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GENOTOXIC RESPONSE OF UNIONID MUSSEL HEMOLYMPH TO HYDROGEN PEROXIDE AND POLYCYCLIC AROMATIC HYDROCARBONS

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ABSTRACT

The single cell gel electrophoresis or comet assay is widely used to detect DNA damage in isolated cells following exposure to genotoxic compounds. This assay, although commonly used with marine bivalve tissue and circulatory fluid, has received little use or demonstration in freshwater mussels of the order Unionida. Because such a large proportion (>70%) of this faunal group is globally imperiled and is being adversely impacted by environmental contaminants, including many genotoxicants, the aim of this study was to assess the applicability of the comet assay in unionid mussel hemolymph sampled non-lethally with a reference genotoxicant, hydrogen peroxide (H_2O_2) and polycyclic aromatic hydrocarbons (PAHs), a class of common environmental pollutants of genotoxic action. DNA damage was evaluated in samples of hemolymph from *Elliptio complanata* in both *in vitro* and *in vivo* exposures and quantified using the endpoints % tail DNA, or the percentage of DNA in the comet tail and OTM or olive tail moment, the product of the fraction of DNA in the tail and tail length. Hemocytes were isolated and the comet assay was performed on control, 160 µM H_2O_2, and PAH treated cells. From the *in vitro* exposures, 160 µM H_2O_2, as well as the 50 and 100 µg/L total PAH treatments yielded statistically significant ($p < 0.05$) levels of DNA damage, with the H_2O_2 yielding an average of 39.7 % tail DNA and 13.3 OTM and the two PAH treatments yielding 40.7 % and 38.6 % tail DNA, and 12.4 and 11.0 OTM, respectively. An *in vivo* PAH exposure with adult *E. complanata* did not detect a similar genotoxic response to that detected with *in vitro* exposure, indicating that additional research and evaluation may be necessary before implementing the widespread use of a non-lethal, unionid mussel hemolymph based genotoxicity screening tool for environmental biomonitoring.

KEY WORDS Comet Assay, Freshwater Mussel, Unionid, Genotoxic, Hemolymph, PAH

INTRODUCTION

Freshwater ecosystems in North America are home to about 12,580 described species of invertebrates, of which 820 are mollusks (Bogan, 1993). There are eighteen families of bivalves (Phylum: Mollusca) that have resided in such freshwater ecosystems. One order in particular that has been the most successful in diversifying is Unionida, with Unionidae and Margaritiferidae being the two predominant families in North America, consisting of approximately 300 recognized species (Williams et al., 1993). However successful Unionida have been, they are also considered extremely sensitive to disturbances (e.g., contaminants) in freshwater ecosystems and are recognized as the most endangered group of mollusks in the world (Neves, 1999).

The cumulative effect of contaminant exposure on native freshwater bivalves is largely unknown during realistic exposure scenarios. Their sedentary, suspension and deposit feeding behaviors combined with a lifespan of 30-130 years (Bauer, 1992) provides numerous opportunities, potentially for an entire lifespan, for exposure and accumulation of anthropogenic contaminants within mussel tissues and circulatory fluid (Cope et al., 2008), including genotoxic compounds. Genotoxic chemicals have the potential of interacting with biologically important molecules and causing a damaging chain of events to DNA. Mollusks, and bivalves in particular, possess a wide range of defenses to mitigate the toxic effects of chemicals at the cellular level, including multi xenobiotic resistance proteins that actively reduce the cellular entrance of toxicants, detoxifying

enzymes, and DNA repair mechanisms (Rocher et al., 2006). In a variety of aquatic animals, DNA damage has been associated with reduced growth, abnormal development and reduced survival of embryos, larvae and adults (Lee & Steinert, 2003).

Hemolymph, the circulatory fluid of bivalves, contains hemocytes, which are collectively involved in a variety of physiological and pathological functions throughout the mussel body such as nutrient transport and digestion, wound and shell repair, internal defense, and exogenous and endogenous material excretion (Giamberini et al., 1996). An evaluation of a nonlethal sampling technique for hemolymph, withdrawn from the anterior adductor muscle sinus of *Elliptio complanata* (Mollusca: Unionidae), demonstrated the lack of negative impacts on survival or growth (Gustafson et al., 2005a). In addition, Rigonato et al. (2005) found hemolymph to be valuable due to the ease of manipulation and efficient response to DNA-stressing compounds in comparison to gill and digestive gland tissue for genotoxicity studies while researching the invasive, non-unionid Asian clam, *Corbicula fluminea*. Sampling hemolymph from native freshwater mussels of the family Unionidae has the potential to provide information pertinent to the health assessment of threatened or endangered individuals or populations without inflicting harm.

The single cell gel electrophoresis or comet assay is widely used to detect DNA damage in isolated cells following exposure to genotoxic compounds (Buschini et al., 2003; Hartl et al., 2004; Lee & Steinert, 2003; Rigonato et al., 2005; Rocher et al., 2006). This assay, although commonly used with marine bivalve tissues (Mitchelmore et al., 1998; Perez-Cadahia et al., 2004; Wessel et al., 2007; Wilson et al., 1998), has received less use or demonstration in unionid mussels (Conners & Black, 2004; Stambuk et al., 2008; 2009). Therefore, the aim of this study was to assess the applicability of the comet assay in hemolymph of the unionid mussel, *Elliptio complanata* sampled non-lethally with a reference genotoxicant (Lee & Steinert, 2003), hydrogen peroxide (H_2O_2), and in laboratory exposures with polycyclic aromatic hydrocarbons (PAHs), a class of common aquatic pollutants containing compounds of known genotoxic action (USEPA, 1986).

METHODS AND MATERIALS

Mussel Collection and Husbandry

For this study, *Elliptio complanata* or eastern elliptio, were collected from a relatively uncontaminated (USGS, 1999), rural forested segment of the Eno River that flows through Hillsborough in Orange County, North Carolina, USA. The Eno River has high biodiversity, an indication of good water and habitat quality (NCDENR, 2009), including the presence of 12 species of native freshwater mussels. Field collection events for this study involved the hand-collection of approximately 30 mussels per sampling trip, held in dive bags and transported (30 min trip) in coolers containing site water to the Aquatic Toxicology Laboratory on the campus of North Carolina State University in Raleigh, NC, USA. Once in the laboratory, the mussels were held within an aerated, recirculating living stream (Frigid Units Inc., Toledo, OH, USA) with reconstituted soft water (pH 7.2-7.6, hardness 40-48 mg CaCO3/L, and alkalinity 30-35 mg CaCO3/L) (ASTM, 2006) at temperatures consistent with river temperatures and fed a commercial mixture of nonviable microalgae prepared from Instant Algae® Shellfish Diet 1800 and *Nannochloropsis* (Nanno 3600) concentrate (Reed Mariculture, Campbell, CA, USA) on a weekly basis. The maximum length of time a group of mussels was held in the living stream and used for in vitro experimentation was two months.

