ABSTRACT

PROCHAZKA, SHARON T. Assessing DNA Damage in Hemocytes of the Freshwater Mussel *Elliptio complanata* with the Comet Assay. (Under the direction of W. Gregory Cope.)

The single cell gel electrophoresis or comet assay is widely used to detect DNA damage following exposure to genotoxic compounds with cells isolated from tissue or circulatory fluids from various organisms. The cell sampling method can often lead to the fatality of the test organism, depending on the type of tissue sampled. The objective of this study was to assess genotoxicity with the comet assay from in vitro and in vivo studies using a non-lethal hemolymph sampling method on *Elliptio complanata*, a native freshwater mussel found abundantly in North Carolina. The hemolymph was withdrawn from the anterior adductor muscle sinus and exposed in vitro to various concentrations (previously tested for cell viability) of selected pesticide formulations, Aatrex® 4L herbicide (atrazine), Roundup® herbicide (glyphosate), Lorsban® 4E insecticide (chlorpyrifos), and Thionex® 3EC insecticide (endosulfan), technical grade copper sulfate and a mixture of 46 different PAHs for 4-hours at 4°C. After exposure, the hemocytes were isolated and the comet assay was performed on the treated cells, control cells, and positive control cells exposed to 160 μM hydrogen peroxide (H_2O_2). For quality assurance purposes, CometAssay Control Cells™ were analyzed throughout each comet assay procedure in the laboratory to ensure the validity of results. The levels of DNA damage measured were expressed as % tail DNA and olive tail moment (OTM). Of all the compounds and concentrations tested during the in vitro exposures, excluding the positive H_2O_2 controls, only the mixture of PAHs yielded statistically significant ($P < 0.05$) levels of DNA damage compared to the controls for both DNA damage

parameters. The DNA damage was observed at 50 and 100 μg/L PAHs, with % tail DNA of 40.70 % and 38.55 %, and OTM of 12.41 and 11.03, respectively. The remaining test compounds yielded no detectable genotoxic effects, regardless of the DNA damage parameter, under the specified concentrations and test conditions. 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 then was detected in vivo, in which only the positive control yielded statistically significant levels of DNA damage.

Thus, under the conditions tested in this study, in vitro exposures with freshwater mussel hemolymph were unable to predict a similar in vivo response, based on the presented data. Nonetheless, a method with a high degree of accuracy was demonstrated during this study with consistent and repeatable levels of DNA damage measured in the CometAssay Control cells™ and positive control treatments. Therefore, we are confident that if the chemicals tested had been genotoxic under the conditions tested, the effects would have been detected. This research investigated the use of a non-lethal genotoxicity screening tool using freshwater mussel hemolymph. Further testing and evaluation is needed before this tool could be widely implemented. Moreover, there is need for a better understanding of freshwater mussel hemolymph and the functions and capabilities of hemocytes in the defense of genotoxic compounds.

Assessing DNA Damage in Hemocytes of the Freshwater Mussel *Elliptio complanata* with the Comet Assay

by

Sharon T. Prochazka

A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Environmental Toxicology

Raleigh, North Carolina

2007

APPROVED BY:

W. Gregory Cope, PhD Chair of Advisory Committee	Heather M. Cheshire, PhD Minor Representative
David B. Buchwalter, PhD Committee Member	Leslie Recio, PhD Committee Member

DEDICATION

To my dear husband Mark, your inexhaustible support, guidance and love has made everything possible. I dedicate this thesis to our son, due to arrive into this world on January 17, 2008. For I can only hope that the preparation of this thesis hasn't caused you any unnecessary stress while in utero. You have provided me with much entertainment (and distraction!) during the writing process, as well as the incentive to meet my deadlines. Your father and I eagerly anticipate your arrival, we can't wait to share life with you, teach you and learn from you.

BIOGRAPHY

"If you follow your bliss, you put yourself on a kind of track that has been there all the while, waiting for you, and the life that you ought to be living is the one you are living. Wherever you are—if you are following your bliss, you are enjoying that refreshment, that life within you, all the time" - Joseph Campbell

When I ask myself what my purpose is in life, what I aspire to, there are many possible answers. However, occupation, defined as the principal business of ones life, is one of the most important and concrete answers. So in keeping with the words of Joseph Campbell, I would like to take this opportunity to share with you my bliss, what led me to the field of Environmental Toxicology.

I believe the field of Environmental and Molecular Toxicology is one of significant purpose. When considering the exponentially expanding global community and the ever-tipping balance between the needs of society, industry and nature, it is clear that I have always been interested in and gravitated towards an intellectual and practical exploration of how to maintain the quality of our collective environment.

Prior to graduate school I worked within the semi-conductor industry. Although it taught me many of the necessary skills for success, including both specific, industry-related knowledge and, on a larger scale, how to succeed outside my comfort zone, I discovered that although financial stability is rewarding, it is not enough. I learned that I needed an enriching occupation with purpose. After extensive research, I realized that the field of

Environmental Toxicology encompassed my innate interests. Some of these interests include the overall health of the environment, especially the air, water and soil in which all living organisms depend, and when mismanaged, all organisms experience the effects. Yet, it became evident that to meaningfully pursue these interests, further education was necessary.

As my graduate studies come to an end, I envision, and am eager to apply Environmental Toxicology as a voice of reason in researching the effects of toxicants on humans and the environment, ideally, while performing Geographic Information Science (GIS) analyses. In addition, I am ready to embark on the next exciting, challenging and fulfilling track of life with my spouse, the bliss of parenthood!

ACKNOWLEDGMENTS

I would like to begin by extending my gratitude to Dr. Robert Bringolf for the many hours you so patiently provided, mentoring me along the way with my research, as well as sharing in life's experiences. Your guidance helped tremendously during the roller coaster ride of graduate school.

I am also extremely grateful for the support of my committee member, Dr. Les Recio, as well as Cathy and John at Integrated Laboratory Systems. If it weren't for your generosity, this research would not have been possible (well, it would have just taken a lot longer!). I can't thank you enough for all the equipment donations and use of the software and microscope. Cathy and John, thanks to you both for all the assistance with the macros and conversation while scoring for hours upon hours in a dark room while listening to NPR.

The best part about using living organisms (especially when you don't have to sacrifice them) is the field trips. So, to my laboratory partners and fellow mussel collectors, I thank you for your time and conversation - Robert, Pete, Shad, Tamara, Kelly, and Natalie. Even when it involved stopping for BBQ and watching you eat! You all have made my experience in graduate school one to remember and fondly reflect on. Also, an extra thank you to Pete for his analytical skills in preparing all of the test chemicals.

I would also like to thank Dr. David Buchwalter for his guidance and Dr. Heather Cheshire. Last but certainly not least, to Dr. Gregory Cope, my advisor throughout the whole process. Thank you for your continual support and patience and for always having an open door. I am thankful for the opportunity to work in your laboratory, the research experience has provided me with so much more then what is contained within this thesis. And to my sweet dog Rizoe, thank you for keeping my feet warm and my spirits up.

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Introduction

Freshwater ecosystems in North America are home to about 12,580 described species of invertebrates, of which 820 are mollusks (Williams *et al.* 1993). There are eighteen families of bivalves (Phylum: Mollusca) that have flourished in such freshwater ecosystems. One order in particular that has been the most successful in diversifying is Unionoida, which is divided into two superfamilies, with Unionidae being one of six constituent families consisting of 165 recognized genera (Bogan 1993). However successful Unionoida have been, they are also considered extremely sensitive to disturbances in freshwater ecosystems and are recognized as the most endangered group of mollusks in the world (Neves 1999). Unless devastation is caused by a traumatic event, such as a flood or toxic spill, impacts on a population may not be immediately detectable. This is in part due to their sessile, filter and deposit feeding behaviors combined with a lifespan of 30-130 years (Bauer 1992). Such behaviors provide numerous opportunities, potentially for an entire lifespan depending on location, for exposure and accumulation of anthropogenic contaminants. The cumulative effect of contaminant exposure on non-target organisms, such as native freshwater bivalves, is largely unknown during realistic exposure scenarios. However, as early as 1909, Ortmann documented the effects of pollution on the destruction of the freshwater molluscan fauna (Ortmann 1909, 1918).

One potential effect of pollution is DNA damage, a consequence of genotoxic compounds interacting with biologically important molecules, resulting in a damaging chain of events. 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). However, if the defenses fail, are overwhelmed, or suppressed, DNA damage can occur, but with uncertain consequences. 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 and Steinert 2003). It is probable that the continual exposure of contaminants can lead to such sublethal effects in bivalves, rather than overt lethality, and over time the decrease in population becomes apparent, yet the exact cause unidentifiable at the time of detection.

Research within the field of mollusk or bivalve genotoxicity has focused mainly on marine species. Most often, *Mytilus sp.* is used as a sentinel species in biomonitoring studies with gill and digestive gland cells or hemolymph collected post in situ exposure, in vivo and in vitro (Gielazyn *et al.* 2003; Lee and Steinert 2003). In vivo experimentation often results in the destruction of the organism due to the invasive nature of cell or tissue collection, especially with gill and digestive gland cells, which are most commonly used with bivalves. Rigonato *et al.* (2005) found hemolymph to be a valuable target tissue 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 freshwater bivalve, *Corbicula fluminea*. Hemolymph, the circulatory fluid of bivalves, contains hemocytes, which are collectively involved in a variety of physiological and pathological functions throughout the mussel body; including 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), conducted by Gustafson *et al.* (2005a) demonstrated the lack of negative impacts on survival or growth. Therefore, sampling hemolymph from bivalves has the potential to provide information pertinent to the health assessment of individual animals or populations without inflicting harm and has thus been used in numerous studies, including genotoxicity studies using the comet assay (Hamoutene *et al.* 2002; Buschini *et al.* 2003; Siu *et al.* 2004; Rocher *et al.* 2006; Rigonato *et al.* 2005).

