Project No. MB2-WQ08

Contamination of Sediment and Crayfish with Benzo[a]pyrene in a Blackstone Valley Stream

Major Qualifying Project

Submitted to the Faculty
of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the
Degree of Bachelor of Science

Submitted by:
Ashley Sutton

Submitted to:
Professor JoAnn Whitefleet-Smith

April 30, 2009

Abstract

Sediment and crayfish samples were collected from several sites of a stream in the Blackstone River Valley in Massachusetts. Assays were performed to test each sample for the presence and level of Benzo[a]pyrene, a potential carcinogen. A drain that carries water from a nearby highway to the stream has acted as a point source for B[a]P, and sediment close to this point source is expected to contain the highest levels. As a bioindicator, crayfish B[a]P levels should correlate to those of sediment samples. When B[a]P levels in sediment collected from the outer edge of the stream (4 or fewer feet from the shore) were compared to levels of the compound in crayfish tail muscle (expressed in terms of ng B[a]P/mg tissue/mm tail length), a correlation was observed. The distribution of B[a]P indicated that the runoff drain acts as a point source for contamination.

Acknowledgements

First and foremost, I would like to thank my advisor, Professor JoAnn Whitefleet-Smith, for sharing with me her knowledge and enthusiasm for biology. Thanks to her, I was pleasantly surprised to find that I actually had *fun* while completing my MQP, and I am very thankful for the time and effort that she spent advising this project.

I would also like to thank Professor Mike Buckholt for enduring cold temperatures and leaky waders to help me collect samples at the stream.

Finally, I owe thanks to Professor Liz Ryder, who willingly shared her insight and advice about data analysis.

Table of Contents

Abstract ii
Acknowledgements iii
Table of Contents iv
Table of Figures v
Table of Tables v
Background 1
A. Benzo[a]pyrene 1
B. Crayfish 3
C. Bioindicators 5
D. Blackstone River Region 5
Introduction 7
Methods 8
A. Sampling site 8
B. Stream sediment sampling 10
C. Preparation of stream sediment samples 10
D. Crayfish 11
E. Preparation of crayfish tissue samples 11
F. Silica gel columns and drying samples 12
G. Assaying with gas chromatography 13
Results 16
A. Sediment 16
B. Crayfish 18
Discussion 20
References 24
Appendix A 26
Appendix B 27

Table of Figures

Figure 1: Chemical structure of Benzo[a]pyrene 1
Figure 2: Chemical structure of Benzo[a]pyrene diol epoxide 2
Figure 3: Crayfish anatomy 4
Figure 4: Blackstone River Watershed with sampling site indicated by red star 8
Figure 5: Drawing of sediment and crayfish sampling sites within the stream 9
Figure 6: Average sediment B[a]P levels at each sampling site 17
Figure 7: Sediment B[a]P level with distance from outer shore at each sampling site .. 17
Figure 8: Assembled nitrogen gas drying apparatus 26

Table of Tables

Table 1: Amount of B[a]P in each sediment sample 16
Table 2: ANOVA of B[a]P levels at each sampling site 16
Table 3: Sediment B[a]P levels for samples 2 and 4 feet from outer shore at each site . 18
Table 4: ANOVA of sediment B[a]P levels ≤ 4 feet from outer shore at each site 18
Table 5: Crayfish tail muscle B[a]P levels normalized to (ng B[a]P/mg tissue) 19
Table 6: Crayfish B[a]P levels normalized to (ng B[a]P/mg tissue/mm tail length) 19
Table 7: Raw sediment data 27
Table 8: Raw crayfish data 27

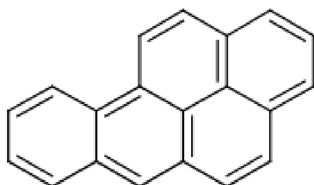
Background

A. Benzo[a]pyrene

Benzo[a]pyrene (B[a]P) is a chemical that is categorized as a polycyclic aromatic hydrocarbon (PAH). PAH's result from the incomplete combustion of organic substances such as coal, gas, oil, and garbage, and are one of the most widespread organic pollutants (ATSDR, 2009). Chemicals in this category are known for their carcinogenic and mutagenic effects; consequently, attempts are now being made to regulate their emissions from fossil fuel-burning engines in developed countries.

B[a]P is technically known as 3,4-benzo[a]pyrene. Its chemical formula is $C_{20}H_{12}$, and it has a molecular weight of 252.3. Figure 1 shows the chemical structure of a molecule of B[a]P.

Figure 1: Chemical structure of Benzo[a]pyrene (Source: National Library of Medicine ChemIDPlus, 2009)



A molecule of B[a]P consists of five benzene rings that are covalently attached to one another, forming a planar molecule. In its purified form, this chemical is yellowish in color and forms crystals in the form of plates and needles (Faust, 1994).

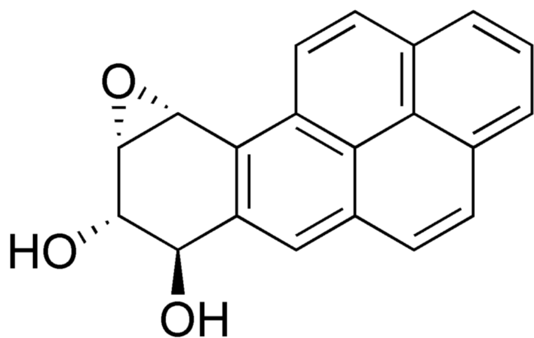
B[a]P can enter the environment in several ways, including through exhausts from gasoline and diesel engines, such as those found in motor vehicles, by-products of furnaces and stoves that burn coal, oil, or wood, cigarette smoke, as well as in other types of industrial or domestic smoke. When released to the environment, benzo[a]pyrene readily adsorbs to sediments and soils due to its highly hydrophobic nature. While B[a]P is occasionally detected in ground water, its strong adsorption to sediment means that there is minimal leaching of the chemical to water sources once it has entered the soil bed (U.S. EPA, 2006).

The strong tendency of B[a]P to adsorb to sediment or particulate substances in water can be both an asset and a detriment to its ability to be removed from environmental elements. While it is possible for the chemical to be removed from water by sunlight degradation, such an event is unlikely to eliminate B[a]P that is protected by a bed of sediment. Within the sediment, the

chemical is resistant to breakdown by microbes or reactive chemicals (U.S. EPA, 2006). The estimated half-life of benzo[a]pyrene in sediment is approximately 5-8 years, which is significantly higher than its estimated half life in any other medium (Faust, 1994). However, any B[a]P that is present in water should theoretically be relatively easily removed by filtration (Environment Agency, 2008). Thus, granular activated charcoal is used as a water treatment for removal of B[a]P from drinking water, a method that is approved by the U.S. Environmental Protection Agency (U.S. EPA, 2006).

Benzo[a]pyrene is a known procarcinogen, meaning that it is converted into a carcinogen through reactions catalyzed by two metabolic enzymes, cytochrome P450 1A1 and epoxide hydrolase. The resulting carcinogen is known as benzo[a]pyrene diol epoxide (Straub, 1977), and its chemical structure is shown in Figure 2.

Figure 2: Chemical structure of Benzo[a]pyrene diol epoxide (Source: NationMaster, 2003)



B[a]P diol epoxide has the ability to intercalate with DNA, meaning that it inserts itself between the stacked nucleotides of DNA's helical structure and is bound there non-covalently, which results in structural changes to the DNA double helix. This shape distortion causes mutations because the DNA cannot be copied in a normal manner.

The carcinogenic effects of B[a]P diol epoxide are suspected to stem from the molecule's tendency to target the transcription factor p53 gene, which regulates the cell cycle and thus prevents tumors. By creating DNA copies in which several important guanine nucleic acids have been changed to thymine within the gene, this transcription factor cannot function normally and lacks the ability to suppress tumor formation within the body (Pfeifer et al, 2002).