In Vitro*: Hemolymph Collection*

At the time of hemolymph collection, mussels were randomly selected from the living stream and weight and length measurements recorded. To collect hemolymph, the mussel was gently pried open with a thin-blade knife just far enough to insert a 5 mm wide flat-end forceps to keep the shell open and expose the anterior adductor muscle, and a small sterile 25-gauge needle on a 1.0 mL syringe (PrecisionGlide™, Becton Dickinson and Company, Franklin Lakes, NJ, USA) was inserted into the anterior adductor muscle sinus. Up to 1 mL of hemolymph was extracted per mussel and expelled from the syringe (with the needle removed to prevent any potential physical damage to hemocytes) into a 20 mL Nalgene® test tube. An equal amount of a modified Alsever's Solution (Sigma-Aldrich, St. Louis, MO, USA), an isotonic, balanced salt solution containing ethylenediaminetetraacetic acid (EDTA), was used as a rinse solution to prevent the spontaneous aggregation of hemocytes (Chen & Bayne, 1995) upon extraction from the mussel. All successive hemolymph samples were immediately pooled in a 20 mL Nalgene® test tube to minimize inter-individual variability, until the necessary volume for the given experiment was obtained. Typically, 7 to 9 mL of hemolymph was collected from 7 to 11 individual mussels, with an equal amount of Alsever's solution, thus the final working volume of the hemolymph-Alsever mixture was between 14 to 18 mL, which will be referred to as the "hemolymph mixture". The hemolymph mixture was used in experiments immediately after extraction.

In Vitro*: Exposure Procedures*

The following procedures were conducted in a laboratory without direct sunlight and the florescent overhead

lights were shielded with an ultraviolet (UV) protective coating to minimize background levels of DNA damage in hemocytes from the UV radiation. All *in vitro* exposures were conducted in triplicate using flat bottom, 18 well, non-tissue culture treated plates (Corning® Costar®, Sigma-Aldrich, St. Louis, MO, USA) with lids. In addition, all exposures included controls and hydrogen peroxide (H_2O_2) as a reference genotoxicant and positive control (VWR International, West Chester, PA, USA), as well as a solvent control of acetone for the PAH exposures. For the in vitro exposures, 650 µL of the hemolymph mixture was aliquoted into the experimental wells of the plate and the H_2O_2 or PAH mixture (Alaskan North Slope crude oil (Battelle, Duxbury, MA, USA) and creosote (CAS # 8001-58-9; AccuStandard Inc., New Haven, CT, USA) containing 48 different PAHs; similar to Thorsen et al., 2004) was added to the appropriate wells. In this study, the Alaskan North Slope crude oil and creosote (3:1, volume:volume) was diluted in acetone. The target concentrations for the sum total 48 PAHs in the two working stock solutions used for the exposures were 0.1 µg/µL and 1.0 µg/µL. The plate was then covered and agitated gently for 1 min, placed in a dark incubator at 4 °C for a 4-h exposure period (Tice et al., 2000). Agitation of the plate was conducted for 1 min at 30-min intervals during the exposure period. Upon exposure completion, the hemolymph mixture was transferred via micropipette from the wells into individually labeled 2 mL microcentrifuge tubes. A Ca2+ and Mg2+ free 1 X phosphate buffered saline (1XPBS) (Cambrex Bio Science, Walkersville, MD, USA) solution mixed 1:1 with Alsever's solution was prepared and used to rinse the wells. The exposed hemolymph mixture was centrifuged at 1100 g for 4 min, supernatant decanted and the hemocyte pellet resuspended in 600 µL of the 1XPBS-Alsever solution, and repeated 2 times. After the final rinse, the exposed hemocytes were brought to a final working volume of 325 µL with 1XPBS-Alsever solution.

In Vitro*: Cell Viability*

A prudent approach for selecting definitive exposure concentration ranges for *in vitro* comet assays is to perform cell viability testing with the compound of interest so that testing concentrations which decrease cell viability by more than 30% (Tice et al., 2000), compared to the control cells can be avoided, as low cell viability negatively influences comet assay results. Cell viability tests were conducted with the CellTiter-Glo® Luminescent assay (Promega, Madison, WI, USA), a fluorometric method for estimating the number of viable cells present based on the quantification of adenosine 5' –triphosphate (ATP), an indicator of metabolically active cells (Crouch et al., 1993). To convert relative luminescence units (RLUs), a measurement of the intensity of the emitted light detected by the luminometer (Fusion™, Packard Instrument Company, Meriden, CT, USA) into ATP concentrations, a linear calibration curve was prepared using 0.025-2.0 µmol/L of 100 mM rATP (Promega, Madison, WI, USA). The calibration curve was used to extrapolate the levels of ATP from the measured RLUs recorded from the unexposed and exposed hemocytes isolated and resuspended in 1XPBS-Alsever buffer solution in 96-well plates (Corning® Costar®, Sigma-Aldrich, St. Louis, MO, USA). Based on the need for data normalization to protein content in the cell viability assay, the Bradford Protein assay (IBI-Shelton Scientific, Peosta, IL, USA), a kit containing 0.5 mg/mL bovine serum albumin (BSA), 0.15 M NaCl and a Bradford Reagent consisting of Coomassie blue, a dye that binds protein, was used to determine the protein concentration in unexposed hemolymph by generating a BSA linear standard curve plotting absorbance at 595 nm (Spectronic® Genesys™, Milton Roy Company, Rochester, NY, USA) versus protein concentration.

