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B[a]P Contamination of Sediment in a Blackstone Valley Streambed

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Benzo[a]pyrene Contamination of Sediment in a Blackstone Valley Streambed

Major Qualifying Project

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Abstract

Benzo[a]pyrene (B[a]P) is a polyaromatic hydrocarbon which has been identified as a potential carcinogen. It enters the environment as a result of industrial emissions, is deposited in the watershed, and is biomagnified in animals living in contaminated waterways. B[a]P was extracted from sediment and crayfish (*O. virilis*) tissues collected from a Blackstone Valley streambed, and gas chromatography was used to quantify contamination levels. Comparisons of B[a]P concentrations at different points in the site gave insight into the deposition patterns of B[a]P from a known point source in a stream, and showed the most relevant test points for future research in this and other streambeds. Gut analysis of crayfishes showed that those living in the stream ecosystem eat sediment. Negative and trace contamination levels in hepatopancreas tissue did not reflect accumulation of B[a]P via the digestive tract, but significant levels of B[a]P detected in muscle tissue suggest that B[a]P may still have a pathway for bioaccumulation between the digestive tract and peripheral tissues. Overall, crayfish analysis confirmed biomagnification of B[a]P in the stream and supported the use of *O. virilis* as an indicator of B[a]P.

Acknowledgments

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I. Background & Introduction

It is a well known fact that worldwide pollution has increased dramatically over the past couple of centuries. Since the onset of the industrial revolution in the 1850's, factories and plants have made significant contributions to pollution, most directly in the beds of the waterways upon which they were often built. The introduction of automobiles and the roads which came with them in the 1920's initiated a new wave of pollution, with car runoff becoming a major area of concern in developed areas. Since this event the effects of industrialization, technology, and infrastructure have reached an alarming magnitude, sullying our resources, environment, health, and quality of life. While the need to find solutions to the pollution problem is an immediate one, little action can be taken without concrete information about the causes, levels, and effects of pollution. This project is a continuation of an effort to secure this very information about a local waterway.

The banks of the Blackstone River have shown considerable industrialization and development over the past two-hundred years, with pollution from industrial sources and car runoff increasing as development and the local population have increased (Blackstone River Watershed Team, 2000). Previous biological assessments have established the contamination of Blackstone waterways by polycyclic aromatic hydrocarbon (PAH) benzo[a]pyrene (B[a]P) (Goscilla et al, 2007), a known carcinogen which is often associated with industrialization and infrastructure ("Benzo(a)pyrene").

In this project a procedure for quantifying the amount of PAH B[a]P deposited in a waterway was created and tested on a Blackstone Rive Valley streambed in Millbury, MA. The gradient of contamination found was correlated with areas of high runoff, then connected to contamination in living tissue of local crayfish to establish bioaccumulation of B[a]P.

I A. Benzo[a]pyrene

B[a]P is categorized as a PAH. PAHs are a group of chemicals which are formed from the incomplete burning of organic material. Generation of PAHs can occur during natural events such as forest fires, but since the inception of the industrial revolution most of the PAHs in the environment are a result of emissions from cars and factories. In fact, it is estimated that less than 15% of PAHs are produced naturally (Benner & Gordon, 1989).

There are several physical and chemical properties which are common to most PAHs. They are generally large, hydrophobic molecules. The molecules are planar, and are formed when covalent bonds are formed between the carbons of benzene rings. PAHs with less than six benzene rings are considered small, and a molecule with six or more rings is usually considered large. The higher the number of benzene rings, the more aromatic and hydrophobic the compound becomes (Douben, 2003). B[a]P has five benzene rings, and is therefore a "small" PAH. The pure, crystallized form is a yellow

color but the chemical is never concentrated enough in nature to detect the color. (Fetzer, 2000)

PAHs entering the environment as a result of industrial burning are initially airborne compounds. They become condensed during precipitation, through which they are deposited in sediment. Because the molecules are hydrophobic, they are not easily washed along with rain once they reach the ground. Because PAHs tend to accumulate in the ground, higher concentrations can often be detected in sediment than in air or water. (Beasley & Kneale, 2004).

