were taken from the contaminated site and associated reference stations. Table 3 shows the maximum priority pollutant PAH concentrations detected in surface waters collected from the on-site creek and the corresponding lowest observed effect concentrations and hazard quotients for amphibians. At the creek, the levels of PAHs detected in the surface water exceeded a hazard index of one ($HI = 20$) (Table 3). A hazard index above one indicates that adverse effects in amphibians from total PAH

exposure is likely. The tissue sample that yielded the highest level of PAHs did not surpass the toxicity reference value (TRV) for benzo[a]pyrene (10,200 ng/wet g) which was used as a surrogate for PAHs in the remedial investigation (CH2MHill 2005) (Table 3). Table 2 shows PAH chemistry from the co-located surface water samples collected in August 2004 during the time of cricket frog sampling. Two water samples from the on-site creek were found to have approximately twice the amount of total PAHs compared to Ref.-3. Water samples from the on-site creek also had approximately fifty times the amount of BaP compared to Ref.- 3 (Table 2).

Sediment On-Site Creek

Sediments were collected from the on-site creek and two reference stations in May 2005. Table 4 shows the priority pollutant chemicals detected in the sediments and their corresponding screening level criteria for the benthic communities at the on-site creek. Sediments collected from the on-site creek, particularly the two downstream collection points (Creek-3,4), had more than 100-fold higher levels of PAHs when compared to the reference samples (Table 5). Comparing contaminated levels in on-site sediments with the highest PAH concentrations to screening level criteria for benthic organisms, it is apparent that PAHs in the sediments exceed the screening level criteria at the on-site creek.

For the remedial investigation, site- and chemical-specific toxicity reference values were derived for benthic invertebrates using *ex situ* toxicity tests. As shown in Table 6, hazard quotients (HQs) for individual chemicals based on the no observed effect concentration (NOEC) were greater for low molecular PAHs (LPAHs, molecular weight less than 200 atomic mass units) than high molecular weight PAHs (HPAHs, molecular weight less than 200 atomic mass units). The total NOEC-based hazard index (HI) for LPAHs, measured as the sum of HQs, ranged from 27 to 181,064 with an average of 19,704 while the HI for HPAHs ranged from 12 to 2,229 with an average of 329. HIs based upon the lowest observed effect concentration (LOEC) were significantly lower. The total LOEC-based hazard index (HI) for LPAHs ranged from 2 to 12,820 with an average of 1,435 while the HI for HPAHs ranged from 0.15 to 684 with an average of 96 (CH2MHill 2005).

Discussion

A series of environmental and biological samples were collected during a one year period from several reference locations and stations on a creek at a facility contaminated with wood preserving waste. Sampling was done to screen for PAH concentrations in the media and biota and to measure levels of genetic damage in the organisms sampled. Biological samples including mosquitofish (*Gambusia affinis*) and cricket frogs (*Acris crepitans*) were obtained from sampling stations. Although the results varied among species, mosquitofish and frogs collected from the contaminated site exhibited increased markers of genotoxic damage as measured by flow cytometry compared to reference specimens. Markers of genetic damage were consistent with increased concentrations of PAHs in tissues compared to reference animals, despite the fact that tissue levels were far below the lowest observed effect concentrations reported in the remedial investigation (CH2MHill 2005). Surface water and sediment sample PAH concentrations exceeded screening level criteria for fish, amphibians, and benthic organisms at the on-site creek.

Genetic damage in mosquitofish collected from the on-site creek was variable over the duration of our studies. This may be a result of changes in PAH concentrations in the water. However, it may also be a result of increased water flow due to heavy rains, and/or due to warmer water temperatures. Although mosquitofish collected in May 2005 showed elevated levels of PAHs in their tissues compared to the reference fish collected in May 2005, levels of genetic damage at the contaminated site were not statistically different than the

references. One of the challenges in performing ecological risk assessments is finding appropriate references. Due to the high variability inherent in ecosystems, references close to the study sites could be considerably different and may also be contaminated. Reference 2 in this study has several mercury advisory signs posted throughout the park. It is known that mercury has the ability to cause chromosome damage (Zucker et al. 1990; De Flora et al. 1994) and may have contributed to elevated genetic damage in the fish and amphibians collected from areas within Ref.-2. While concerns about mercury advisories at Ref.-2 would seem to preclude its use in this type of study, this contamination is likely the result of airborne deposition and not via a point-source. This entire region has mercury advisories in virtually all of the monitored public lakes, suggesting that other sites, including small private lakes and streams, would likely be similarly contaminated. There has been debate about whether to use references close to the experimental sites that might have contamination from aerial deposition or other anthropogenic sources, or try to find distant sites with little contamination of any kind. Given that studies at highly contaminated sites are generally concerned with the effects of the point-source pollution at the site, we chose the close reference with “normal background” levels of pollution.