Range-finding cell viability tests were performed with the reference genotoxicant H_2O_2 (positive control) to determine the appropriate concentrations for the 4-h *in vitro* exposures. Once the optimum H_2O_2 concentrations (0-640 µM H_2O_2) were determined, the PAH mixture underwent the same range-finding cell viability tests to confirm an acceptable exposure concentration range (0-200 µg/L total PAH). The concentration of acetone (solvent control) required for complete PAH solubility was determined by using the greatest concentration of acetone required in the preparation of the PAH treatments and ensuring cell viability was within the acceptable level. Cell viability was expressed as the changes in intracellular ATP levels, or µmol ATP/µg of protein converted to a percentage and compared to the unexposed hemolymph or baseline levels measured immediately after extraction.

In Vitro*: Genotoxicity*

Once cell viability was determined to be no less than 75% below baseline levels for all exposure concentrations and the 4-h *in vitro* exposure period was complete, the comet assay was performed using rinsed, isolated, and resuspended hemocytes. The procedures differed slightly from the traditional comet assay methods developed by Singh et al. (1988) in that CometSlides™ (Trevigen, Gaithersburg, MD, USA), specially treated two-well microscope slides were used in accordance to the manufacturer's protocol (Trevigen, 2007).

During processing, the humidity of the laboratory was monitored and maintained below 60%. The isolated hemocytes, suspended in 1X PBS-Alsever solution at approximately 1 X 105 mL-1 were combined with 500 µL molten 37 °C low melting agarose (LMA) (Trevigen, 2007). Then, 50 µL of the hemocyte-LMA mixture was

pipetted onto each of the two sample wells of the coded CometSlide™ and repeated in duplicate. Each exposure, including the controls and solvent controls, was represented by a total of six slides. The prepared CometSlides™ were placed on slide trays and incubated at 4 °C for 30 min before immersion in pre-chilled cell lysis solution (Trevigen, 2007) in coplin jars and incubated at 4 °C for 60 min. After cell lysis, the slides were drained and transferred to coplin jars containing freshly prepared pH > 13 alkaline solution containing NaOH (Mallinckrodt Baker Inc., Paris, KY, USA) and 200 mM EDTA (Trevigen, 2007) for 20 min to unwind and denature the DNA. Next, the slides were placed on a recirculating, horizontal electrophoresis apparatus (Fisher Scientific, Pittsburgh, PA, USA), voltage set to 1 volt/cm, and freshly prepared alkaline electrophoresis solution (pH > 13, 300 mM NaOH, 1 mM EDTA) was added until the current reached 300 mA. Electrophoresis was performed for 40 min and once complete, the slides were rinsed 3X in distilled water, fixed in 70% ethanol (EMD Chemicals, Inc., Gibbstown, NJ, USA), and stored flat in desiccators to dry.

When the CometSlides™ were ready for analysis, each sample well was stained with 50 µL SYBR® Gold Nucleic Acid gel stain (Molecular Probes, Eugene, OR, USA), a fluorescent DNA intercalating dye, and visualized by epifluorescence microscopy. A computer imaging analysis system, Komet™ 5.5 (Andor Technology, South Windsor, CT, USA) was used to capture and analyze, or score, the levels of DNA damage. The parameters measured and reported include % tail DNA, the percentage of DNA in the comet tail, and olive tail moment (OTM), the product of the fraction of DNA in the tail and tail length. The hemocytes were scored 25 cells per well (50 cells per slide) in duplicate per treatment with three replicates per treatment for a total of 300 cells per exposure concentration. Each slide was labeled with a code unrelated to treatment and processed randomly to reduce potential bias during image analysis.

In Vivo *PAH: Exposure Concentrations and Experimental Design*

The same 3:1 mixture of Alaskan North Slope crude oil and creosote dissolved in acetone with the 48 different PAHs that was screened *in vitro* was also tested *in vivo* through aqueous exposures. The concentration range for the sum total 48 PAHs in this test was determined based on measured concentrations commonly reported within freshwater, aquatic environments (USGS, 1999) and was similar to that used in the *in vitro* tests (0-200 µg/L total PAH). All PAH preparations were dissolved in acetone and all test exposure concentrations of total PAHs in the *in vitro* and *in vivo* tests were validated with empirical measurements using standard analytical methods, as previously described (Thorsen et al., 2004). All measured total PAH concentrations in the test treatments from this study averaged 98% (range 96-101%) of the target concentrations at test initiation.

For this experiment, mussels were collected from the Eno River as previously stated. Immediately upon collection from the river, two mussels were randomly selected to represent baseline or background levels of DNA damage. Hemolymph was sampled from these mussels as previously described and they were not used for further experimentation. The remaining mussels were returned to the lab and were acclimated in aerated coolers containing river water to the test temperature of 20 °C. The mussels were not fed during the 3-d acclimation or during the 3-d experiment. Once acclimated, the shells of the mussels were gently cleaned of debris with a soft-bristled brush and returned to clean, aerated coolers containing reconstituted soft water (ASTM, 2006). Upon start of the experiment, mussels were selected at random, weight and length measurements recorded, and distributed to labeled, aerated, glass aquaria, containing 2-L of soft water (ASTM, 2006). The exposure consisted of adding the Alaskan North Slope crude oil and creosote mixture in concentrations of 1, 10, 50, 100, and 200 µg/L total PAHs into the appropriate 2-L glass aquarium with a glass syringe, for a total exposure duration of 3 d (similar to Thorsen et al., 2004). A positive control consisting of 30% H_2O_2 was also used in this experiment, but the optimum peroxide concentration from the *in vitro* exposures was increased to 1500 µM H_2O_2 for the *in vivo* exposure. The increased peroxide concentration was chosen based on an *in vivo* study performed with *Mytilus edulis* exposed for 1 h at concentrations up to 1000 µM H_2O_2 (Wilson et al., 1998). All exposure concentrations, including the controls, H_2O_2, and acetone solvent controls were conducted in triplicate. A 48-h renewal of test concentrations and exposure water was conducted for all treatments with measurements of water chemistry, following standard methods, to analyze dissolved oxygen, conductivity and temperature using a YSI Model 556 MPS (Yellow Springs Instruments, Yellow Springs, OH, USA) calibrated multiprobe meter. Analysis of pH was performed with a Beckman Model Φ 240 (Beckman Instruments, Fullerton, CA, USA) calibrated meter. Alkalinity was determined by titration with 0.02 N H2SO4 to pH 4.5, and hardness by titration with 0.01 M EDTA.

