

ABSTRACT

HUMPHRIES, III, LEROY FRIAR. Effects of Polycyclic Aromatic Hydrocarbon Exposure on Three Life Stages of Freshwater Mussels (*Bivalvia*: Unionidae). (Under the direction of Jay F. Levine and W. Gregory Cope, Co-chairs)

Freshwater mussels (*Bivalvia*: Unionidae) are among the most threatened aquatic species in the world. One of the major issues implicated in this decline is water pollution. Polycyclic aromatic hydrocarbons (PAHs) are a suite of hydrophobic environmental pollutants common in terrestrial and aquatic ecosystems. These compounds are largely derived from petroleum related sources (e.g., gasoline, oil) and are of major concern from transportation-related runoff to aquatic systems due to the acute and chronic (e.g., mutagenic and carcinogenic) toxic properties of many members of this class. The effects of exposure to PAHs have been investigated in many species of bivalves; however, to date no comprehensive study of the effects of exposure to these compounds on all life stages of native freshwater mussels have been completed. The goals of this study therefore were to investigate the effects of exposure to PAHs on all life stages of freshwater mussels and to develop diagnostic tests that are rapid, accurate, inexpensive, and of minimal impact to the mussels. This study examined the acute (48 h) toxicity of PAHs to the glochidial (larval) and juvenile stages of mussels and the sub-acute (7 d) toxic effects on adult mussels. Additionally, the study examined the use of genetic damage as a biomarker of exposure of mussels to PAHs by utilizing the Comet assay to determine levels of DNA strand breakage following aqueous exposure. Finally, mussels were collected from areas of high and low environmental levels of PAHs and were analyzed to validate laboratory findings and to examine relations to previously obtained field PAH mussel, water and sediment measurements. We found that there were no acute toxic effects of PAHs on glochidia or juveniles of the two species of freshwater mussels examined, up to concentrations approaching water solubility, and well exceeding those commonly measured in the streams of North Carolina. Experiments with adult *Elliptio complanata*, both in the laboratory and from the field, indicated that genetic damage due to PAH exposure was likely present, however the results were highly variable and the potential for biological, ecological, and toxicological consequences were uncertain. Further development and improvement of assay methods may reduce this variation.

Generally, mussels from streams with higher average daily traffic counts (ADTC) exhibited greater levels of genetic damage compared to mussels from streams with lower ADTC values. Data obtained from the laboratory study generally showed increasing DNA damage relative to increasing PAH concentration. Based on the data generated, however, PAHs are not likely contributing to acute toxicity of mussels in North Carolina streams, but the chronic, long-term pervasive effect of PAHs on native freshwater mussels remains uncertain.

**EFFECTS OF POLYCYCLIC AROMATIC HYDROCARBON EXPOSURE ON
THREE LIFE STAGES OF FRESHWATER MUSSELS (BIVALVIA:
UNIONIDAE)**

by
LEROY F. HUMPHRIES, III

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

COMPARATIVE BIOMEDICAL SCIENCE

Raleigh, NC

2006

APPROVED BY:

Jay F. Levine, DVM, MPH

W. Gregory Cope, Ph.D.

Co-Chair of Advisory Committee

Co-Chair of Advisory Committee

Arthur E. Bogan, Ph.D.

Dedication

This work is dedicated to my wife Diane, and my children Leah and Tyler, without whose support and love I would be lost, and to my grandmother, the late Mary Winifred K. Solomons, who always believed in me and encouraged me to do my best and to reach my goals. I also wish to dedicate this work to the memories of Mrs. Faye Battle and Mr. Barry Butts, both of whom inspired me as a student and a teacher.

Biography

I am a 1987 graduate of Mullins High School in Mullins, SC. Following graduation, I enlisted in the United States Navy and served aboard *USS Richmond K. Turner (CG-20)* for 4 years. During my tour of duty I participated in “Operation Desert Shield/Desert Storm” and “Operation Provide Comfort” in the Middle East. Upon returning to the States after the war, I received an Honorable Discharge from active duty and entered active reserve service, during which I participated in numerous drug interdiction exercises in the Caribbean Sea. I attended Horry-Georgetown Technical College where I earned an Associates degree in Science in 1995. After graduation from HGTC I enrolled at Coastal Carolina University where I earned a Bachelors of Science degree in Marine Science with minors in Biology and Environmental Science in 1998. Following graduation from CCU I moved to North Carolina to work as a Laboratory Technician for the NC Division of Marine Fisheries in Morehead City, NC where I conducted testing to determine the prevalence of the oyster parasite *Perkinsus marinus* in NC waters. In 2001 I came to NCSU and enrolled in Dr. Levine’s Comparative Biomedical Science graduate program. Currently I teach Biology at Overhills High School in Springlake, NC. My wife and I reside in Angier, NC with our two children.

Acknowledgements

I wish to thank my co-authors and mentors, Drs. Jay Levine, Greg Cope, and Arthur Bogan for all their advice, assistance, and, above all, their patience and understanding. I also wish to thank Chris Eads, Dr. Robert Bringolf, Peter Lazaro, and the rest of the “Mussel Crew” for all their help in sample collection and processing. Furthermore, I wish to acknowledge Dr. Ray Tice from Integrated Laboratory Systems in Research Triangle Park, NC for technical advice on the use of the Comet Assay. The NC Department of Transportation funded this work.

Table of Contents

List of Tables	vi
List of Figures	vii
1. Introduction and Literature Review	1
2. Materials and Methods	10
2.1. Study organisms	10
2.2. Collection of study organisms	11
2.3. Field Study Site selection	12
2.4. Test solutions and supplies	13
2.5. Test protocols	14
2.5.1. Glochidial tests.....	14
2.5.2. Juvenile tests	15
2.5.3. Adult tests	15
2.5.3.1.Positive Control Experiment.....	15
2.5.3.2.Laboratory Exposure Study	16
2.5.3.3.Field Study	17
2.6. Test procedures	18
2.6.1. Comet Assay	19
2.6.2. Contaminant Analysis	20
3. Statistical Analyses	21
3.1. Acute Toxicity Tests on Glochidia	21
3.2. Juvenile Tests	22
3.3. Comet Assay	22
4. Results	22
4.1. Glochidial Tests	22
4.2. Juvenile Tests	23
4.3. Adult tests	23
4.3.1. Positive Control Experiment	23
4.3.2. Adult Mussel PAH Experiment	23
4.3.3. Field Study	24
5. Discussion.....	26
6. Conclusions.....	31
7. References.....	32
8. Appendix 1: Tables.....	39
9. Appendix 2: Figures.....	41

List of Tables

		Page
Table 1	Sites selected for use in this study	39
Table 2	Measured levels of PAHs in glochidia and juvenile test solutions in $\mu\text{g/L}$	39
Table 3	Tissue levels of PAHs in mussels exposed in the laboratory and water concentrations (day 14).	40
Table 4	Average sum of PAH contamination measured from PSDs	40

List of Figures

	Page
Figure 1	Tail and Olive Moment values (μm) for <i>E. complanata</i> hemocytes exposed for 4 and 24 hours to 4-NQO compared to unexposed cells41
Figure 2	Tail (2a) and Olive Moment (2b) values for laboratory study41
Figure 3	Percent DNA in comet tails per treatment.....42
Figure 4	Regression of mussel tissue PAH concentration versus Log Tail Moment (μm) from the Laboratory study42
Figure 5	Regression of Average Daily Traffic Count versus estimated water PAH contamination ($\mu\text{g/L}$) from PSD data43
Figure 6	Sum of 48 PAH in 20 streams estimated from PSD residue43
Figure 7	Mean Petrogenic/pyrogenic PAH ratio in the 20 streams used in this study44
Figure 8	Tail and Olive Moment (μm) values for the streams sampled for the field portion of the study.....44
Figure 9	Predicted concentrations (ng/g lipid) of total PAHs in mussel tissue estimated from passive sampling devices (PSDs) placed at the study sites45

1. Introduction

Polycyclic aromatic hydrocarbon (PAH) compounds are a class of hydrophobic environmental pollutants widespread in terrestrial and aquatic environments. Many of the compounds in this group are of major concern to environmental agencies and researchers worldwide due to their mutagenic and carcinogenic properties (Baumard et al., 1999). Polycyclic aromatic hydrocarbons enter the environment via natural (biogenic) processes (e.g., forest or grass fires, natural petroleum seeps, etc.), and anthropogenic processes, including accidental spills or releases of petroleum compounds into the environment (e.g., tanker spills, oil platform releases) and high temperature combustion (petrogenic and pyrolytic) processes (e.g., the burning of fossil fuels, industrial activities) (Eisler, 1987; Fernandes et al., 1997; Baumard et al., 1998; Piccardo et al., 2001). Higher molecular weight PAH compounds are mainly generated by high temperature combustion of organic matter, therefore anthropogenic activities are generally considered to be the major source of higher molecular weight PAH environmental contamination (Piccardo et al., 2001). Low molecular weight PAH compounds may be produced by fossil fuel combustion, but are also major components of petroleum products (Fernandes et al., 1997), and natural processes (Eisler, 1987).

Polycyclic aromatic hydrocarbon compounds are considered to be highly hazardous to the environment and to human health. Higher molecular weight four- to seven-ring PAHs are highly mutagenic and carcinogenic, and lower molecular weight two- to three-ring PAHs, although less mutagenic, can be highly toxic (Eisler, 1987, Fernandes et al., 1997). In many cases the parent compounds are relatively inert, but the metabolites exert toxicity. Low molecular weight PAHs, dominant in fossil fuel

assemblages, are more labile and readily volatilize into the atmosphere from the air/water interface. With increasing molecular weight comes decreasing water solubility, with the result that low molecular weight PAHs are preferentially adsorbed to particles in the water column (Baumard et al., 1999). Low molecular weight PAH compounds in the water column may therefore be more bioavailable to organisms. Polycyclic aromatic hydrocarbons can interact with cells to produce toxic responses by binding to lipophilic sites in cells and interfering with cellular processes (Neff, 1979). In light of the toxicity and carcinogenicity issues to terrestrial organisms, aquatic organisms, and to humans, the monitoring of PAH contamination in the environment is critical.

Polycyclic aromatic hydrocarbons enter aquatic environments by many routes, including domestic and industrial effluents, surface runoff from land, atmospheric deposition, and spillage from petroleum operations (Eisler, 1987; Piccardo et al., 2001). Runoff from impervious surface can be one of the main carriers of these pollutants to surface waters, and a wide range of organic and heavy metal contamination has been detected in waters adjacent to paved roads (Federal Highway Administration, 1981; Hoffman et al., 1985). Maltby and co-workers (1995) demonstrated that stormwater runoff from a motorway in the United Kingdom was toxic to the benthic amphipod *Gammarus pulex*. Heavy metals and PAHs at levels that could significantly impact aquatic biota were detected in runoff from a bridge in Canada (Marsalek et al., 1997). Beasley and Kneale (2002) noted that there is considerable evidence that heavily trafficked roads are an important source of toxicants to streams. Hallhagen (1973) and Wakeham (1977) indicated that urban storm water runoff was responsible for a significant level of hydrocarbon contamination to aquatic systems. Data from a previous

study of roadway crossing structures in North Carolina found that there were elevated levels of PAH compounds downstream of 18 bridges and 2 culverts, and that this increase in contaminant levels correlated to decreased freshwater mussel abundance in stream reaches directly downstream of the crossing structure (Shea et al. 2004).