Moreover, water levels in the on-site creek appear to correspond to increased levels of genetic damage. When water levels were at their highest in May 2004, tissue body burden in mosquitofish was also at its highest. In August 2004 and February 2005, water levels were higher than during May 2005 which might help to explain the increased levels of genetic damage seen in the fish in August 2004 and February 2005 compared to May 2005. This result is likely related to the isolation of the most contaminated section of the creek. During dry periods mosquitofish would not be able to move through this section of the creek. Undoubtedly, mosquitofish would have been trapped in this large highly-contaminated section of the on-site creek. However, we were unable to find evidence of any living organisms within the most contaminated pool when water levels were low. Since mosquitofish were clearly able to survive in even smaller pools of water just upstream of the contaminated stretch of the creek, we concluded that their absence was likely a direct result of the PAH contamination. It is impossible to say whether this was through direct toxicity, starvation resulting from a lack of invertebrate prey, or some other more complex combination of factors. The highest levels of genetic damage were observed in mosquitofish collected from the most contaminated areas of the on-site creek during flood conditions. These observations suggest that the temporal variability in contaminant levels and genetic damage may result from alterations in environmental conditions produced by heavy rainfall events. These data confirm the critical influence environmental conditions can have on environmental monitoring and biomarker status, and also the value of collecting samples at multiple time points and under different environmental conditions. We might have missed significant biomarker responses had we collected samples only once. A strength and weakness of *in situ* biomarker testing is the inclusion of the many variables present at contaminated sites. While this type of testing allows for all of these variables to be included, this complexity can confound interpretation of observed responses.

With regard to the amphibians, frogs are biphasic. As tadpoles they live in the water and feed on detritus. As adults they feed on insects and other organisms. They can be highly susceptible to contaminants and are exposed via dietary routes and from dermal absorption through their permeable skin. Frogs are an essential link in the food chain in terms of being predators themselves and also being preyed upon by larger aquatic and terrestrial predators such as fish, snakes, foxes, raccoons and hawks found at the site investigated. Changes in their individual fitness due to contaminant exposure could have effects at the population levels and disrupt ecosystem balance at these sites.

The results of genotoxicity testing, *ex situ* toxicity tests and chemical analysis indicate that ecological receptors are impacted by residual contaminants at this former wood preserving facility. Although genotoxicity data were not used to generate remediation goals, the flow cytometry data coupled with chemical analysis demonstrated that genotoxicity tests can be incorporated as lines of evidence into ecological risk assessment provided they are considered early in the process and specifically designed to be part of the remedial investigation. This collaborative effort may help improve the risk assessment process and enhance our understanding of the impact that certain xenobiotics have on genetic stability in contaminated ecosystems.

The use of *in situ* animal testing provides a unique opportunity to test for the interactive effects of all of the local stressors and the environment on wildlife populations. While utilizing biomarkers of effect in risk analysis is a complicated process, it is easy to recognize the relevance of these data. In situations where complex mixtures of contaminants are involved, which we would argue is very common, *in situ* animal testing may be the only method to adequately test for biological effects. Clearly there are still many issues to be worked out regarding the relative sensitivities of different ecological receptors. As we build up more extensive databases of wildlife biomarker responses to contaminants, there will be less uncertainty involved in calibrating data to account for differential susceptibility. The utility of *in situ* biomarker data is dramatically enhanced when multiple ecological receptors are examined, particularly when species are included that represent different trophic levels, exposure histories, habitats, etc. The difficulty will primarily involve weighting observed biomarker responses in an ecological risk assessment.

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Figure 1.
Map of the contaminated site showing sampling locations for mosquitofish, cricket frogs, and surface water and sediment samples.

Figure 2.

Total PAH concentration in mosquitofish (*Gambusia affinis*) composite tissue samples from the contaminated on-site creek and two reference sites (Ref.-1, $n = 2$; Ref.-2, $n = 9$; Creek, $n = 7$). Samples were collected between May 2004 and May 2005. Data were analyzed with an ANOVA followed by Fisher's LSD *post hoc* pairwise comparisons. Samples that were determined to be significantly different ($p < 0.05$) are represented by different letter codes above the bars.

Figure 3.