In Vivo *PAH Exposure: Hemolymph Collection and The Comet Assay*

On day 3 of the *in vivo* PAH exposure, 300 µL of hemolymph was extracted per mussel and expelled into individually labeled microcentrifuge tubes containing 300 µL Alsever's solution. The hemocytes were isolated by

centrifugation at 1100 g for 4 min, supernatant decanted, and the hemocyte pellet rinsed 2X with 1XPBS-Alsever solution and brought to a final working volume of 300 µL with 1XPBS-Alsever solution. As described previously, the comet assay procedures were performed immediately following hemocyte isolation. Quantification of DNA damage was performed as described for the *in vitro* experiments.

Quality Control

CometAssay Control Cells™ (Trevigen) were used to assess the comet assay procedure in the laboratory and to ensure the validity of results. The Control Cells™ consisted of a negative control (CC0) and three DNA damaged cell treatments (CC1, CC2, and CC3) that had been pretreated with increasing concentrations of etoposide (a model genotoxicant) and cryopreserved. When electrophoresed, the Control Cells™ exhibit a dose-response of DNA damage. The results obtained with the Control Cells™ were compared to the results published by Trevigen (2007) within their protocol. The Control Cells™ were run in conjunction with the mussel hemocytes during all *in vitro* comet assay procedures, and produced the following levels of DNA damage, reported as % tail DNA (SD in parenthesis): CC0 11.4 % (3.4), CC1 29.0 % (2.6), CC2 39.4 % (2.6), and CC3 49.6 % (3.5). The levels published by Trevigen were the following in % tail DNA: CC0 5.8 % (7.7), CC1 28.4 % (14.0), CC2 39.7 % (21.8), and CC3 56.8 % (23.6). All results obtained within this study using the Control Cells™ were reflective of the mean % tail DNA values established by Trevigen.

Statistical Analysis

Differences in the amount of DNA damage detected and quantified by image analysis, using the parameters of % tail DNA and OTM, were performed with JMP Statistical Analysis software (version 5.1, SAS Institute, Cary, NC, USA) by use of analysis of variance (ANOVA) followed by a Dunnett's test for means comparison ($\alpha = 0.05$) between the control and each treatment.

RESULTS

In Vitro*: Cell Viability and Genotoxicity*

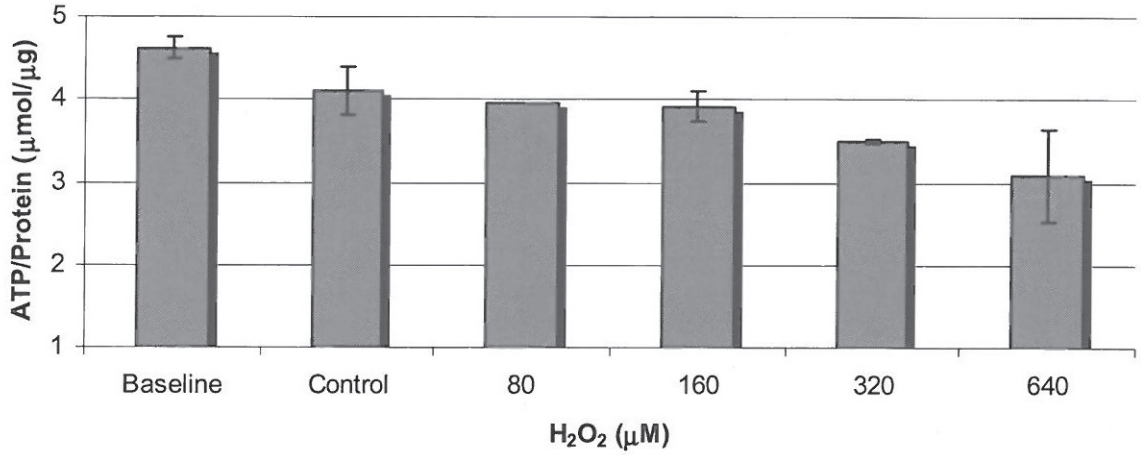
Hemolymph from a total of 139 *Elliptio complanata* was used during the *in vitro* experiments. The average weight of test mussels was 81.2 g (range 44.8 - 149.6 g) and the average length was 300.8 mm (range 188.3 - 322.7 mm). The concentration range tested to establish a suitable positive control using 30% H_2O_2 was 80 to 640 µM. These concentrations yielded a concentration response decrease in cell viability from 86% to 67% relative to baseline (n = 3) levels (Figure 1). Thus, 160 µM H_2O_2 was chosen as the positive control concentration because it provided an acceptable level of cell viability, approximately 80%, and yielded a statistically significant ($p < 0.05$) amount of genotoxicity in comparison to the controls for all *in vitro* exposures. Overall genotoxicity results are reported as % tail DNA and OTM in Figure 2, for all controls and 160 µM H_2O_2, processed in triplicate per *in vitro* experiment. The controls yielded a mean % tail DNA of 17.9 % (2.6) and OTM of 4.2 (0.8) (n = 18). The 160 µM H_2O_2 yielded a mean % tail DNA of 39.7 % (4.3) and OTM of 13.3 (2.1) (n = 17).

In accordance with the comet assay protocol (Tice et al., 2000), each comet assay experiment included 5 to 8 test concentrations of total PAHs (minimum of 3 recommended). All of the concentration ranges chosen yielded cell viability levels greater than 85%, and were thus used for the 4-h *in vitro* comet assay exposures, these included total PAH mixture; 0.05 to 200 µg/L total PAHs. Of all the concentrations tested during the 4-h *in vitro* exposure, excluding 160 µM H_2O_2, only the 50 and 100 µg/L total PAH mixture yielded statistically significant ($P < 0.05$) levels of DNA damage compared to the controls for both DNA damage parameters, with % tail DNA of 40.7 % (2.1) and 38.6 % (0.08) and an OTM of 12.4 (2.1) and 11.0 (0.3), respectively, shown in Figure 3. No other PAH concentrations elicited statistically significant levels of DNA damage in comparison to the controls.