Biomonitoring of environmental conditions, especially in relation to measurements of hydrophobic contaminants, is more cost effective and accurate compared to direct environmental sampling. For instance, direct analyses of water samples for PAH contaminations are time-consuming, require large sample sizes (and are therefore innately more expensive), and do not necessarily represent the bioavailable fraction present in the water column (Gewurtz et al., 2002). Beasley and Kneale (2002) state that “snapshot” monitoring represents conditions only at a single point in time and may imply a greater grade of water quality than actually present. Additionally, these types of samples may miss periods of high contamination due to pulsed events (i.e., storm runoff). Sentinel organisms, however, concentrate contaminants within their tissues making trace levels of contaminants easier to monitor (Baumard et al., 1998). According to Pereira and co-workers (1996), bed sediments and lipid tissues of aquatic organisms integrate hydrophobic contaminants over seasonal or yearly timeframes, indicating that the biota of a stream may be more effective as monitors of water quality than the water in which they reside. However, this accumulation of organic contaminants is a complex function of the physiochemical properties of the contaminant, its distribution within the system, and the feeding behavior and metabolism of the aquatic organism used as biomonitors.

Due to their primarily sessile lifestyles and filter feeding activities, bivalve mollusks are among the most sensitive aquatic species to environmental contamination (Dame, 1996; Suavé et al., 2002). In fact, some mollusks may be more susceptible to the effects of PAHs compared to vertebrate species due to the lack of efficient enzymes for metabolizing and detoxifying PAH compounds and metabolites (Eisler, 1987). The Cytochrome P-450 (CYP450) mono-oxygenase system is an apparently universally distributed system involved in the metabolism of xenobiotics, including PAH compounds. Increases in the activity of the CYP450 system are routinely used as a means of detecting exposure to PAHs and other pollutants in fish, although the response in bivalves is less obvious. Porte and co-workers (2001) attempted to develop an integrated monitoring strategy using CYP450 activity, benzo(a)pyrene hydroxylase (BPH) activity, and stress-70 proteins. They found that exposure to PAH-contaminated environments did not elicit a CYP450 response, but there was a clear induction of stress proteins (stress-70). It is important to note, however, that stress proteins are induced by a great number of environmental factors (e.g., UV light, salinity, temperature, oxidizing agents, etc.) in addition to contaminants (Sanders, 1993). In one study, no clear evidence of changes in activity of respiratory enzymes due to exposure to petroleum hydrocarbons were observed in the blue mussel (*Mytilus edulis planatus*) (Long et al., 2003).

The use of bivalves as sentinel organisms in aquatic environments has proved to be an effective method of monitoring chemical contaminant levels. Oysters (*Crassostrea sp.*) and mussels (*Mytilus sp.*) are commonly used sentinel organisms in marine ecosystems (Baumard et al., 1999; Piccardo et al., 2001; Geffard et al., 2002). For many years freshwater bivalves have been used as biomonitors of pollutant contamination in

waterways (Renaud et al., 1995; Gagné et al., 2002; Gewurtz et al., 2002). Cataldo and co-workers (2001) used juvenile *Corbicula fluminea* survival to monitor sediment pollutant levels in Argentina. They determined that *C. fluminea* mortality rates corresponded well with sediment pollutant levels. Gewurtz and co-workers (2002) used the mussel *Elliptio complanata* to perform quantitative biomonitoring of PAHs. In another study, *E. complanata* and *Dreissena polymorpha* were used to study the effects of exposure to pollutants dispersed in a municipal effluent plume (Gagné et al., 2002). Assays of tissue body burden conducted on *Mytilus sp.*, *Anodonta anatina*, *Unio tumidus*, and *E. complanata* have demonstrated that mussels bioaccumulate PAHs and are reliable sentinel organisms (Cossu et al., 1997; Anderson et al., 1999; Gewurtz et al., 2002; Hyötyläinen et al., 2002; Thorsen et al., 2004).

A major objective of toxicology-related epidemiological testing is to provide reliable and specific information concerning the effects of exposure to a particular agent. The possibility of using biomarkers of exposure to substitute for classical endpoints (e.g., disease incidence or mortality) in molecular epidemiological studies is promising (Bonassi and Au, 2002). However, documentation of exposure may be non-existent or difficult to obtain in many cases. Bonassi and Au (2002) suggested that the use of biomarkers of exposure may provide a more precise method of obtaining that information, and that the data, if correctly collected, may be utilized to calculate the internal exposure doses and to determine the dose-response relationship. According to Porte et al. (2001), mussels exhibit a series of sub-lethal biochemical responses to pollutants, making them excellent choices for pollution monitoring studies of chemical

analysis of tissue burden and biomarkers of exposure. In instances of non-lethal exposures, biomarkers may be used to determine exposure level of sentinel organisms.

The primary routes of exposure for bivalves are across the gill and digestive gland membranes. Biomarkers of PAH exposure for bivalves have included growth and development (Geffard et al., 2002; Widdows et al., 2002), CYP450 induction (Anderson et al., 1999; Porte et al., 2001; Gagné et al., 2002), respiratory enzymes (Long et al., 2003), embryogenesis and larval development (Beiras et al., 1998; Geffard et al., 2002), hemocyte phagocytosis (Fournier et al., 2000; Blaise et al., 2002), antioxidant enzymes, glutathione and lipid peroxidation (Cossu et al., 1997; Doyette et al., 1997), digestive acini (Le Penneec and Le Penneec, 2001), and DNA damage in hemocytes, digestive tissues and somatic cells (Sasaki et al., 1997; Wilson et al., 1998; Pavlica et al., 2001; Coughlan et al., 2002; Hamoutene et al., 2002; Large et al., 2002; Rank and Jensen, 2003; Klobučar et al., 2003; Siu et al., 2004). In one study, RNA arbitrarily primed PCR was used to look for genomic aberrations in digestive tissues of *Unio tumidus* exposed to effluent from a cokery plant on the Fensch River (France) known to be responsible for PAH contamination (Rodius et al., 2002).

Previous studies of biomarkers of contaminant exposure have utilized gill and digestive gland dissected from mussels (Cossu et al., 1997; Doyotte et al., 1997; Long et al., 2003) or whole body analyses (Anderson et al., 1999; Porte et al., 2001) for CYP450 and stress-70 protein induction (Porte et al., 2001), respiratory enzyme activity (Long et al., 2003), and hemocyte phagocytosis (Fournier et al., 2000; Blaise et al., 2002) in mussels exposed to contaminants. However, the results of some of these experiments have been inconclusive. Cytochrome P450 activity in bivalves is believed ineffective in

relation to the metabolism of PAH compounds, hence the propensity of these chemicals to bioaccumulate in mollusks (Eisler, 1987). Stress-70 proteins are non-specific (i.e., elicited by a variety of stressors) and are thus offer little predictive value in determining exposure to specific contaminants (Porte et al., 2001). Additionally, the use of respiratory enzymes has proved to be an unreliable measure of contaminant exposure in bivalve mollusks (Long et al., 2003). A comparison of lethal and non-lethal biomarker techniques is needed. One such non-lethal technique, the sampling of bivalve hemocytes may yield reliable results with minimal impact on the animals (Gustafson, et al., 2005). Hemocytes may be sampled from the hemolymph extracted from the adductor muscle of bivalves with minimal effort and adverse impact to the animal.

Bivalve mollusks are vital members of aquatic ecosystems. They function as living filters, trapping food and particles in the water column as they filter-feed (Dame, 1996). Particles not ingested are excluded in pseudo-feces and effectively removed from the water column, enhancing the removal of particle-associated contaminants from the system. Native freshwater mussels (Bivalvia: Unionidae) filter large volumes of water on a daily basis, removing suspended particles and pollutants at a rate faster than accounted for through normal settling. Furthermore, freshwater mussels are important components of aquatic food webs, forming a major portion of the diet of muskrats, otters, raccoons, and other carnivorous animals that use rivers and streams as feeding areas. Biomonitoring of freshwater mussels may provide an early detection of potential problems arising from exposure to environmental contaminants. This would allow a potential pollution problem to be addressed prior to reaching levels within the system that would pose a threat to humans, agricultural animals, and other wildlife.

As a group, native freshwater mussels are among the most threatened aquatic animal species in North America (Williams et al., 1993; Lydeard et al., 2004). The National Native Mussel Conservation Committee (1998) estimated that 67% of the nearly 300 species of native North American mussels are either vulnerable to extinction or are already extinct, and recognized water pollution as a major factor in unionid decline. Despite this speculation, little documentation of the effects of major aquatic pollutants on these animals exists (Moulton et al., 1996). Major sources of water pollution in streams and rivers home to freshwater mussels include agricultural runoff containing various pesticides and chemicals, roadway runoff, municipal wastewater treatment plants (Goudreau et al., 1993), and industrial effluent. In particular, roadway runoff and municipal wastewater discharges can carry heavy loads of PAHs into an aquatic system. This may be particularly hazardous to mussels during their reproductive period.

Freshwater mussels have a stage of development during which they are obligate parasites on fish (Huebner and Pynnönen, 1992; Pynnönen, 1995; McMahon and Bogan, 2001). The female mussel broods her larvae, called glochidia, inside specially adapted chambers within her gills known as marsupia. When mature, the female mussel will either release the glochidia into the water in a mucosal conglutinate packet or attempt to attract an appropriate host organism using a section of her mantle as a lure designed to mimic a prey item (Jacobson et al., 1997; McMahon and Bogan, 2001) depending on the species of mussel. When the glochidia come into contact with a potential host organism they rapidly snap their valves together and attach themselves to either a fin or to the gills of the host. Once attached, the host rapidly forms a cyst around the glochidia. The period of encystment on the host varies between species and many species of mussel have

a specific suite of fish hosts. Upon encystment on the host fish, the mussel glochidia are assumed to be well protected from stressful environmental conditions. However, in the period of development prior to or just after release into the environment, glochidia may be at risk of exposure to toxic compounds in the water column.

Experiments utilizing the glochidia of freshwater mussels have demonstrated sensitivity of glochidia to many toxic compounds, including PAHs. Huebner and Pynnönen (1992), Pynnönen (1995), and Hanstén et al. (1996) demonstrated that glochidia of *Anodonta sp.* were sensitive to sub-lethal exposure concentrations of heavy metals and low pH, and that these exposures could significantly impact viability and survival. Keller et al. (1998) tested the toxicity of diesel fuel contaminated sediments on the glochidia of *Lampsilis siliquoidea* and *Lasmigona costata* and juvenile *Villosa villosa*, with ambiguous results. It should be noted, however, that the contaminant levels in these experiments were below the documented 'lowest effects level' from the literature. Weinstein (2000) tested the glochidia of *Utterbackia imbecillis* to characterize the acute toxicity of photo-activated fluoranthene. He found that the glochidia rapidly accumulated the contaminant within their tissues and the presence of low UV intensities made the glochidia >45 times more sensitive to fluoranthene. In 2001, Weinstein and Polk repeated this experiment with anthracene and pyrene on the same species of mussel with similar results. Tests on juveniles of many bivalve species have produced comparable results (McKinney and Wade, 1996; Ahrens et al., 2002). However, little is known about the toxicity of PAHs found at relatively low levels in streams with little urbanization in the watersheds.

This study was conducted as part of a larger study funded by the NC Department of Transportation examining the impact of crossing structures on freshwater mussels and their habitat. The primary goal of this effort was to examine the effects of exposure to PAHs on various life stages (glochidia, juvenile, and adult) of freshwater bivalves. Mussels at each of the three different life stages were analyzed to assess toxicity and to evaluate biomarkers of exposure and genotoxic effects resulting from exposure to PAH compounds. The secondary goal of this study was to explore and develop non-lethal sampling regimes and test procedures for working with this rapidly declining group of aquatic macro-invertebrates. The specific objectives of this study were:

- 1) To quantify in the laboratory and the field the effects of exposure to PAH compounds on all life stages of freshwater mussels;
- 2) To explore the use of non-lethal techniques that are accurate, rapid, inexpensive, and have minimal adverse impact on the animals being sampled in determining exposure history of freshwater bivalves to PAH compounds.

