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The effects of urbanization on *Lepomis macrochirus* using the comet assay

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ABSTRACT

Urbanization has been linked to increased concentrations of polycyclic aromatic hydrocarbons in natural waterways. This study was designed to examine the impact of urbanization and a wastewater treatment plant by investigating the impact on field-collected bluegill (*Lepomis macrochirus*). Results show a significant increase in DNA strand breaks in blood cells (comet assay) linked to urbanization and a reduction in DNA strand breaks downstream of the WWTP, likely the result of dilution. A laboratory study exposing *L. macrochirus* to the known mutagen, methyl methanesulfonate, was performed to validate the comet assay endpoints in this species. Results of the laboratory study showed that the comet assay endpoints of tail length and tail extent moment responded in a dose- and time-dependent manner. Habitat quality assessments, along with chemical concentrations of polycyclic hydrocarbons in sediments showed that habitat quality between all sites were similar and that hydrocarbons likely contributed to the DNA strand breaks observed.

1. Introduction

Increased sediment concentrations of polycyclic aromatic hydrocarbons (PAHs) have been directly linked to increase in urbanization (Soclo et al., 2002; Foster and Cui, 2008). PAHs are ubiquitous organic pollutants in the environment, some with mutagenic and carcinogenic properties (Aas et al., 2000; Neff, 1985). PAHs can be formed by the incomplete combustion of fuels (Rogge et al., 1993) and are present in the exhaust from motor vehicles (Halsall et al., 1994). Other sources of PAHs associated with urban runoff can be the wearing of asphalt pavement surfaces, car tires, and rainwater from the impervious surfaces (Grynkiewicz et al., 2002).

The single cell gel electrophoresis or comet assay is a method that measures DNA strand breaks at the individual cell level (Singh et al., 1988) by analysis of DNA migration in gel-embedded cells following electrophoresis (Bombail et al., 2001). The name, 'comet', refers to the formation of a long comet-like tail of DNA fragments that are formed as a result of DNA damage. This assay has been used as an indicator of DNA damage in fish collected from sites contaminated with a variety of compounds including PAHs, polychlorinated biphenyls (PCBs), pesticides, and heavy metals (Bony et al., 2008; Cestari et al., 2004; Chang et al., 2005; Devaux et al., 1998; Ferraro et al., 2004; Inzunza et al., 2006; Winter et al., 2004).

The overall goal of the present study was to investigate the effects of urbanization on field-collected bluegill (*Lepomis macrochirus*), specifically the effects of urban runoff and discharge from a WWTP. The specific objectives were to: (1) validate the comet assay in bluegill using a laboratory exposure, (2) measure DNA strand breaks in bluegill via the comet assay and (3) investigate the relationship between DNA strand breaks and PAH concentrations at each field site.

2. Material and methods

2.1. Sampling sites

This study was conducted on a 16 km reach of the Reedy river, located in South Carolina, USA (Fig. 1). The Reedy river supports at least 33 fish species from eight families, with centrarchids (*Lepomis* spp.), cyprinids (*Notropis* and *Nocomis* spp.), and ictalurids (*Ameiurus* spp.) representing the majority of species and individuals and has an average annual discharge of approximately 100 cubic feet per second.

Four reaches of approximately 100 m each of the river were selected for this study. Site selection was based on land use and point-source inputs from both upstream and surrounding each sites.

The reference area (site 1) was chosen as a reach of the Reedy river just above the start of urbanization; the surrounding land use was mainly rural and forested with no known point source inputs. Site 2 was approximately 200 m downstream of the major urbanized area of the city of Greenville (population approximately

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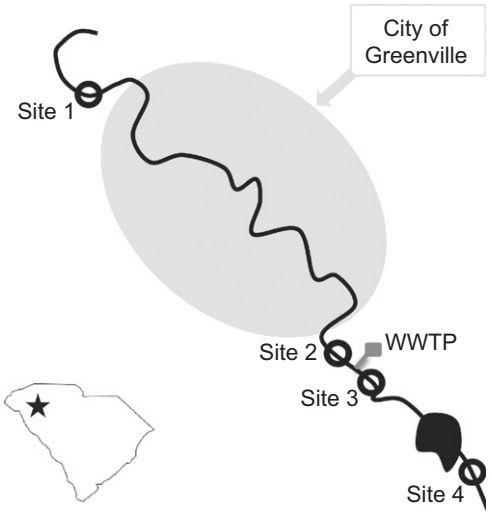

Fig. 1. Locations of sampling sites on the Reedy river in South Carolina, USA. WWTP=wastewater treatment plant.