In Vivo*: PAH Genotoxicity*

A total of 26 *Elliptio complanata* were used during the *in vivo* 3 d PAH experiment, and had an average weight of 103.1 grams (range 75.6 - 128.7 g) and average length of 309.4 mm (range 302.5 - 317.6 mm). The baseline, controls, and solvent controls all yielded similar levels of DNA damage (% tail DNA and OTM), 10.4 % (1.3) and 1.8 (0.4), 11.6 % (3.4) and 2.3 (0.9), and 11.8 % (4.3) and 2.0 (1.2), respectively (n = 3), shown in Figure 4. In contrast, the 1500 µM H_2O_2 yielded statistically significant ($P < 0.05$) levels of DNA damage with % tail DNA of 21.9 % (1.2) and OTM of 5.2 (0.4). However, none of the PAH exposure concentrations elicited statistically significant levels of DNA damage in comparison to the controls under the tested conditions. The level of DNA damage for the total PAH exposures, reported as % tail DNA ranged from 10.9 to 15.4 % and OTM of 1.6 to 2.9.

(a)



(b)

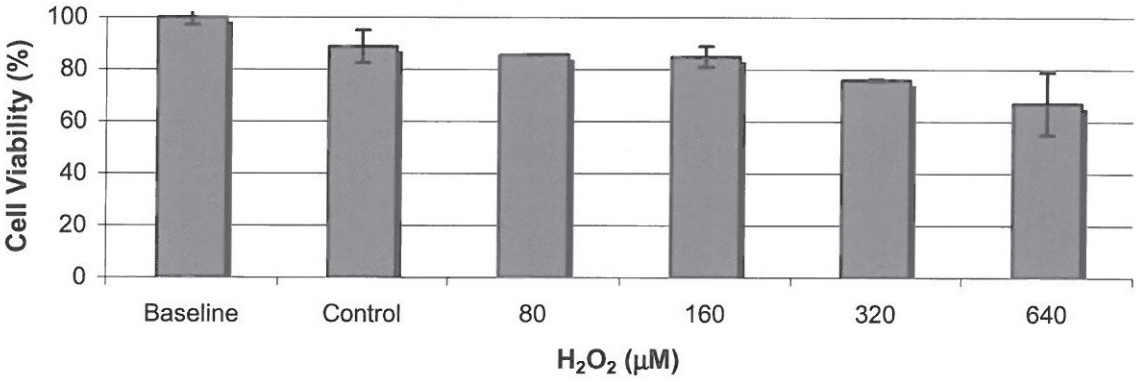


FIGURE 1

The concentration range for the positive control (n = 3), hydrogen peroxide (H_2O_2), during the *in vitro* exposure of freshwater mussel hemolymph with cell viability expressed as (a) µmol ATP/µg of protein and (b) converted from µmol ATP/µg of protein to cell viability (%) in comparison to baseline levels.

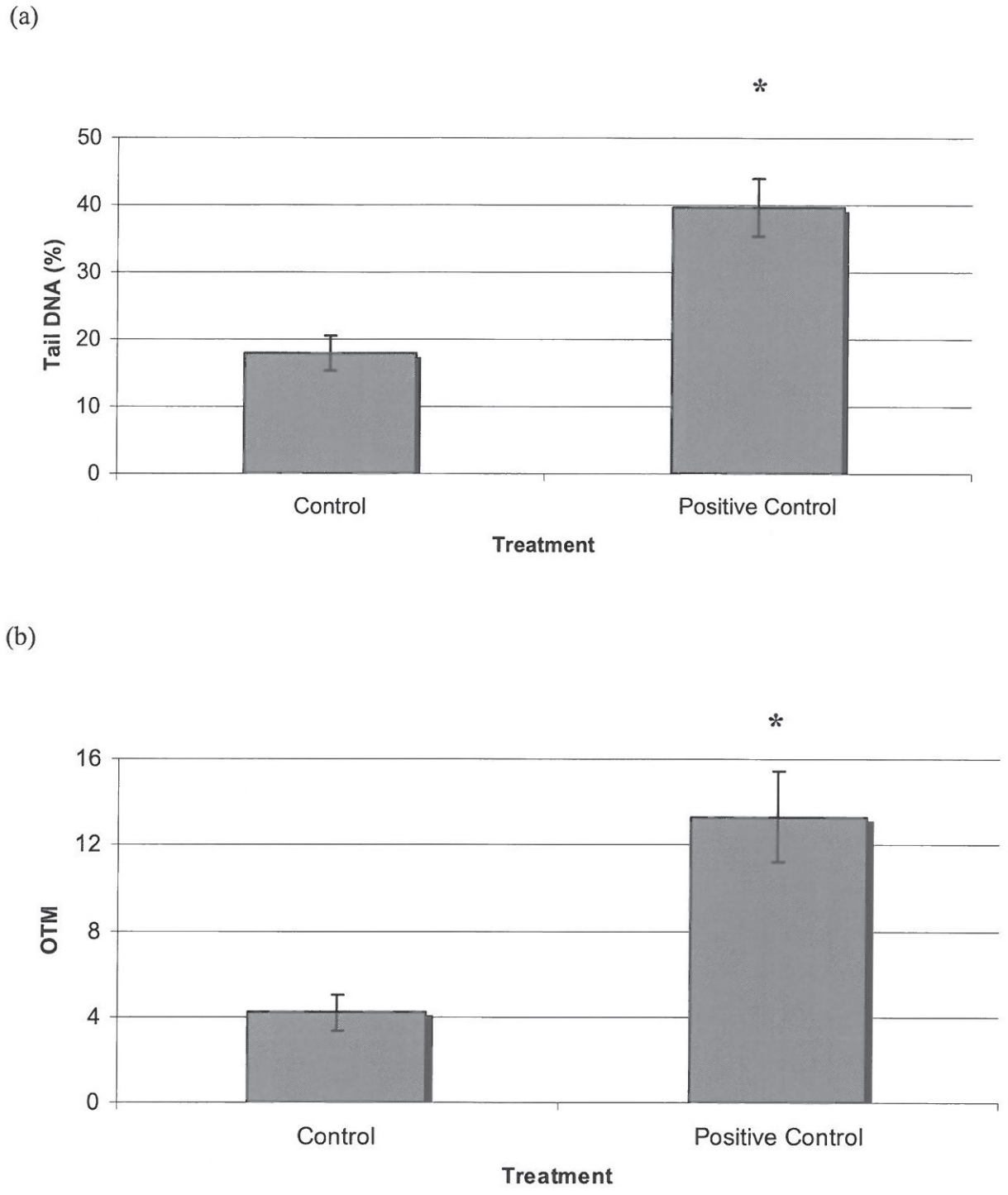


FIGURE 2

Overall mean genotoxicity of the *in vitro* controls (n = 18) and positive control, hydrogen peroxide (H_2O_2) (n = 17), in freshwater mussel hemolymph, (a) % tail DNA, (b) olive tail moment; OTM. *Indicates significantly different from the control ($P < 0.01$).