2. Materials and Methods

2.1 Study organisms

Three species of unionid mussels (*Elliptio complanata*, *Lampsilis fasciola*, and *Lampsilis siliquoidea*) were used in glochidia, juvenile, and adult tests. *Lampsilis fasciola* and *Lampsilis siliquoidea* were used in glochidia and juvenile tests, and *Elliptio complanata* was used in adult tests. *Lampsilis fasciola* glochidia were obtained from gravid females collected from the Little Tennessee River near Franklin, NC. Juvenile *L. fasciola* were obtained from individuals transformed in the Freshwater Mussel Rearing

Facility at the NCSU College of Veterinary Medicine. *Lampsilis siliquoidea*, an interior drainage mussel found in the Midwestern United States, glochidia and juveniles were obtained from Dr. Chris Barnhart at Missouri State University in Springfield, MO. *Elliptio complanata* is a common mussel found in many Atlantic slope drainage streams in North Carolina, and is a tachytictic brooder (Bogan, 2002). *Lampsilis siliquoidea* and *L. fasciola* are sexually dimorphic and are bradytictic brooders. *Elliptio complanata* and *L. fasciola* represent different reproductive strategies and mussel habitats found in North Carolina. Voucher specimens of all species used were obtained from the North Carolina Museum of Natural History archive collection to define the standards and verify species identification.

2.2 Collection of study organisms

For glochidial tests, gravid mussels were collected by hand and kept damp, cool, and dark for transport to the laboratory where they were placed in an indoor closed, recirculating holding facility at an ambient air temperature of 21°C and a 12:12 light:dark cycle. Gravid mussels brought into the laboratory were placed into a tank equipped with a chiller system to maintain a water temperature of 12°C to reduce the possibility of premature release of glochidia prior to use. Collection time of gravid mussels varied between species, based on the time of year for the maturation of glochidia within the marsupia.

All adult mussels brought into the lab were measured (total length to the nearest mm), weighed (to the nearest g), and marked with an identifying number with a rotary grinding tool. Mussels were held in the laboratory prior to testing in closed, recirculating tanks with aerated tap water from the City of Raleigh conditioned with

sodium thiosulfate to remove chloramine ions. Mussels in the laboratory were fed a diet of *Chlorella sp.* cultured at our facility in 150L batches. Glochidia used for testing were flushed directly from the marsupia of gravid females collected from the field after the depuration period. Female mussels were returned to their native stream following extraction of glochidia.

Adult *Elliptio complanata* collected for use in the adult PAH toxicity test were obtained from a relatively uncontaminated stream in Central North Carolina (based on data obtained in a previous NCDOT funded study) and transported to the laboratory in a 45.5L cooler filled approximately half full with ambient water from their native stream. The mussels were acclimated by replacing roughly half the volume of ambient water with ASTM moderately hard re-constituted water (ASTM, 1993) every hour until the entire volume had been replaced. Mussels were weighed and measured as previously described, and their shells were scrubbed to remove attached debris. The mussels were randomly assigned a number (I or II) and following acclimation overnight, one mussel from each number group was randomly distributed to an experimental unit (test chamber).

2.3 Field Study Site selection

Twenty streams in North Carolina were randomly selected from the 50 streams utilized in the NCDOT funded study for use in the Toxicology portion of the study. The sites for the intensive field study were a subset of 6 randomly selected streams out of the 20 used in the Toxicology portion of the study (Table 1). At all 20 sites, passive sampling devices (PSDs) were deployed upstream and downstream of the crossing structure to determine baseline levels of stream contamination with PAH compounds. Passive sampling devices have been shown to be a good surrogate for mussel tissues in

determining PAH contaminant levels within a stream (Shea et al., 2004). Toxicity data was compared to that obtained from 18 bridges and 2 culverts in a previous study.

2.4 Test solutions and supplies

Baseline data from a previous study funded through NCDOT was used to determine polycyclic aromatic hydrocarbon (PAH) levels in test solutions (1, 10, 50, 100, and 200 µg/L). The test concentrations were based on the mean PAH levels measured at relatively uncontaminated sites (agricultural/rural/forested) and highly contaminated sites (urban) in a previous study, and designed to cover a range of potential contaminant levels (Shea, et al., 2004), up to solubility of most of the higher molecular weight PAH compounds in water. The stock PAH test solutions were prepared using a mixture of Alaskan North Slope crude oil and creosote dissolved in dichloromethane (DCM). Test concentrations consisted of stock solutions diluted with ASTM moderately hard re-constituted water (ASTM, 2002). Controls consisted of ASTM water and 200µl DCM + ASTM water. Positive control treatments consisted of 4-Nitroquinoline-N-oxide + ASTM water. All test treatments were conducted in triplicate.

Test containers were borosilicate glass dishes washed with HPLC grade reverse-osmosis water, acetone-rinsed, and oven-baked between trials to remove organic residues and other contaminants. Glass containers were used to minimize loss of PAH compounds due to adsorption onto the surface of containers. Test containers consisted of 120 x 90mm dishes for glochidia and juvenile tests, and 3L glass jars for adult trials.

Glochidia were gently flushed from one marsupia of each female mussel using a 50cc hypodermic syringe with a 10-gauge needle and ASTM water. Glochidia and juvenile experiments were conducted at 21°C with a 12:12 light:dark cycle. Adult

experiments were conducted at 20°C ambient air temperature and aerated gently with a 16:8 light:dark cycle. Water quality variables (temperature, dissolved oxygen, pH, and conductivity) were measured daily in each test chamber. Mussels were not fed during any of the experiments.

2.5 Test protocols

2.5.1 Glochidial tests

Acute (48h) toxicity tests were conducted on glochidia during summer 2004, depending on the mussel species and availability of glochidia. Each brood was tested for viability with the addition of 2-3 drops of saturated NaCl solution to a sub-sample of the brood. When exposed to NaCl solution glochidia snap closed, viability is determined based on the percent of glochidia within the sub-sample that close following NaCl exposure. Broods with less than 90% viability were not used in experiments. Once viability was determined the broods were pooled to minimize any between animal associated bias and about 150 glochidia were added to each test chamber. Glochidia were added to the test containers by gently swirling the holding container and withdrawing ~0.5cc into a borosilicate glass pipette to obtain a random sample. At 24 and 48h of exposure to PAHs, a sub-sample of ~50 glochidia was tested for viability using the NaCl method, and the test solutions were renewed ($\frac{2}{3}$ volume) with new stock solution in ASTM water. *Lampsilis fasciola* glochidia not used in the acute toxicity tests were used to infest fish hosts (largemouth bass, *Micropterus salmoides*) to obtain laboratory-reared juveniles. Concentrations of PAHs in test solutions were measured as described below to ensure that the target contaminant level was reached.

Largemouth bass were infested with glochidia by either pipetting glochidia directly onto the gills or by placing the fish with glochidia in a 10-gallon aquarium rapidly aerated to mix the water well. The period of encystment of the glochidia on the host fish varies per species of mussel, but lasts only a few weeks (<http://news.fws.gov/mussels.html>). During the encystment period the fish hosts were maintained in recirculating 10-gallon aquaria in the Mussel Barn at the NC State University College of Veterinary Medicine. Aquaria were siphoned daily beginning one week post-infestation to collect transformed juvenile mussels.

2.5.2 Juvenile tests

Acute toxicity testing was performed on recently (<30 day old) transformed mussels of both species, depending on transformation success from fish hosts and availability of juveniles from the supplier, during summer 2004, and on >60d old *L. siliquioda*. Test duration was 96h and viability assessment was conducted at 48 and 96h of exposure. Viability was determined during a 5-minute observation period and based on foot movement inside or outside of the shell. Seven juvenile mussels were used per replicate, and all PAH treatments were conducted in triplicate. PAH test solutions were renewed daily ($2/3$ volume) with new stock solution in ASTM water.

2.5.3 Adult tests

2.5.3.1 Positive Control Experiment

Adult *E. complanata* (N=4) were sampled from a relatively uncontaminated reference site (Richland Creek, Wake County, NC) on 16 Feb 2005. Approximately 0.5ml of hemolymph was drawn from the anterior adductor muscle of each mussel (Gustafson et al., 2005). Hemolymph samples were pooled to account for between

animal variation, and allocated in 100µl aliquots to 4 tubes for a positive control experiment using 4-nitroquinoline-N-oxide, a known genotoxic compound (Le Penneec and Le Penneec, 2001; Connors and Black, 2004). Treatments consisted of 2 control (100µl untreated hemolymph) tubes and 2 treatment tubes (100µl hemolymph + 10µl 0.25mg/ml 4-nitroquinoline-N-oxide) and placed in a refrigerator. One tube from each treatment was sampled after 4h exposure and the second tube was sampled at 24h. Two samples were taken from each tube for comet assay analysis.

2.5.3.2 Laboratory Exposure Study

Adult *E. complanata* (N=62, 6 per treatment, 8 treatments, plus an additional 13 to obtain baseline data) collected on 03 March 2005 from a relatively uncontaminated reference site on the Eno River were exposed in the laboratory to the PAH test concentrations for 14d following a 24h depuration and acclimation period in the laboratory. Pre-exposure hemolymph samples were taken to determine baseline levels of genetic damage in the population. Three mussels were sampled in the field to determine pre-acclimation levels of genetic damage. Hemolymph was removed from the anterior adductor muscle and placed in 1ml plastic tubes and stored dark and cold for transport to the laboratory. Once in the lab, these samples were processed immediately for the Comet assay to minimize loss due to cellular degradation. The remaining mussels were acclimated to laboratory conditions for 24h prior to use. Mussels were scrubbed with a soft bristled brush to remove particulate matter attached to the shells to prevent particle adsorption of test solutions and randomly labeled with either an "I" or "II" for allocation to treatments. Following the 24h acclimation period, one mussel from each group was randomly allocated to each of the treatments (control, positive control, solvent control,

PAH 1, 10, 50, 100, and 200 μ g/L). Hemolymph (0.25ml) was drawn from 10 mussels post-acclimation and processed immediately for Comet assay. These same 10 mussels were removed from their shells, and the tissues frozen at -80°C for later tissue PAH analysis. Hemolymph from each of the experimental mussels was repeatedly sampled on days 3, 7, and 14. On d14 all experimental mussels were removed from their shells, and frozen at -80°C for PAH tissue analysis. Test solutions of PAH were renewed daily ($2/3$ volume) with the exception of the positive control which was only renewed on d7. Water quality measurements (temperature, dissolved oxygen, and pH) were taken daily in each test chamber and water in the test containers was completely changed on d7. Composite waters samples (100ml per treatment) were taken for PAH analysis on d0, d7, and d14.

2.5.3.3 Field Study

Adult *E. complanata* (N=6 per stream, 36 total) were collected from 6 streams (Table 1) out of the 20 chosen for study in a NC Department of Transportation (NCDOT) funded study examining the effects of culvert style crossing structures on freshwater mussels. Mussels were collected between 25 – 50m upstream and downstream of each of the road crossing structures from 15–17 December 2004. Two streams were considered reference sites and corresponded to a low average daily traffic count (e.g., <500 vehicles). Two streams were from suburban areas and corresponded to moderate average daily traffic volume (e.g., 500-1000 vehicles). The remaining 2 streams were from high traffic areas (>10,000 ADTC): one stream passed beneath Interstate 40 between Raleigh and Research Triangle Park, the other passed beneath Interstate 40 at Raleigh Durham International Airport and is directly beneath the runway flight path of the airport. Streams chosen for this portion of the study were matched as closely as possible

regarding geomorphological structure (e.g., drainage area, flow, size, substrate composition) to minimize potential variation due to non-contaminant related variables. Mussels collected from these streams were processed immediately for testing. Mussels were weighed and measured as previously described, and ~1.0ml of hemolymph was drawn to obtain hemocytes for use in the Comet assay.