Since the late 1700's when an elevated rate of scrotal cancer was observed in chimney sweeps ("Soots"), much research has been done on the health risks associated with burnt organic materials. While evidence exists for the carcinogenic properties of several PAHs, only a few have been conclusively identified as toxic to animals and humans. B[a]P is one such PAH which has been associated with cancer. Many studies have shown elevated rates of cancer in humans who spend considerable times near sources of B[a]P. Exposure of rats to B[a]P in several experiments has resulted in toxic depression, hemorrhaging, and increased tumor formation. One study studied the effects of applying B[a]P to the skin of rats in order to mimic the accumulation of B[a]P-containing soot on industry workers' skin. The treatment resulted in the formation of malignant tumors on the rats' skin (Ruggeri et al, 1993) Other studies have also used B[a]P to induce cancer in tracheal and tissue shown the formation of cancerous tumors in (Ashurst et al, 1983) (Little & O'Toole, 1974) (Yan et al, 2005).

B[a]P is a procarcinogen, which means that it is a precursor for a carcinogenic molecules. By the action of various enzymes within cells, B[a]P is converted to a molecule called benzo[a]pyrene diol epoxide (B[a]P diol epoxide) (Straub et al, 1977). Figure 1 depicts the oxidation reaction which produces B[a]P diol epoxide.

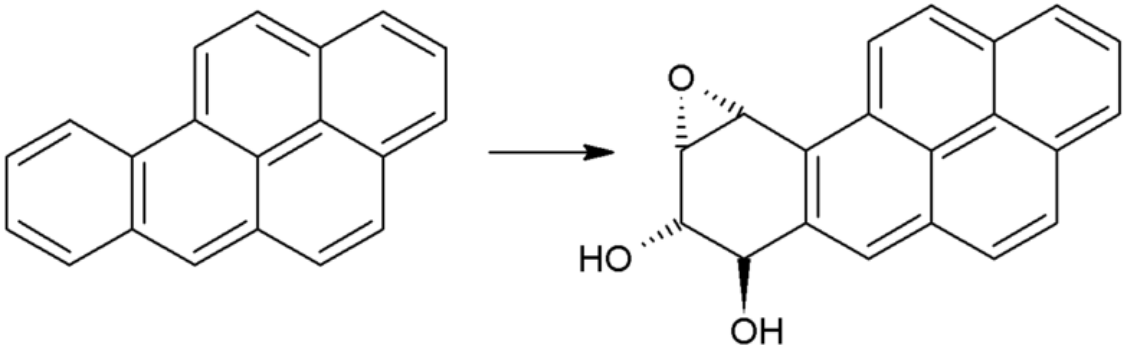

Figure 1: B[a]P (left) and B[a]P diol epoxide (right)(adapted from images in "Benzopyrene")

Like its precursor, B[a]P diol epoxide is a planar molecule, which enables it to intercalate into DNA. This alters the shape of the double helix and can lead to incorrect synthesis of a complementary strand of DNA resulting in guanine to thymine mutations, as well as transposons which cause genes to become "scrambled." B[a]P has been strongly linked to mutations in the p53 gene, a tumor suppressor gene which regulates cell death

(Preferential formation of benzo[a]pyrene adducts at lung cancer mutational hotspots in P53) (Xin-Hai Pei et al, 1999).

Evidence of these genetic effects has been recorded in several labs. It has been shown that treating cultured human bronchial epithelial cells with metabolites of B[a]P to simulate exposure through breathing contaminated air will cause the cells to show the same types of mutations on the p53 gene as cells extracted from lung tumors. (Mikhail et al, 1996)

The direct mutagenic effects of B[a]P on DNA has been proven and quantified. A Finnish research team measured the mutation rate of two loci in the DNA of workers in an iron foundry and compared it to the amount of B[a]P detected in their workplace breathing air, on the surface of their skin, and in their urine. A significant correlation was observed between the mutant frequencies and level of exposure to B[a]P. (Perera et al, 1993).