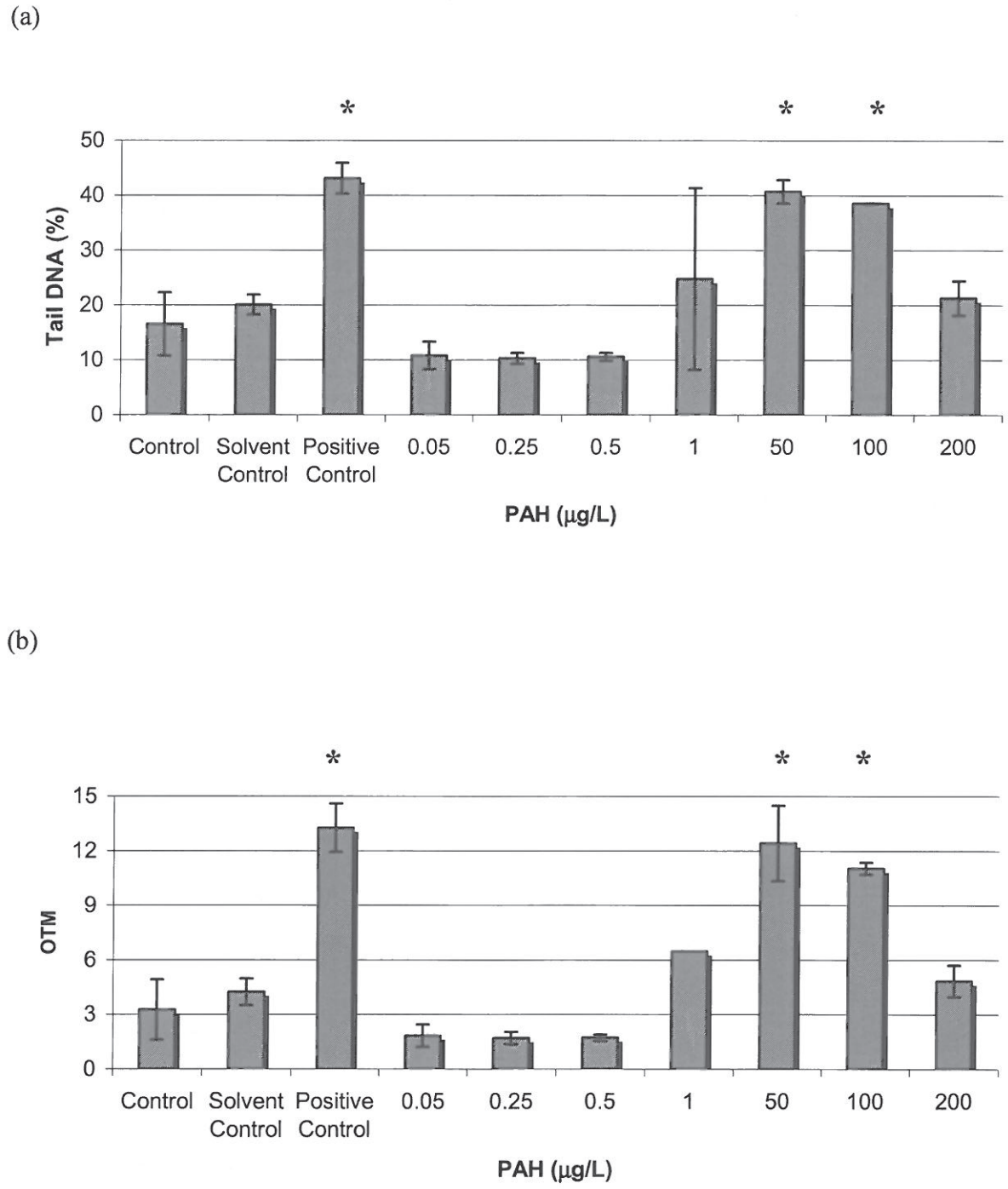


FIGURE 3

In vitro genotoxicity of total PAHs in freshwater mussel hemolymph (n = 3) (a) % tail DNA, (b) olive tail moment; OTM. *Indicates significantly different from the control ($P < 0.01$).