Passive sampling devices (PSDs) were deployed at these study sites upstream and downstream of the crossing structure following the methods of Shea et al. (2004). Briefly, PSDs were constructed using approximately 12.7 μ m thick low-density polyethylene (PE) tubing, containing no plasticizers or additives. The PE tubing (5cm x 30cm, surface area of 300cm²) was pre-extracted with hexane for 48h prior to use and fixed inside a protective polyethylene cage. Two PSDs were placed in each cage and deployed within a 50m zone upstream and downstream from the crossing structure and retrieved approximately 30d later. Previous work has demonstrated that a 30d deployment time allows the 12.7 μ m PE to reach equilibrium with water. Following deployment, one of the PSDs was archived at -20°C and the second was cleaned with de-ionized water and a soft brush, followed by a rinse in acetone to rigorously solvent remove material from the surface of the LDPE prior to extraction. Data collected from the PSDs, directly related to PAH contaminant levels found within the streams, was used for comparison with DNA damage levels in adult mussels sampled from the same stream.

2.6 Test procedures

This study utilized acute toxicity and DNA strand breakage to explore the effects of PAH exposure on the glochidial, juvenile, and adult life stages of freshwater mussels and to explore the use of non-traditional tissue types (i.e., hemocytes) for use in the

Comet assay for determination of levels of genetic damage in relation to exposure level. The goal was to develop accurate, rapid, and cost effective non-lethal sampling procedures to determine effects of exposure of mussels to PAHs.

Hemolymph was drawn from the anterior adductor muscle of adult mussels. Following hemolymph extraction, mussels were dissected and the tissue frozen at -80°C for tissue body burden analysis in the Environmental Toxicology Laboratory at NCSU.

2.6.1 Comet Assay

The single-cell gel electrophoresis assay (Comet assay) was performed to determine the extent of genetic damage due to exposure to PAHs. This assay measures the level of DNA damage in single cells and has been reliably used on a variety of organisms (Cotelle and Féraud, 1999). Slides were prepared using an adaptation of the methods outlined by Woods et al. (1999) and Coughlan et al. (2002). Microscope slides were prepared by dipping each slide in 1.5% normal-melting agarose in phosphate buffered saline followed by air-drying and storage in a desiccator until use. All of the following steps were conducted under low light conditions to prevent confounding DNA damage due to ultra-violet radiation exposure. To prepare the sample, 100µl of mussel hemolymph was mixed with 100µl of 1.3% low melting point agarose (LMPA). The tubes were vortexed gently to mix the sample then 100µl were drawn off and placed on the slide, a 40 x 60 mm coverslip added, and the gels allowed to set on ice. Once the cell layer had set, the coverslips were removed, a third layer of 1.5% NMA was added and allowed to set as before.

Once the gels were set, the cover slips were removed and the cells lysed in a high salt buffer (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 1% (v/v) Triton X-100, and 10%

(v/v) DMSO, pH 10.0) for at least 90 min to 8h in coplin jars at 4°C in the dark. Following the lysis period, the slides were rinsed 3 times with DI water for 5 minutes and gently placed in a horizontal electrophoresis tank and covered with an alkaline solution (0.3 M NaOH, 1 mM EDTA; pH >12) for 15 min at 4°C in the dark to allow for unwinding of the DNA. Without changing the electrolysis solution, a 25 V, 300 mA current was applied for 15 min, followed by neutralization three times with Tris buffer (0.4 M Tris-HCl; pH 7.4) at 5-minute intervals followed by rinsing with cold EtOH. Slides were then stored in a desiccator until visual microscopic analysis. When ready to be read, slides were stained with 2–3 drops of ethidium bromide for 5 min, the coverslips were replaced and randomly selected nucleoids were photographed at 100x magnification using an Olympus BH-2 epifluorescence microscope fitted with a Fuji Finepix S5100 digital camera. DNA damage was expressed in terms of tail moment (TM, determined as the product of the tail length and the fraction of DNA in the tail), olive moment (OM, the summation of Tail Intensity profile values multiplied by their relative distances to the Head Center, divided by Total Comet Intensity), and percent DNA in tail .

2.6.2 Contaminant Analysis

Mussel tissue and PSD samples were extracted for PAH analysis as described by Thorsen et al. (2004) and Luellen and Shea (2003). Samples were shaker-extracted (200 rpm) for 24-h using dichloromethane (DCM) for mussels and PSDs. Concentrated extracts were fractionated using high performance gel permeation chromatography to remove high molecular weight matrix components (e.g., lipids, polyethylene waxes). The extracts were solvent exchanged into hexane and then further purified on a 3-g silica column. Mussel lipid content was determined by passing extracts through a gel

permeation chromatography (GPC) column, collecting the lipid fraction, evaporating and weighing. Samples were analyzed for 48 PAH analytes including the 16 USEPA priority PAHs.

Instrumental analysis on water samples and tissue and PSD extracts was conducted following the methods outlined in Shea et al. (2004). Briefly, the purified extracts were analyzed for total PAHs using an Agilent 6890 gas chromatograph (GC) connected to an Agilent 5973N MSD utilizing a Restek 30m x 0.25mm Rtx-5 (film 5 thickness 0.25 μm) MS w/Integra-Guard column. The pressure was ramped to 40 psi before injection with a 1-min hold time. The flow was then dropped to give a constant flow of 1mL/min for the duration of the run. The temperature program for PAH analysis was as follows: initial temperature 40 °C for 1 min with a ramp of 6 °C /min to 290 °C and a final hold time of 30 min; injector temperature 300 °C, detector temperature 280 °C. Selected ion monitoring (SIM) was used for analysis.

3. Statistical Analyses

3.1 Acute Toxicity Tests on Glochidia

Data from the 48h acute toxicity tests on glochidia were used to determine “No Observed Effects Concentration” (NOEC) and “Lowest Observed Effects Concentration” (LOEC) curves using PROC PROBIT in SAS based on 48h survival (Weinstein, 2000; Weinstein and Polk, 2001). Additionally, ToxStat software (Gulley and WEST, Inc., 1994) was used to determine LC₅₀ values using the Spearman-Kärber method and 95% confidence intervals. Tests were considered valid if mortality was <20% in the controls during the duration of the test. In the event that no total kills were observed, EC₅₀ values were calculated instead of LC₅₀.

3.2 Juvenile Tests

Data from the 96h acute toxicity tests on juvenile mussels were used to determine NOEC and LOEC curves using PROC PROBIT in SAS (Weinstein, 2000; Weinstein and Polk, 2001) and the Spearman-Karber method in ToxStat (Gulley and WEST, Inc., 1994) based on 96h survival, as stated previously. Again, if no total kills were observed EC₅₀ values were used instead of LC₅₀ values.

3.3 Comet Assay

Comet images were analyzed using CometScore™ software (TriTek Corporation, <http://tritekcorp.com>). Data were exported from CometScore into Microsoft Excel and then to JMP (SAS Corporation, Cary, NC) for statistical analysis. The average distance of strand migration of ~50 nuclei per slide were used in data analysis using a one-way analysis of variance (ANOVA). When conducting the comet assay, the slides, not the individual cells on the slide, were considered the smallest unit of measure (i.e. the means of all of the cells measured on a given slide are used for analysis, not the individual cells). The Tukey-Kramer HSD method for pairwise comparisons of means between treatments was used for statistical analysis. This procedure requires a single value for judging the significance of differences between measured parameters. Statistical significance was considered at $p < 0.05$. Data were normalized by logarithmic transformation, where necessary.

4. Results

4.1 Glochidial Tests

In repeated tests, no significant mortality was observed in glochidia exposed to any of the experimental concentrations of PAHs, therefore no LOEC curves could be

calculated. Experimental exposures with *L. fasciola* and *L. siliquoides* indicated EC₅₀ values greater than solubility of most PAH compounds in water (e.g., EC₅₀ > 200µg/L for both species). Measured concentrations of contaminants were on target at time 0, but declined some over time (Table 2). It is important to note however that samples for contaminant analysis were taken just prior to replenishment of the solutions in the test chambers.

4.2 Juvenile Tests

Studies with *L. fasciola* and *L. siliquoides* juveniles (n = 14) indicated no acute toxicity to any PAH treatment after 96h of exposure. Acute toxicity tests indicated LC₅₀ values greater than solubility of most PAH compounds in water (EC₅₀ > 200µg/L for both species). Although not quantified as a test endpoint, some lethargy was observed in mussels exposed to the greatest PAH concentration (200µg/L).

4.3 Adult tests

4.3.1 Positive Control Experiment

Hemocytes exposed to 4-NQO for only 4 hours exhibited significantly greater levels of genetic damage compared to controls (Fig. 1) expressed in terms of tail moment and olive moment (as defined previously). This trend continued at 24h, although levels of genetic damage in both the control and treatment samples were reduced.

4.3.2 Adult Mussel PAH Experiment

Samples of hemolymph (1.0ml) were taken from test mussels on days 3, 7, and 14. However, the first set of slides made from the d14 samples was compromised when nearly all of the gels slipped off of the slides during the 24h lysis period, possibly due to an error in slide preparation. The slides were immediately remade utilizing new

ingredients, however the cells appeared to have degraded and therefore, the data obtained from the d14 samples has not been reported. Most comet parameters at d7 showed distinct trends towards increasing levels of DNA damage with increasing PAH exposure levels. Trends in tail moment and olive moment increased with increasing PAH concentration over time, compared to control values (Fig. 2). The data were highly variable resulting in low levels of statistical significance in both comet parameters.

Solvent control treatments did not exhibit any significant difference from control treatments. Levels of DNA damage in the positive control treatments did vary significantly from control treatments, particularly in samples from d3.

Other comet parameters demonstrated similar increasing trends with exposure level and time. Most notably, the percent DNA in the comet tails (%DNA in Tail) increased over time compared to controls (Fig. 3).

Tissue analysis on exposed mussels demonstrated that the mussels were taking up and bioaccumulating PAHs in the test chambers. The tissue concentrations exhibited a clear concentration dependent relationship, increasing in the mussels with respect to exposure level (Table 3). Water samples from day 14 indicated that the desired levels of PAH concentration in the water in the treatment chambers were slightly off target, however the samples were taken 24 hr after replenishment (Table 3). Levels of genetic damage, in terms of Tail Moment, expressed a positive correlation to mussel tissue PAH levels (Fig. 4)

4.3.3 Field Study

Data obtained from PSDs deployed upstream and downstream of the 20 crossing structures indicated a general positive relationship between contamination level and

average daily traffic count (Fig. 5). Stream G29 was omitted from the analysis of the PSD data because sewer line construction and paving in the vicinity lead to concentrations of PAHs that were unusually high compared to other streams with similar traffic loads. When site G29 was included in the analysis the regression equation was:

$$y = 0.0027x + 112.11 (R^2 = 0.1244).$$

Estimated water concentrations for 48 PAHs are shown in Fig. 6. There was no significant difference between petrogenic and pyrogenic PAHs between low, medium, or high ADTC groups of streams, although there were differences between individual streams, even within ADTC groups (Fig. 7).

Levels of genetic damage in mussel hemocytes from field-collected mussels generally increased with average daily traffic count (ADTC) (Fig. 8), measured as vehicle crossings per day. As in the laboratory adult mussel PAH exposure study, the data were highly variable, but the trend towards increasing genetic damage in relation to water column PAH concentration was distinct. The lone exception to this trend was stream A338. This stream represented the lowest average daily traffic count of any site in the field study (Table 1), but the PSD data indicated an extremely high level of PAH contamination relative to streams of comparable ADTC (Table 4). Despite the high PAH contamination at this site, mussels sampled from A338 exhibited the lowest levels of DNA damage measured in the field study. Stream O263 had the second highest ADTC of the streams in this study (Table 1), yet the PSD data indicated that the PAH levels were slightly less than streams with significantly lower ADTC values (Table 4). Levels of DNA damage in mussels sampled at this location, however, reflected the trend of

increasing levels of genetic damage with increasing ADTC. The predicted levels of mussel tissue PAH concentration based on PSD data are shown in Fig. 9.