Fractional rank transformed full peak coefficient of variation (FPCV) data in mosquitofish, a flow cytometry method for estimating chromosomal damage. Data represent mosquitofish collected from the on-site creek and two references during both flood and drought conditions. Sample sizes are provided within the bars on the graph. (A) Samples collected during periods of active rainfall and thus a vigorously flowing creek. This allowed for the collection of mosquitofish throughout the creek collection zone noted on Figure 1. Data were analyzed with an ANOVA followed by Fisher's LSD *post hoc* pairwise comparisons. Samples that were determined to be significantly different ($p < 0.05$) are represented by different letter codes above the bars. (B) Samples collected during low rainfall period with no observable flowing water. Creek was represented by a series of isolated pools of water. As noted on Figure 1, no fish were found within the more contaminated downstream section of the creek (Creek-3,4) during this collection. As a result, these data are only representative of the less contaminated upstream sites on the creek (Creek-1,2). The on-site creek had lower estimates of chromosomal damage than Ref.-2 for this collection; however, these data were not statistically significant (Welch's *t*-test, $p = 0.288$).

Figure 4.

Half peak coefficient of variation (HPCV) data in cricket frogs as a measure of chromosomal damage. Data represent cricket frogs collected in August 2004. Frogs collected from the banks of the on-site creek and the top of the capped waste cell were compared to two separate references. An ANOVA followed by Fisher's LSD *post hoc* pairwise comparisons were used to evaluate differences among sites. Samples that were determined to be significantly different ($p \leq 0.05$) are represented by different letter codes above the bars. Sample sizes are provided within the bars on the graph.

Figure 5. Total PAH concentration in cricket frog (*Acris crepitans*) tissues grouped according to low and high HPCV values for the contaminated site and two reference sites. Samples represent individuals collected in August, 2004 ($n = 1$ composite sample for each HPCV group).

Table 1

Priority pollutant PAHs in surface water from site creek and corresponding screening level criteria for fish. The composite mosquitofish sample collected in May 2004 represents the maximum tissue concentration of priority pollutant PAHs from the site creek

^a Chemical Class	^b Chemical	^c Maximum Concentration (surf. water) ng/L	^d Screening Level Criteria ng/L	Maximum Tissue Concentration ng/wet g
LMW PAH	Naphthalene	301	250,000	147
LMW PAH	Acenaphthylene	4,000	5,800	7
LMW PAH	Acenaphthene	290	2,300	675
LMW PAH	Anthracene	10,300	300	21
LMW PAH	Fluorene	2,320	11,000	543
LMW PAH	Dibenzofuran	238	NSL	511
LMW PAH	Phenanthrene	32,700	30,000	954
HMW PAH	Fluoranthene	58,300	6,160	174
HMW PAH	Pyrene	45,600	7,000	72
HMW PAH	Benz[a]anthracene	19,000	34,600	4
HMW PAH	Chrysene	31,300	7,000	6
HMW PAH	Benzo[b]fluoranthene	36,200	27	4
HMW PAH	Benzo[k]fluoranthene	11,600	27	1
HMW PAH	Benzo[a]pyrene	13,300	14	12
HMW PAH	Indeno[1,2,3-c,d]pyrene	4,830	27	1
HMW PAH	Dibenzo[a,h]anthracene	1,250	5,000	0.3
HMW PAH	Benzo[g,h,i]perylene	3,330	7,640	1

^aLMW PAH = low molecular weight (< 200 atomic mass units) PAH, HMW PAH = high molecular weight (> 200 atomic mass units).

^b**Bold** denotes carcinogenic PAHs (cPAHs)

^c**Bold italics** denote tissue concentrations that are above screening level criteria for fish.

^dScreening level criteria for surface waters were derived from sources detailed in the remedial investigation (CH2MHill, 2005), NSL = no screening level.

Table 2

Summary chemical analysis of co-located surface water samples collected from the onsite creek and corresponding references during August 2004 and May 2005 sampling events. Data were averaged for locations with more than 1 sample collected. For averaged samples, data are presented as mean ng/L $\pm$ SEM

Sample date & location	<i>n</i>	BaP ng/L	cPAHs ng/L	ppPAHs ng/L	tPAHs ng/L
Aug. 2004					
Ref.-1	2	11 $\pm$ 0.05	191 $\pm$ 35	4,707 $\pm$ 2,721	7,598 $\pm$ 4,070
Ref.-2	3	21 $\pm$ 15	393 $\pm$ 286	5,129 $\pm$ 1,687	8,979 $\pm$ 3,238
Ref.-3	3	20 $\pm$ 7	500 $\pm$ 100	6,700 $\pm$ 1,600	10,400 $\pm$ 2,000
Creek-2	1	86	714	3,649	5,881
Creek-3	1	1,140	8,281	16,168	24,377
Creek-4	1	828	6,081	15,572	22,719
May 2005					
Ref.-2	2	5 $\pm$ 4	88 $\pm$ 44	1,813 $\pm$ 1,100	2,977 $\pm$ 1,763
Creek-1	1	23	383	1,405	2,283
Creek-2	1	303	3,942	6,531	10,613
Creek-3	1	2,390	38,434	121,121	186,555
Creek-4	1	13,300	117,480	274,859	555,372