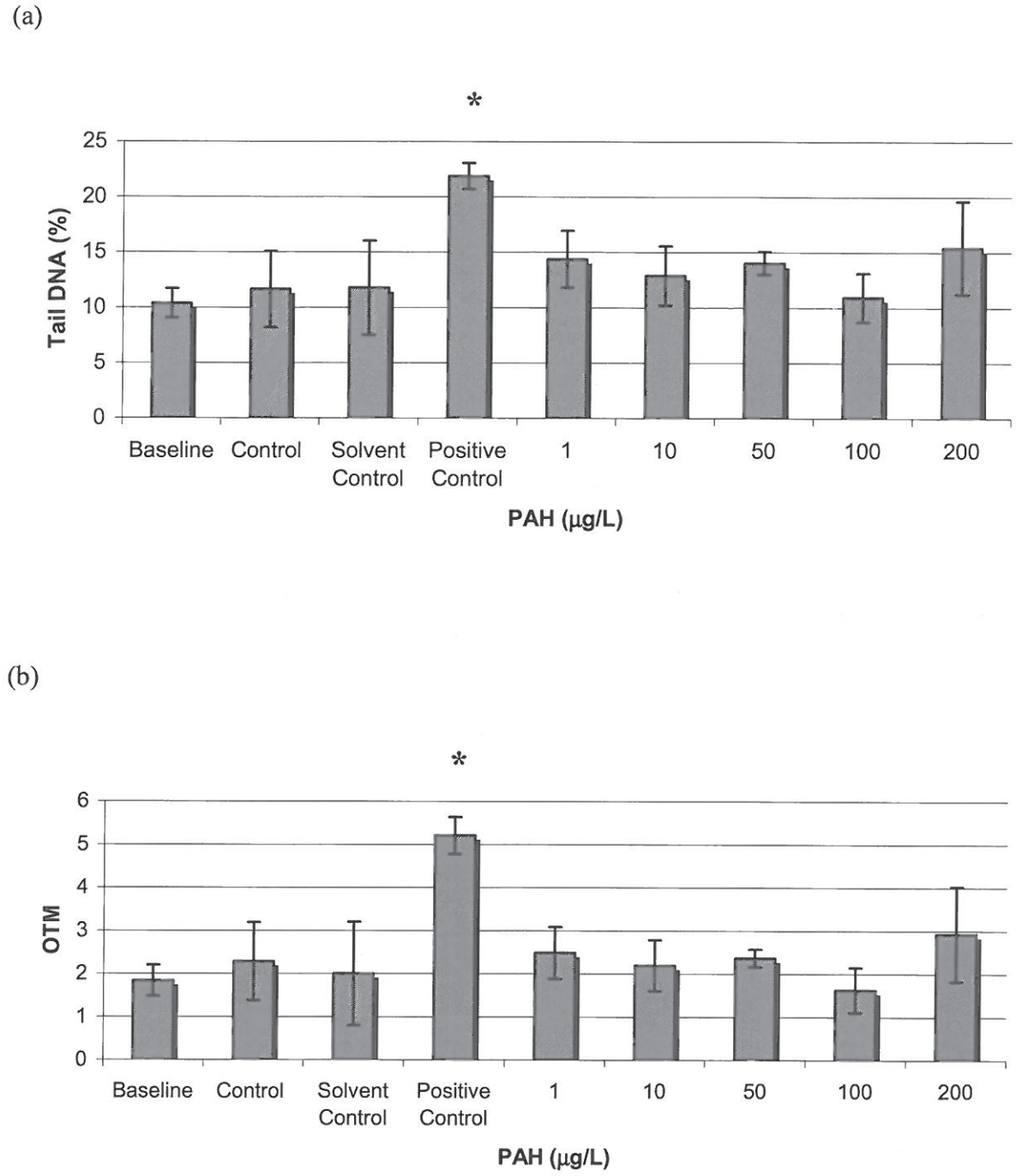


FIGURE 4

In vivo genotoxicity of total PAHs in freshwater mussel hemolymph (n = 3) (a) % tail DNA. *Indicates significantly different from the control (P = 0.0027), (b) olive tail moment; OTM *Indicates significantly different from the control (P = 0.0011).

DISCUSSION

This study demonstrated the *in vitro* and *in vivo* use of hemolymph, sampled non-lethally from a unionid mussel (*Elliptio complanata*) to detect DNA damage, or genotoxicity using the comet assay upon exposure to a reference genotoxicant (H_2O_2) and to environmentally relevant concentrations of a known aquatic ecosystem contaminant. This study also extends the previous research on genotoxicity assessment with unionid mussels (Conners & Black, 2004; Stambuk et al., 2008; 2009) by incorporating CometAssay Quality Control Cells™ (Trevigen, 2007) into the protocol. Most commonly, genotoxicity experimentation results in the destruction of the organism due to the invasive nature of cell or tissue collection, especially when gill, liver or digestive gland cells are used. Therefore, the successful use of hemolymph sampled non-lethally from a unionid mussel is an important finding from this study because of the global imperilment of this fauna and the fact that they have been rarely utilized in genotoxicity assessments. To date, research within bivalve genotoxicity has focused mainly on marine species. Most often, *Mytilus sp*. is used as a sentinel species in biomonitoring studies (Rocher et al., 2006; Lee & Steinert, 2003; Wilson et al., 1998), but Stambuk et al., (2009) have recently used a unionid species (Unio pictorum) placed in cages in two Croatian rivers to assess genotoxicity in polluted freshwaters. There are many advantages to the use of mussel hemolymph with the comet assay, for example, few cells are required and mussel hemolymph contains numerous hemocytes, with a median level of 1018 cells/µL (Gustafson et al., 2005b). Moreover, the results provided by mussel hemocytes, as demonstrated by the overall mean of the control and positive control data (Figure 2), were extremely reproducible throughout all tests. In addition, minimal manipulation of the hemolymph and hemocytes was required for the comet assay, thus creating less opportunity for error.

The PAHs and hydrogen peroxide were genotoxic in *in vitro* exposures of whole hemolymph under our testing conditions and two total PAH concentrations (50 and 100 µg/L) produced statistically significant levels of DNA damage, or genotoxicity in comparison to the controls. Because genotoxicity was detected during the *in vitro* exposure with PAHs, an *in vivo* exposure with PAHs was performed to assess the predictive capabilities of the *in vitro* test. The *in vitro* PAH exposure produced a much greater genotoxic response with both parameters (% tail DNA and OTM) than was detected *in vivo*, in which only the hydrogen peroxide yielded statistically significant levels of DNA damage. Thus, under the conditions tested in this study, *in vitro* exposure was unable to predict a similar *in vivo* response. This may be due in part to the inability to definitively determine or measure the exact exposure concentration of PAHs reaching the hemolymph through the waterborne exposure route. A 3-d *in vivo* PAH exposure allowed for *Elliptio complanata* to reach steady state with the PAHs (Thorsen et al., 2004), however, the actual exposure concentration of the hemocytes to PAHs *in vivo* remains unknown. Nonetheless, our results demonstrated a high degree of method accuracy, evident in the consistent levels of DNA damage measured in the CometAssay Control Cells™ and hydrogen peroxide treatments. For those reasons, we are confident that if the concentrations of PAHs were genotoxic under the tested conditions, the effects would have been detected. A variable that may have influenced the outcome of the *in vivo* tests with the PAHs is the physiological role of mode of action and metabolism. Of particular concern is when the mode of action causing genotoxicity is dependent on the formation of reactive metabolites or metabolic activation. For example, PAHs are well known genotoxic agents, demonstrated to cause DNA damage in marine mussels, *Mytilus sp*., either by direct DNA strand breakage via the generation of reactive oxygen species or indirectly by the formation of reactive intermediates that form unstable DNA adducts (Mitchelmore et al., 1998; Hartl et al., 2004). The extent to which the PAHs were inhibited in their mode of action and/or metabolism or caused toxicity other than DNA damage in whole hemolymph exposed in this study is unclear and requires further research.