The link between benzo[a]pyrene and cancer was first suspected when, in the 1700's, men working as chimney sweeps, who were frequently exposed to burning organic materials, were found to have elevated incidences of scrotal cancer (Ruggeri, 1993). Since then, it has been extensively studied and found to have a strong correlation to the incidence of cancer. In 1962, Huggins and Yang reported that of 9 female Sprague-Dawley rats that were force-fed a single 100 mg dose of benzo[a]pyrene, 8 developed mammary tumors. Similarly, a 1967 study by Neal

and Rigdon showed that male and female CFW-Swiss mice fed diets containing 40 ppm or greater concentrations of benzo[a]pyrene developed forestomach papillomas and carcinomas in statistically significant incidences over control mice. This study also reported that the incidence of tumors was proportional to the dietary concentration of B[a]P and the number of doses administered (Faust, 1994).

Ingestion is not the only suspected route of exposure to the carcinogenic effects of B[a]P. In 1981, Thyssen et al allowed Syrian hamsters to inhale benzo[a]pyrene at several concentrations for a given number of hours per day, and a set number of days. Papillomas and squamous cell carcinomas developed in the respiratory tracts of mice that inhaled concentrations of 9.5 mg/m^3 and greater, and upper digestive tract tumors developed in mice exposed to the maximal concentration of 46.5 mg/m^3 (Faust, 1994).

In addition, several injection studies performed by the ATSDR (Agency for Toxic Substances and Disease Registry) in 1990 in which benzo[a]pyrene was injected into mice showed developmental and reproductive effects of the chemical. Mice injected interperitoneally had statistically greater incidences of stillbirths, resorptions, and deformed offspring, as well as testicular changes and decreased growth of corpus lutea and follicles. Direct embryonal injection of B[a]P resulted in a lower rate of fetal survival in mice, and subcutaneous injection in rats led to increased rates of resorption (Faust, 1994).

Due to the links between benzo[a]pyrene exposure and adverse health effects, the U.S. EPA has taken several measures to reduce the risk of human exposure to the chemical. It has set the maximum contaminant level of B[a]P in drinking water to 0.2 parts per billion, and acknowledges that the safest level of B[a]P is zero in drinking water (U.S. EPA, 2006). The EPA has also classified benzo[a]pyrene as a level B2 chemical, meaning that it is a probable human carcinogen (U.S. EPA, 2008).

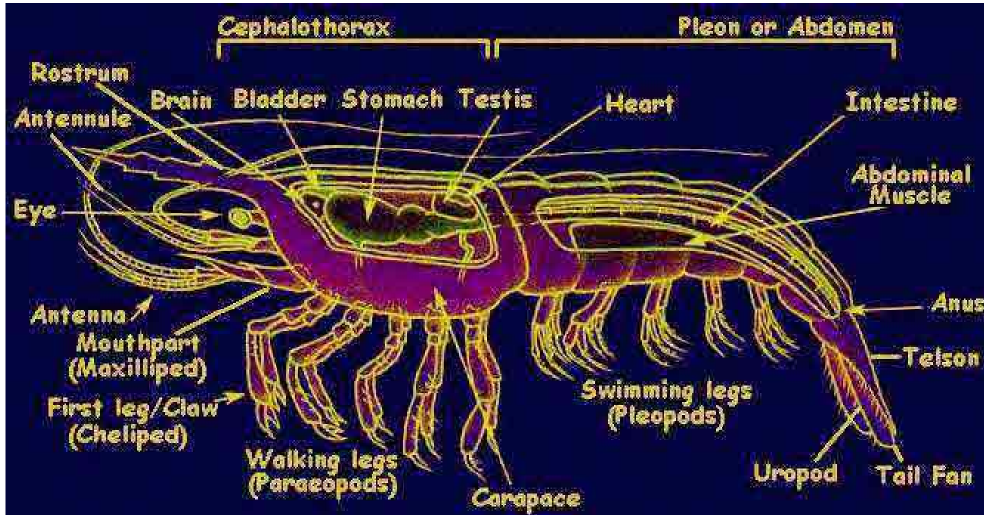
B. Crayfish

Crayfish are crustacean animals, and belong to the order Decapoda. There are three families of crayfish, with each family inhabiting a specific region of the world. The Cambaridae family can be found in eastern Asia and eastern North America, and with 330 species represented, has the greatest diversity of all the crayfish families. For this study, any crayfish that were captured in the testing region were used as samples, since no data is available suggesting that specific species would suit the purposes of the study better than others. Visual examination of the sample crayfish indicates that the species used in this study were *Orconectes virilis* and similar, comparable New England species.

The anatomy of a crayfish is very similar to that of a lobster, although the two animals are distinct and separately classified. Crayfish species used in this study usually measure 3-8 cm in length, and have a mean mass of 15 g. The anatomy of a crayfish is can be seen in Figure 3, but

the animal is characterized as having a tough exoskeleton, one large and one smaller pincer, ten legs, and a tail that fans at the end to aid in movement.

Figure 3: Crayfish anatomy (Source: Decapoda Anatomy and Biology)



The lifecycle of the crayfish species used in this study is typically between two to three years long, and the animals tend to mature when they are one to two years in age. Previous studies have shown that there is a strong correlation between the sizes and ages of crayfish (Harm, 2002). For the purpose of simplification in this study, crayfish tail length was assumed to be proportional to age; thus animals with longer tails were assumed to be older than those with shorter abdomens.

Crayfish activity levels depend on the season, especially in New England, where the temperature and climate vary so greatly depending on the time of year. Activity is highest for these crayfish during the spring, summer, and early fall seasons. A 1964 study on the “home ranges” of freshwater crayfish indicated that even during time periods of peak activity, crayfish tend to stay well within 100 feet of their specific “home” point (Black, 1964). This means that a crayfish captured in a particular area has likely spent the majority of its life dwelling within 100 feet of the point of capture, and therefore as only been significantly exposed to materials present within that area. Thus, if a crayfish were found to have accumulated a given substance by ingestion, it can be reasoned that the substance was present in the environment within 100 feet of where the crayfish was found.

Crayfish are omnivores, meaning that their diet can consist of plants, animals, and decaying organisms. The diets of juvenile crayfish are limited by their small size. Animals that feed on crayfish vary greatly, but include fish, birds, small mammals such as mink, weasels, and otters, snakes, and even humans.

C. Bioindicators

Bioindicators are animal species that offer a representative signal of the biological conditions within a certain area of the environment (U.S. EPA, 2009). These sentinel species can be tested for levels of chemicals that have bioaccumulated within them. Bioaccumulation is the buildup of a substance or chemical within an organism of part of an organism. These substances can be sequestered in the organism by ingestion, respiration, epidermal contact, or any other means by which the chemical enters the organism's tissues. The resulting concentration of the substance within the organism is greater than the substance's concentration in the surrounding environment, because it has accumulated within the organism (U.S. Geological Survey, 2006).

The presence of a bioaccumulated chemical within a bioindicator species indicates the movement of the chemical through the food web. For instance, if a given chemical is found to be bioaccumulated in an organism, then it can be assumed that predators of that organism have been, and will continue to, ingest the chemical of interest, and will be likely to bioaccumulate the chemical within their tissues as well, unless they possess a specific metabolic capability of breaking the substance down that their prey did not have. For this reason, bioindicators are usually at or near the bottom of their food chain. Also, bioaccumulation of a harmful chemical within an organism provides more compelling evidence of the need for concern from environmental groups such as the U.S. Environmental Protection Agency, than does the presence of the chemical in water or sediment alone (U.S. EPA, 2007).

The possibility of crayfish in a stream being bioindicators of benzo[a]pyrene levels seems intuitive. Since crayfish must search for and ingest food within the stream sediment bed, there is a distinct possibility that they could ingest B[a]P-containing sediment along with their intended food. In addition, the materials that crayfish purposefully feed on may also be contaminated with benzo[a]pyrene. Research performed by Goscila et al in 2007 determined that polycyclic aromatic hydrocarbons do bioaccumulate in the tissues of *Orconectes* organisms, and all tested tissue types (blood, hepatopancreas, and muscle) had equal ability to accumulate PAH's (Goscila et al, 2007). In a 2008 study, Gikas determined that the crayfish hepatopancreas was not a conclusive site of accumulation of B[a]P. This study did show that analysis of tail muscle of sample crayfish proved freshwater crayfish to be useful bioindicators of contamination of a streambed with B[a]P. However, the small number of crayfish samples (four individual organisms) in this study call the overall conclusions yielded into question, and necessitate further study with a larger sample size to create evidence proving or disproving the study's claims (Gikas, 2008).