BaP = total benzo[a]pyrene, cPAHs = total carcinogenic polycyclic aromatic hydrocarbons (*n* = 7)

ppPAHs = total priority pollutant PAHs (*n* = 17)

tPAHs = total PAHs.

Table 3

Priority pollutant PAHs in surface water and corresponding LOEC HQ values for amphibians at the on-site creek. The composite cricket frog sample with the maximum tissue concentrations of priority pollutant PAHs from the contaminated site is also shown

Chemical	Maximum Conc. Creek (surf. water) ng/L	Species (used to derive LOEC) (CH2MHill, 2005)	Receptor Life Stage	Normalized LOEC ng/L (CH2MHill, 2005)	^a LOEC HQ Creek	Maximum Tissue Conc. ng/wet g
Naphthalene	301	<i>Xenopus laevis</i>	Tadpole	210,000	0.0014	10
Acenaphthylene	4,000	<i>Xenopus laevis</i>	Tadpole	210,000	0.0190	1
Acenaphthene	290	<i>Xenopus laevis</i>	Tadpole	210,000	0.0014	49
Anthracene	10,300	<i>Rana pipiens</i>	Embryo	2,500	4.1200	2
Fluorene	2,320	<i>Xenopus laevis</i>	Tadpole	210,000	0.0111	29
Phenanthrene	32,700	<i>Xenopus laevis</i>	Tadpole	210,000	0.1557	144
Dibenzofuran	238	NA	NA	NA	NA	25
Fluoranthene	58,300	<i>Rana pipiens</i>	Tadpole	9,000	6.4778	74
Pyrene	45,600	<i>Rana pipiens</i>	Tadpole	14,000	3.2571	50
Benz[a]anthracene	19,000	<i>Pleurodeles waltl</i>	Embryo	50,000	0.3800	7
Chrysene	31,300	<i>Pleurodeles waltl</i>	Embryo	50,000	0.6260	33
Benzo[b]fluoranthene	36,200	<i>Pleurodeles waltl</i>	Embryo	50,000	0.7240	5
Benzo[k]fluoranthene	11,600	<i>Pleurodeles waltl</i>	Embryo	50,000	0.2320	2
Benzo[a]pyrene	13,300	<i>Pleurodeles waltl</i>	Embryo	50,000	2.6600	0.4
Indeno[1,2,3-c,d]pyrene	4,830	<i>Pleurodeles waltl</i>	Embryo	50,000	0.0966	1
Dibenzo[a,h]anthracene	1,250	<i>Pleurodeles waltl</i>	Embryo	50,000	0.0250	0.2
Benzo[g,h,i]perylene	3,330	<i>Pleurodeles waltl</i>	Embryo	50,000	0.0666	0.5
				^b HI=	20	

^aLOEC HQ = lowest observed effect concentration hazard quotient; formula = maximum surface water concentration ng/L / normalized LOEC ng/L.

^bHI = Hazard Index (HI = HQ₁ + HQ₂...HQ₁₆ for each priority pollutant listed).

NA = Not available

Table 4

Priority pollutant chemicals (PAHs and PCP) in sediments collected in May 2005 and corresponding screening level criteria for benthic communities at the on-site creek

Chemical	^a Maximum Concentration Creek ng/dry g sed.	^b Screening Level Criteria ng/dry g sediment
Acenaphthylene	<i>1,491</i>	6
Acenaphthene	<i>39,023</i>	7
Anthracene	<i>47,487</i>	57
Fluorene	<i>143,059</i>	77
Dibenzofuran	<i>110,103</i>	NSL
Phenanthrene	<i>214,214</i>	204
Fluoranthene	<i>168,525</i>	423
Pyrene	<i>113,848</i>	195
Benz[a]anthracene	<i>83,139</i>	108
Chrysene	<i>62,467</i>	166
Benzo[b]fluoranthene	<i>51,082</i>	6
Benzo[k]fluoranthene	<i>20,747</i>	240
Benzo[a]pyrene	<i>7,408</i>	150
Indeno[1,2,3-c,d]pyrene	<i>6,891</i>	200
Dibenzo[a,h]anthracene	<i>2,007</i>	33
Benzo[g,h,i]perylene	<i>3,191</i>	170
Total PAH	<i>1,548,687</i>	12,200

^a Bold italics denote sediment concentrations that are above screening level criteria for the benthic community.