60,000). The land use between the reference area and site 2 was entirely urbanized. Site 3 was approximately 400 m downstream of site 2 and approximately 100 m downstream of the principal wastewater treatment plant (WTTP) that serves the city of Greenville. The WWTP has an average flow of 21 million gallons daily (MGD)—1.7 MGD from industrial, 2.1 MGD from commercial, and 17.2 MGD from residential sources and can be a major contributor to the volume of the Reedy river, especially during months with little rainfall. Site 4 was approximately 3 km downstream of site 3 and approximately 200 m downstream of the only impoundment (and barrier to fish movement) between sites 3 and 4, Lake Conestee. Lake Conestee is a heavy polluted reservoir with high concentrations of PAHs, metals, and pesticides in the sediments (Zapata Engineering and Pinnacle Consulting Group, 2003; Schreiber et al., 2006).

2.2. Habitat assessment

An evaluation of habitat quality at each site was performed according to United States Environmental Protection Agency's rapid bioassessment protocols for low-gradient streams (Barbour et al., 1999). A team of two researchers arrived at a consensus on the quality for each habitat parameter.

2.3. Fish collection

Bluegill were chosen as the species of interest in this study based on their abundance in the Reedy river and their relatively small home range (~100 m) in lotic ecosystems (Gatz and Adams, 1994; Smithson and Johnston, 1999). The small home range of this species would minimize the possibility of collecting non-resident fish from a specific site. It was presumed that all fish obtained within a particular site were residents of that site and, therefore, subject to all physical and chemical stressors within that site.

Bluegill were collected at all sites by electrofishing on June 20, 2005. Fifteen individuals weighing between 4–10 g (7–13 cm in total length) were collected from each site. After collection, each fish was measured, and approximately 50 μl of blood was collected by caudal sever into a heparinized hematocrit tube, transferred to a 0.5 ml microcentrifuge tube containing sodium heparin and placed on ice until comet assay analysis was performed (within 48 h of collection). Following blood collection, the age class (adult or juvenile) of each fish collected was determined based on fish size (less than 10 cm in total length) and gonadal inspection (performed by two independent researchers).

2.4. Laboratory exposure

Commercially raised bluegill (3–8 g) were obtained from Aquatic Research Organisms (Hampton, NH). Fish were dosed via a single intraperitoneal injection with methyl methanesulfonate in a phosphate-buffered saline carrier (Sigma Chemical, St Louis, MO, USA) at doses of 0 (control), 2.0 and 4.0 μg/g in a dose volume of 5 μl/g body weight. Fifteen fish per treatment were placed in 40 l aquaria with aeration and particulate/carbon filtration. Moderately hard reconstituted water was replaced daily. At 4, 24, and 72 h post injection, five fish per concentration were removed and blood was drawn with a heparinized syringe and needle via caudal vein puncture. Blood samples were then stored in ice until comet assay analysis was performed (within 24 h of collection).

2.5. Comet assay

The comet assay was performed as described by Singh et al. (1988) and Tice (1995), with modifications as follows. Three microliters of whole fish blood were diluted with 1 ml of cold mincing solution (10% dimethyl sulfoxide in Hank's balanced salt solution with 5 mM Na_2-ethylenediaminetetraacetic acid). Duplicate 10 μl volumes of this mixture were mixed with 75 μl of 0.5% low-melting agarose (LMA) at 37° C and pipetted onto a microscope slide that had been pre-coated with a layer of 1.2% normal melting agarose. The slide was cover-slipped, and placed on an ice-chilled tray for 15 min to allow the agarose gel to solidify. The slide was covered with a second layer of 75 μl of LMA. After gelling, the slides were placed in ice-cold, freshly prepared lysing solution (2.5 M NaCl, 100 mM Na_2-ethylenediaminetetraacetic acid, 10 mM Tris, 10% dimethyl sulfoxide, 1% sodium sarcosinate, 1% Triton X-100; Sigma St. Louis, MO, USA) at pH 10 in glass screw-cap Coplin jars (Fisher Scientific, Pittsburgh, PA, USA) and incubated overnight at 4° C in darkness.