D. Blackstone River Region

The stream from which all sediment and crayfish samples were collected in this study is located in the Blackstone River Valley region of Millbury, Massachusetts. The climate in this region is typical for New England, and has hot, humid summers, wet springs, and cold winters. The

surrounding area is known for being highly industrialized, although there are a few residential areas scattered nearby as well. The stretch of stream which was the focus of this study is located below a bridge, which allows route 146 to cross above the stream. A large drain empties directly into the upstream portion of the sampling area, presumably carrying water and other substances from the one or several nearby roadways into the stream. All of these factors contribute greatly to why this site is an ideal location to collect samples and test them for B[a]P levels, because factors in the surrounding area provide an excellent potential source of pollution by PAH's.

In addition, the sampling site of the stream contained both types of sediment bed that are necessary for crayfish to inhabit and to be useful for this study. Areas of the stream with sediment beds consisting of large rocks provide ideal shelter for crayfish to live in. However, areas of the stream with a sediment bed comprised of fine-grain particles also provides opportunity for feeding crayfish to ingest sediment, along with the chemicals contained by the sediment.

Introduction

This study had several important goals that it aimed to achieve. One important objective was to find data that either supported or weakened the hypothesis that crayfish are bioindicators of benzo[a]pyrene within the stream site of interest, and that bioaccumulation of the chemical occurs within the crayfish tail muscle, as indicated by previous studies (Goscila et al, 2007) (Gikas, 2008). Such evidence of crayfish as bioindicators would appear in the resulting data as a direct correlative relationship between the amounts of B[a]P detected in crayfish tail muscle tissue and sediment samples from the same or similar sampling site points.

Additionally, this study will test the result of research done by Gikas in 2008, indicating that the samples collected from immediately below the point source (drain) had the highest B[a]P concentrations, and concentrations of the chemical at downstream collection sites decreased with increasing distance from the drain. The storm drain that is a suspected point source for contamination is presumably supplied water by one or several of the nearby roadways (including Route 146, Route 20, the Massachusetts Turnpike, or local roads in the area's immediate vicinity). Many of the roadways suspected of contributing runoff water to the drain are frequently travelled by large, commercial trucks, as well as commuters. In accordance with the hypothesis that crayfish are bioindicators of B[a]P, the concentrations indicated by the data should show the same patterns in both crayfish tail muscle tissues and sediment samples.

A final goal of this study was to develop a more clear relationship between the levels of B[a]P detected in crayfish tail muscle tissues and sediment samples, or whether such a relationship truly exists. While Goscila et al (2007) and Gikas (2008) conducted experiments that aimed to develop a protocol for defining this relationship, a pattern between detected levels of B[a]P in crayfish tissues and sediment samples was not well established by the data resulting from either study, suggesting the need to make adjustments to their protocols in an effort to normalize data and establish meaningful relationships.

In this study, sediment samples and crayfish tail muscle tissue samples were collected from several specific sites within a designated stretch of a stream in Blackstone River Valley, Massachusetts. The collection sites were designed to indicate the distance of the site from the point source, the drain. Several specific steps were taken for each type of sample to extract the benzo[a]pyrene present within the sample. Then, gas chromatography was performed with the extracted material from each sample to determine the chemical distribution. Specifically of interest were the detected concentrations of benzo[a]pyrene in each sample. The data was analyzed to establish whether crayfish act as bioindicators of B[a]P in this stream, and whether distance from the point source did in fact correlate to the B[a]P levels detected in crayfish tail muscle tissue and sediment samples.

Methods

A. Sampling Site

A portion of a stream in the northwest region of Blackstone River Watershed in Massachusetts was the site of all sample collections in this study. The particular stream portion sampled lies on the town line between Worcester and Millbury, beside the Blackstone River Bikeway. See Figure 4 for a map of the Blackstone River Watershed, in which the sampling site is labeled.

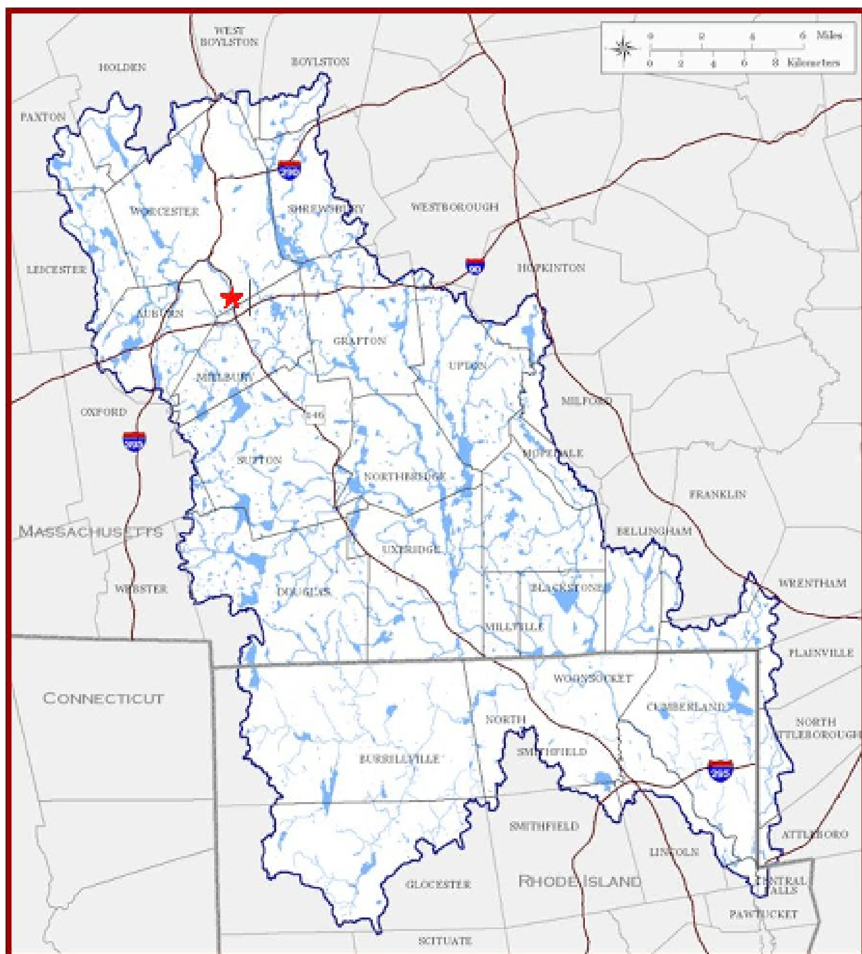
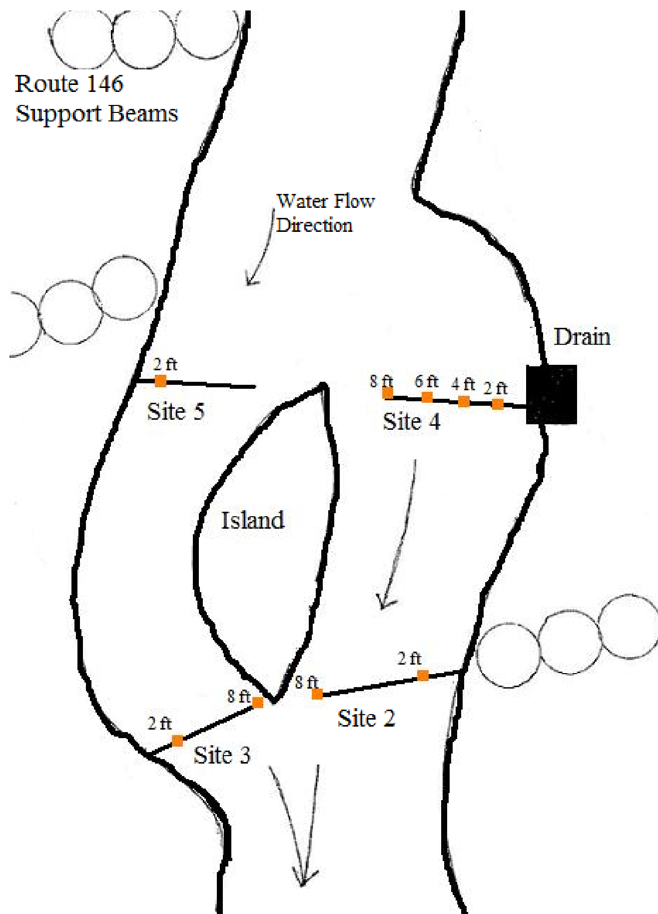


Figure 4: Blackstone River Watershed with sampling site indicated by red star (Goscilla et al, 2007)

The sampling site was chosen for several reasons. First, it has been the subject of two previous studies in regards to its contamination with B[a]P, as it was used as a testing site by Goscilla et al in 2007, and Gikas in 2008. This site also provides several important features required for such studies. It is shallow enough to safely wade across its entire width in order to collect necessary samples. In addition, its location under a highway bridge, which is suspected to drain directly into the stream via a large drain pipe, provides a likely source of contamination from roadway runoff.