^b Screening level criteria for sediments were derived from sources detailed in the remedial investigation (CH2MHill, 2005).

Table 5

Summary chemical analysis of co-located sediment samples collected from the on-site creek and corresponding references during May 2005 sampling events. Data were averaged for locations with more than 1 sample collected. For averaged samples, data are presented as mean ng/dry g sediment $\pm$ SEM

Sample date & location	N	BaP ng/dry g sediment	cPAHs ng/dry g sediment	ppPAHs ng/dry g sediment	tPAHs ng/dry g sediment
May 2005					
Ref.-2	2	12 $\pm$ 4.5	581 $\pm$ 511	1,984 $\pm$ 893	2,897 $\pm$ 1,267
Ref.-3	3	5 $\pm$ 0.4	47 $\pm$ 7	548 $\pm$ 261	828 $\pm$ 315
Creek-1	1	84	648	4,747	6,442
Creek-2	1	521	6,316	10,460	17,166
Creek-3	1	12,474	110,404	329,695	519,244
Creek-4	1	7,408	233,740	1,076,066	1,548,689

BaP = benzo[a]pyrene, cPAHs = total carcinogenic polycyclic aromatic hydrocarbons ($n = 7$), ppPAHs = total priority pollutant PAHs ($n = 17$), and tPAHs = total PAHs.

Table 6

Hazard quotients for benthic invertebrates using site-specific data from the remedial investigation

Chemical Group	Parameter	Final NOEC	Final LOEC	Min NOEC HQ	Avg NOEC HQ	Max NOEC HQ	Min LOEC HQ	Avg LOEC HQ	Max LOEC HQ
LPAH	2-Methylnaphthalene	0.41	0.68	0.53	258.39	2,139.02	0.32	156.95	1,299.26
LPAH	Acenaphthene	0.19	2.25	1.25	939.31	7,513.51	0.10	77.40	619.15
LPAH	Acenaphthylene	0.12	NA	1.81	28.74	242.98	0.00	0.00	0.00
LPAH	Anthracene	0.43	0.72	0.54	67.19	490.65	0.32	40.22	293.71
LPAH	Fluorene	0.23	1.92	1.02	591.07	4,330.40	0.12	69.88	511.98
LPAH	Naphthalene	0.01	0.18	20.66	17,398.25	163,207.55	1.21	1,021.73	9,584.49
LPAH	Phenanthrene	0.88	5.40	0.26	420.84	3,139.93	0.04	68.50	511.11
HPAH	Benzo(a)anthracene	1.17	NA	0.49	28.24	195.73	0.00	0.00	0.00
HPAH	Benzo(a)pyrene	0.79	NA	0.73	13.96	97.47	0.00	0.00	0.00
HPAH	Benzo(b)fluoranthene	0.98	NA	0.59	13.42	87.19	0.00	0.00	0.00
HPAH	Benzo(g,h,i)perylene	0.28	NA	1.95	10.97	63.21	0.00	0.00	0.00
HPAH	Benzo(k)fluoranthene	0.83	NA	0.70	12.10	81.63	0.00	0.00	0.00
HPAH	Chrysene	2.02	NA	0.29	14.39	98.02	0.00	0.00	0.00
HPAH	Dibenz(a,h)anthracene	0.13	NA	4.18	17.10	66.79	0.00	0.00	0.00
HPAH	Fluoranthene	1.80	3.99	0.13	111.51	811.11	0.06	50.30	365.91
HPAH	Indeno(1,2,3-c,d)pyrene	0.30	NA	1.80	11.32	68.42	0.00	0.00	0.00
HPAH	Pyrene	1.28	2.66	0.18	95.59	659.38	0.09	46.08	317.89
LPAH HI				27.44	19,703.80	181,064.04	2.22	1,434.69	12,819.70
HPAH HI				11.51	328.59	2,228.95	0.15	96.39	683.81
TPAH HI				38.96	20,032.39	183,292.99	2.37	1,531.07	13,503.50

All concentrations are in mg/kg, HPAH = high molecular weight PAHs (> 200 atomic mass units), LPAHs = low molecular weight PAHs (< 200 atomic mass units), NOEC = no observed effect concentration, LOEC = lowest observed effect concentration, HQ = hazard quotient (measured concentration divided by NOEC or LOEC), HI = hazard index (Sum of HQs for individual chemicals)