After incubation, the jars were drained and the slides were transferred to a horizontal electrophoresis tray, and then submerged in an electrophoresis chamber containing freshly made buffer (300 mM NaOH and 1 mM Na_2-ethylenediaminetetraacetic acid), pH > 13. The DNA was allowed to denature in the alkaline buffer for 15 min and subjected to electrophoresis (25 V, 300 mA) for 10 min at room temperature. After electrophoresis, the slides were neutralized by three 5 min washes in a neutralizing solution (0.4 M Tris, pH 7.5), fixed by immersion in cold methanol for 5 min, and air-dried.

After staining with ethidium bromide (50 μl, under cover slip, of 20 μg/ml solution), slides were analyzed using a fluorescence microscope (BH-2; Olympus Optical, Tokyo, Japan) under 400× magnification. The microscope was equipped with a B excitation filter, EY455 supplementary excitation filter, and 530 nm barrier filter. Fifty cells per slide and a total of 100 cells per fish were analyzed randomly with a computerized image-analysis system (Komet 4.0; Kinetic Imaging, Liverpool, UK). The comet assay analysis parameters were percentage DNA in the tail and extent tail moment (TM), which is calculated as comet tail length multiplied by the relative DNA content of the tail.

2.6. Sediment PAH analysis

Superficial (top 8 cm) sediment samples were taken from three locations at each field site. Samples were placed in Teflon-lined amber jars, stored in ice immediately, and stored at 4° C until analysis (within 6 h collection). Samples were extracted (Sample Extraction Kit User Guide, Strategic Diagnostics, Newark, DE) and analyzed for PAHs via EPA method SW-846 #4035 (Strategic Diagnostics, Newark, DE) on a RPA-1™ RaPID Photometric Analyzer (Strategic Diagnostics, Newark, DE). Briefly, this method is an immunoassay that utilizes antibody coated magnetic particle as a screening assay total PAHs. Phenanthrene standards of 2, 10, 25, and 50 mg/kg were used for assay verification during each analysis. All PAH screening results are reported as mg phenanthrene equivalents per kg sediment (wet weight).

2.7. Statistical analysis

To test for differences in biomarker endpoints and PAH concentrations among sites a one-way analysis of variance (ANOVA) was performed. Analysis of comet assay endpoints in the laboratory study was performed using a two-way ANOVA (variables of dose and time). Post hoc comparisons (Tukey's) were used to discriminate differences between mean values. All data are reported as mean ± standard error of the mean. Significant differences were defined as having a p-value less then 0.05.

3. Results

3.1. Comet assay

The known mutagen, methyl methanesulfonate, induced DNA strand breaks in laboratory fish as early as 4 h following a single intraperitoneal dose. For percent tail DNA a significant effect of time ($F_{(2,36)}=70.368$, $p<0.001$) and dose ($F_{(2,36)}=259.246$, $p<0.001$) were observed (Fig. 2a). Likewise for TM a significant effect of time ($F_{(2,36)}=76.792$, $p<0.001$) and dose ($F_{(2,36)}=172.566$, $p<0.001$) were observed (Fig. 2b). Post hoc analysis revealed significant increases in percent tail DNA and TM at 4, 24 and 72 h post injection in all treated groups when compared to the control.

In field-collected fish, significant differences in percent tail DNA ($F_{(3,22)}=6.1544$, $p=0.0034$) (Fig. 3a) and TM ($F_{(3,22)}=6.7096$, $p=0.0022$) (Fig. 3b) were detected based on site. For percent tail