There are also multiple procedural steps in the comet assay that involve the factor of time, all of which have varied considerably from study to study (Fairbairn et al., 1995). As a consequence, the influence of time could potentially impede the detection of genotoxicity. For this study, the length of time for cell lysis, alkaline unwinding, and electrophoresis were partially dictated by the manufacturer's protocol (Trevigen, 2007). The protocol suggests conducting cell lysis for 30 min to 1-h and alkaline unwinding for 20 min to 1-h, whereas Tice et al., (2000) state that a minimum cell lysing of 1-h and unwinding of 20 min is preferred. Therefore, cell lysing was conducted for 1-h and alkaline unwinding was 20 min. Longer lysing times, up to 24-h, were evaluated, but the specially coated slides could not withstand the high salts and detergents of the solution, reflected in the degradation of the agarose gel. Thus, our confidence is maintained that the times used for cell lysing and alkaline unwinding in this study provided a sufficient amount of time to liberate and unwind the DNA. Electrophoresis is another influential and variable step, where the conditions of which have varied from laboratory to laboratory based on time, temperature, size of electrophoresis unit, power supply, and set voltage

(Tice et al., 2000). The manufacturer's protocol recommended 20 to 40 min (Trevigen, 2007), whereas Singh et al., (1988) performed electrophoresis for 20 min, and the guidelines (Tice et al., 2000) report a range of 5 min to 40 min, stating that 20 min is sufficient. Through preliminary research using the CometAssay Control Cells™ that were run as a measure of quality control with every *in vitro* experiment in this study, we determined that 40 min of electrophoresis was optimal to reach the reported means for % tail DNA, a time well within the recommendations. Moreover, the same electrophoresis unit and power supply, set to a constant voltage of 1.0 V/cm2 and brought to 300 mA was used throughout the study. Therefore, minimal variation was expected to have arisen from the electrophoresis procedure because all variables were kept constant, partially evident in the negligible standard deviations of the CometAssay Control Cells™.

The intent of this study was to evaluate the genotoxicity of a class of environmentally relevant compounds (i.e., PAHs) at ecologically relevant concentrations; given that mussels are facing peril within their own habitats (Cope et al., 2008). Although testing realistic exposure concentrations of PAHs was of importance, future genotoxicity studies with unionids and PAHs might benefit from an expanded and higher concentration range, as well as evaluating mixtures of these and other compounds, which would represent an even greater realistic exposure scenario. The genotoxic potential of PAHs has been extensively studied, as mentioned previously, in mixture form or singly, most notably benzo[a]pyrene (B[a]P]). The concentration range of total PAHs used in our study was similar to that used in an in vivo experiment with the Pacific oyster, *Crassostrea gigas*, which demonstrated adverse effects on fertilization capability and larval development (Jeong & Cho, 2005). This could be explained by the results obtained from another study with *C. gigas*, in which embryos were used to investigate the relationship between the embryotoxic and genotoxic effects of B[a]P (Wessel et al., 2007). A positive and significant correlation was demonstrated in the oyster embryos between genotoxicity and embryotoxicity; such a connection between embryotoxicity and genotoxicity caused by the PAH B[a]P, a widespread aquatic contaminant, is of great concern at the individual and community level. It is probable that the near persistent exposure to B[a]P can lead to sub-lethal effects in bivalves and over time decrease their population, yet the exact cause may be unidentifiable at the time of the observed decline.

Although variables associated with the exposure conditions, method, test concentrations or mode of action may have influenced the detection of genotoxicity in this study, the significant hydrogen peroxide and CometAssay Control Cell™ data demonstrate the accuracy and reliability of the results obtained. We, therefore, remain confident that if the concentrations of PAHs tested during both the *in vitro* and *in vivo* experiments had been genotoxic, the effects would have been detected with the assay. This research investigated the use of a non-lethal genotoxicity screening tool using unionid mussel hemolymph. Based on our results, additional testing and evaluation is needed before this tool could be widely implemented in biomonitoring programs to detect all potential classes of genotoxicants. Moreover, there is need for a better understanding of unionid mussel hemolymph and the functions and capabilities of hemocytes in their defense and repair of genotoxic compounds.

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OUR PURPOSE

The Freshwater Mollusk Conservation Society (FMCS) is dedicated to the conservation of and advocacy of freshwater mollusks, North America's most imperiled animals. Membership in the society is open to anyone interested in freshwater mollusks who supports the stated purposes of the Society which are as follows:

1) Advocate conservation of freshwater molluscan resources;

2) Serve as a conduit for information about freshwater mollusks;

3) Promote science-based management of freshwater mollusks;

4) Promote and facilitate education and awareness about freshwater mollusks and their function in freshwater ecosystems;

5) Assist with the facilitation of the National Strategy for the Conservation of Native Freshwater Mussels (Journal of Shellfish Research, 1999, Volume 17, Number 5), and a similar strategy under development for freshwater gastropods.

OUR HISTORY

The FMCS traces it's origins to 1992 when a symposium sponsored by the Upper Mississippi River Conservation Committee, USFWS, Mussel Mitigation Trust, and Tennessee Shell Company brought concerned people to St. Louis, Missouri to discuss the status, conservation, and management of freshwater mussels. This meeting resulted in the formation of a working group to develop the National Strategy for the Conservation of Native Freshwater Mussels and set the ground work for another freshwater mussel symposium. In 1995, the next symposium was also held in St. Louis, and both the 1992 and 1995 symposia had published proceedings. Then in March 1996, the Mississippi Interstate Cooperative Research Association (MICRA) formed a mussel committee. It was this committee (National Native Mussel Conservation Committee) whose function it was to implement the National Strategy for the Conservation of Native Freshwater Mussels by organizing a group of state, federal, and academic biologists, along with individuals from the commercial mussel industry. In March 1998, the NNMCC and attendees of the Conservation, Captive Care and Propagation of Freshwater Mussels Symposium held in Columbus, OH, voted to form the Freshwater Mollusk Conservation Society. In November 1998, the executive board drafted a society constitution and voted to incorporate the FMCS as a not-for-profit society. In March 1999, the FMCS held it's first symposium "Musseling in on Biodiversity" in Chattanooga, Tennessee. The symposium attracted 280 attendees; proceedings from that meeting are available for purchase. The second symposium was held in March 2001 in Pittsburgh, Pennsylvania, the third in March 2003 in Raleigh, North Carolina, the fourth in St. Paul, Minnesota in May 2005, the fifth in Little Rock, Arkansas in March 2007, and the sixth in Baltimore, Maryland in April 2009. The society also holds workshops on alternating years, and produces a newsletter three times a year.

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