The single cell gel electrophoresis assay or comet assay is a method commonly used to analyze and quantify DNA damage in individual eukaryotic cells. The comet assay involves embedding a single cell suspension in agarose onto microscope slides, which are then lysed to liberate the DNA from the cells. Following lysis, the slides are immersed in a solution to unwind the DNA and then electrophoresed on a horizontal electorophoresis apparatus. The damaged, unwound DNA migrates out of the cell towards the anode and the slides are stained with a fluorescent DNA binding agent for comet visualization via fluorescence microscopy. Thus, the greater the extent of DNA that is liberated from the cell nucleus and pulled into a "comet" is directly proportional to the amount of DNA damage. The techniques were first developed by Rydberg and Johanson (1978) and later modified by Ostling and Johanson (1984) to detect double-stranded DNA breaks. Further adaptations were made by Singh *et al.* (1988) to include more alkaline conditions (pH > 13) so that even

lower levels of DNA strand breaks could be detected with greater sensitivity**.** At an International Workshop on Genotoxicity Test Procedures held in Washington, DC, an expert panel developed guidelines for the use of the comet assay in genetic toxicology (Tice *et al.* 2000). The guidelines were based on the structure used in the current Organisation for Economic Co-operation and Development (OECD) guidelines for in vitro genetic toxicity testing (Tice *et al*. 2000). The panel agreed that the optimal version of the assay was the more alkali (pH > 13) version, operated under alkaline DNA unwinding and electrophoresis conditions, allowing for the detection of DNA single-strand breaks (SSB), but also alkali-labile sites, DNA-DNA/DNA-protein crosslinking and SSB associated with incomplete excision repair sites (Singh *et al.* 1988; Tice *et al.* 2000). Our laboratory chose to adhere to the recommendations in the guideline to contribute towards standardizing procedures across research laboratories.

Research involving the genotoxicity of current use pesticides on native freshwater mussels is currently limited. Therefore, the objective of this study was to combine the alkali comet assay with hemolymph collected from the abundant, freshwater mussel *Elliptio complanata* to determine the DNA damaging effects of environmentally relevant, freshwater contaminants at realistic exposure concentrations. The chemicals selected were chosen on the basis of their extensive use and frequency of detection in U.S. surface waters (USGS 1999). Specifically, the following pesticide formulations, with their active ingredients where appropriate, were selected for testing: Aatrex® 4L (atrazine), Roundup® (glyphosate), Lorsban® 4E (chlorpyrifos), Thionex® 3EC (endosulfan), and technical grade copper sulfate.

Atrazine is a widely used triazine herbicide, with approximately 76.5 million pounds applied to control broadleaf and grassy weeds in agricultural and non-agricultural applications (USEPA 2001). Glyphosate, a phosphanoglycine non-selective herbicide is also used to control broadleaf and grassy weeds in food, non-food crops and non-crop areas like roadsides. Chlorpyrifos, a restricted-use compound, is the second most common organophosphorous broad-spectrum insecticide used in the United States, with 11-16 million pounds applied annually in 2001 for applications such as agricultural crops, livestock, and mosquito control (USEPA 2001). Endosulfan, an organochlorine pesticide used as a broad-spectrum contact insecticide and acaricide, is registered for use on a wide variety of food and non-food crops. Copper sulfate, an essential metal that is a component of many inorganic fertilizers and fungicides, is registered as a general use pesticide and classified as highly toxic.

In addition to pesticides, the genotoxicity of a mixture of Alaskan North Slope crude oil and creosote (Thorsen *et al.* 2004)**,** containing 46 different polycyclic aromatic hydrocarbons (PAHs) was investigated. PAHs are a group of environmentally toxic and persistent chemicals that enter the aquatic environment through many natural and anthropogenic processes. For instance, forest fires, volcanic eruptions, coal and oil-fired power plants, and waste incinerators generate emissions of PAHs, which can then enter the aquatic environment during precipitation events, carried in rainfall and runoff. Included in the mixture were six of the seven PAHs listed by the United States Environmental Protection Agency (USEPA) in the 1986 guidelines as probable carcinogens: benzo[a]anthracene,

benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenz[a,h]anthracene, and chrysene (USEPA 1986).

In an effort to increase the use of non-lethal and in vitro methods, the overall intent of this research was to determine whether the in vitro exposure of freshwater mussel hemolymph has the potential to forecast results of in vivo exposure using the comet assay. When a test substance yielded statistically significant levels of genotoxicity or DNA damage in vitro, an in vivo experiment was performed using equivalent exposure concentrations. Hence, an attempt was made to develop a non-lethal, in vitro, genotoxicity-screening tool using native freshwater mussels.

Materials and methods

2.1 Sample collection and animal husbandry

For this study, *Elliptio complanata* or eastern elliptio, were collected from a relatively uncontaminated (Thorsen *et al*. 2004), rural forested segment of the Eno River that flows through Hillsborough in Orange County, North Carolina. The Eno River is forty miles long, flowing through a mix of habitat, including urban areas. It has high biodiversity, an indicator of good water and habitat quality, including 12 species of freshwater mussels. Field collection events for this study occurred from May 2005 through August 2007, and involved the hand-collection of ~30 mussels per sampling trip, held in dive bags and transported in coolers containing site water to the laboratory on the campus of North Carolina State University in Raleigh, NC. Once in the laboratory, the mussels were held within an aerated,

recirculating living stream (Frigid Units Inc., Toledo, OH, USA) with soft water (ASTM 2006; pH 7.2-7.6, hardness 40-48 mg $CaCO_3$/L, and alkalinity 30-35 mg $CaCO_3$/L) at temperatures consistent with river temperatures and fed a commercial mixture of nonviable microalgae prepared from Instant Algae® Shellfish Diet 1800 and *Nannochloropsis* (Nan 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 was two months, after which time, the mussels used for in vitro experimentation (i.e., hemolymph withdrawl) were returned unharmed to the Eno River downstream of the original collection site to avoid repeat sampling.

2.2 In vitro experimentation

2.2.1 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 and a sterile 1.0 mL syringe (PrecisionGlide™, Becton Dickinson and Company, Franklin Lakes, NJ, USA) with a 25-gauge needle was inserted into the anterior adductor muscle sinus. Up to 1 mL of hemolymph was extracted per mussel. The needle was removed from the syringe prior to expelling the hemolymph into a 20 mL Nalgene® test tube. An equal amount of Alsever's Solution (Sigma-Aldrich, St. Louis, MO, USA), an isotonic, balanced salt solution containing ethylenediaminetetraacetic acid (EDTA) and glucose, used to prevent the spontaneous aggregation of hemocytes upon extraction from the mussel (Bachere *et al.* 1988; Chen and Bayne 1995), was drawn into the syringe for rinsing before

adding it to the 20 mL test tube. All successive hemolymph samples were immediately pooled in the test tube to minimize inter-individual variability, until the necessary volume for the 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 added, thus the final working volume of the hemolymph-Alsever mixture was between 14 to 18 mL.

2.2.2 Test chemicals and exposure concentrations

The following pesticide formulations were obtained from retail suppliers for the in vitro exposures: Aatrex® 4L herbicide (40.8% active ingredient, atrazine; Syngenta), Roundup® herbicide (50.2% active ingredient, glyphosate, isoproplyamine salt; Monsanto), Lorsban® 4E insecticide (44.9% active ingredient, chlorpyrifos; Dow AgroSciences, LLC), and Thionex® 3EC insecticide (33.7% active ingredient, endosulfan; MANA). In addition, technical grade copper sulfate (Fisher Scientific, Fair Lawn, NJ, USA) and an Alaskan North Slope crude oil (Battelle, Duxbury, MA, USA) and creosote (AccuStandard Inc., New Haven, CT, USA) mixture containing 46 different PAHs (Thorsen *et al.* 2004) were screened in vitro.

The concentration range for each test compound was determined based on measured concentrations commonly reported within freshwater, aquatic environments (USGS 1999). In addition, the test concentration range was further refined based on results published from toxicity tests performed on the early life stages (glochidia and juveniles) of freshwater mussels (Bringolf *et al.* 2007a,b,c). The target exposure concentrations were based on the active ingredient, especially within the formulations, sometimes up to concentrations

approaching water solubility. All test preparations were dissolved or suspended in distilled water, except the PAH mixture, which required acetone. All test exposure concentrations of pesticides, copper and PAHs were validated with empirical measurements using standard methods, as previously described (Thorsen *et al.* 2004; Bringolf *et al.* 2007a,b,c).

2.2.3 Exposure setup

The remaining procedures were conducted within a laboratory without sunlight and the florescent overhead lights were shielded with yellow light covers to protect hemolymph from ultraviolet radiation. This was done to minimize background levels of DNA damage. 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 positive controls (hydrogen peroxide), as well as a solvent control (acetone), when necessary. When hemolymph collection was complete, the in vitro experiment required 650 μL of the homogenous, pooled hemolymph-Alsever mixture, which was aliquoted from the 20 mL test tube into the appropriate wells of the plate. The test compound was added to the corresponding well from low to high concentration, agitated gently, covered, and incubated in the dark at 4°C for the 4-hour exposure period. Agitation of the well plate was conducted for 1 minute at 30-minute intervals during the exposure period. The incubation temperature was based on a previous study conducted by Cheung *et al.* (2006) and the length of the exposure period was in accordance with the panel of expert guidelines (Tice *et al.* 2000), which state that cells in vitro tests should be in contact with the test solution for 3 to 6 hours. While the exposure was in process, a Ca^{2+} and Mg^{2+}

free 1 X phosphate buffered saline (1XPBS) (Cambrex Bio Science, Walkersville, MD, USA) solution was prepared and mixed 1:1 with Alsever's solution. Upon exposure completion, the hemolymph-Alsever-test compound mixture within the designated wells, were transferred via micropipette into individually labeled 2 mL microcentrifuge tubes. Then 600 μL of the 1XPBS-Alsever solution was used to rinse the well, and added to the microcentrifuge tube. The exposed hemolymph mixture was centrifuged at 1100 g for 4 minutes, the supernatant decanted and the hemocyte cell pellet resuspended in 600 μL of the 1XPBS-Alsever solution, and repeated 2 times. After the final rinse, the hemocyte cell pellets were brought to a final working volume of 325 μL.

2.2.4 Cell viability

The approach used when selecting exposure concentrations in an in vitro comet assay is to avoid testing concentrations that decrease cell viability by more than 30%, compared to the control cells. Cell viability tests were conducted using 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, Sokolova *et al.* 2004). 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 μmol l^{-1} 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 non-exposed and exposed

hemocytes, isolated and resuspended in 1XPBS-Alsever buffer solution in 96-well plates (Corning® Costar®, Sigma-Aldrich, St. Louis, MO, USA). 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 protein concentration in the unexposed, pooled hemolymph-Alsever mixture. Standards were prepared using the BSA to generate a linear standard curve by plotting absorbance at 595 nm (Spectronic® Genesys™, Milton Roy Company, Rochester, NY, USA) versus protein concentration. In triplicate, 10, 15 and 20 µL of the hemolymph-Alsever mixture was aliquoted into separate microcentifuge tubes, brought to a volume of 100 µL with 0.15 M NaCl, and 1mL of the Bradford Reagent was added. The microcentrifuge tubes were vortexed and allowed to stand at room temperature for 2 minutes. The samples were then transferred to 1 mL microcuvettes, absorbance measured at 595 nm, and protein concentration determined in µg of protein based on the BSA standard curve.