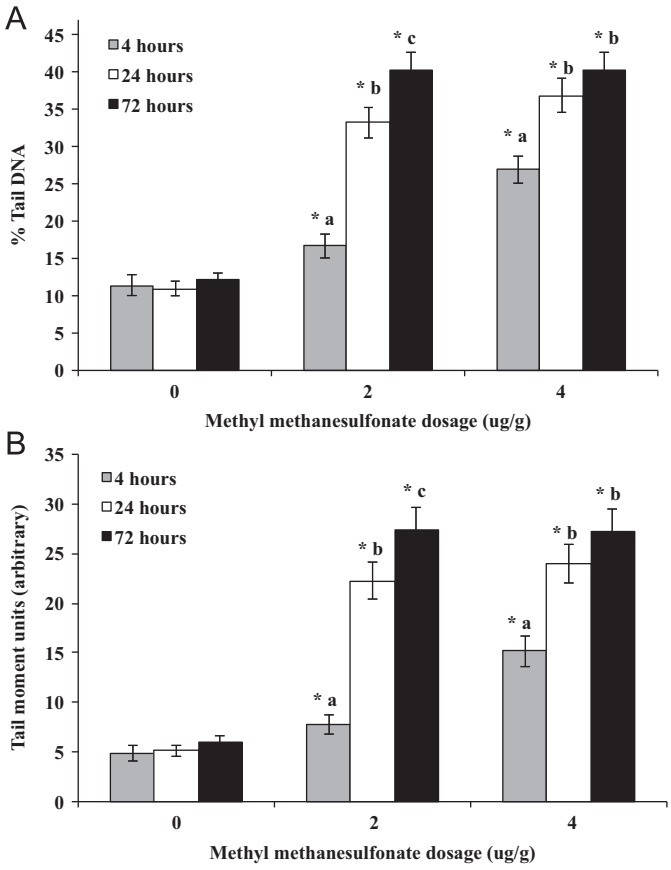

Fig. 2. Comet assay results in laboratory-treated bluegill following a single intraperitoneal injection of methyl methanesulfonate for (A) percent tail DNA and (B) tail moment. An asterisk (*) indicates significant differences compared to time specific controls ($p < 0.05$, ANOVA). Significant differences between time points within the same dosage group are indicated by different lower case letters ($p < 0.05$, ANOVA). Data presented as mean ± standard error mean.

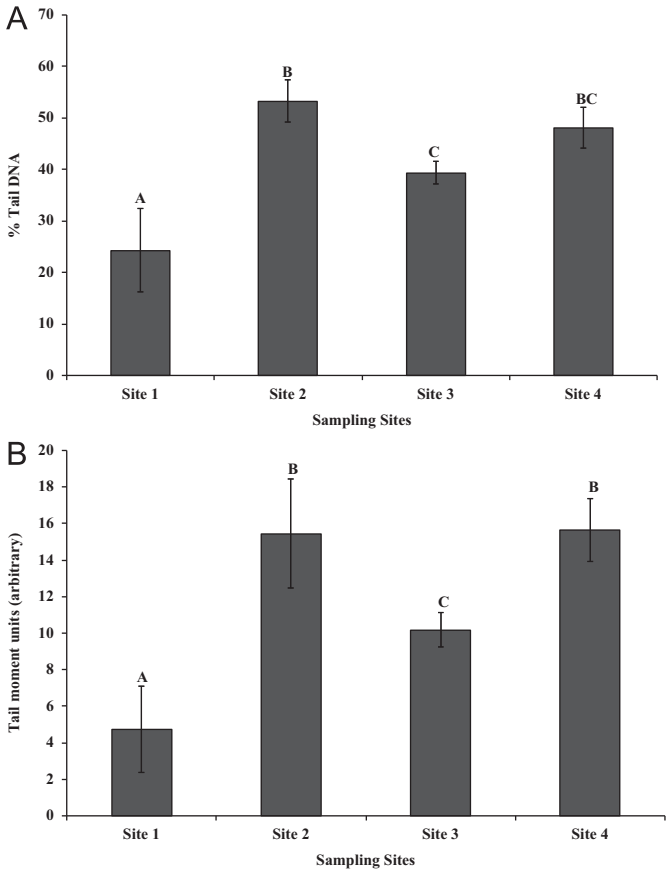

Fig. 3. Comet assay results in feral bluegill for (A) percent tail DNA and (B) tail moment. Significant differences between sites are indicated by different letters ($p < 0.05$, ANOVA). Data presented as mean ± standard error mean.

DNA significant increases were found at sites 2 ($p=0.0059$) and 4 (0.0064) compared to the reference site. Site 3 was not significantly different from the reference site ($p=0.1322$), site 2 ($p=0.2317$), or site 4 ($p=0.4206$) for percent tail DNA values. The exact same pattern existed for TM values, with significant increases at sites 2 ($p=0.016$) and 4 ($p=0.0023$) compared to the reference site and no significant differences between site 3 and the reference site ($p=0.2124$), site 2 ($p=0.3294$), or site 4 ($p=0.1102$).