5. Discussion

Freshwater mussel populations are in decline throughout their range within North America (Bogan 1993; Williams et al., 1993; Lydeard et al., 2004). Although the decline is well documented and extensive, the factors associated with these changes in population abundance and density are poorly understood. In some locations, urban and suburban development has been associated with declining populations, and road and bridge construction are often components of the related land-use changes. In many of these instances, polycyclic aromatic hydrocarbons are generally present in alarmingly high concentrations due to paving activities, fuel spills and leaks, or burning of debris. Polycyclic aromatic hydrocarbons have previously been associated with decreases in embryo growth, development, and survival, and adult mortality in a variety of aquatic species. Bivalve mollusks have been utilized for many studies of the effects of PAH compounds in aquatic ecosystems due to their primarily sessile lifestyles and lack of efficient metabolizing enzymes. Freshwater mussels are of particular concern as many of their habitats are in high traffic or developing areas.

Data from the glochidial and juvenile tests appeared to contradict previous published information, however these studies were conducted with other freshwater mussel species. Weinstein (2000) and Weinstein and Polk (2001) reported high sensitivity and mortality of *U. imbecillis* glochidia to relatively high levels of several different PAH compounds (fluoranthene, pyrene, and anthracene) following photoactivation with ultraviolet light. This study utilized total PAHs and used a 16:8

light/dark cycle with no UV photoactivation of the PAHs. The measured levels of fluoranthene, pyrene, and anthracene from rivers used in this study were 0.01073, 0.01022, and 0.00027 $\mu\text{g/L}$ respectively. Weinstein's studies indicated sensitivity of *U. imbecillis* glochidia to levels of these same compounds that were significantly higher (lowest concentrations 1.2, 0.9, and 0.7 $\mu\text{g/L}$ respectively). Based on the field data, the levels of the individual PAHs used in this study were considerably lower than the concentrations reported by the previous works. It is possible, therefore, that this study presents a more natural scenario (i.e., more like the naturally occurring conditions) than the Weinstein studies, as levels of individual PAHs measured in streams in NC are lower than experimental values.

The experiments with glochidia did not yield any evidence of acute toxicity to PAHs and suggested that LC_{50} levels for total PAHs may be above solubility of the compounds in water. The measured endpoint, however, was simply survival of glochidia during a 48h exposure. It is possible that sub-lethal effects, or the EC_{50} value, (e.g., delayed response to stimuli, lethargy, genetic damage, or delayed development) occurred due to exposure, although no quantification attempts were made. No attempt at measuring single strand DNA breaks using the Comet assay with glochidia or juveniles was successful. In methods development trials with *U. imbecillis*, attempts were made to duplicate the methods utilized by Connors and Black (2004) to test for genetic damage with limited success. Further work in refining methods of removing tissue from the minute shell fragments of the glochidia and juveniles will present greater opportunities for determining genotoxic effects on these life stages of mussels. More complete separation of tissue from shell fragments could be accomplished using chemical or

mechanical means (i.e., use of chemicals to disassociate tissue from shell fragments, or more complete pulverization of the minute shells).

Experiments with juvenile mussels did not yield any evidence of acute toxicity of PAHs; therefore no LC_{50} value could be calculated. Mortality in PAH treatments was not significantly different from that of controls. Although some lethargy was observed, no quantification of this or other sub-lethal effects (e.g., response to stimuli, time to movement, growth and development) were made in the studies. A direct method of quantification of sub-lethal effects due to exposure would be to measure time to first movement. Lethargy could thereby be quantified and related to exposure level. In the wild, lethargic responses due to exposure to contaminants could directly impact the survival of juvenile mussels by delaying closing response initiated by the proximity of a potential predator.

The data obtained from the positive control experiment indicate that mussel hemocytes may be affected by exposure to environmental genotoxic contaminants and therefore may be a viable alternative to traditionally sampled tissue types such as gill or digestive gland tissues from mussels. The decrease in levels of genetic damage over the 24h period of the positive control experiment may be due to a reduction in cell viability over time. The data concur with the findings of Siu and co-workers (2004) and Klobučar and co-workers (2003). Both of these studies found that hemocytes were sensitive to genotoxins and that the use of hemocytes was a sensitive and valuable tool in monitoring of these compounds in the environment. Additionally, hemocytes are rapidly and easily sampled with minimal impact on the organism (Gustafon et al., 2005). During the laboratory portion of this study, 0.25ml of hemolymph was sampled from mussels 3

times during a 2-week period. No mussels died during the experiment, suggesting that repeated sampling of small amounts of hemolymph is not detrimental to short-term survival of the mussel.

The PAH exposure study with adult eastern elliptio demonstrated clear time and concentration dependant effects on levels of genetic damage in mussel hemocytes, although the results exhibited a high degree of variation. Previous in vivo studies (Siu et al., 2004; Rank and Jensen, 2003; Klobučar et al., 2003) have found that mussel hemocytes withdrawn from exposed mussels are as sensitive as tissues (gill, digestive gland, etc.) in detecting DNA damage in the mussels. However, based on observations from this study, hemocytes drawn from mussels should be utilized soon after collection, as viability tends to decrease over time. Still, the results indicate that rapid, cost effective, and non-lethal hemolymph sampling (Gustafson et al., 2005) may be a viable alternative to whole mussel or tissue sampling methods for assessing the effects of genotoxic compounds. Levels of DNA damage, expressed in terms of Tail Moment corresponded well to levels of PAH tissue contamination, which were high due to bioaccumulation of the PAHs over the course of the experiment. This indicates that chronic exposure to PAHs is detrimental to the long-term health of the animals and may relate to survival of animals in contaminated streams.

Data obtained from the field portion of the study demonstrated a distinct trend in increasing levels of genetic damage in relation to average daily traffic load, and thus presumably PAH exposure. This concurs with evidence presented by Maltby and co-workers that motorway runoff was toxic to the benthic amphipod *G. pulex*. Other researchers have presented evidence that PAH levels in streams crossed by heavily

trafficked roads were at levels that could significantly impact biota within the waterway (Marsalek et al., 1997; Beasley and Kneals, 2002; Hallhagen, 1973, Wakeham, 1977) The results of this study appear to reinforce this evidence. Generally, ADTC on a roadway corresponded well to PAH concentrations within the stream. The exceptions to this relationship were likely due to other factors such as land use patterns in the watershed, atmospheric deposition influenced by regional weather patterns, or other anthropogenic activities upstream of the crossing structure. Therefore, based on the data, sampling and analysis of mussel hemocytes for genotoxic compounds may yield important information about contaminant loading in a stream and its effects on the biota within the stream.

The data obtained from the laboratory and field portions of this study indicate that there is a good correlation between traffic load on a roadway, water contaminant load, and tissue body burden of mussels in the stream. The Comet assay data, although variable, indicates a positive correlation between tissue burden of contaminants and DNA damage in the mussels (Fig. 4). Shea and coworkers (2004) found that there was a positive correlation between contaminant loading in streams in North Carolina and a decrease in mussel biomass downstream of the roadway crossing structure. The data from this study indicate that this decrease is not due to acute effects of exposure, but due to chronic effects (e.g., reduction in DNA viability over time due to exposure).

Although much of the data obtained from the Comet assay in this study were highly variable, the positive control exposure experiment indicates that mussel hemocytes present a potential alternative to lethal methods of testing. The data obtained from the laboratory and field portions of this study indicate that mussel hemocytes are sensitive to PAH exposure in the environment. However, methods need to be refined and attempts

made to reduce variability. Although additional testing is required to refine assay methods, this study indicates that the methods are robust and that PAH contamination in streams may be negatively affecting freshwater mussels.

6. Conclusions

Overall, we found that there were no acute toxic effects of PAHs on glochidia or juveniles of the two species of freshwater mussels examined, up to concentrations approaching water solubility, and well exceeding those commonly measured in the streams of North Carolina. Experiments with adult *Elliptio complanata*, both in the laboratory and from the field, indicated that genetic damage due to PAH exposure was likely present, however the results were highly variable and the potential for biological, ecological, and toxicological consequences were uncertain. Further development and improvement of assay methods may reduce this variation. Generally, mussels from streams with higher average daily traffic counts (ADTC) exhibited greater levels of genetic damage compared to mussels from streams with lower ADTC values. Data obtained from the laboratory study generally showed increasing DNA damage relative to increasing PAH concentration. Based on the data generated, however, PAHs are not likely contributing to acute toxicity of mussels in North Carolina streams, but the chronic, long-term pervasive effect of PAHs on native freshwater mussels remain uncertain.

References

- Ahrens MJ, Nieuwenhuis R, Hickey CW (2002) Sensitivity of Juvenile *Macomona liliana* (Bivalvia) to UV-Photoactivated Fluoranthene Toxicity. *Enviro Tox*, 17: 567 – 577.
- American Society for Testing and Materials (ASTM) (2002) Standard guide for conducting acute toxicity tests on test materials with fishes, macroinvertebrates, and amphibians (ASTM E729-96). ASTM annual book of standards volume 11.05, ASTM, West Conshohocken, PA.
- Anderson JW, Jones JM, Steinert S, Sanders B, Means J, McMillin D, Vu T, Tukey R (1999) Correlation of CYP1A1 induction, as measured by the P450 RGS biomarker assay, with high molecular weight PAHs in mussels deployed at various sites in San Diego Bay in 1993 and 1995. *Mar Enviro Res*, 48: 389 – 405.
- Baumard P, Budzinski H, Garrigues P (1998) PAHs in Arcachon Bay, France: Origin and Biomonitoring with Caged Organisms. *Mar Poll Bull*, 36 (8): 577 – 586.
- Baumard P, Budzinski H, Garrigues P, Narbonne JF, Burgeot T, Michel X, Bellocq J (1999) Polycyclic aromatic hydrocarbon (PAH) burden of mussels (*Mytilus sp.*) in different marine environments in relation with sediment PAH contamination, and bioavailability. *Mar Enviro Res*, 47: 415 – 439.
- Beasley G, Kneale P (2002) Reviewing the impact of metals and PAHs on macroinvertebrates in urban watercourses. *Prog Phys Geo*, 26(2): 236 – 270.
- Beiras, R, His E, Seaman MNL (1998) Effects of storage temperature and duration on toxicity of sediments assessed by *Crassostrea gigas* oyster embryo bioassay. *Enviro Tox & Chem*, 17(10): 2100 – 2105.
- Blaise C, Trottier S, Gagné F, Lallement C, Hansen P-D (2002) Immunocompetence of Bivalve Hemocytes as evaluated by a Miniaturized Phagocytosis Assay. *Enviro Tox*, 17: 160 – 169.
- Bogan, AE (1993) Freshwater bivalve extinctions (Mollusca:Unionoida): a search for causes. *American Zoologist*, 33: 599-609.
- Bogan AE (2002) *Workbook and key to the freshwater bivalves of North Carolina*. North Carolina Museum of Natural Sciences, Raleigh, NC, 101 pp, 10 color plates.
- Bonassi S, Au WW (2002) Biomarkers in molecular epidemiology studies for health risk prediction. *Mut Res*, 511: 73 – 86.
- Cataldo D, Columbo JC, Boltovskoy D, Bilos C, Landoni P (2001) Environmental toxicity assessment in the Parana River delta (Argentina): simultaneous evaluation of selected pollutants and mortality rates of *Corbicula fluminea* (Bivalvia) early juveniles. *Enviro Poll*, 112: 379 – 389.