To establish the cell viability procedure, range-finding cell viability tests were performed using the positive control, 30% hydrogen peroxide (H_2O_2) (VWR International, West Chester, PA, USA) to determine the appropriate concentration for the 4-hour in vitro exposures. Once the positive control concentration was determined, the test compounds underwent the same range-finding cell viability tests to confirm acceptable exposure concentration ranges. The concentration of acetone for the solvent control was determined by using the greatest concentration of acetone required in the preparation of the chemical 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. This applied for all exposure concentrations, including the controls, positive controls and solvent controls, and was compared to the unexposed, pooled hemolymph-Alsever subsample or baseline levels measured immediately after extraction, to minimize time for the natural degradation of ATP.

2.2.5 Genotoxicity

Once cell viability was determined to be no more than 75% below baseline levels for all exposure concentrations and the 4-hour in vitro exposure period was complete, the single cell gel electrophoresis assay, or 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), two-well microscope slides that allow for the processing of two samples on a single slide and specially treated to promote agarose adherence, were used in accordance to the manufacturer's protocol (Trevigen 2007).

During processing, the humidity of the laboratory was monitored and maintained below 60% with a dehumidifier. The isolated hemocytes, suspended in 1X PBS-Alsever solution at approximately 1 X 10^5 mL^{-1} were combined with 500 μL molten 37°C low melting agarose (LMA) (Trevigen). 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. Therefore, each exposure, including the controls, positive controls and solvent controls, was represented by a total of six slides. The prepared CometSlides™ were placed flat on slide

trays and incubated at 4°C for 30 minutes to increase the adherence of the samples on the slides. Then the slides were immersed in prechilled lysis solution (Trevigen) in coplin jars and incubated at 4°C for 60 minutes. After cell lysis, the slides were drained to remove residual salts and transferred to clean coplin jars containing freshly prepared pH > 13 alkaline solution containing NaOH (Mallinckrodt Baker Inc., Paris, KY, USA) and 200 mM EDTA (Trevigen) for 20 minutes to unwind and denature the DNA. Next, the slides were placed onto a recirculating, horizontal electrophoresis apparatus (Fisher Scientific, Pittsburgh, PA, USA) and freshly prepared alkaline electrophoresis solution (pH > 13, 300 mM NaOH, 1 mM EDTA) was poured into the side wells of the apparatus until the solution just covered the CometSlides™. The voltage on the power supply unit was set to 1 volt/cm, buffer was added or removed until the current reached 300 mA, and electrophoresis was performed for 40 minutes. Once complete, the slides were transferred from the electrophoresis chamber to slide holders, rinsed 3X in distilled water, then fixed in 70% ethanol (EMD Chemicals, Inc., Gibbstown, NJ, USA) for 5 minutes, 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 two DNA damage parameters measured and reported include % tail DNA, the percentage of DNA in the comet tail, and olive tail moment or OTM, the product of the fraction of DNA in the tail

and tail length, a unitless measurement. The hemocytes were scored 25 cells per well, for a total of 50 cells per slide in duplicate per treatment with three replicates per treatment. A total of 300 cells were scored per exposure concentration. Each slide was labeled with a code unrelated to treatment and processed randomly to reduce potential bias during image analysis.

2.2.6 Quality control

As a measure of quality control, CometAssay Control Cells™ (Trevigen) were used to assess the comet assay procedure in the laboratory and 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 genotoxin) 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): CCO 11.43 % (3.37), CC1 28.96 % (2.59), CC2 39.39 % (2.64), and CC3 49.62 % (3.48). The levels obtained by Trevigen are the following in % tail DNA: CC0 5.75 % (7.72), CC1 28.37 % (14.01), CC2 39.74 % (21.82), and CC3 56.8 % (23.59). All results obtained within this study using the Control Cells™ were reflective of the mean % tail DNA values established by Trevigen. The data are displayed in Figure 9. The values reported by Trevigen represent one slide, with 50

cells counted per slide, and the standard deviation is in reference to the 50 individual cells scored. The values presented from NCSU represent six separate comet assay experiments, with one slide prepared per experiment for each treatment and 50 cells scored per slide. Thus, the standard deviation reported in this study references the deviation between the six independent experiments, with a total of 300 cells scored. The Control Cells™ were prepared on CometSlides™ as previously described in the testing protocol and included in all successive procedural steps of the comet assay alongside the CometSlides™ prepared with mussel hemocytes for all in vitro comet assay experiments.

2.3 In vivo PAH exposure

2.3.1 Exposure set up

Field collection of mussels occurred in August of 2007 and upon return to the lab, the mussels were acclimated in aerated coolers containing site water to the test temperature, 18-23°C. The mussels were not fed during the 3-day acclimation or during the 3-day experiment. Once acclimated, the shell of the mussels were gently cleaned of debris with a soft-bristled brush and returned to clean, aerated coolers containing 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 similar to the in vitro exposure, 1, 10, 50, 100, and 200 μg/L of PAHs into the appropriate 2-L glass aquarium with a glass syringe, for a total exposure duration of 3 days. In addition, a positive control consisting of 30% H_2O_2 was used,

however, the peroxide concentration of 160 μM from the in vitro exposure 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 hour at concentrations up to 1000 μM H_2O_2 (Wilson *et al.* 1998). All exposure concentrations, as well as the controls, positive controls and acetone solvent controls were conducted in triplicate. A 48-hour renewal of test concentrations and exposure water was conducted for all treatments. Measurements of water chemistry, following standard methods were also conducted every 48 hours 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.

2.3.2 Hemolymph collection and the comet assay

Following collection from the river, two mussels were randomly selected to represent baseline or background levels of incoming DNA damage. Hemolymph was sampled as previously described and these mussels were not used for further experimentation. Thus, hemolymph was sampled on two separate occasions, once from the mussels chosen to represent baseline levels and then on day 3 of the PAH in vivo exposure. Hemolymph collection from the in vivo PAH exposed mussels was modified slightly from the in vitro collection, in that only 300 μL of hemolymph was extracted per mussel and expelled into individually labeled microcentrifuge tubes containing 300 μL Alsever's solution. The

hemocytes were then isolated by centrifugation at 1100 g for 4 minutes, supernatant decanted, and the cell pellet rinsed 2X with 1XPBS-Alsever solution and brought to a final working volume of 300 μL. As described previously in the genotoxicity section, the comet assay and the quantification of DNA damage procedures were followed immediately after hemocyte isolation.

2.4 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 the treatments.

Results

3.1 In vitro

3.1.1 Negative and positive control exposure concentration, cell viability, and genotoxicity

The concentration range tested to establish a suitable positive control, hydrogen peroxide, 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 levels (n = 3) (Figure 1a and Figure 1b). Thus, 160 μM H_2O_2 was chosen as the positive control concentration given that it provided an acceptable level of cell viability, ~80%, with a statistically

significant ($P < 0.05$) amount of genotoxicity in comparison to the controls for all in vitro exposures. Overall genotoxicity results, reported as % tail DNA and OTM, for all controls and positive controls, processed in triplicate per in vitro experiment, are displayed in Figure 2a and Figure 2b. The controls yielded a mean tail DNA of 17.91 % (2.62) and OTM of 4.20 (0.82) ($n = 18$). The positive controls yielded a mean % tail DNA of 39.68 % (4.30) and OTM of 13.32 (2.13) ($n = 17$).

3.1.2 Test chemical exposure concentrations, cell viability and genotoxicity

Hemolymph from a total of 139 *Elliptio complanata* was used during all in vitro experimentation. The average weight of test mussels was 81.2 grams (range 44.8 - 149.6 g) and the average length was 300.8 mm (range 188.3 - 322.7 mm). In accordance with the comet assay protocol (Tice *et al.* 2000), each comet assay experiment included 5 to 8 test concentrations (minimum of 3 required). All of the concentration ranges chosen yielded cell viability levels greater than 85%, and were thus used for the 4-hour in vitro comet assay exposures, these included, Aatrex® 4L herbicide; 1 to 16 mg/L atrazine, Roundup® herbicide; 0.8 to 12.5 mg/L glyphosate, Lorsban® 4E insecticide; 0.13 to 2 mg/L chlorpyrifos, Thionex® 3EC insecticide; 0.25 to 10 μg/L endosulfan, copper sulfate; 2.25 to 72 μg/L $CuSO_4$, and total PAH mixture; 0.05 to 200 μg/L PAHs.

Of all the compounds and concentrations tested during the 4-hour in vitro exposure, excluding the positive control, only the mixture of 46 different PAHs yielded statistically significant ($P < 0.05$) levels of DNA damage compared to the controls for both DNA damage parameters. This was observed at 50 and 100 μg/L PAHs, with % tail DNA of 40.70 % (2.12)

and 38.55 % (0.08), with an OTM of 12.41 (2.08) and 11.03 (0.33), respectively (Figure 3a and Figure 3b). No other PAH concentrations elicited statistically significant levels of DNA damage in comparison to the controls. For the five remaining test compounds, no genotoxic effects were detected under the specified experimental conditions, regardless of the DNA damage parameter.

Atrazine, or Aatrex® 4L herbicide, the level of DNA damage ranged from 12.58 to 18.31 % tail DNA and OTM of 3.09 to 5.26 (Figure 4a and Figure 4b). Glyphosate, or Roundup® herbicide, the level of DNA damage ranged from 16.97 to 18.45 % and OTM of 3.86 to 5.17 (Figure 5a and Figure 5b). Chlorpyrifos, or Lorsban® 4E insecticide, the level of DNA damage ranged from 15.76 to 18.03 % tail DNA and OTM of 3.82 to 4.62 (Figure 6a and Figure 6b). Endosulfan, or Thionex® 3EC insecticide, the level of DNA damage ranged from 16.43 to 21.32 % tail DNA and OTM of 4.22 to 5.79 (Figure 7a and Figure 7b). Technical grade copper sulfate yielded levels of DNA damage in the range of 16.56 to 18.44 % tail DNA and OTM of 3.26 to 3.54 (Figure 8a and Figure 8b).

3.2 In vivo PAH genotoxicity

A total of 26 *Elliptio complanata* were used during the in vivo 3 day 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), 10.37 % (1.33) and 1.84 (0.36), 11.64 % (3.45) and 2.28 (0.91), and 11.77 % (4.26) and 2.00 (1.20), respectively (n = 3) (Figure 10a and Figure 10b). In contrast, the positive controls yielded statistically

significant ($P < 0.05$) levels of DNA damage, in comparison to the controls, with % tail DNA of 21.88 % (1.17) and OTM of 5.21 (0.43). However, none of the PAH exposure concentrations elicited statistically significant levels of DNA damage in comparison to the controls under the specified conditions. The level of DNA damage for the PAH exposures, reported as % tail DNA ranged from 10.93 to 15.42 % and OTM of 1.62 to 2.92.