3.2. Sediment PAHs

A significant difference in sediment PAH concentration was detected based on site ($F_{(3,8)}=147.71$, $p < 0.001$) (Fig. 4). Significantly higher PAH concentrations were observed at site 4 compared to the reference site, site 2, and site 3 ($p < 0.001$ for each site). No other significant differences were observed between sites.

3.3. Habitat assessment

Habitat assessments performed at each site found no major scoring differences between any sites (Table 1). The two factors determined to have the most variability between sites were available cover and sediment deposition. Available cover (also known as epifaunal substrate) was defined as the relative quantity and variety of natural structures in the stream, such as cobble, large rocks, fallen trees, and undercut banks, available as refugia, feeding or sites for spawning and nursery functions of aquatic macrofauna (Barbour et al., 1999). Sediment deposition was defined as the amount of sediment that had accumulated in pools and the changes that have occurred to the stream bottom as a result of deposition (Barbour et al., 1999).

4. Discussion

Fish that live in waterways surrounded by urban land use must deal with a variety of stressors, both chemical and physical. Physical stressors such as flow and habitat loss can affect fish health and fish community structure (An et al., 2002; Miltner et al., 2004; Scott, 2006). In the present study habitat quality assessments show that, overall, all sites were similar (Table 1). Because of the methods used for assessing habitat quality no statistical test could be performed on the data. Based on similar scores for both individual habitat parameters and total habitat, it was concluded that the habitats of all sites were relatively similar and that the differences observed in the biomarker endpoints were linked to chemical stressors rather than physical stressors.

The comet assay has been used in multiple freshwater and marine fish species, including butterfish (*Pholis gunnellus*), chub (*Leuciscus cephalus*), mullet (*Mugil* sp.), common carp (*Cyprinus carpio*), brown bullhead (*Ameiurus nebulosus*), and sea catfish (*Netuma* sp.) as an indicator of DNA damage (Andrade et al., 2004; Bombail et al., 2001; Chang et al., 2005; Devaux et al., 1998; Winter et al., 2004; Yang et al., 2006).

Numerous studies have shown DNA in tail and TM to be reliable measurements for the comet assay (Bombail et al., 2001; Fairbairn et al., 1995; Flammarion et al., 2002; Mitchelmore and Chipman, 1998. Winter et al. (2004) demonstrated an elevation of

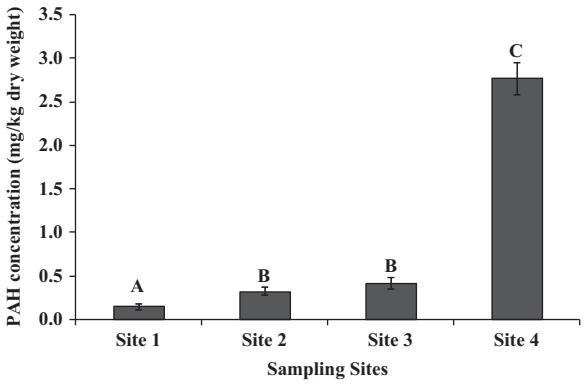

Fig. 4. Concentration of PAHs in the sediments of sampling sites. Significant differences between sites are indicated by different letters ($p < 0.05$, ANOVA). Data presented as mean ± standard error mean.

Table 1
Habitat quality assessment.

Habitat parameter	Site 1	Site 2	Site 3	Site 4
Epifaunal substrate/available cover	20	12	19	19
Pool substrate characterization	15	10	17	14
Pool variability	10	17	16	15
Sediment deposition	14	9	15	18
Channel flow status	20	18	20	20
Channel alteration	20	19	19	19
Channel sinuosity	19	18	19	19
Bank stability				
Left bank	7	9	8	9
Right bank	7	9	9	9
Vegetative protection				
Right bank	9	10	10	10
Left bank	9	10	10	10
Riparian vegetative zone width				
Left bank	10	7	10	9
Right bank	8	6	10	8
Total	168	154	182	179

All scores range from 1–20 (20–16=optimal; 15–11=suboptimal;10–6=marginal; 5–0=poor)

percent tail DNA in both caged and field-collected chubs (*L. cephalus*) with a decrease in water quality in United Kingdom. Devaux et al. (1998) used TM as the endpoint in field-collected and laboratory chubs (*L. cephalus*) and concluded that TM was a valuable technique for the assessment of genotoxicity in aquatic organisms.