I B. Pollution in the Blackstone region

This study focused on levels of B[a]P in a fifty-meter stretch of a stream in Millbury, Massachusetts, which lies in the Blackstone Valley region. The stream was ideal for B[a]P testing owing to its location in an area of known industrialization and pollution, but also for it's location under a bridge. Route 146 runs above the testing site, and a runoff drain pours water from the highway directly into the stream. B[a]P contamination of the sediment and crayfish inhabiting the area has also been confirmed by a previous study, though no exhaustive data on concentration across the stream site or correlation between sediment and bioindicators was produced (Goscilla et al, 2007)

The sediment in the stream has both rocky portions which provide a dwelling place for crayfish (the bioindicators used for the study), as well as more fine-grain sediment which crayfish often ingest. The range of sediment types also allowed for comparisons of the effectiveness of B[a]P extraction methods used in this project on different types of sediment. The site was also fairly convenient. The water was just shallow enough for safe wading across the entire stream, which allowed cross-sections of the stream to be tested at several points.

The specific test points were selected with several goals in mind. Multiple sections of the stream were tested across the 50-meter length of the site. The sections selected were fairly evenly spaced, though concerns for safety and convenience prevented entirely equal distances. This range allowed for the change in concentration as the water moved downstream to be determined. Also, the selection of sections which were upstream, downstream, and directly in front of the runoff drain allowed the effect of the runoff to be determined. At each of the sections, four points were tested to create a cross-section view of contamination at that section of the stream. This showed whether there was a change in deposition levels across the stream bed.

I C. Detection of B[a]P through sediment sampling

The testing of sediment samples was decided upon as the best means of B[a]P contamination of inorganic elements in the environment for this study. There were multiple advantages to testing the sediment as opposed to the water or air. First, B[a]P would be most concentrated in the sediment. B[a]P in the air is concentrated into the water during precipitation. The hydrophobic B[a]P molecules are then deposited and concentrated in the fine grain sediment in the streambed (Letellier & Budzinski, 1999). Secondly, the sediment is fairly stationary, allowing for the testing of specific points. Finally, the fact that crayfish ingest sediment, coupled with its concentrated levels of B[a]P, makes it the most direct link between crayfish and B[a]P in their habitat. To confirm that crayfish were ingesting sediment, gut content analysis was performed.

I D. Movement of B[a]P through food web and use of bioindicators

The ultimate motivation to research deposition of B[a]P into stream sediment is its capacity to spread throughout through the environment. As indicated previously, airborne B[a]P makes its way to stream sediment through a water intermediate which helps to concentrate it. B[a]P moves up the food chain in a similar fashion, becoming more concentrated as it progresses through the trophic levels. This occurs through two distinct processes: bioaccumulation, and biomagnification.

Bioaccumulation is the process by which a foreign and often dangerous substance amasses in a living system. This occurs through the process of bioconcentration, which is when the volume of the substance taken up by the organism is greater than the amount which is excreted. (Smith & Smith, 2001) Substances which are bioaccumulated through sediment are typically lipid-soluble and hydrophobic chemicals, such as PAHs. When bioaccumulation takes place in an organism which is then eaten by predator, biomagnification may occur. This is when a toxic substance moves through the food chain, becoming more concentrated as it works its way up the food chain. (Kelly et al, 2007) While toxins tend to become more concentrated as the trophic level rises, it is important to remember that physiological aspects of each organism the toxin passes through (filtration efficiency, storage, metabolism, excretion rates, etc.) make it impossible to apply a comprehensive rate of magnification to any pathway (Hendrickx et al 2003).

While it is possible to detect trace amounts of such toxic pollutants in inorganic elements of the environment, researchers often look for the substances in the tissues of certain organisms. More specifically, they look for an increase of the chemical over time. These sentinel species, often called bioindicators, serve two purposes. First, they show whether or not the pollutant is able to work its way into the food web; and second, the appearance of the pollutant in a food chain serves as a warning which is often taken more seriously than the appearance of pollution in sediment or water. The Environmental Protection Agency's (EPA's) general definition of a bioindicator is "…a numeric value derived from actual measurements…whose trends over time represent or draw attention to underlying

trends in the condition of the environment." (U.S. EPA, *Biological Indicators of Watershed Health*)