Discussion

To our knowledge, this is the first study to demonstrate the in vitro and 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 environmentally relevant concentrations of known aquatic ecosystem contaminants. The successful use of hemolymph sampled non-lethally from a native, freshwater mussel is an important finding from this study because of the global imperilment of this fauna (Lydeard *et al.* 2004) and the fact that they have been rarely represented in genotoxicity assessments. There are many advantages in 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 2a and Figure 2b) 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. However, during establishment of the in vitro procedures,

the use of an antiaggregation solution (Alsever's) became necessary due to the tendency of hemocytes to aggregate upon extraction from the mussel. The dissociation of the hemocytes into a homogenous sample was difficult without the use of Alsever's solution. The benefit of the antiaggregation solution was evident when the hemocyte cell pellet resuspended into solution after centrifugation and the standard deviations associated with the cell viability and protein assays decreased. Clusters of aggregated hemocytes were reduced from prepared comet slides, thereby allowing for more accurate scoring. The inability to score clustered cells interferes with the results of the comet assay, because cells should be scored on a first come basis so that no bias in cell selection is introduced from the scorer.

The in vitro affect of all of the selected contaminants on whole hemolymph, from which hemocytes were isolated post exposure, was not genotoxic under our specified testing conditions, except for the positive control and two concentrations of PAHs. Both of which produced statistically significant levels of DNA damage, or genotoxicity in comparison to the controls. The PAHs demonstrated similar statistically significant genotoxic effects at 50 and 100 μg/L during the vitro exposure. Because genotoxicity was detected during the in vitro exposure with PAHs, an in vivo exposure with PAHs was also performed to assess 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) then was detected in vivo, in which only the positive control 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, based on the presented data. In part due to the inability to measure

the concentration of PAHs reaching the hemolymph, as a result the actual exposure concentration of the hemocytes in vivo is unknown. Nonetheless, our results demonstrated a high degree of method accuracy, evident in the consistent levels of DNA damage measured in the CometAssay Control Cell™ and positive control treatments. For those reasons, a high degree of confidence was instilled such that if the test chemicals were genotoxic under the specified conditions, the effects would have been detected.

Alternatively, there may have been testing variables, conditions or method artifacts that impeded in the detection of genotoxicity with these chemicals. For example, one primary set of variables with the potential to influence the outcome was the conditions selected for the exposure. These include the concentration of the test chemical, length of exposure, incubation temperature, and use of antiaggregation solution. The intent of this study was to test the genotoxicity of environmentally relevant chemicals at ecologically relevant concentrations, given these organisms are facing peril within their own habitats. Testing realistic exposure concentrations was of importance, not simply genotoxicity testing with high concentrations, as is frequently done when evaluating the potential genotoxicity of select compounds. In the future however, it would be a benefit to conduct genotoxicity studies with the same chemicals at higher concentrations with mussel hemolymph, as well as to evaluate mixtures of these chemicals, which would represent an even greater realistic exposure scenario. The 4-hour in vitro exposure period and incubation temperature of 4°C was chosen based on previous studies (Tice *et al.* 2000; Cheung *et al.* 2006), in conjunction with cell viability experiments. A 3-day in vivo exposure allowed the mussels to reach steady

state with the PAHs, as determined by a previous study with *Elliptio complanata* (Thorsen *et al.* 2004) and thus comparable to a 4-hour in vitro exposure. Yet, it is possible that a shorter or longer exposure period, incubated at other temperatures may have produced a different outcome for both in vitro and in vivo exposures, as demonstrated when the zebra mussel *Dreissena polymorpha* was exposed in situ to melphalan, a model genotoxic compound, under a range of temperatures from 4°C to 37°C (Buschini *et al.* 2003). The potential for mussel hemocytes, a group of immune responsive cells, to respond metabolically different during temperature fluctuations also exists. Therefore, the manipulation of temperature within in vitro experimentation requires consideration. Lastly, in taking into account exposure condition variables, the use of Alsever's solution, as stated earlier, greatly improved the antiaggregation behavior of the hemocytes. However, Pipe *et al.* (1997) noted the potential inhibiting affect of glucose and EDTA on the cellular processes of phagocytosis and release of reactive oxygen metabolites in *Mytilus edulis* hemocytes. Hypothesizing that a requirement for divalent cations or cell-to-cell contact was necessary, they resuspended the hemocytes in an antiaggregation buffer without EDTA and glucose, and such cellular processes were restored. However, the positive control (hydrogen peroxide) treatment always elicited statistically significant levels of DNA damage compared to the controls. This result could be a function of the strong mutagenic effects of hydrogen peroxide via oxidative damage. Whereas a compound with lesser mutagenic affects via oxidative damage or other mode of action could have been overwhelmed by the inhibiting affect of the solution. For instance, atrazine was determined to be a weak genotoxin in *Utterbackia imbecillia* glochidia

at 22.55 mg/L (Conners and Black 2004), a concentration slightly greater then our highest of 16 mg/L. Because EDTA, a component of the Alsever solution, is a known metal chelator and has been demonstrated to interact with oyster, *Crassostrea gigas,* hemocytes and copper during in vitro exposures (Auffret and Oubella 1997), it is plausible that EDTA interfered with the genotoxicity of copper sulfate (or the other chemicals tested in our study) by binding the metal, thus making it unavailable. Especially because Bolognesi (1999) reported significant levels of Cu-induced DNA strand breakage in the marine mussel, *Mytilus galloprovincialis*. Also, recent studies have demonstrated that copper undergoes redox cycling resulting in the production of reactive oxygen species (Guecheva *et al.* 2001), which can lead to enhanced lipid peroxidation, DNA damage, and altered calcium and sulpohydryl homeostasis. Therefore, future studies involving the in vitro use of hemolymph should investigate the use of an antiaggregation solution that does not contain EDTA, specifically if metal compounds are used.

Another variable that may have influenced the outcome of the in vitro tests with the selected compounds is the physiological role of mode of action (MOA) and metabolism. Of particular concern is when the MOA 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 (Mitchelmore *et al.* 1998) or indirectly by the formation of reactive intermediates that form unstable DNA adducts (Hartl *et al.* 2004). In contrast, organophosphorous insecticides have been shown to

exhibit cholinesterase-inhibiting effects in adductor muscle tissue collected from *Elliptio complanata* exposed to similar concentrations used in our study (Moulton *et al.* 1996). Therefore, it is probable that the toxicity of chlorpyrifos, an organophosphorous insecticide, is not detectable with the comet assay, because the primary mode of action is other than DNA damage. The extent to which the PAHs and the other compounds studied were inhibited in their MOA and/or metabolism or caused toxicity other than DNA damage in whole hemolymph exposed in vitro is unclear and requires further study.

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 minutes to 1-hour and alkaline unwinding for 20 minutes to 1-hour, whereas the recommended guidelines (Tice *et al.* 2000) state that a minimum cell lysing of 1-hour and unwinding of 20 minutes is preferred. Therefore, cell lysing was conducted for 1-hour in this study. Longer lysing times, up to 24-hours, were tested, but the specially coated slides could not withstand the high salts and detergents of the solution, reflected in the degradation of the agarose gel. The length of time for alkaline unwinding was 20 minutes, which is typical, according to the guidelines (Tice *et al.* 2000) and Singh *et al.* (1988) protocol for the alkaline comet assay. Thus, confidence is maintained that the times used for cell lysing and alkaline unwinding in our study provided a sufficient amount of time

to liberate and unwind the DNA. Electrophoresis is another influential and variable step, where the liberated and unwound, single-stranded DNA in the gels produce comets with the potential to influence the outcome. 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 minutes (Trevigen 2007)**,** whereas Singh *et al.* (1988) performed electrophoresis for 20 minutes, and the guidelines (Tice *et al.* 2000) report a range of 5 minutes to 40 minutes, stating that 20 minutes is sufficient. Because the CometAssay Control Cells™ were run as a measure of quality control with every in vitro experiment, preliminary research was conducted with these cells to establish the electrophoresis conditions necessary to reach the reported means for % tail DNA. We determined that 40 minutes of electrophoresis was optimal, a time well within the recommendations (Tice *et al.* 2000). Moreover, the same electrophoresis unit and power supply, set to a constant voltage of 1.0 V/cm^2 and brought to 300 mA was used throughout the study. Therefore, within our laboratory, minimal variation is expected to have arisen from the electrophoresis procedure because all variables were kept constant, partially evident in the negligible standard deviations of the Control Cells™ (Figure 9). However, the potential remains that an increased time for cell lysing and alkali-unwinding incubation could have liberated more DNA.

Studies have indicated genotoxicity, as well as additional toxicity endpoints, in bivalves and other aquatic organisms with the chemicals tested in this study. Roundup® was acutely toxic to the early life stages of the freshwater mussel *Lampsilis siliquoidea*, with a

48-hour EC50 of 2.9 mg/L, which was attributed to the surfactant and liberation of ammonia from the amine group of the glyphosate IPA salt, the active ingredient (Bringolf *et al*. 2007b). However, in glochidia of the freshwater mussel *Utterbackia imbecillis*, no genotoxicity was detected at 2.5 and 5 mg/L (Conners and Black 2004). In the freshwater goldfish *Carassius auratus*, exposed in vivo to 5, 10, and 15 mg/L of Roundup®, there was significant concentration-dependent increases in the frequencies of micronuclei, nuclear abnormalities as well as DNA strand breaks on peripheral erythrocytes (Cava and Konen 2007). Thus, due to the lack of genotoxicity data and differences in those reported, further studies are necessary to elucidate the potential genotoxicity of Roundup®. Especially because glyphosate is the most widely used herbicide in the world (Woodburn 2000) with mg/L concentrations reported from streams in agricultural regions (Giesy 2000).

The genotoxic potential of PAHs has been extensively researched, as mentioned earlier, in mixture form or singly, most notably benzo[a]pyrene. An in vivo exposure of a marine mussel, *Mytilus galloprovincialis* to a PAH mixture over a 12 day period yielded a significant amount of DNA damage, with a reported mean of 6.17 % tail DNA in gill cells, at 55 μg/L (Perez-Cadahia *et al.* 2004). The level of DNA damage in % tail DNA detected during our 3 day in vivo PAH exposure yielded slightly higher levels. However, our control levels of DNA damage were greater than the 12-day study (yet reflective of baseline), thus not resulting in significant levels of DNA damage. Endogenous levels of DNA damage can differ between species and cell types of different tissues due to variation in excision repair activity, metabolic activity, and anti-oxidant concentrations (Lee and Steinert 2003).