- Conners DE, Black MC (2004) Evaluation of lethality and genotoxicity in the freshwater mussel *Utterbackia imbecillis* (Bivalvia: Unionidae) exposed singly and in combination to chemicals used in lawn care. *Arch Environ Contam Toxicol*, 46(3): 362-371.
- Cossu C, Doyette A, Jacquin MC, Babut M, Exinger A., Vasseur P (1997) Glutathione Reductase, Selenium-Dependent Glutathione Peroxidase, Glutathione Levels, and Lipid Peroxidation in Freshwater Bivalves, *Unio tumidus*, as Biomarkers of Aquatic Contamination in Field Studies. *Ecotox Environ Safety*, 38: 122-131.
- Cotelle S, Férard JF (1999) Comet Assay in Genetic Ecotoxicology: A Review. *Environ Mol Mutat*, 34: 246 – 255.
- Coughlan BM, Hartl MGJ, O'Reilly SJ, Sheehan D, Morthersill C, van Pelt FNAM, O'Halloran J, O'Brien NM (2002) Detecting genotoxicity using the Comet assay following chronic exposure of Manila clam *Tapes semidecussatus* to polluted estuarine sediments *Mar Pollut Bull*, 44: 1359 – 1365.
- Dame RF (1996) *Ecology of Marine Bivalves: an Ecosystems Approach*. CRC Press, Boca Raton, FL.
- Doyette A, Cossu C, Jacquin M-C, Babut M, Vasseur P (1997) Antioxidant enzymes, glutathione, and lipid peroxidation as relevant biomarkers of experimental or field exposure in the gills and the digestive gland of the freshwater bivalve *Unio tumidus*. *Aqua Toxicol*, 39: 93 – 110.
- Eisler R (1987) Polycyclic aromatic hydrocarbon hazards to fish, wildlife, and invertebrates: a synoptic review. U.S. Fish Wildlife Service Biological Report 85(1.11). 81 pp.
- Federal Highway Administration (1981) Constituents of highway runoff. Vol. VI. Executive Summary Final Report. Report No. FHWA/RD-G1/047. Environmental Division, Federal Highway Administration, Washington, DC.
- Fernandes MB, Sicre M-A, Boireau A, Tronczynski J (1997) Polyaromatic Hydrocarbon (PAH) Distributions in the Seine River and its Estuary. *Mar Pollut Bull*, 34(11); 857 – 867.
- Fournier M, Cyr D, Blakely B, Boermans H, Brousseau P (2000) Phagocytosis as a Biomarker of Immunotoxicity in Wildlife Species Exposed to Environmental Xenobiotics. *Amer Zoo*, 40: 412 – 420.
- Gagné F, Blaise C, Aoyama I, Luo R, Gagnon C, Couillard Y, Cambell P, Salazar M (2002) Biomarker Study of a Municipal Effluent Dispersion Plume in Two Species of Freshwater Mussels. *Environ Toxicol*, 17: 149 – 159.
- Geffard O, Budzinski H, His E (2002) The Effects of Elutriates from PAH and Heavy Metal Polluted Sediments on *Crassostrea gigas* (Thunberg) Embryogenesis, Larval Growth, and Bioaccumulation by the Larvae of Pollutants from Sedimentary Origin. *Ecotox*, 11: 403 – 416.
- Gewurtz SB, Drouillard KG, Lazar R, Haffner GD (2002) Quantitative Biomonitoring of PAHs Using the Barnes Mussel (*Elliptio complanata*). *Arch Environ Contam Toxicol*, 43: 497 – 504.

- Goudreau SE, Neves RJ, Sheehan RJ (1993) Effects of wastewater treatment plant effluents on freshwater bivalves in the upper Clinch River, Virginia, USA. *Hydrobiologia*, 252(3): 211-230.
- Gulley DD, WEST, Inc. (1994) TOXSTAT 3.4. WEST, Inc., Cheyenne, WY.
- Gustafson LL, Stoskopf MK, Bogan AE, Showers W, Kwak TJ, Hanlon S, Levine JF (2005) Evaluation of a nonlethal technique for hemolymph collection in *Elliptio complanata*, a freshwater bivalve (Mollusca: Unionidae). *Dis Aqua Org*, 65: 159-165.
- Hallhagan A (1973) Survey of present knowledge and discussion of input of petroleum to the marine environment in Sweden. Paper presented at workshop on Inputs, Fates, and Effects of Petroleum in the Marine Environment, National Academy of Sciences.
- Hamoutene D, Payne JF, Rahimtula A, Lee K (2002) Use of the Comet assay to assess DNA damage in hemocytes and digestive gland cells of mussels and clams exposed to water contaminated with petroleum hydrocarbons. *Mar Enviro Res*, 54: 471 – 474.
- Hanstén C, Heino M, and Pynnönen K (1996) Viability of glochidia of *Anodonta anatina* (Unionidae) exposed to selected metals and chelating agents. *Aqua Tox*, 34: 1 – 12.
- Hoffman EJ, Latimer JS, Hunt CD, Mills GL, Quinn JG (1985) Stormwater runoff from highways. *Water, Air, and Soil Pol*, 25: 349 - 364.
- Huebner JD, Pynnönen KS (1992) Viability of glochidia of two species of *Anodonta* exposed to low pH and selected metals. *Can J Zoo*, 70: 2348 – 2355.
- Hyötyläinen T, Karels A, Oikari A (2002) Assessment of bioavailability and effects of chemicals due to remediation actions with caging mussels (*Anodonta anatina*) at a creosote-contaminated lake sediment site. *Water Res*, 36: 4497 – 4504.
- Jacobson PJ, Neves RJ, Cherry DS, Farris JL (1997) Sensitivity of glochidial stages of freshwater mussels (Bivalvia: Unionidae) to copper. *Enviro Tox Chem*, 16(11): 2384 – 2392.
- Keller AE, Ruessler DS, Chaffee CM (1998) Testing the toxicity of sediments contaminated with diesel fuel using glochidia and juvenile mussels (Bivalvia: Unionidae). *Aqua Eco Health Manage*, 1: 37 – 47.
- Klobučar GIV, Pavlica M, Erben R, Papeš D (2003) Application of the micronucleus and comet assays to mussel *Dreissena polymorpha* haemocytes for genotoxicity monitoring of freshwater environments. *Aqua Tox*, 64: 15 – 23.
- Large AT, Shaw JP, Peters LD, McIntosh AD, Webster L, Mally A, Chipman JK (2002) Different levels of mussel (*Mytilus edulis*) DNA strand breaks following chronic field and acute laboratory exposure to Polycyclic aromatic hydrocarbons. *Mar Enviro Res*, 54: 493 – 497.
- Le Pennec G, Le Pennec M (2001) Evaluation of the toxicity of chemical compounds using the digestive acini of the bivalve mollusk *Pecten maximus* L. maintained alive in vitro. *Aqua Tox*, 53: 1 – 7.

- Long SM, Ryder KJ, Holdway DA (2003) The use of respiratory enzymes as biomarkers of petroleum hydrocarbon exposure in *Mytilus edulis planatus*. *Ecotox Enviro Saf*, 53: 261 – 270.
- Luellen DR, Shea D (2003) Semipermeable membrane devices accumulate conserved ratios of sterane and hopane petroleum biomarkers. *Chemo*, 53:705-713.
- Lydeard C, Cowie RH, Ponder WF, Bogan AE, Bouchet B, Clark SA, Cummings KS, Frest TJ, Gargominy O, Herbert DG, Hershler R, Perez KE, Roth B, Seddon M, Strong EE, Thompson FG (2004) The global decline of nonmarine mollusks. *Bioscience*, 54(4): 321 – 330.
- Maltby L, Alistair ABA, Boxall BA, Forrow DM, Calow P, Betton CI (1995) The effects of motorway runoff on freshwater ecosystems: 2. Identifying major toxicants. *Enviro Tox Chem*, 14(6): 1093 – 1101.
- Marsalek J, Brownlee B, Mayer T, Lawal S, Larkin GA (1997) Heavy Metals and PAHs in Stormwater Runoff from the Skyway Bridge, Burlington, Ontario. *Water Qual Res J Canada*, 32(4): 815-827.
- McKinney AD, Wade DC (1996) Comparative response of *Ceriodaphnia dubia* and juvenile *Anodonta imbecillis* to pulp and paper mill effluents discharged to the Tennessee River and its tributaries. *Enviro Tox*, 15(4): 514 – 517.
- McMahon RF, Bogan AE (2001) Mollusca: Bivalvia. In Ecology and Classification of North American Freshwater Invertebrates, 2nd Edition. Academic Press, pp. 331 - 430.
- Moulton CA, Fleming WJ, Purnell CE (1996) Effects of two cholinesterase-inhibiting pesticides on freshwater mussels. *Enviro Tox Chem*, 15(2): 131-137.
- National Native Mussel Conservation Committee (1998) National Strategy for the Conservation of Native Freshwater Mussels. *J Shell Res*, 17(5): 1419-1428.
- Neff JM (1979) Polycyclic Aromatic Hydrocarbons in the Aquatic Environment: Sources, Fates, and Biological Effects. Applied Science, Barking, Essex.
- Pavlica M, Klobučar GIV, Mojaš N, Erben R, Papeš D (2001) Detection of DNA damage in haemocytes of zebra mussel using comet assay. *Mut Res*, 490: 209 – 214.
- Pereira WE, Domagalski JL, Hostettler FD, Brown LR, Rapp JB (1996) Occurrence and accumulation of pesticides and organic contaminants in river sediment, water, and clam tissues from the San Joaquin River and tributaries, California. *Enviro Tox Chem*, 15(2): 172 – 180.
- Piccardo MT, Coradeghini R, Valerio F (2001) Polycyclic Aromatic Hydrocarbon Pollution in Native and Caged Mussels. *Mar Pol Bul*, 42(10): 951 – 956.
- Porte C, Biosca X, Solé M, Albaigés J (2001) The integrated use of chemical analysis, cytochrome P450 and stress proteins in mussels to assess pollution along the Galician coast (NW Spain). *Enviro Pol*, 112: 261 – 268.

- Pynnönen K (1995) Effect of pH, hardness and maternal pre-exposure on the toxicity of Cd, Cu, and Zn to the glochidial larvae of a freshwater clam *Anodonta cygnea*. *Water Res*, 29(1): 247 – 254.
- Rank J, Jensen K (2003) Comet assay on gill cells and hemocytes from the blue mussel *Mytilus edulis*. *Ecotox Enviro Saf*, 54: 323-329.
- Renaud CB, Kaiser KLE, Comba ME, Metcalfe-Smith JL (1995) Comparison between lamprey ammocoetes and bivalve molluscs as biomonitors of organochlorine contaminants. *Ca. J Fish Aquat Sc.*, 52: 276-282.
- Roduis F, Hammer C, Vasseur V (2002) Use of RNA Arbitrarily Primed PCR to Identify Genomic Alterations in the Digestive Gland of the Freshwater Bivalve *Unio tumidus* at a contaminated site. *Enviro Tox*, 17: 538 – 546.
- Sanders BM (1993) Stress proteins in aquatic organisms: an environmental perspective. *Crit Revs Tox*, 23: 49 – 75.
- Sasaki YF, Izumiyama F, Nishidate E, Ishibashi S, Tsuda S, Matsusaka N, Asano N, Saotome K, Sofuni T, Hayashi M (1997) Detection of genotoxicity of polluted seawater using shellfish and the alkaline single-cell gel electrophoresis (SCE) assay: a preliminary study. *Mut Res*, 393: 133 - 139.
- Sauve S, Brousseau P, Pellerin J, Morin Y, Senecal L, Goudreau P, Fournier M (2002) Phagocytic activity of marine and freshwater bivalves: in vitro exposure of hemocytes to metals (Ag, Cd, Hg and Zn) *Aqua Tox*, 58(3-4): 189 – 200.
- Shea D, Cope WG, Lazaro P, Thorsen W, Forestier D (2004) Highway Runoff as a Source of Contaminants to Freshwater Mussels. Final report to NC Dept. of Transportation. NCSU Dept. of Environmental and Molecular Toxicology, 59 pp.
- Siu WHL, Cao J, Jack RW, Wu RSS, Richardson BJ, Xu L, Lam PKS (2004) Application of the comet and micronucleus assays to the detection of B[a]P genotoxicity in haemocytes of the green-lipped mussel (*Perna viridis*). *Aqua Tox*, 66: 381- 392.
- Thorsen WA, Cope WG, Shea D (2004) Bioavailability of PAHs: Effects of Soot Carbon and PAH Source. *Environ Sci Technol*. 38: 2029-2037.
- Wakeham SG (1977) A characterization of the sources of petroleum hydrocarbons in Lake Washington. *J Water Pol Control Fed*, 49: 1680 – 1689.
- Weinstein JE (2000) Characterization of the acute toxicity of photoactivated fluoranthene to glochidia of the freshwater mussel, *Utterbackia imbecillis*. *Environ Tox Chem*, 20(2): 412 – 419.
- Weinstein JE, Polk KD (2001) Phototoxicity of anthracene and pyrene to glochidia of the freshwater mussel *Utterbackia imbecillis*. *Environ Tox Chem*, 20(9): 2021 – 2028.
- Widdows J, Donkin P, Staff FJ, Matthiessen P, Law RJ, Allen YT, Thain JE, Allchin CR, Jones BR (2002) Measurement of stress effects (scope for growth) and contaminant levels in mussels (*Mytilus edulis*) collected from the Irish Sea. *Mar Environ Res*, 53: 327-356.