Bioindicators are usually plants or small animals which are at the bottom of their food chain. Some of the most commonly used bioindicators for aquatic ecosystems from lowest to highest place on their food chains are algae, water plants, mollusks, crustaceans, fish and birds. When choosing a bioindicator for a study, one must consider the specific system to be tested, as well as the goals of the study. Periphyton, or attached algae, is a commonly used bioindicator of PAH contamination in aquatic ecosystems because toxins usually accumulate in the algae before it concentrates in any other organism (Lavoie et al., 2004). Because the stream tested in this study flows too strongly for algae to accumulate on the stream bed, algae was dismissed as a bioindicator. When researchers cannot or do not wish to use periphyton as bioindicators they often use macroinvertebrates, which have the lowest trophic level of any animals in the ecosystem. Benthic, or bottom-dwelling, macroinvertebrates have several characteristics which make them ideal indicators for aquatic ecosystems. They are easy to catch, large enough to identify, very closely linked to the pollutant-containing elements of their ecosystem, and stay in one general area for their lifetime (U.S. EPA, "Invertebrates as indicators"). These characteristics, particularly the their tendency to stay in one area throughout their entire life, made macroinvertebrates an ideal bioindicator for this project.

I E. Crayfish as bioindicators

1. Choice of crayfish

When considering the food web present in the Blackwater stream ecosystem, the most prevalent macroinvertebrate in the area is the crayfish. Not only are crayfish readily available as specimen in the stream, they have many characteristics beyond those generally seen in all macroinvertebrates which make them a perfect specimen for this project. First, crayfish have an ideal lifespan for a sentinel species. Because they usually live for about 2-3 years, year-to-year fluctuations in pollution will not dramatically skew results. This is useful in studies where contaminant levels cannot be monitored every year, and when the contaminant of interest has a slow degradation rate, both of which are the case with this study. This makes data obtained from their tissue more reliable. An added benefit is that this lifespan is unaffected by pollutants such as B[a]P, especially in invasive species (Gadzala-Kopciuch et al, 2004). Second, their physiological complexity provides several testable tissues. Whereas algae or a physiologically simpler mollusk would provide limited information about how and where B[a]P might accumulate in the body, comparisons of contamination levels in varying types of tissue provides insight into the types of tissues which may be affected by B[a]P.

The ecological role of crayfish also makes them an excellent choice for a bioindicator in this study. When choosing a biological indicator, it is imperative that one consider the ecological roles of candidates. The selection of birds as bioindicators of mercurial poisoning of the environment as a whole is an excellent example. The fact that birds drink contaminated water, breathe contaminated air, and eat animals living in both the water and on land makes them an excellent indicator of overall contamination in an ecosystem. While this is desired in some studies, the project at hand calls for an indicator

which will reflect contamination of stream sediment as directly as possible. Mercury has a very similar pathway into birds as B[a]P does in the Blackstone stream ecosystem (Babbit & Groat, 1999). One major similarity between the two toxins is that both can be initially internalized by organisms through both water and breathing air. This necessitates the selection of an aquatic bioindicator. Because crayfish occupy the highest aquatic trophic level in the stream ecosystem, they will have the most concentrated levels of B[a]P absorbed from the sediment. Additionally, they are a significant link between the aquatic and terrestrial parts of the food web. Because terrestrial organisms such as birds prey on crayfish, they serve as an excellent sentinel species for impending bioaccumulation of B[a]P in the rest of its food chain.

Crayfish also have a lifestyle which makes using them as indicators more convenient than other members of the ecosystem. They can be quickly uncovered from the rocks they hide under, and are easily scared out of their shallow burrows. Crayfish also tend to stay near their burrows, and therefore have low mobility rate relative to other aquatic organisms (Füreder and Reynolds, 2003). This means that contamination levels observed in them are more directly linked to the sediment by which they are found. Aquatic organisms such as fish would not fill this requirement at all, nor could any terrestrial animals which are considerably more mobile than crayfish.