Therefore, the lack of correlation between the DNA damage to hemocytes and that of gill cells demonstrates the potential difference that exists in cellular responses. The concentration range of PAHs used during our study were similar to those used in an in vivo experiment with the Pacific oyster, *Crassostrea gigas*, which demonstrated adverse affects on fertilization capability and larval development (Jeong and 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 benzo[a]pyrene and endosulfan (a known xenoestrogen) (Wessel *et al.* 2007). A positive and significant correlation was demonstrated in the oyster embryos between genotoxicity and embryotoxicity for both compounds. The connection between embryotoxicity and genotoxicity caused by these compounds is of concern at the individual and community level. Even though endosulfan did not cause significant levels of genotoxicity in this study, further studies are warranted on the early life stages of freshwater mussels (glochidia and juveniles), considering the potential severity of the effects.

Although variables associated with the exposure conditions, method, test concentrations or mode of action may have influenced the detection of genotoxicity in the in vitro studies with the selected compounds (except PAHs), the significant positive control and CometAssay Control Cell™ data demonstrate the accuracy and reliability of the results obtained. We are confident that if the chemicals tested had been genotoxic, the effects would have been detected. This research investigated the use of a non-lethal genotoxicity screening tool using freshwater mussel hemolymph. Further testing and evaluation is needed before this

tool could be widely implemented. Moreover, there is need for a better understanding of freshwater mussel hemolymph and the functions and capabilities of hemocytes in the defense of genotoxic compounds.

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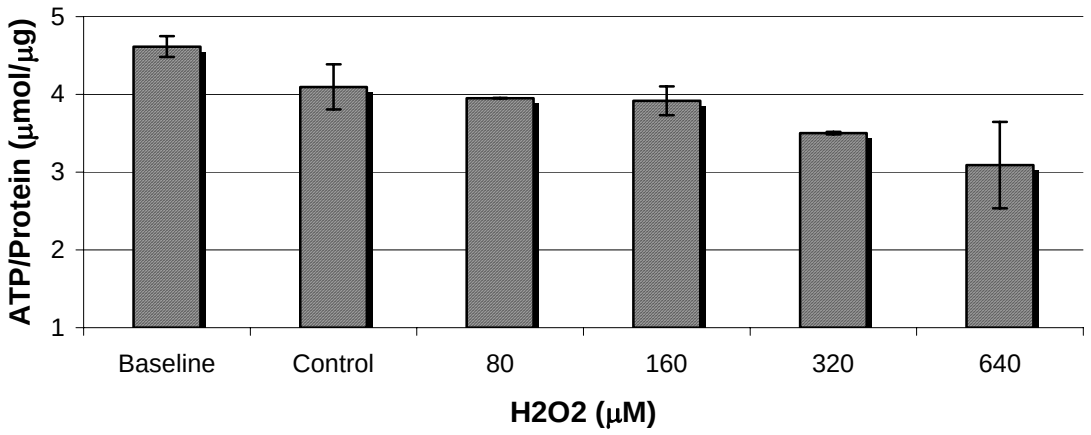

Figure 1a. Cell viability, expressed as μmol ATP/ μg of protein, of the concentration range for the positive control, hydrogen peroxide (H_2O_2), during the in vitro use of freshwater mussel hemolymph (n = 3).

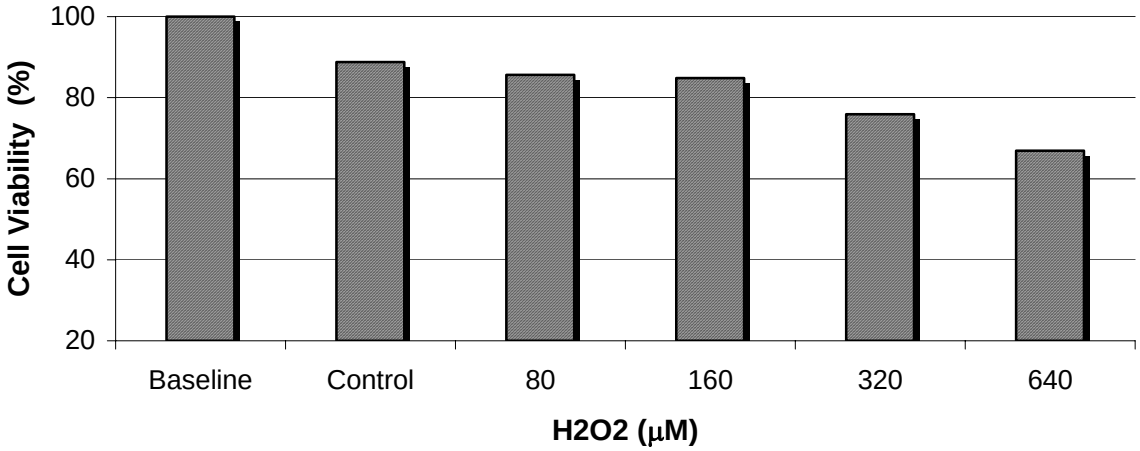

Figure 1b. Cell viability, converted from μmol ATP/ μg of protein to cell viability (%), of the concentration range for the positive control, hydrogen peroxide (H2O2), in comparison to baseline levels during the in vitro use of freshwater mussel hemolymph (n = 3).

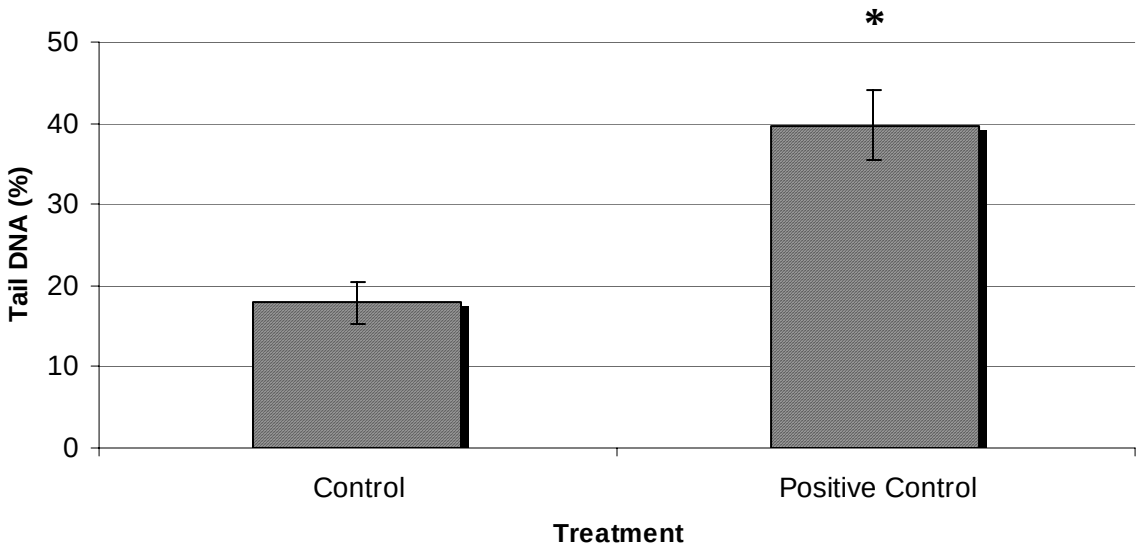

Figure 2a. Overall mean of the genotoxicity (% tail DNA) of the in vitro controls (n = 18) and positive control, hydrogen peroxide (H_2O_2) (n = 17), in freshwater mussel hemolymph. * Indicates significantly different from the control ($P < 0.01$).

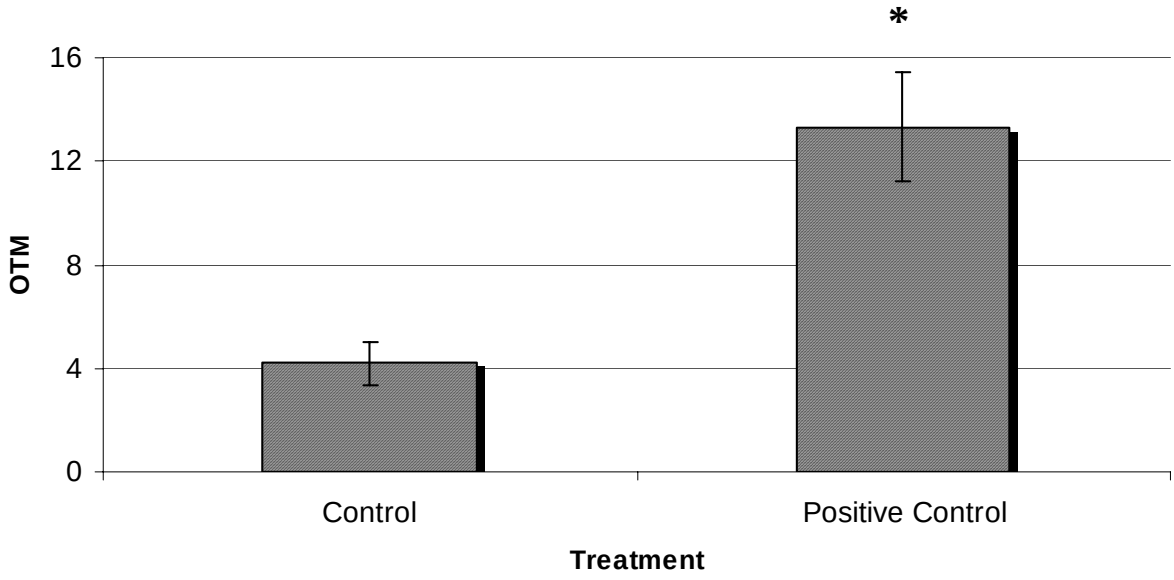

Figure 2b. Overall mean of the genotoxicity (olive tail moment; OTM) of the in vitro controls (n = 18) and positive control, hydrogen peroxide (H_2O_2) (n = 17), in freshwater mussel hemolymph. * Indicates significantly different from the control ($P < 0.01$).