- Williams JD, Warren ML, Cummings KS, Harris JL, Neves RJ (1993) Conservation status of freshwater mussels of the United States and Canada. *Fisheries*, 18: 6 – 22.
- Wilson JT, Pascoe PL, Parry JM, Dixon DR (1998) Evaluation of the comet assay as a method for the detection of DNA damage in the cells of a marine invertebrate, *Mytilus edulis* L. (Mollusca: Pelecypoda). *Mut Res*, 399: 87 – 95.
- Woods JA, O’Leary KA, McCarthy RP, O’Brien NM (1999) Preservation of comet assay slides: comparison with fresh slides. *Mut Res*, 429: 181 – 187.

Appendix

Appendix 1: Tables

Table 1: Sites selected for use in this study. Sites where mussels were sampled for Comet assay are highlighted in gray.

River Basin	County	Bridge Number	Creek	Road	Average Daily Traffic Volume (vehicles/day)
Cape Fear	Alamance	74	UT to Back Creek	Jimmie Kerr Road	55
Cape Fear	Randolph	339	Reedy Creek	Jugtown Rd	90
Dan	Person	211	Mayo Creek	Mayo Lake Rd.	90
Cape Fear	Alamance	338	Poppaw Creek	Foster's Store Rd	100
Neuse	Person	38	Lick Creek	Willie Gray Road	130
Tar	Granville	177	Shelton Cr	Sunset Rd	< 500
Tar	Granville	9	Coon Creek	Mountain Road (Horner Siding Rd)	440
Cape Fear	Randolph	459	Reed Creek	Low Bridge Road	1100
Cape Fear	Alamance	204	Rock Creek	Friendship Patterson Rd (Walt Shoe Rd)	1500
Cape Fear	Chatham	12	Terrell's Creek	NC 87	2300
Cape Fear	Chatham	18	Dry Creek	NC 87	2550
Cape Fear	Alamance	20	Mary's Creek	NC 87	3500
Tar	Granville	28	North Fork	US 158	3500
Neuse	Orange	30	North Fork Little River	NC 57	3600
Tar	Franklin	62	Fox Creek	NC 56	10000
Neuse	Johnston	2052	Buffalo Creek	NC 42	13000
Tar	Granville	29	Coon Creek	Business 158 (in Oxford, NC)	13000
Neuse	Wake	561	Terrible Creek	US 401	24000
Cape Fear	Orange	263	New Hope Creek	I-40	56000
Neuse	Wake	49	Brier Creek	I-40	126000

Table 2: Measured levels of PAHs in glochidia and juvenile test solutions in µg/L. Concentrations in test solutions at t0 were on target. Note that the t48 and t96 water samples were taken post-incubation just prior to replenishment.

Date	Type	Treatment and Time							
		PAH50 t0	PAH100 t0	PAH200 t0	PAH50 t48	PAH100 t48	PAH200 t48	PAH 100 t96	PAH 200 t96
19-Jul-04	Glochidia		105.07	206.81		44.40	70.54		
11-Aug-04	Glochidia		91.58	219.40		22.83	54.52		
5-Mar-04	Glochidia	46.73	90.34	164.32	12.82	18.52	34.38		
2-Aug-04	Juvenile		96.14	201.71		20.25	42.68	19.69	50.16

Table 3: Tissue levels of PAHs in mussels exposed in the laboratory and water concentrations (day 14).

Target Conc. (ug/L)	Mussel Tissue Conc. (ug/g)	Water Conc. (ug/L)
CTRL	0.203	Not Sampled
PAH 1	3.977	Not Sampled
PAH 10	25.812	1.326
PAH 50	101.464	Not Sampled
PAH 100	223.637	14.515
PAH 200	289.309	69.002

Table 4: Average sum of PAH contamination measured from PSDs. Sampling devices were deployed above and below the crossing structure for streams where mussels were sampled for Comet assay testing. Streams are listed in order of ADTC.

Stream	Ave. Sum PAH (ng/L)
A338	300.37
G177	25.11
C12	63.11
O30	58.80
O263	48.81
W49	486.38

Appendix 2: Figures

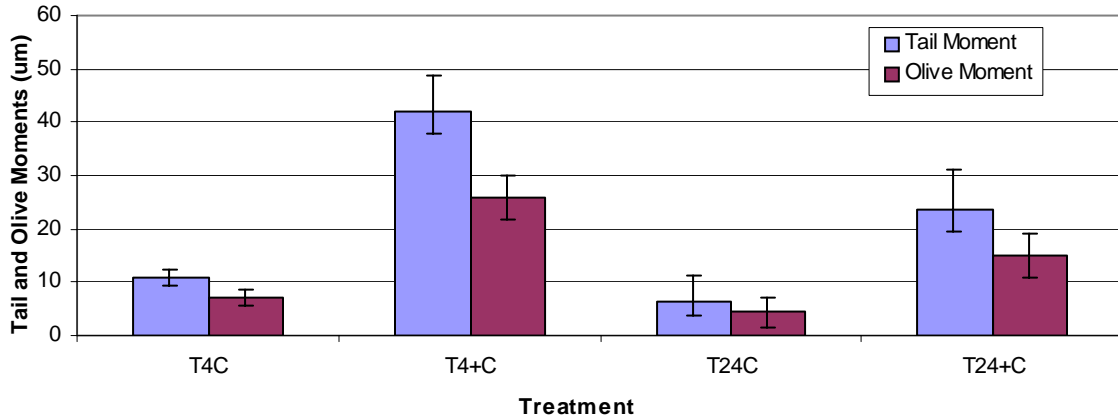
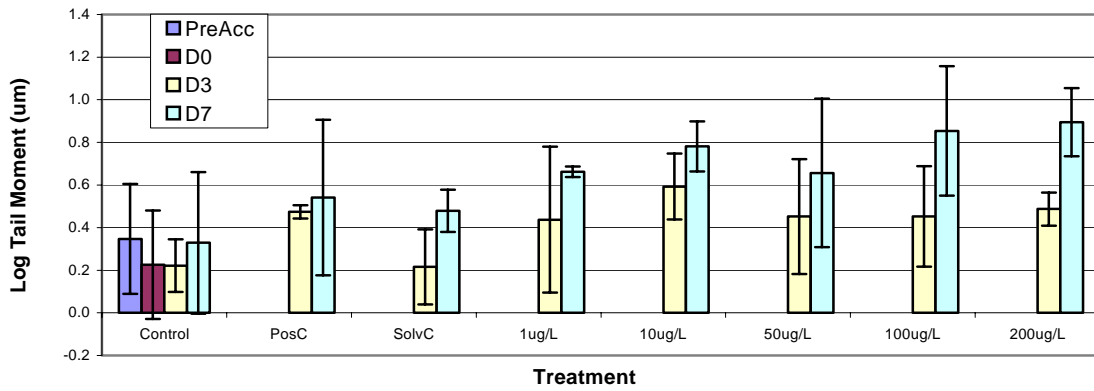


Figure 1. Tail and Olive Moment values (μm) for *E. complanata* hemocytes exposed for 4 and 24 hours to 4-NQO compared to unexposed cells, with 95% confidence intervals. (C = Control sample, +C = Positive Control)

2a



2b

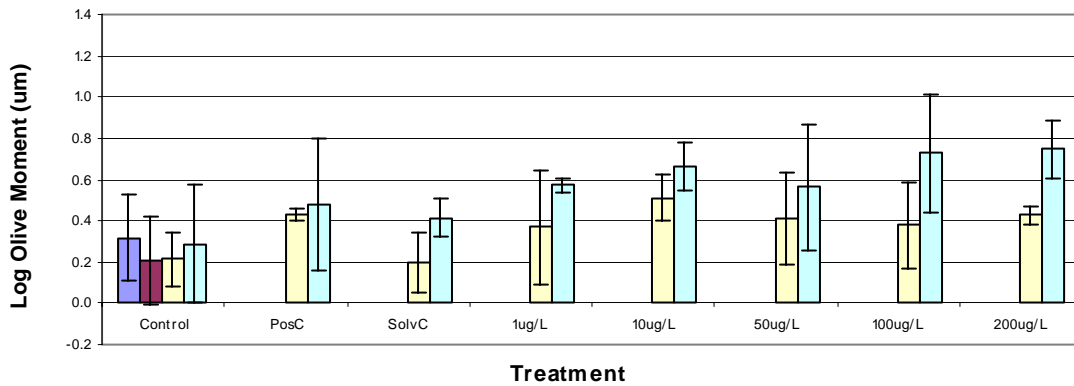


Figure 2. Tail (2a) and Olive Moment (2b) values for laboratory study, logarithmically transformed with 95% confidence intervals.

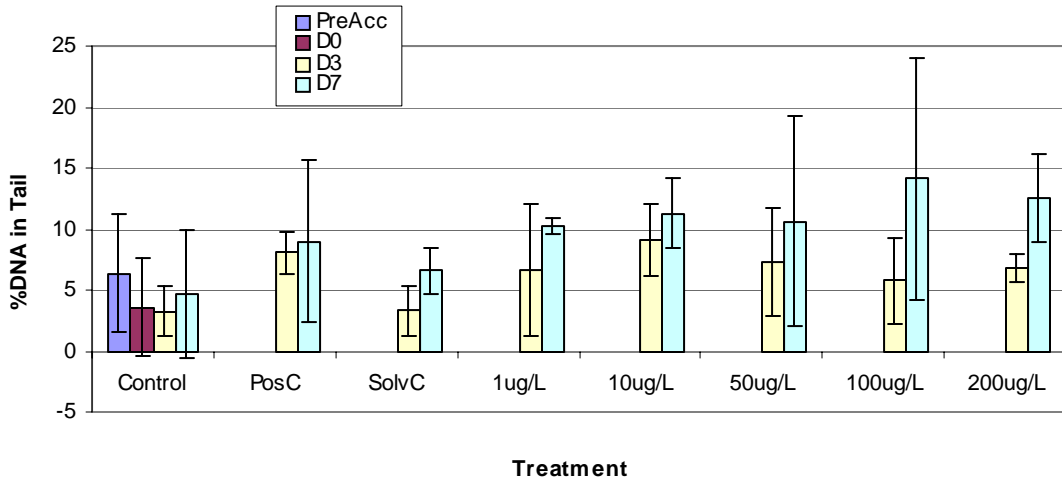


Figure 3. Percent DNA in comet tails per treatment, with 95% confidence intervals from the laboratory study.