Four cross-sectional sites were located in the stream for sampling of both sediment and crayfish. A drawing of the stream, the four cross-sectional sampling sites, and important landmarks such as the roadway runoff drain and an island, is shown in Figure 5. The drain and Route 146 support beams were used as visual markers of the site locations. Several sediment samples were collected at each site, at measured distances from the outer shore of the stream (the shore that was furthest from the island). Locations of sediment collection sites are represented by orange dots in Figure 5. Although sediment samples would ideally have been collected at 2, 4, 6, and 8 feet distances from the outer shore at all four sampling sites, the terrain of the stream's bottom or depth of the stream limited collection sites to those indicated by the figure using the available collection methods. Captured crayfish were labeled according to which of the four main sites they were located, since it was assumed that a crayfish likely moved about within and around the site.

Figure 5: Drawing of sediment and crayfish sampling sites within the stream



B. Stream Sediment Sampling

Sediment was collected at each sampling site. Any large rocks that would obstruct sampling were moved aside with a metal spade if necessary. Then, a plastic beaker was pushed about 5 inches into the soil bed, and turned in a way that would prevent contents from being lost when the scoop was withdrawn. The scoop was removed from the stream, and its contents were poured into a glass sample jar through a plastic funnel with an opening that was approximately 1 inch in diameter. This funnel allowed any large rocks in the sample to be removed. A metal spatula was used as needed to facilitate the sediment's passage through the funnel's opening. This process was repeated as necessary until the sample jar was sufficiently filled with sediment from that particular site. The jar was then closed and labeled with the site number, and refrigerated within 2 hours at 4° Celsius.

C. Preparation of Stream Sediment Samples

Sediment samples were transferred to appropriately sized centrifuge containers. They were centrifuged at 10,500 g for 10 minutes, so that a sediment pellet formed within the containers. Supernatant was poured off, and care was used to avoid loss of sediment during this process.

Each sample was transferred to a labeled 900 mL lyophilization flask, using a minimal amount of reagent grade water to rinse all of the sediment from the centrifuge container to the flask. The flasks were covered with rubber lyophilization caps, and stored at 4°C for later use.

Each sediment sample was frozen in its lyophilization flask using liquid nitrogen. The contents of the flask were shaken to create the most even consistency prior to being shell frozen. When the entire contents of the flask were completely frozen, they were lyophilized. The lyophilization process was carried out overnight, and monitored for completion.

When a sample had been entirely dried, the flask was removed from the lyophilizer. Each sample was then filtered through a 2 mm ×2 mm mesh wire colander to remove all but the fine grain sediment. This sediment was carefully transferred to a glass jar labeled with the sampling site, sealed, and stored at 4°C.

10 grams of sediment were placed in a clean, labeled 100 mL glass bottle. Hexane was added to the bottle until the total volume in the bottle was approximately 150% of the volume of the sediment alone. The bottles were fitted with Teflon-lined caps and closed tightly, then shaken at 300 rpm at 40ºC for 3 hours.

When the shaking was complete, the sediment samples were filtered. The filtration apparatus consisted of a sheet of Q5 filter paper that had been folded and inserted into a clean glass funnel. The filter paper was saturated with hexane, and then the apparatus was suspended using a ring stand so that it filtered into a 40 mL vial. Individually, the contents of each shaken bottle were poured into the filtration apparatus, allowing the liquid to drain into an appropriately labeled vial.

5 mL of hexane was used to thoroughly rinse the inside of the 100 mL shaking bottle, and then this hexane was poured over the sediment remaining in the filter paper, as a means of rinsing the sediment. This rinse was performed two more times, using 5 mL of hexane for each rinse. The filtered extract was stored in labeled vials at 4ºC until it was needed. The samples were prepared for analysis by gas chromatography by being run over silica gel columns, and concentrated in 2 mL amber vials via drying with nitrogen gas, as described in part F of this Methods section. Finally, the samples were dissolved in 40 µL hexane, and stored at 4ºC in the labeled amber vials until needed for gas chromatography.

D. Crayfish

Several methods were employed in catching crayfish. The first made use of hand nets, which were held so that the bottom of the net lay on the bottom of the stream. Then, large rocks and debris several feet upstream of the net were disturbed so as to dislodge any crayfish that were present in this area, which would then be carried into the net by the water flow of the stream. The contents of the net were periodically observed, and any crayfish that had been caught were removed and stored. Similarly, the same process of moving rocks to dislodge crayfish was used upstream of a seine, which was held across the stream to catch debris caught in the water current. Traps were also used to catch crayfish. Cylindrical traps commonly used to catch minnows or crayfish were baited with raw strips of salmon meat. Lines were attached to the traps, and the traps were positioned and tied so that they were submerged in water shallow enough to allow crayfish to crawl into them. Contents of the traps were checked daily, and any trapped crayfish were collected at this time.

When a crayfish was caught, it was placed into a Ziploc bag with a small amount of stream water. The bag was labeled with the site number in which the crayfish was found. Within 2 hours, the crayfish were transported to their storage site. Just prior to storage, the stream water was removed from the Ziploc bags. The crayfish were then euthanized by freezing them at -21° C. They were stored at this temperature in the Ziploc bags until dissection.

E. Preparation of Crayfish Tissue Samples

Tail muscle tissues were dissected from crayfish for testing. The tails of the crayfish were cut from the body using a large pair of dissecting scissors. The tails were thawed, and then small dissecting scissors were used to snip along the sides of the ventral exterior shell of the tail. The ventral exterior shell was peeled away from the muscle using thumb forceps. Next, the entire tail muscle was removed as a whole from the shell, and needle forceps were used to pull the nerve cord and intestine from the dorsal side of the muscle if they were still intact. The crayfish tail muscles were stored in clean, labeled 20 mL glass vials at -21ºC.

For each sample, a portion of tail muscle with mass between 30 and 60 mg was ground with 1.0 g anhydrous sodium sulfate using a glass mortar and pestle until a uniform, free-flowing powder was formed.

The cotton plug, if present, was removed from a 5 inch Pasteur pipet, and a small amount of glass wool was inserted and pushed down gently to block the tip. A small, clean glass funnel was rinsed with hexane, and then attached to the top of the Pasteur pipet using a minimal amount of rubber tubing. The column apparatus was suspended above a clean, labeled 40 mL glass vial, using a ring stand, so that the elute from the column was captured by the vial. The ground sample powder was poured into the funnel so that it rested above the glass wool in the pipet. Then, 2 mL of hexane was used to rinse the mortar and pestle, and this hexane was poured into the funnel to enter the column. This rinse was performed four more times. The sodium sulfate column eluent vials were labeled with the tissue sample number and stored at 4ºC until needed.

F. Silica Gel Columns and Drying Samples

All samples (tissue and sediment) were processed further using silica gel columns and nitrogen gas drying.

To prepare the silica gel slurry, grade 2 silica gel was baked at 150-160ºC for a minimum of 3-4 hours, with occasional gentle stirring. 6% (weight/volume) rdH_2O was added. The mixture was shaken vigorously, and then allowed to sit at 21ºC for at least 24 hours. Next, hexane was added to the gel in enough volume to create a slurry comprised of approximately 1 part silica gel: 3 parts hexane.