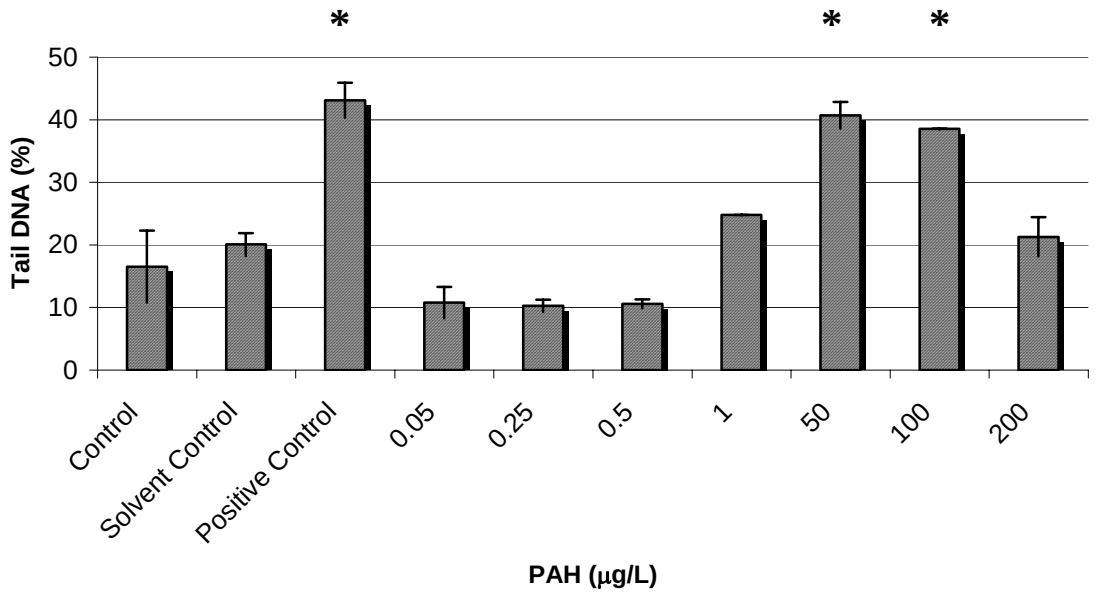

Figure 3a. In vitro genotoxicity (% tail DNA), of PAHs in freshwater mussel hemolymph (n = 3). * Indicates significantly different from the control ($P < 0.01$).

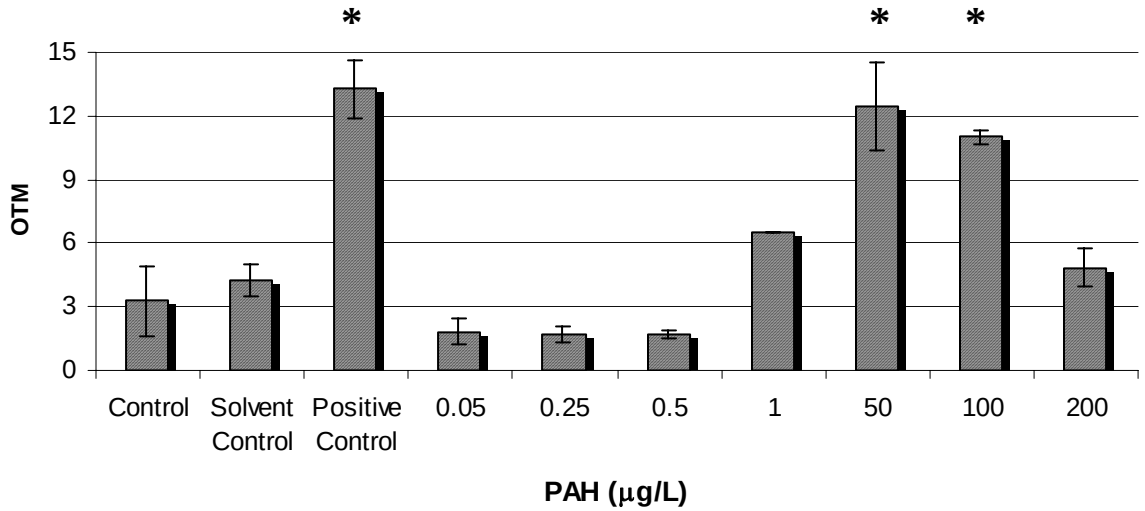

Figure 3b. In vitro genotoxicity (olive tail moment; OTM) of PAHs in freshwater mussel hemolymph (n = 3). * Indicates significantly different from the control ($P < 0.01$)

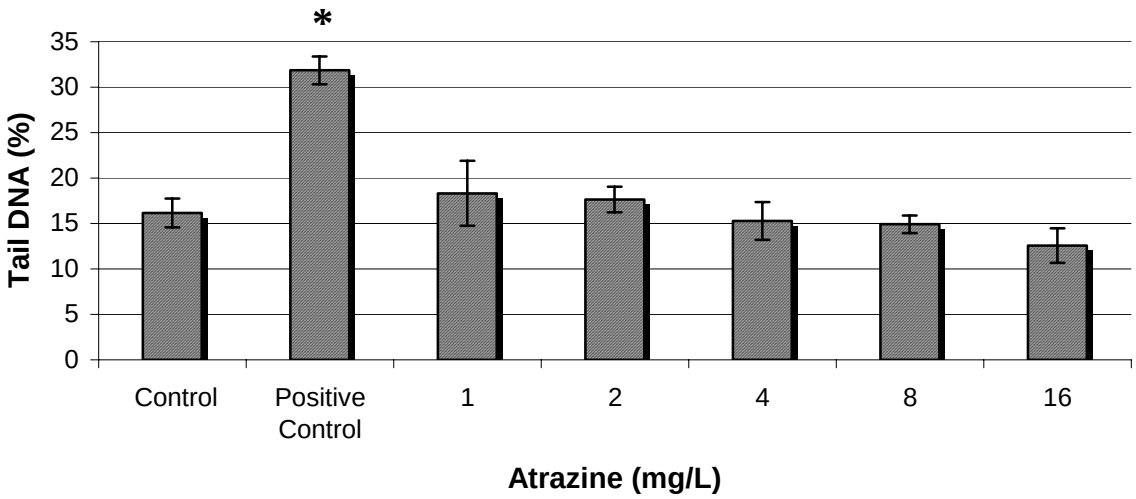

Figure 4a. In vitro genotoxicity (% tail DNA), of atrazine or Aatrex® 4L herbicide, in freshwater mussel hemolymph (n = 3). * Indicates significantly different from the control ($P < 0.0001$).

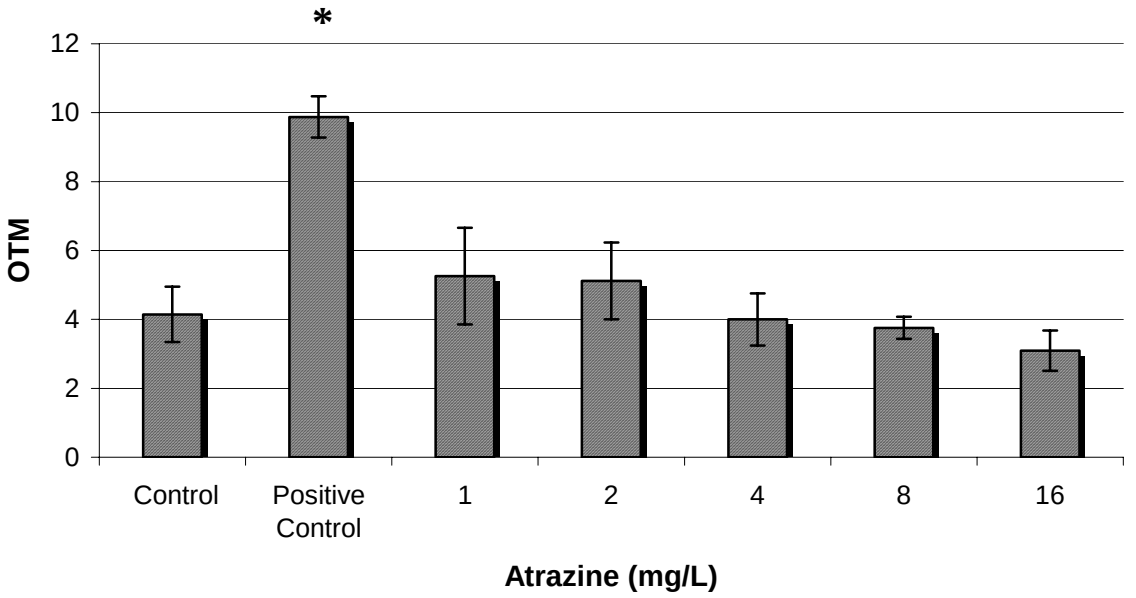

Figure 4b. In vitro genotoxicity (olive tail moment; OTM), of atrazine or Aatrex® 4L herbicide, in freshwater mussel hemolymph (n = 3). * Indicates significantly different from the control ($P < 0.0001$).

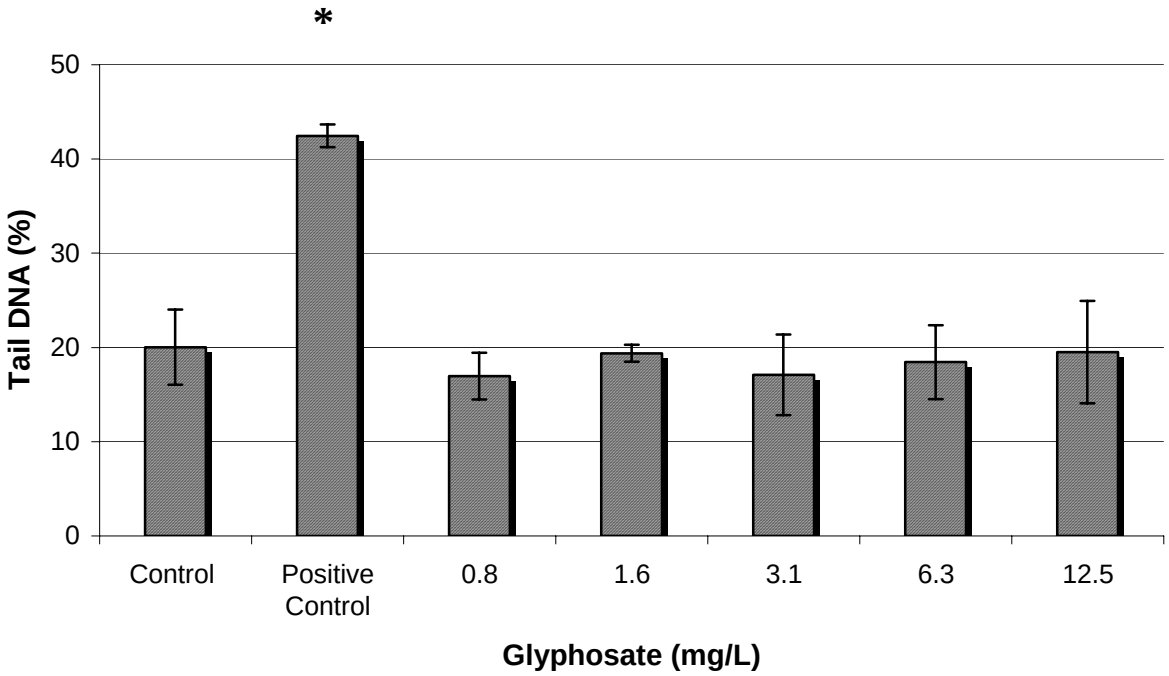

Figure 5a. In vitro genotoxicity (% tail DNA) of glyphosate, or Roundup® herbicide in freshwater mussel hemolymph (n = 3). * Indicates significantly different from the control ($P < 0.0001$).