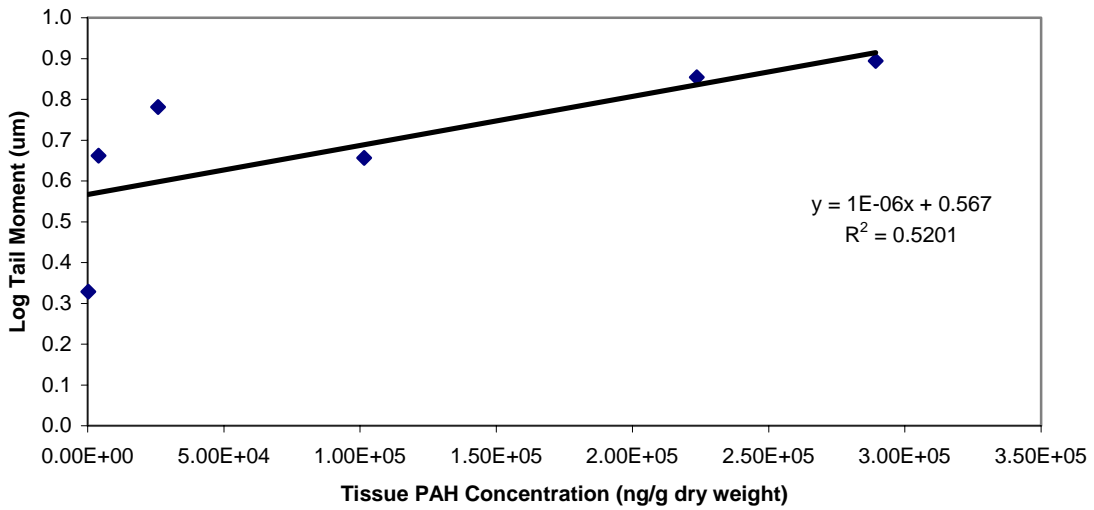


Figure 4. Regression of mussel tissue PAH concentration versus Log Tail Moment (um) from the Laboratory study.

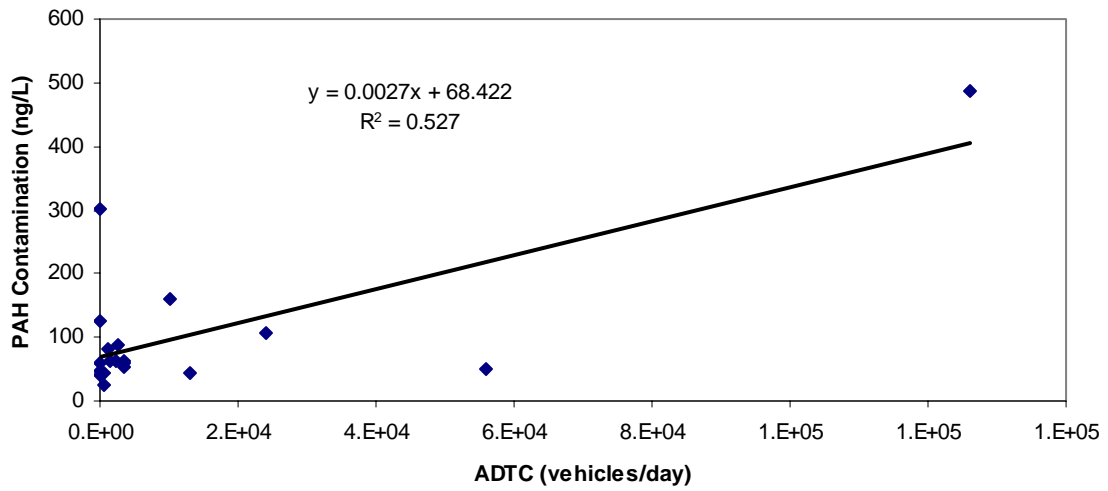


Figure 5. Regression of Average Daily Traffic Count versus estimated water PAH contamination ($\mu\text{g/L}$) from PSD data. Stream G29 was omitted from this regression.

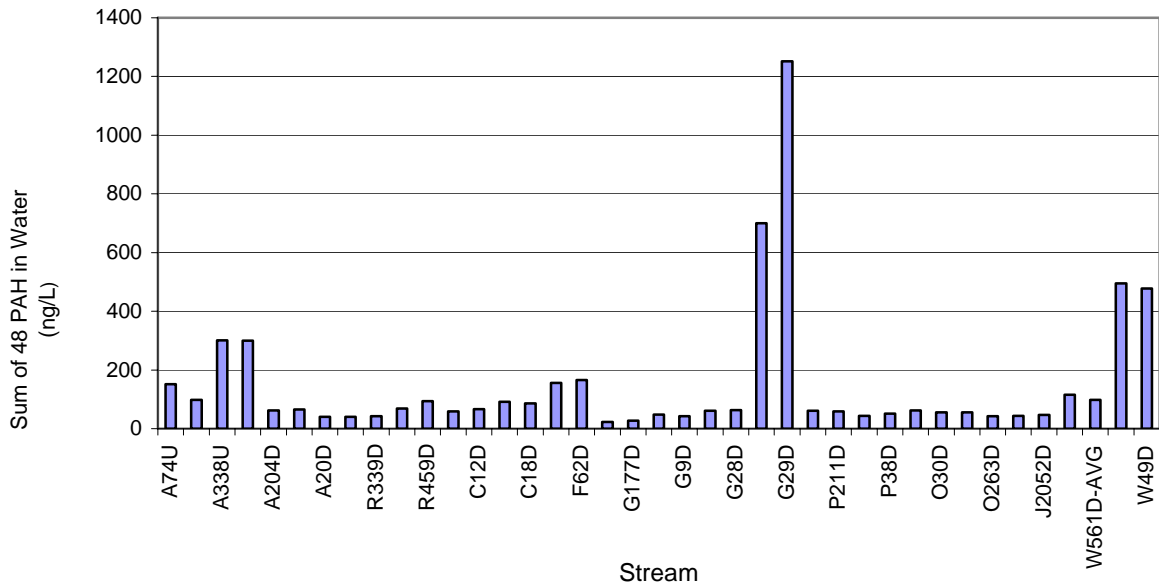


Figure 6. Sum of 48 PAH in 20 streams estimated from PSD residue. Streams are categorized from lowest to highest ADTC.

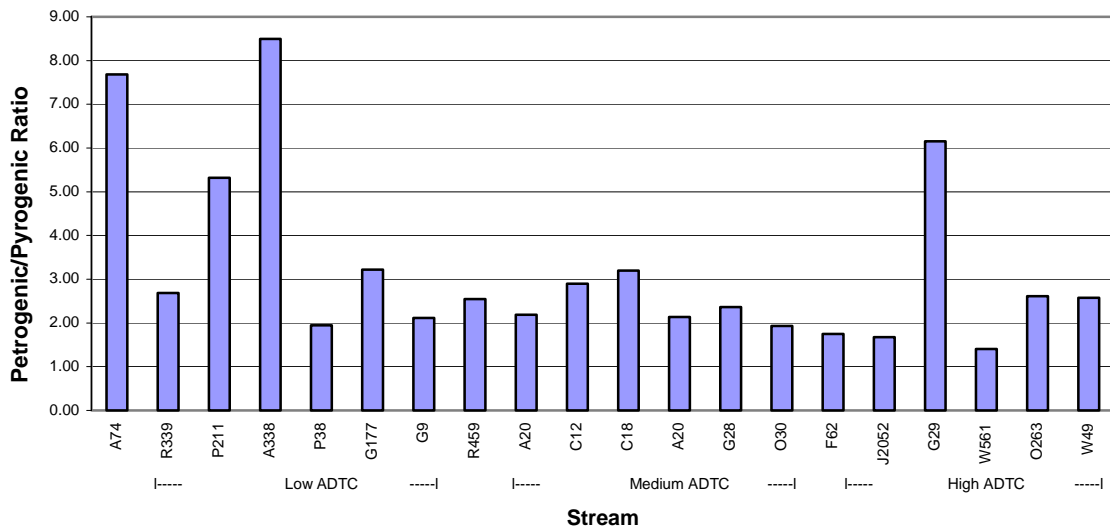


Figure 7. Mean Petrogenic/pyrogenic PAH ratio in the 20 streams used in this study. Streams are categorized from lowest ADTC to highest. There is no significant difference between ADTC groups.

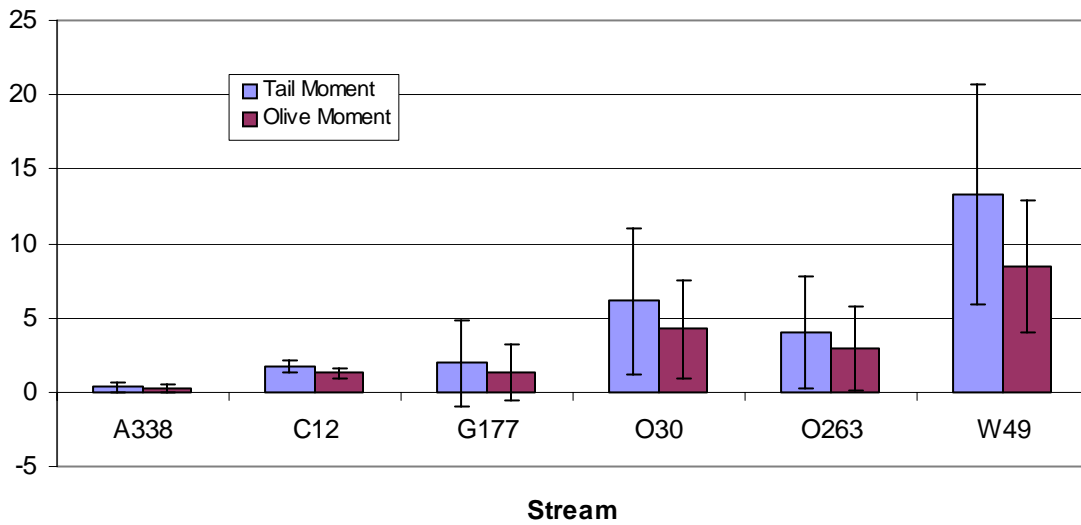


Figure 8. Tail and Olive Moment (μm) values for the streams sampled for the field portion of the study, with 95% confidence intervals.

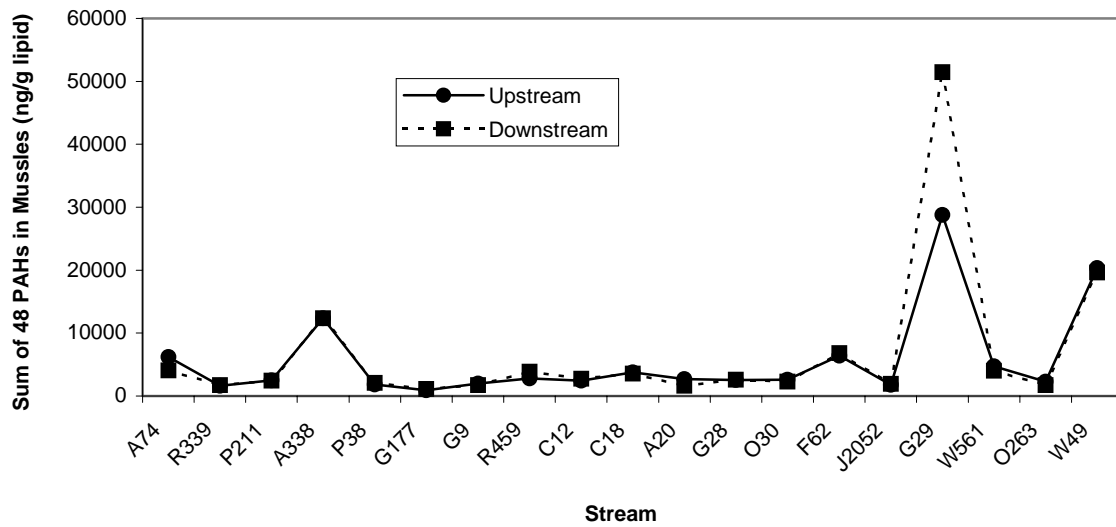


Figure 9. Predicted concentrations (ng/g lipid) of total PAHs in mussel tissue estimated from passive sampling devices (PSDs) placed at the study sites, using the empirical relation between measured concentrations of PAHs in mussel tissue and PSDs [$\text{PSD (ng)} = 0.29 \times \text{mussel (ng/g lipid)}$] from an earlier study (Shea et al. 2004).