Finally, because *O. virilis*, the species used for this study, has a very widespread habitat, they will be accessible for researchers across the nation, allowing them to use this protocol with a degree of confidence.

Crayfish have proved to be useful as bioindicators in numerous studies. One Dutch study used crayfish in conjunction with water and sediment samples to test for a number of organic pollutants, including PAHs, and found that crayfish can serve as excellent bioindicators for PAHs. Not only were PAHs found in the tissue of crayfish who lived in contaminated areas (as proven by positive sediment and water samples), significant trends in concentration of PAHs in tissue show that levels in crayfish are reflective of actual concentrations in the environment. (Schilderman et al, 1999). Previous research on sediment and tissues of crayfish collected at the Millbury stream site has shown concentrations of B[a]P in both, though no exact correlation was determined (Goscilla et al, 2007).

2. Orconectes virilis

The crayfish species used in this study is called *Orconectes virilis*. It is a decapod crustacean, and therefore has ten legs, one large pincer, and a characteristic tail fan General physiology can be seen in Figure 2. *O. virilis* is a moderately sized species, typically measuring 3-7 cm in length, and weighing about 15 g. Individuals usually mature between the ages of one to two years, with total lifespan lasting about two to three years. Individuals living in New England are usually most active from spring to early fall (late May to September). (Fetzner, 2002)

Figure 2: Orconectes virilis (Fullerton)

O. virilis have a distinct role in their ecosystem. They are opportunistic feeders, and will eat most any organic matter which finds its way to them. This usually consists of plant material, but could include dead animals or even other crayfish. Crayfish are the largest freshwater invertebrates, which makes them ideal prey for a range of predators which includes fish, birds, reptiles, amphibians, some small mammals, and even humans (Fetzner, 2002).

O. virilis is native to the Midwestern states, with its original habitat stretching from Utah to Ohio, and from the Canadian to the Mexican border. Due to a high fecundity rate and relatively early maturation age, *O. virilis* is extremely competitive with other species with niches similar to its own. It is believed that they were first introduced to new areas in the 1960's through bait-bucket release, and since that time they have invaded 17 non-native states (see Fig. 3). Their current habitat includes all of New England.

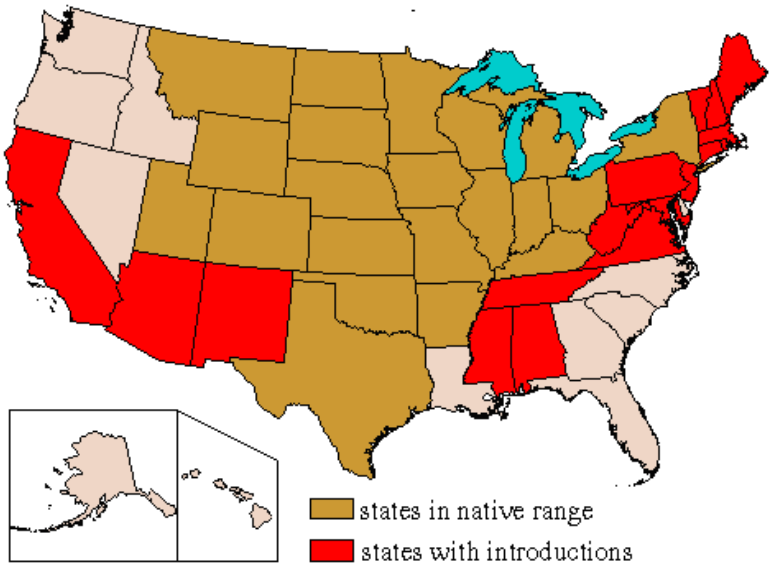

Figure 3: Habitat of O. virilis (Benson & Fuller, 2006)

It can be inferred from Figure 3 that *O. virilis* can thrive in a variety of different climates. However, there are some constants in the *O. virilis* habitat. These crayfish always live in bodies of freshwater, whether they be small streams or large rivers (Benson & Fuller, 2006). They thrive on any substrate, but do especially well in rocky substrates which allow for protective cover and easy burrowing. The burrows of *O. virilis* are about 3-4 inches deep, and are used as a hide-out during the daylight hours.