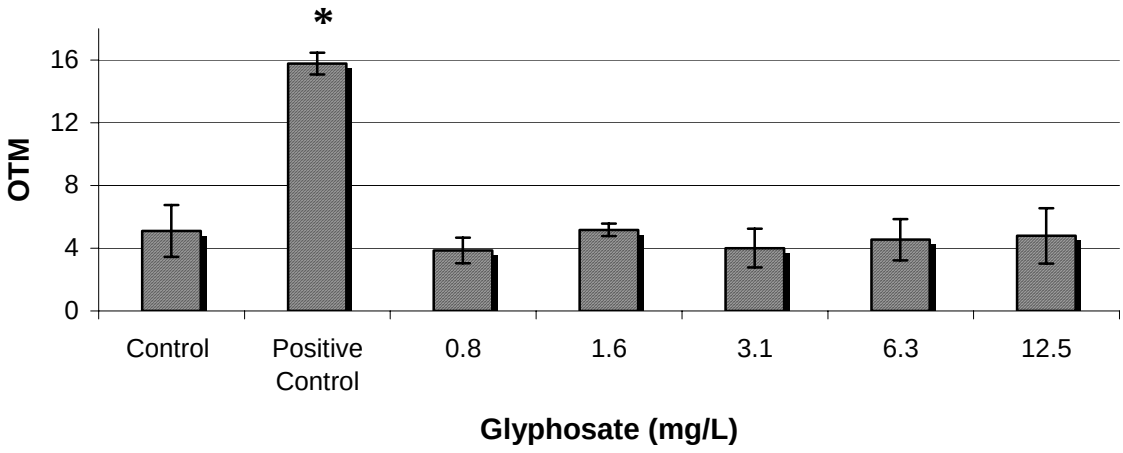

Figure 5b. In vitro genotoxicity (olive tail moment; OTM) of glyphosate, or Roundup® herbicide in freshwater mussel hemolymph (n = 3). * Indicates significantly different from the control ($P < 0.0001$).

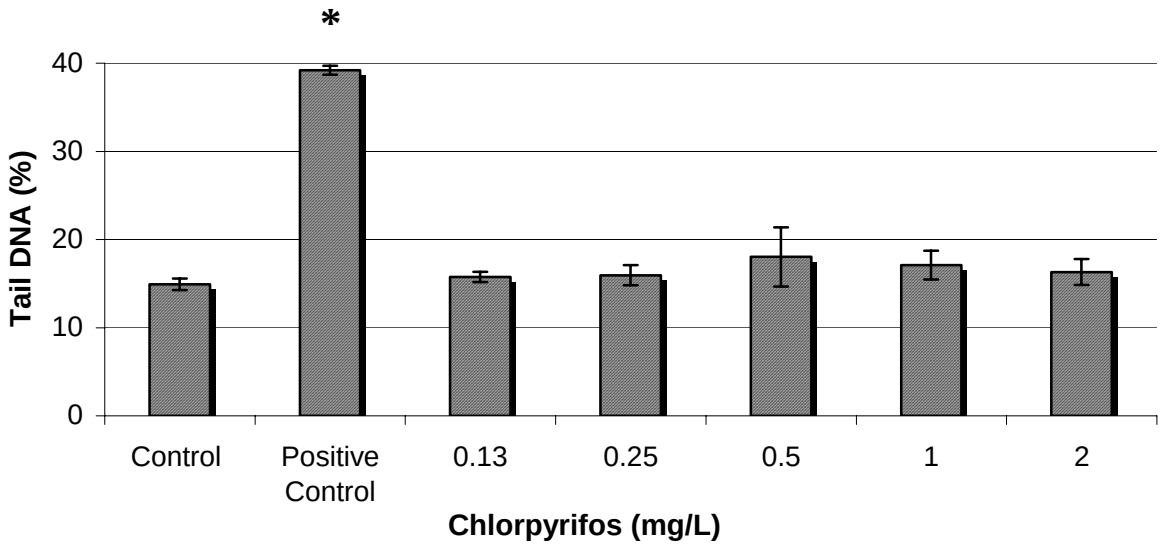

Figure 6a. In vitro genotoxicity (% tail DNA) of chlorpyrifos, or Lorsban® 4E insecticide, in freshwater mussel hemolymph (n = 3). * Indicates significantly different from the control ($P < 0.0001$).

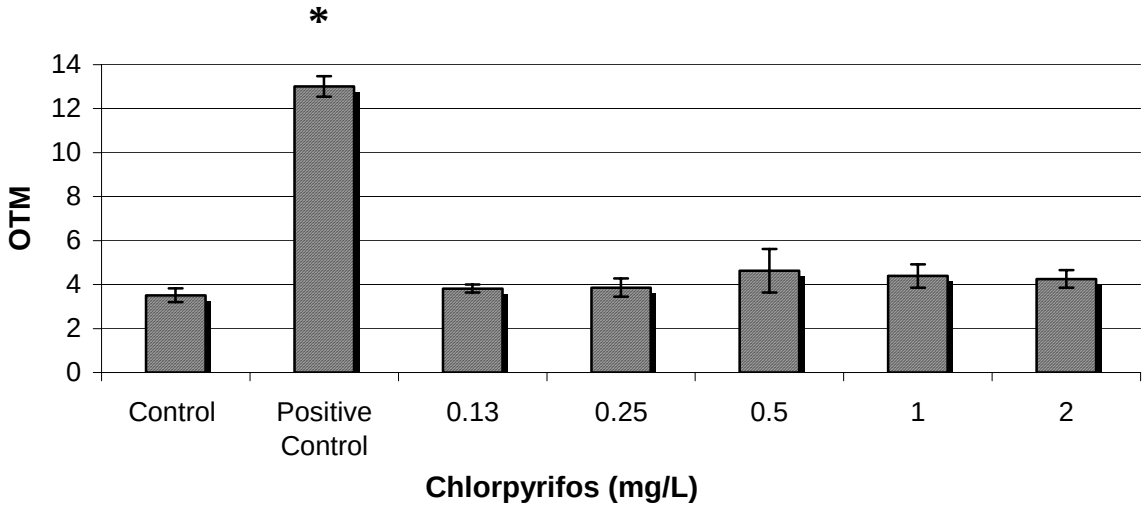

Figure 6b. In vitro genotoxicity (olive tail moment; OTM) of chlorpyrifos, or Lorsban® 4E insecticide, in freshwater mussel hemolymph (n = 3). * Indicates significantly different from the control ($P < 0.0001$).

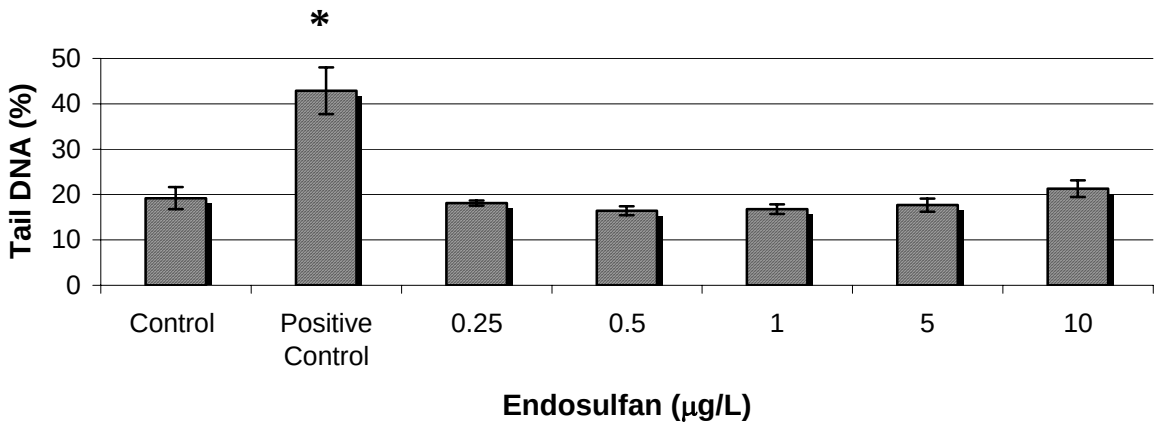

Figure 7a. In vitro genotoxicity (% tail DNA) of endosulfan, or Thionex® 3EC insecticide, in freshwater mussel hemolymph (n = 3). * Indicates significantly different from the control ($P < 0.0001$).

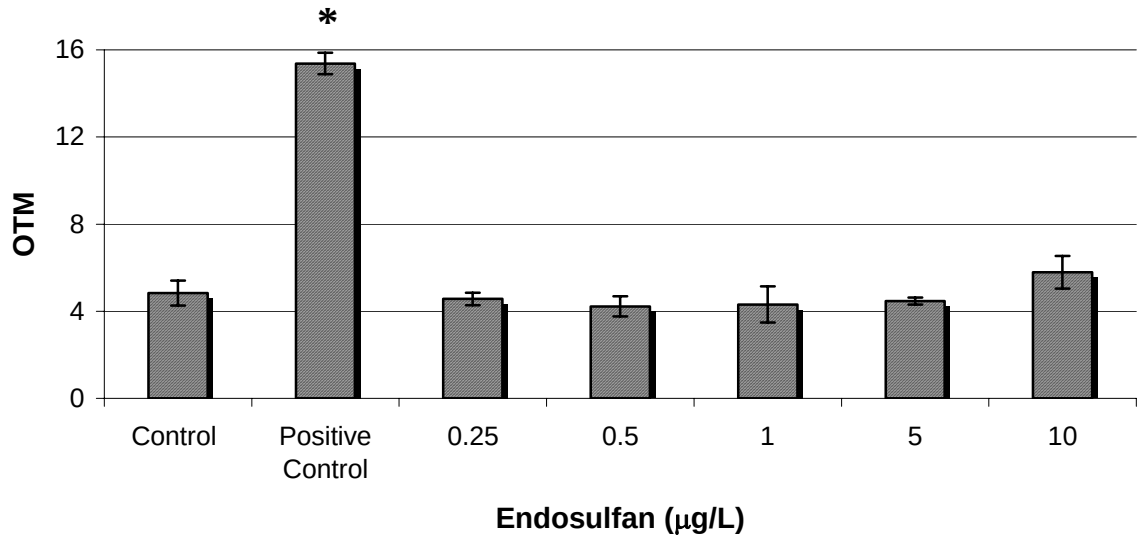

Figure 7b. In vitro genotoxicity (olive tail moment; OTM) of endosulfan, or Thionex® 3EC insecticide, in freshwater mussel hemolymph (n = 3). * Indicates significantly different from the control ($P < 0.0001$).