The cotton plug, if one was present, was removed from a 5 inch glass Pasteur pipet, and enough glass wool was inserted into the pipet to block the tip. A permanent marker was used to mark the outside of the pipet a distance of 4.5 cm from the top of the glass wool. A ring stand was used to suspend the pipet above a waste container of hexane.

The silica gel slurry was shaken to create as uniform a solution as possible, then a relatively small amount was funneled into a clean 120 mL Erlenmeyer flask to fill the flask approximately halfway. The slurry was swirled within the flask, then a 2 mL glass pipet and pipetman were used to immediately transfer between 1 and 2 mL of the slurry to the Pastuer pipet, creating a silica gel column. The hexane was allowed to drip through the column into the waste container of hexane. If necessary, the Erlenmeyer flask was again swirled, and small amounts of slurry were transferred to the column until the silica gel reached the marked 4.5 cm height. When the gel reached the appropriate height within the column and the hexane had exited the column, the waste container of hexane was capped and replaced by a clean, 40 mL glass vial that was labeled with the number of the sample that was to be eluted.

Each tissue and sediment sample was run over a fresh silica gel column. A clean glass pipet and pipetman were used to add the sample to the top of the column, and the eluent was captured in the glass vials below the column. The labeled glass vials containing the silica gel eluents for each sample were then stored at 4ºC until needed.

Vials of silica gel column eluent were uncapped and placed in a test tube rack below a drying apparatus (see Appendix A for picture and detailed description of its assembly). The pipet ends of the apparatus were cleaned with ethanol and Kimwipes, then, each valve was slowly turned to a level so that a minimal amount of nitrogen gas was flowing through the pipet end. The pipet end of the tube was positioned within the vial so that it hung above the level of the elute liquid. Care was taken to avoid splashing the samples out of the vials.

When there was no liquid remaining in a vial, the gas valve was turned off, and the vial was removed from below the drying apparatus and capped. Two clean 250 µL syringes were rinsed with hexane. Then, one of the syringes was used to transfer 250 µL of hexane to the dried vial, allowing the hexane to run down the sides of the vial as much as possible. The vial was capped and swirled, to allow the hexane to rinse the inside of the vial. The other syringe was then used to draw up the hexane from the vial and transfer it to a clean, labeled 2 mL amber vial. This rinse was performed a total of 6 times, using a single syringe to insert clean hexane, and a separate syringe to draw up hexane from the rinsed vial and insert it into the amber vial.

The amber vials were then completely dried with nitrogen gas using the previously described protocol. 40 µL of hexane was added to each labeled vial, and the vials were tightly capped and stored at 4ºC until assayed.

G. Assaying with Gas Chromatography

Prior to assaying, the amber vials containing the samples dissolved in hexane were floated in a 37ºC water bath for at least 30 minutes, until no solid material could be seen inside the vial.

A Perkin-Elmer Sigma 3 Gas Chromatography (GC) machine that had a flame ionization detector was used for separation. The machine used a Supelco SPB-5™ wide bore glass capillary column, which had these characteristics:

ID = 0.53 mm, Length = 30 m, dF = 0.50 µm, Beta value = 265.0

The following GC parameters were used for the analyses:

Oven Temp = 100ºC
Inj Temp = 300ºC
Det Temp = 300ºC
Time 1 = 1 min
Rate = 8ºC/min
Time 2 = 15 min

GC results were analyzed using a Hewlett Packard 3395 Integrator. The following parameters were used to define a "BAP1" method for the integrator:

Run Parameters

ZERO = 0
ATT2^ = 10
CHT SP = 0.5
AR REJ = 0
THRSH = 0
PK WD = 0.04

Timetable Events

0.000 ZERO = 0
0.000 INTG = 8
0.000 INTG = 2
6.000 ATT2^ = 6
20.000 PK WD = 0.20
35.000 STOP

A 10 µL gas-tight syringe was thoroughly rinsed with Chromosolve hexane. The syringe's needle was then used to pierce the septum cap of an amber vial containing sample dissolved in hexane, and liquid from the vial was drawn up and ejected several times to mix the contents. Next, 1.0 µL of liquid was drawn into the syringe, and care was taken to ensure that no air bubbles were present. The needle was withdrawn from the amber vial's septum cap, and about 1.0 µL of air was additionally drawn into the syringe. The sample was loaded into the gas chromatography machine, and the integrator was started simultaneously. The prescribed temperature program was immediately initiated.

To remove any lingering contaminants from the GC column, after every fourth sample was analyzed, the machine was brought up to a temperature of 300ºC for at least 10 minutes.

GC integrator plots were examined to determine which peak, if any, represented B[a]P. An extensive set of B[a]P standards performed by Letourneux (2009) revealed that the average retention time for B[a]P under these parameters was 28.662 minutes. The standard deviation from this average was 0.367 minutes. This was the accepted range of retention times in which a peak representing B[a]P was considered to occur. In addition, due to the variability of GC and integrator instruments used, a B[a]P standard (in hexane) was run daily to ensure that the B[a]P peak would fall within the accepted retention time range. The B[a]P peak was also identified based on the fact that the plotted peak tended to have a narrow and sharp spike, rather than one that was broad.

If there was further uncertainty as to which peak, if any, was representative of B[a]P, the sample was spiked with a known amount of B[a]P, and GC analysis of this spiked sample was performed. The peak that had an increase in area that was proportional to the amount of B[a]P added to the sample was identified. The plot for the original sample was examined and the peak that corresponded to the spiked B[a]P peak was identified as B[a]P.

Once a B[a]P peak was identified, the amount of B[a]P present in 1 μL sample was quantified using the equation for a B[a]P standard curve. The equation of this curve was: (Area under peak)= 4389.6 × (ng B[a]P) – 55456 (Letourneux, 2009). For sediment samples, the units were converted from (ng B[a]P/μL sample) to (ng B[a]P/g sediment). Tissue data was normalized according to the mass of tail muscle tissue used in a particular sample, as well as the length of the individual's tail, so that the final units of B[a]P were expressed in (ng B[a]P/mg tissue/mm tail length).

Results

Raw data for both sediment and crayfish samples including B[a]P peak areas can be found in Appendix B.

A. Sediment

Table 1 shows the calculated amount of B[a]P in each sediment sample tested.

Table 1: Amount of B[a]P in each sediment sample

Site Number	Distance from outer shore (in feet)	Amount of B[a]P (ng B[a]P/g sediment)
2	2	136.40
	8	108.96
3	2	102.48
	8	92.88
4	2	577.84
	4	427.48
	6	97.32
	8	94.60
5	2	127.36
	2	124.96

Table 2 shows the output of a single factor analysis of variance (ANOVA) performed to determine whether there is a statistically significant difference in B[a]P levels in sediment between the four sampling sites. Since the P value yielded by this analysis is 0.483349, which is higher than the accepted range of P values that would indicate a significant difference (P value ≤ 0.05), the difference between the average B[a]P levels at each site is not considered significant.

Table 2: ANOVA of B[a]P levels at each sampling site

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	5127.965	3	1709.322	0.92629	0.483349	4.757063
Within Groups	11072.05	6	1845.342			
Total	16200.01	9				

Figure 6 is a bar graph of the average amount of B[a]P per gram of sediment for each site. Although the ANOVA in Table 2 reports that the difference between average B[a]P levels at the sites is not statistically significant, it is clear from this graph that the average level of B[a]P is considerably higher at site 4 than at the other sites.