As the crayfish dig through the sediment, they often ingest a good deal of the substrate. This is the aspect of their lifestyle which links them directly to B[a]P contamination in the stream sediment. While there is little information which directly compares observed B[a]P levels in crayfish tissue to contamination of the sediment they live in, a precursor study to this project did find comparable levels of B[a]P in both *O. virilis* and stream sediment samples (Goscilla et al, 2007)

3. Sampling of crayfish

Two procedures were used for testing Blackstone stream crayfish for B[a]P content. First, gut analysis was performed to establish the entry of B[a]P into the food chain via sediment eaten by the crayfish. A 'traditional" approach to gut analysis was used for this study in that visual observations of the sediment under a dissecting scope were the only results gleaned from this procedure (McIntyre et al, 2006).

Second, tissue extractions were taken from crayfish and analyzed for B[a]P content. Commonly used tissues for such testing include hepatopancreas, antennal gland, muscle, and blood (Varanasi, 1989). Figure 4 depicts the general anatomy of a crayfish, in which several of these structures can be seen. Each type of tissue has its own advantages and reflects different processes, and it was ultimately decided that antennal gland and blood tissue would not be used as indicators, but the hepatopancreas and muscle tissue would be extracted.

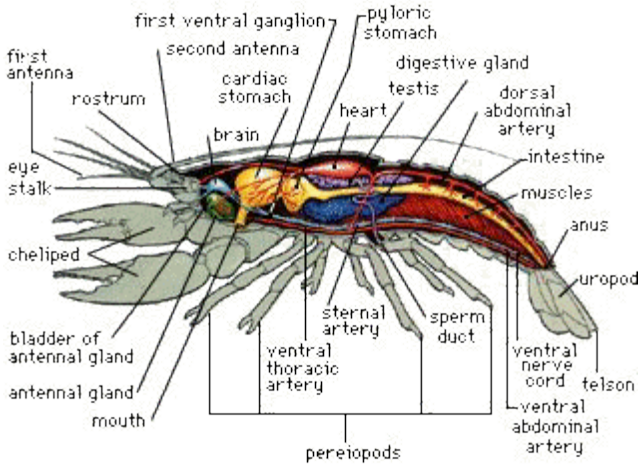

Figure 4: Anatomy of a crayfish (Fetzner, 2002)

The hepatopancreas is an organ unique to arthropods, some mollusks, and fish which, as its name implies, serves a dual function performed by the liver and pancreas in humans. The organ is located off of the gastrointestinal tract and is a storage organ for fatty acids,

as well as toxins filtered from food (Styrishave & Andersen, 2000). These two characteristics make the hepatopancreas a likely place for hydrophobic pollutants to bioaccumulate, and it has been used to reflect bioaccumulation in many studies. (Hendrickx et al, 2003) fed fruit flies contaminated with cadmium to wolf spiders for a period of time, then analyzed hepatopancreas cadmium content and excretion level of cadmium to determine bioconcentration via ingestion. High concentrations of cadmium were found in the spider hepatopancreas tissue, establishing the hepatopancreas as a part of cadmium's pathway for biomagnification. Another study investigated accumulation of B[a]P after long-term ingestion in crabs which, like *O. virilis*, belong to the phylum arthropoda. It was found that even after transfer to freshwater for 20 days, B[a]P was still present at fairly high concentration. A more controlled study of lobsters (crustaceans) observed the fate of a single periocardial dose of B[a]P. Researchers found that even 6.5 weeks later, over 80% of the original B[a]P and a slew of metabolites were present in the hepatopancreas. Very little excretion of B[a]P was observed during this time. (Varanasi, 1989)

The antennal gland (also called the green gland) is responsible for urine production, osmotic control, and ionic concentrations (Lin et al, 1989). It is often compared to the vertebrate kidney, but the tissue has many significant physiological differences from nephrons (Fuller et al, 1989). Another distinguishing factor is the antennal gland's role in detoxifying the blood and catabolism of toxins. Hydrophobic substances such as steroids and fatty acids are frequently detected in the antennal gland, and B[a]P has been detected in exposed individuals. A major difference between the hepatopancreas and antennal gland is that enzymes in the antennal gland will metabolize B[a]P at a quicker rate than that observed in the hepatopancreas (Harm, 2002). The antennal glad is also more indirectly connected to the gastro-intestinal tract, and could easily contain B[a]P which entered the blood through the respiratory system instead of the digestive (Ueno & Inoue, 2008). This fact, combined with the small size of the organ and difficulty level in extracting it, led to the decision not to use antennal gland tissue as an indicator of B[a]P contamination.