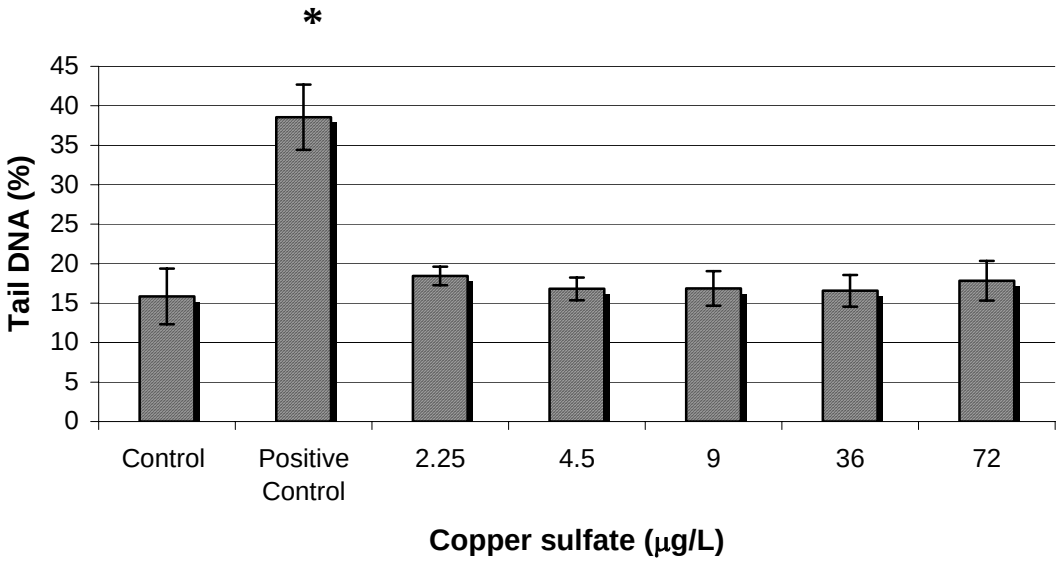

Figure 8a. In vitro genotoxicity (% tail DNA) of copper sulfate in freshwater mussel hemolymph (n = 3). * Indicates significantly different from the control (P < 0.0001).

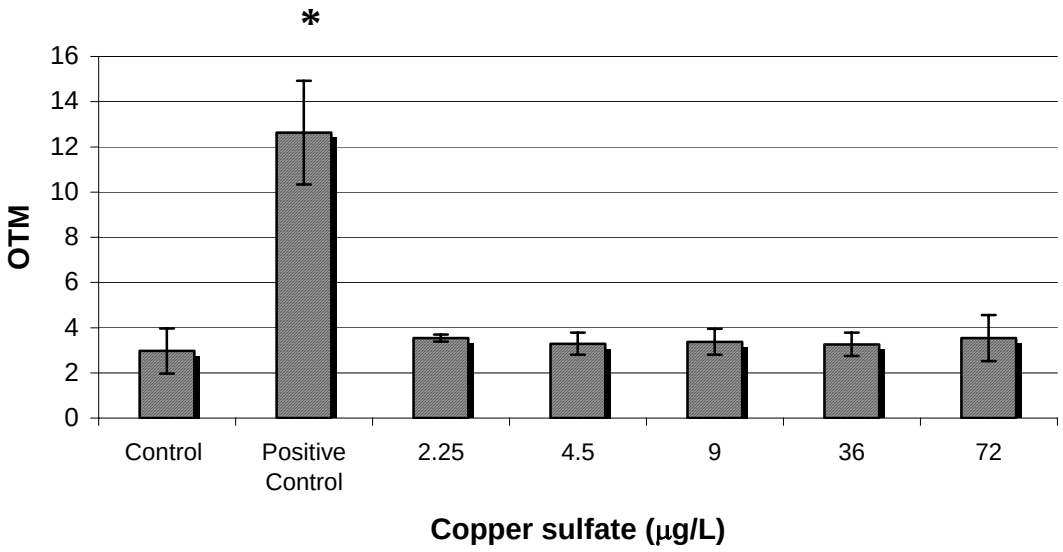

Figure 8b. In vitro genotoxicity (olive tail moment; OTM) of copper sulfate in freshwater mussel hemolymph (n = 3). * Indicates significantly different from the control (P < 0.0001).

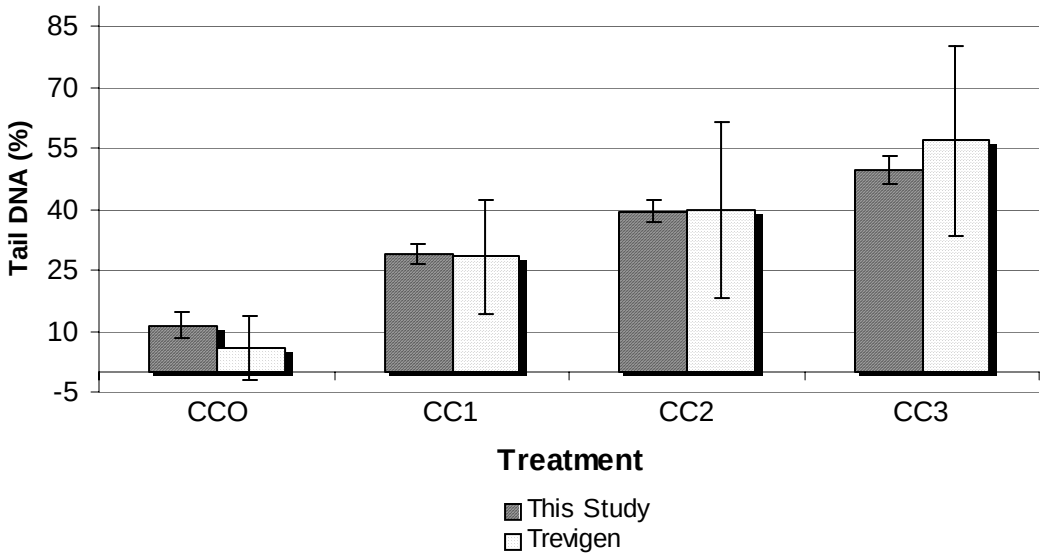

Figure 9. Certified range of DNA damage in CometAssay Control Cells™ used for quality assurance, Trevigen (n = 50), compared to the values obtained in this study (n = 6).

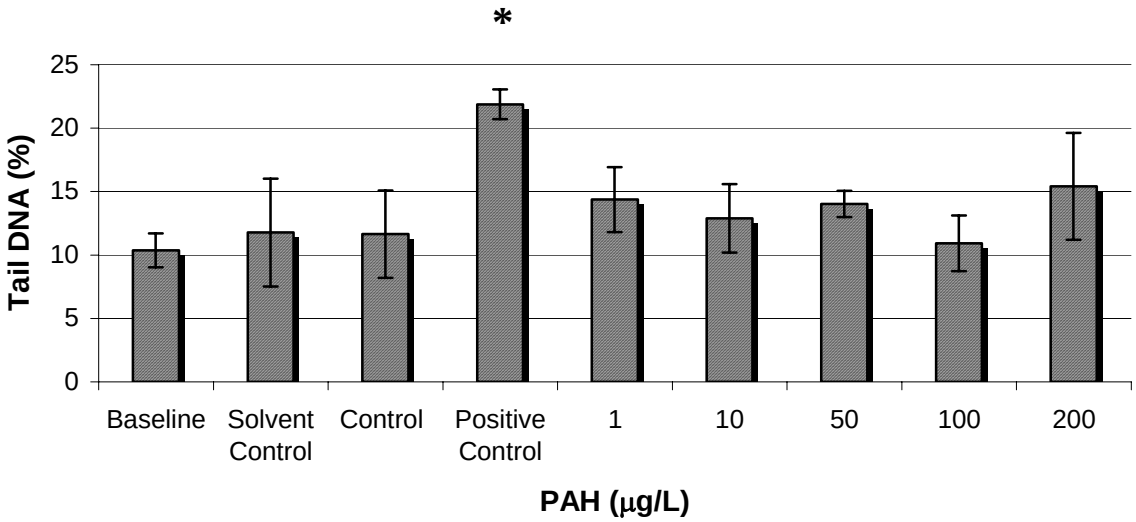

Figure 10a. In vivo genotoxicity (% tail DNA) of PAHs in freshwater mussel hemolymph (n = 3). * Indicates significantly different from the control (P = 0.0027).

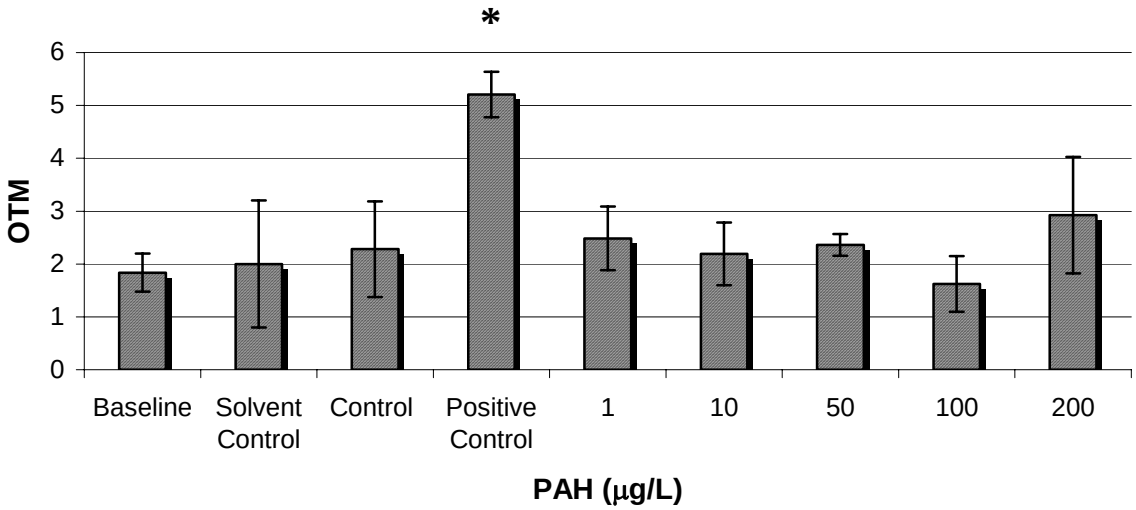

Figure 10b. In vivo genotoxicity (olive tail moment; OTM) of PAHs in freshwater mussel hemolymph (n = 3). * Indicates significantly different from the control (P = 0.0011).