Figure 6: Average sediment B[a]P levels at each sampling site

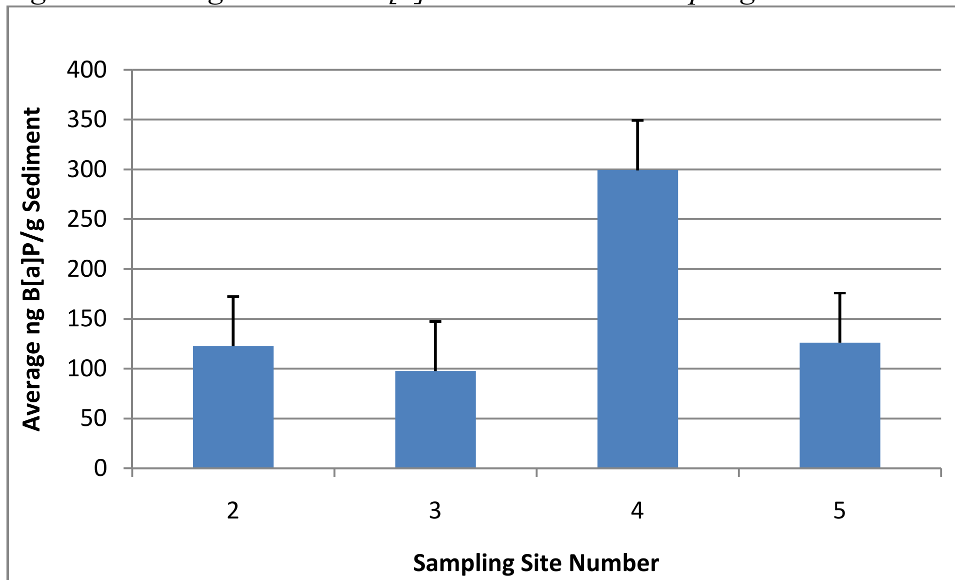


Figure 7 shows a graphical representation of the sediment B[a]P contamination level at each site plotted versus sample distance from outer shore. It should be noted that since two separate samples were collected 2 feet from the outer shore at site 5, the amounts of B[a]P found in each of these samples was averaged for the purposes of this graph. It is clear that at distances of 2 and 4 feet from the outer shore at each site, the highest B[a]P levels are found at site 4.

Figure 7: Sediment B[a]P level with distance from outer shore at each sampling site

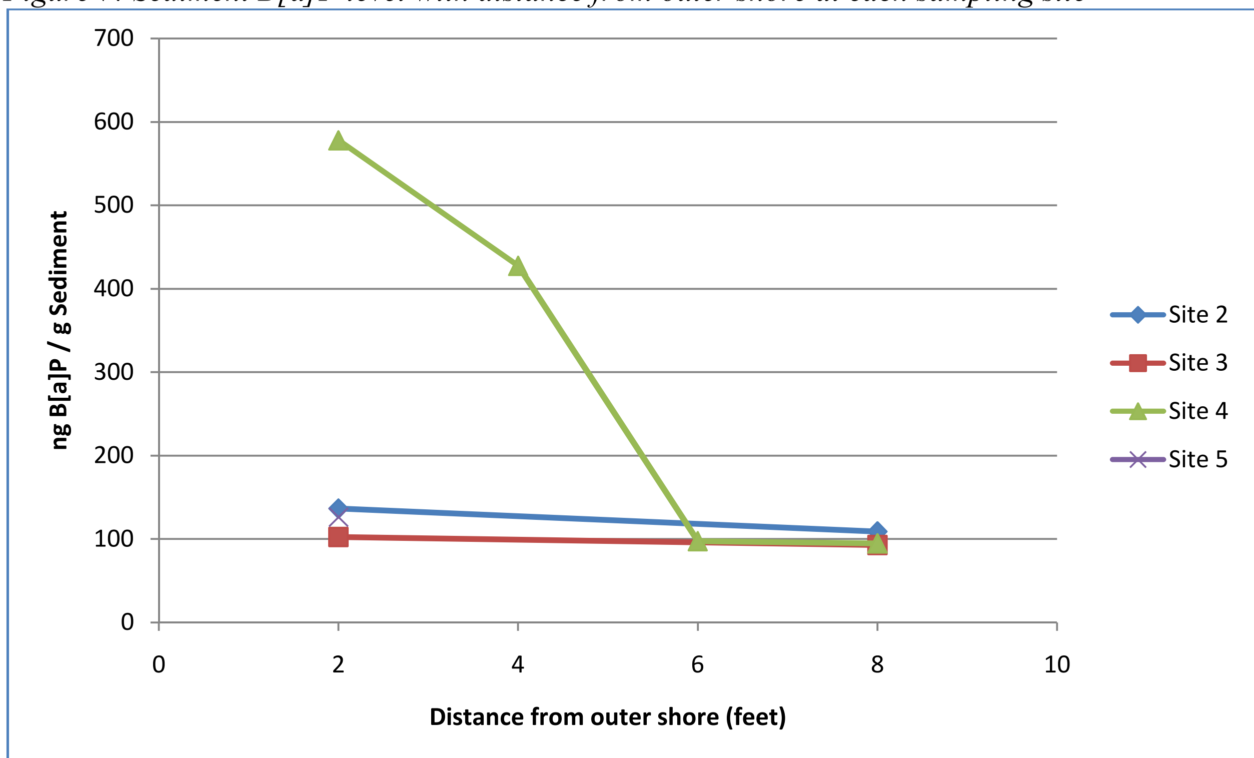


Table 3 shows the sediment data points used when only samples collected at distances of 2 and 4 feet from the outer shore were considered. Sediment samples collected at distances of 6 and 8 feet from the outer shore at each site were excluded from this table. This was done in consideration of the fact that crayfish samples were captured (due to capture methods and animal distribution) toward the outer shores of their respective sites.

Table 3: Sediment B[a]P levels for samples 2 and 4 feet from outer shore at each site

Site Number	Distance from outer shore (in feet)	Amount of B[a]P (ng B[a]P/g sediment)
2	2	136.40
3	2	102.48
4	2	577.84
	4	427.48
5	2	127.36
	2	124.96

Table 4 shows the output of a single factor ANOVA performed on the data in Table 3, to test if there is a statistically significant difference in the average level of B[a]P in sediment in the outer 4 feet of each sampling site. The P value yielded by this analysis is 0.08185, which indicates that the difference between the average B[a]P levels in sediment at each site is closer to significant when only data from samples collected at distances ≤ 4 feet from the outer shore are used, rather than when samples collected at distances ranging from 2 to 8 feet from the outer shore are used, as in Tables 1 and 2.

Table 4: ANOVA of sediment B[a]P levels ≤ 4 feet from outer shore at each site

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	193011.9	3	64337.31	11.38014	0.08185	19.16429
Within Groups	11306.94	2	5653.472			
Total	204318.9	5				

B. Crayfish

Table 5 shows calculated amounts of B[a]P in tail muscle tissue of each crayfish normalized so that they are expressed in units of (ng B[a]P/mg tissue). The “letter” assigned to each crayfish was used for identification purposes only. A T-test to determine whether there is a significant difference in B[a]P levels between crayfish collected at site 4 versus site 5 yields a P value of 0.55, which is much higher than the accepted range (P value ≤ 0.05) that would indicate a significant difference between the averages of levels at each site.

Table 5: Crayfish tail muscle B[a]P levels normalized to (ng B[a]P/mg tissue)

Collection Site	Letter ID	Amount of B[a]P in tail muscle (ng B[a]P/mg tissue)
4	A	0
	B	0.78
	C	0.41
	D	0.41
	E	0.38
	F	0.53
	G	0.50
5	A	0.31
	B	0.36
	C	0.44

Table 6 shows calculated amounts of B[a]P in tail muscle tissue of each crayfish normalized so that they are expressed in units of (ng B[a]P/mg tissue/mm tail length). This was done in as a means of accounting for the fact that the older a crayfish was, the more exposure that individual would have had to contaminants in their environment, and thus the greater the chance of bioaccumulation in their tissue. Similarly, an individual's metabolism could play a role in the bioaccumulation of B[a]P that takes place in their tissues. In order to eliminate age and metabolism as factors that would affect B[a]P levels in crayfish, tail length was used as a means of representing the age and metabolism of a particular crayfish. A T-test performed with data normalized in this manner to test whether there is a significant difference in B[a]P levels of tissue between crayfish at sites 4 and 5 yields a P value of 0.04, which falls in the accepted range (P value ≤ 0.05) to indicate a significant difference between groups. Thus, the difference between the average ng B[a]P/mg tissue/mm tail length of crayfish at sites 4 and 5 is statistically significant.