In this study, the comet assay was validated for use with bluegill using the known mutagen methyl methanesulfonate (Fig. 2a and b). The method successfully detected increasing DNA strand breaks in whole nucleated blood samples throughout the 72 h study and indicated the amount of DNA damage was linked to both dosage and time. Results of this investigation are similar to those of other researchers, such that the comet assay endpoints responded in a dose-dependent manner to increasing concentrations of methyl methanesulfonate (Gontijo et al., 2003; Andrade et al., 2004).

For field-collected bluegill a general pattern was observed for the comet assay measurements percent tail DNA (Fig. 3a) and TM(Fig. 3b). This trend indicated a significant increase in both endpoints at all sites compared to site 1 (reference), a significant increase at site 2 compared with site 3, and an increase from sites 3 to 4 (only significant for TM). The DNA stand breaks seen at site 2 could be attributed to point and non-point source contaminants originating in the urbanized area between sites 1 and 2. The significant decrease in percent tail DNA and TM between sites 2 and 3 was most likely due to a dilution of the causative agent(s) by the large input of water (approximately 21 MGD) from the WWTP. The observed effects at site 4 were likely due to causative agent(s) present in Lake Conestee, a United States government designated Superfund site located between sampling sites 3 and 4. Previous investigations have demonstrated that PAHs in Lake Conestee are bioavailable to largemouth bass (Schreiber et al., 2006) and that high concentrations of PAHs, metals, and pesticides were found in the sediments (Schreiber et al., 2006; Zapata Engineering and Pinnacle Consulting Group, 2003). This man-made impoundment was originally constructed around 1900 and has been collecting and storing sediments and runoff from the city of Greenville for over 100 years. The history of Lake Conestee, combined with the previous research and the data collected during this study, indicate the main sources of DNA strand breaks to bluegill are originating in the urbanized section of the study area.

The comet assay has been previously reported as a measure of genotoxicity at field sites contaminated with PAHs, polychlorinated biphenyls, pesticides and heavy metals (Bony et al., 2008; Cestari et al., 2004; Chang et al., 2005; Devaux et al., 1998; Ferraro et al., 2004; Inzunza et al., 2006; Winter et al., 2004). Flammarion et al. (2002) observed a significant relationship between TM and benzo[a]pyrene concentration in the sediment. In the present study, sediment-screening concentrations of PAHs were measured in an attempt to link PAHs as the causative agent responsible for the observed DNA strand breaks. Sediment analysis showed significantly higher PAH concentrations at all sites compared to the reference site with the furthest downstream study site (site 4) having a eighteen-fold higher concentration than the reference site (Fig. 4). Although there was a significant difference in PAH concentration between sites 2 and 4, there was no significant difference between these sites for DNA strand breaks, indicating that PAHs were not the sole source of DNA damage. In addition, there was a significant change in DNA strand break endpoints between sites 2 and 3; however, there was no significant difference between PAH concentrations at these sites. Overall, the PAH results from this study suggest that PAHs were a potential causative agent for DNA damage, but not the only agent in the system responsible for the damage. Given the quantity and diversity of contaminants (point and non-point source) entering the Reedy river, it is not surprising that PAH sediment concentrations did not entirely explain the DNA strand break results observed in bluegill. Other contaminants commonly found in urban receiving streams and known to be potentially genotoxic, such as certain pesticides (Bony et al., 2008; Chang et al., 2005) and lead (Cestari et al., 2004; Ferraro et al., 2004). Further investigations into specific PAHs and other contaminate known to cause DNA damage are warranted to determine their role in the DNA strand breaks observed in this study.

In conclusion, this investigation found that the comet assay technique, validated with cultured bluegill in the laboratory, was easily applied to samples obtained from field-collected bluegill. The highest amount of DNA strand breaks in field-collected bluegill was observed at sites below the urbanized section of the study area. Sediment screening concentrations of PAHs analyzed at each site indicated that PAHs may be contributing the DNA strand breaks observed, but they were likely not the only genotoxic compounds present in the system.

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reviewed in accordance with United States Environmental Protection Agency policy and approved for publication. Approval does not signify that the contents necessarily reflect the views or policies of the Agency, nor does the mention of trade names or commercial products constitute endorsement or recommendation for use. This study was conducted with the approval of the Clemson University Animal Research Committee, under the Office of Research Compliance and is in accordance with national and Clemson University guidelines for the protection of human subjects and animal welfare.

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