Table 6: Crayfish B[a]P levels normalized to (ng B[a]P/mg tissue/mm tail length)

Collection Site	Letter ID	Tail length (mm)	Amount of B[a]P in tail muscle (ng B[a]P/mg tissue/mm tail length)
4	A	25	0
	B	35	0.022
	C	22	0.019
	D	20	0.021
	E	17	0.023
	F	32	0.017
	G	30	0.017
5	A	33	0.0093
	B	40	0.0090
	C	44	0.010

Discussion

The first hypothesis that was being tested was that the runoff drain from Route 146 acts as a point source for benzo[a]pyrene contamination in the area of interest in the Blackstone Valley stream. Sediment samples were collected at several sites in the stream, at measured distances from the outer shore of the stream. Gas chromatography was used to determine the chemical composition of each sediment sample, and a standard curve was used to quantify the amount of B[a]P per gram of sediment for each sample.

Within sampling site 4, which is located directly below the suspected point source, the level of B[a]P contamination of sediment is highest (577.84 ng B[a]P/g sediment) at a distance of 2 feet from the outer shore (where the drain is located). Four feet from the drain, the second highest B[a]P level is observed (427.48 ng B[a]P/g sediment). The sediment samples collected at 6 and 8 feet from the drain had B[a]P levels of 97.32 ng B[a]P/g sediment and 94.60 ng B[a]P/g sediment, respectively. This trend of relatively high B[a]P levels at site within close proximity to the drain, which decrease drastically as distance from the drain increases, is consistent with results previously observed by Gikas (2008). This trend is supportive of the hypothesis that the runoff drain acts as a point source for B[a]P contamination, because sediment contamination levels are highest in samples collected closest to the drain's location.

The average levels of B[a]P in all samples of sediment collected at sites 2, 3, 4, and 5 are 122.68 ng B[a]P/g sediment, 97.64 ng B[a]P/g sediment, 299.32 ng B[a]P/g sediment, and 126.16 ng B[a]P/g sediment, respectively. Comparison of these values reveals that the highest level of sediment contamination is present at site 4, which is closest to the drain. This data supports the hypothesis that the drain acts as a point source for B[a]P contamination, since the highest average sediment contamination level is at the site that is immediately below where the drain empties into the stream.

The fact that elevated B[a]P levels are observed at close proximities (2 and 4 foot distances) to the drain but then drastically decrease to relatively low levels similar to those seen much further away from the drain's location indicates that the compound is not being carried more than a few feet by the flow of water in the stream before it comes to rest in the sediment of the streambed, assuming that the drain is the source of B[a]P contamination. Since benzo[a]pyrene is known to be a very hydrophobic molecule, it would seem logical that it would quickly enter the sediment bed in order to evade the water of the stream.

A single-factor analysis of variance between the average sediment B[a]P levels at each site indicates that there is no statistically significant difference (P value of 0.483) in overall contamination levels of sediment between the four sites. The fact that site 4 has such variability in B[a]P levels, depending on the sample distance from the drain, undoubtedly affects the overall average level of site contamination.

However, crayfish samples in this study were only captured toward the outer banks of the stream, within their respective sites. This was due to limited means of capture. There is also a good possibility that the streambed terrain toward the middle of the stream made the areas near the outer banks more habitable for the animals. Whatever the reason, the crayfish captured for this analysis were captured toward the outer banks of sites 4 and 5. Thus, to get a good representation of the B[a]P contamination trend that would be expected in crayfish (the hypothesized bioindicator organism), it is appropriate to exclude data from sediment samples collected toward the center of the stream (at distances greater than 4 feet from the outer shore).

When the B[a]P levels in sediment samples collected only at distances of 2 and 4 feet from the outer shore in their respective sites are used, a single-factor analysis of variance between the sites yields a P value of 0.08. Although this value is still above the accepted significant range of $P \leq 0.05$, it is much closer to a significant value than was observed when all site sample data points were used. This test indicates that there is nearly a statistically significant difference in average sediment B[a]P levels between the four sites. Simple observation of the data for the sites reveals that if one site had a different average contamination level than the others, it would be site 4, which has elevated B[a]P levels in sediment samples collected both 2 and 4 feet from the outer shore.

A second hypothesis that was tested is that crayfish act as bioindicators of benzo[a]pyrene levels in their environment. Crayfish have been shown by Goscila et al to have the potential to act as bioindicators of polycyclic aromatic hydrocarbons (2007) such as B[a]P. As bioindicators, trends in tail tissue contamination with B[a]P are expected to be similar to those observed in sediment samples, with respect to sampling site. Since sediment collected at site 4 was shown to have considerably higher average B[a]P contamination levels, especially closer to the outer stream shore, where crayfish were captured, than at any other site, it was predicted that crayfish collected at site 4 would have higher average levels of tail tissue contamination than crayfish collected at site 5.

Crayfish contamination levels expressed in units of ng B[a]P/mg tissue were calculated for each specimen. A T-test was performed to determine if the average level of contamination with B[a]P of crayfish from site 4 was significantly different from that of crayfish from site 5. The P value yielded by this test was 0.55, indicating that there was no significant difference in tissue B[a]P levels between crayfish collected at sites 4 and 5. This data would suggest that the hypothesis that crayfish act as bioindicators of B[a]P is incorrect.

However, crayfish data expressed only in units of ng B[a]P/mg tissue does not account for the fact that the crayfish analyzed in this study very likely had different ages (and thus, exposure times to environmental contaminants), as well as different metabolic rates at which they could store and/or break down benzo[a]pyrene. Thus, it is necessary to take these factors into account as much as possible in analyzing the levels of B[a]P for each individual. For example, if two crayfish were collected from a given site, one of which had been alive and inhabited the site (and

therefore been exposed to the contaminants there) for two years, and the other of which was only one year old, it is a reasonable assumption that the two-year-old crayfish has twice the potential for bioaccumulation of that of the one-year-old crayfish. The same scenario is theoretically possible for crayfish that have different metabolic rates; a crayfish that consumes twice the amount of food (and thus, twice the sediment) will potentially have twice the bioaccumulation of B[a]P in its tissues of another crayfish inhabiting the same area. It is also reasonable to predict that the crayfish that consumes more will also be larger.

For the purposes of this study and in an attempt to eliminate the effect of age and thus exposure time on crayfish tail B[a]P levels, the data was further normalized according to tail length of each individual, under the assumption that crayfish tail length is proportional to the age of the individual. This eliminated the effect of crayfish age and exposure time on the level of B[a]P reported, since the levels were expressed per unit of tail length (age). A T-test on crayfish tail B[a]P levels expressed in units of ng B[a]P/mg tissue/mm tail length yielded a P value of 0.043, which was considered significant. This indicates that there is a statistically significant difference between the average B[a]P levels in tails of crayfish captured at site 4 versus those captured at site 5. The average B[a]P level in crayfish at site 4 is 0.017 ng B[a]P/mg tissue/mm tail length, which is considerably higher than that of crayfish at site 5, which is 0.0094 ng B[a]P/mg tissue/mm tail length. This supports the hypothesis that crayfish act as bioindicators of B[a]P levels in their environments, since the trend of higher contamination with B[a]P at site 4 than at site 5 is observed in both sediment and crayfish tail muscle tissue.

A final goal of this study was to define an analysis protocol that was appropriate for comparison of B[a]P levels in crayfish tail muscle and sediment. When crayfish levels are expressed in units that account for the difference in tail length (a measure of age for these purposes), a significant P value of 0.043 indicates that there is a distinct difference in B[a]P levels of crayfish collected at sites 4 versus site 5. This difference also reflects the trend observed in sediment B[a]P levels at sites 4 and 5, leading to the conclusion that the analysis protocol of normalizing crayfish data using crayfish tail length as a measure of age is appropriate.

There are several possible sources of error in this study. The normalization of crayfish data to units of B[a]P/mg tissue/mm tail length is based on the assumption that tail length is proportional to age and thus exposure time to B[a]P and potential for bioaccumulation. However, if crayfish specimen groups consisted of more than one species of crayfish (which may have different size characteristics), then the method of normalization described may not be an appropriate way to take age into account.

Another possible source of error may have come from the slight variability of the retention time of B[a]P during GC analysis. Despite having a clearly define accepted range of retention times, the possibility of outliers is always present, especially due to the variability of the instruments used. While questionable samples were spiked in order to identify the B[a]P peak, these additional measures were not taken with all samples. Certainly this lends itself to the suggestion

that all samples should be spiked in future studies, in order to ensure that the correct peak has been identified as B[a]P.

Small sample size, both of crayfish and sediment, is another area of concern in consideration of the results of this study. In order for the data to be more statistically significant, several sediment samples must be collected at each sampling point; many more crayfish are also needed from each site.

Based on the results of this study, as well as the questions it raises, several recommendations can be made concerning the direction of further research in this area. Certainly one of the most important issues in consideration of crayfish as bioindicators of B[a]P levels is the factors that affect B[a]P bioaccumulation in crayfish tissue. As mentioned in this study, age and metabolism likely play a role in bioaccumulation. This role could be examined by studying the accumulation of B[a]P in tissues of "clean" crayfish individuals, which have had no previous exposure to the compound. The accumulation with time and other factors could be closely studied in this way.

Another idea would be to track the movement of crayfish within a freshwater stream, to determine where most of their time is spent, where they tend to eat, etc. This would give a clearer picture as where each individual is likely to have maximum exposure to environmental contaminants.

To normalize crayfish data further, a study could be conducted to determine whether crayfish species (and their resulting physical characteristics) play a role in bioaccumulation of B[a]P within tissues. The species of collected crayfish could be determined, and comparisons of crayfish B[a]P levels at each site could be performed using each species individually. For example, if crayfish of species A and B were collected at sites 4 and 5, levels of B[a]P in tissues of species A could be compared at sites 4 and 5. The same could be done for species B at the two sites. If the sample size is large enough, P values from the two comparisons could indicate whether crayfish species affects bioaccumulation potential. If the P values of the comparisons are similar, then it is unlikely that species has a significant effect; if the P values are dissimilar, this indicates that crayfish species plays a role in bioaccumulation of B[a]P in tissues.

Finally, more research should be done as to the source of benzo[a]pyrene contamination in the environment. If the drain in this study acts as a point source for contamination with the compound, do similar streams also have point sources for contamination from which B[a]P is distributed? If crayfish do, in fact, bioaccumulate B[a]P in their tissues, there is a very real possibility that B[a]P is able to move upward in the food web, where it may also be biomagnified. Research into whether biomagnification of B[a]P is occurring as a result of crayfish B[a]P bioaccumulation would provide a better picture of the effects of benzo[a]pyrene on individual organisms and the ecosystem as a whole.

References

ATSDR (Agency for Toxic Substances and Disease Registry) (2009). *Toxicological Profile for Benzo(a)pyrene*. Retrieved January 30, 2009, from: http://www.atsdr.cdc.gov/substances/toxsubstance.asp?toxid=25

Black, J. B. (1964).Observations on the home range of stream-dwelling crawfishes. *Ecology*. *44*, 592-595.

"Decapoda Anatomy and Biology." Retrieved March 1, 2009, from: http://www.shrimpcrabsandcrayfish.co.uk/Shrimp.htm?Anatomy.htm~mainFrame

Environment Agency, (2008). *Benzo-[a]-pyrene*. Retrieved January 29, 2009, from: http://www.environment-agency.gov.uk/business/topics/pollution/39407.aspx

Faust, R. A. (December, 1994). Toxicology summary for benzo[a]pyrene. *Oak Ridge Reservation Environmental Restoration Program*, Retrieved February 10, 2009, from: http://cira.ornl.gov/documents/Benzoapyrene.pdf

Gikas, Penny E., (2008). Benzo[a]pyrene contamination of sediment in a Blackstone Valley streambed. Worcester Polytechnic Institute.

Goscila, Jennifer et al, (2007). Crayfish (*Orconectes virilis*) as bioindicators of polycyclic aromatic hydrocarbon contamination in the Blackstone River Valley. Worcester Polytechnic Institute.

Harm, P. (2002). Biology of Freshwater Crayfish. Chapter 15: *Orconectes*. (Holdich, D.M, ed.). *Blackwell Science Ltd*. Oxford, England.

Letourneux, James, (2009). Benzo[a]pyrene in river sediment. Worcester Polytechnic Institute.

National Library of Medicine ChemIDPlus, (2009). *Benzo(a)pyrene*. Retrieved January 29, 2009, from: http://chem.sis.nlm.nih.gov/chemidplus/ProxyServlet?objectHandle=DBMaint&actionHandle=default&nextPage=jsp/chemidheavy/ResultScreen.jsp&ROW_NUM=0&TXTSUPERLISTID=000050328

NationMaster, (2003). Benzopyrene. Retrieved March 15, from: http://www.nationmaster.com/encyclopedia/Benzopyrene

Pfeifer GP, Denissenko MF, Olivier M, Tretyakova N, Hecht SS, Hainaut P. Tobacco smoke carcinogens, DNA damage and p53 mutations in smoking-associated cancers. *Oncogene*. 2002 October 21;21(48):7435-51.

Ruggeri, B. et al (1993). Benzo[a]pyrene-induced murine skin tumors exhibit frequent and characteristic B to T mutations in the p53 gene. *Proceedings of the National Academy of Sciences, 90:* 1013-1017.

Straub, K. M. et al (1977). Identification of the major adducts formed by reaction of benzo[a]pyrene diol epoxide with DNA in vitro. *Biochemistry, 74(12):* 5285- 5289.

U.S. Environmental Protection Agency (2006). *Consumer fact sheet on: Benzo[a]pyrene.* Retrieved: December 10, 2008, from: http://www.epa.gov/OGWDW/contaminants/dw_contamfs/benzopyr.html

US Environmental Protection Agency (2007). *Biological indicators of watershed health.* Retrieved April 1, 2009, from: http://www.epa.gov/bioindicators/

U.S. Environmental Protection Agency (2008). *Integrated risk information system: Benzo[a]pyrene (BaP) (CASRN 50-32-8.* Retrieved December 10, 2008, from: http://www.epa.gov/ncea/iris/subst/0136.htm#woe

U.S. Environmental Protection Agency (2009). *Bioindicators: Indicator species.* Retrieved December 13, 2008, from: http://www.epa.gov/bioiweb1/html/indicator.html

U. S. Geological Survey (2006). *Toxic substances hydrology program: bioaccumulation.* Retrieved February 21, 2009, from: http://toxics.usgs.gov/definitions/bioaccumulation.html

Appendix A

The following is a description of the assembly of the drying apparatus that was used for drying samples as described in the Methods section of this report.

The apparatus was made by attaching rubber tubing pieces to the outlets of an aquarium aeration unit, which had a valve capable of turning each individual vent on and off. Tips were made for the ends of each vent tube by melting Pasteur pipets a few millimeters beyond where the neck broadens, and stretching the melted section to form a tube portion with a smaller diameter. The pipets were cooled, and then carefully broken at the stretched section. The stretched ends of the mini-pipets were inserted into the rubber tubing of the aeration units, so that the pipet necks extended outward from the tubes. Next, the aeration units were attached to a ring stand, and rubber tubing was used to connect the units. Finally, the completed unit was attached to a tank of nitrogen gas (Gikas, 2008). Figure 8 shows the setup of the assembled unit.

Figure 8: Assembled nitrogen gas drying apparatus

Appendix B

Table 7: Raw sediment data

Site Number	Distance from outer shore (in feet)	Area of B[a]P peak	Calculated ng B[a]P/µL sample
2	2	94233	34.10
	8	64119	27.24
3	2	56994	25.62
	8	46454	23.22
4	2	578675	144.46
	4	413668	106.87
	6	51330	24.33
	8	48348	23.65
5	2	84328	31.84
	2	81676	31.24

Table 8: Raw crayfish data

Site Number	Letter ID	Area of B[a]P peak
4	A	0
	B	101296
	C	32246
	D	13443
	E	30377
	F	26476
	G	30228
5	A	3787
	B	7529
	C	55522