While the hepatopancreas provided conclusive evidence as to the bioaccumulation of B[a]P in crayfish, another tissue had to be selected as evidence of biomagnification. Because the hepatopancreas' function is to filter toxins out of food, because it makes up a very small proportion of the body, another tissue was needed to show that B[a]P is present in enough of the crayfish's body to be taken up by predators. Muscle tissue serves this purpose well, since it has no role in storing or metabolizing B[a]P and is the part of a crayfish's body which is most likely to be eaten by predators (especially humans). Previous studies have had success with using muscle as an indicator of B[a]P contamination through ingestion of contaminated food and water in crayfish. While B[a]P was not as concentrated in the muscle tissue as it was in the hepatopancreas, the study demonstrated that long-term exposure should result in correlating levels in the two types of tissues. (Gossiaux & Landrum, 2005).
Overall, sampling of crayfish gut and tissue will establish three important facts: 1) crayfish do eat sediment, and B[a]P contamination is therefore partially related to sediment contamination (contaminated water being the other source), 2) B[a]P ingested

with sediment bioaccumulates in the crayfish body, 3) biomagnification of B[a]P can occur whenever crayfish are preyed upon.

I F. Gas Chromatography to measure B[a]P levels

A gas chromatograph (GC) was used to isolate B[a]P present in extractions from sediment and living tissue samples. Gas-liquid chromatography uses the flow of an inert gas to carry a liquid sample through a chromatographic column which contains a liquid stationary phase. The liquid sample transitions from the liquid to gas phase as it passes through this column at extremely high temperatures. Once the liquid sample reaches the GC detector, the machine measures the amount of each chemical and an integrator provides a graph showing the time at which the detection level for the chemical level peaked, referred to as the retention time (RT), and provides the area under each peak. Chemicals can be recognized by their RT, and the area can be analyzed to determine the mass of the chemical injected into the GC. (Bobbit et al, 1968)

I G. Predictions and Goals

This study was designed to produce several pieces of information regarding sediment contamination. Confirmation that B[a]P is present in the Blackstone Valley stream due partly to highway runoff and that B[a]P and can be extracted from sediment at the test site would be gained, as well as insight as to how contamination levels vary across different points in the stream. It was expected that the transsection at the point source would have the highest levels of contamination, and that contamination of points downstream of the source would decrease with distance. The transsection upstream of the source was expected to have the lowest contamination of all. It was also predicted that test points in the middle of each transsection would be more contaminated than those closer to the stream since more water runs over the center of the streambed, and the banks of the stream bed might not have any water during especially dry times of the year.

The importance of crayfish as bioindicators was also reflected in the protocol. Gut analysis was performed to establish ingestion of sediment as a point of entry for B[a]P into living tissue, and potential for bioaccumulation in crayfish. It was predicted that bioaccumulation within the bodies of crayfish would be heaviest in the hepatopancreas because of its proximity to the original source as well as its role in detoxification and storage, and so the tissue was dissected and processed for B[a]P to show evidence of bioconcentration. Muscle tissue was also processed to determine whether or not B[a]P can enter the bloodstream and contaminate tissues outside of the digestive tract. Overall contamination was expected to be higher in larger crayfish, since they are presumably older and therefore have had longer exposure time.

The major goal of this study was to create and test a protocol for efficiently detecting and measuring deposition of B[a]P in stream sediment, and connecting it to bioaccumulation of B[a]P in crayfish.

II. Methods

II A. Sampling Site

All stream sediment samples and crayfish were collected from a stream in the northwestern region of the Blackstone River Watershed (Figure 5)

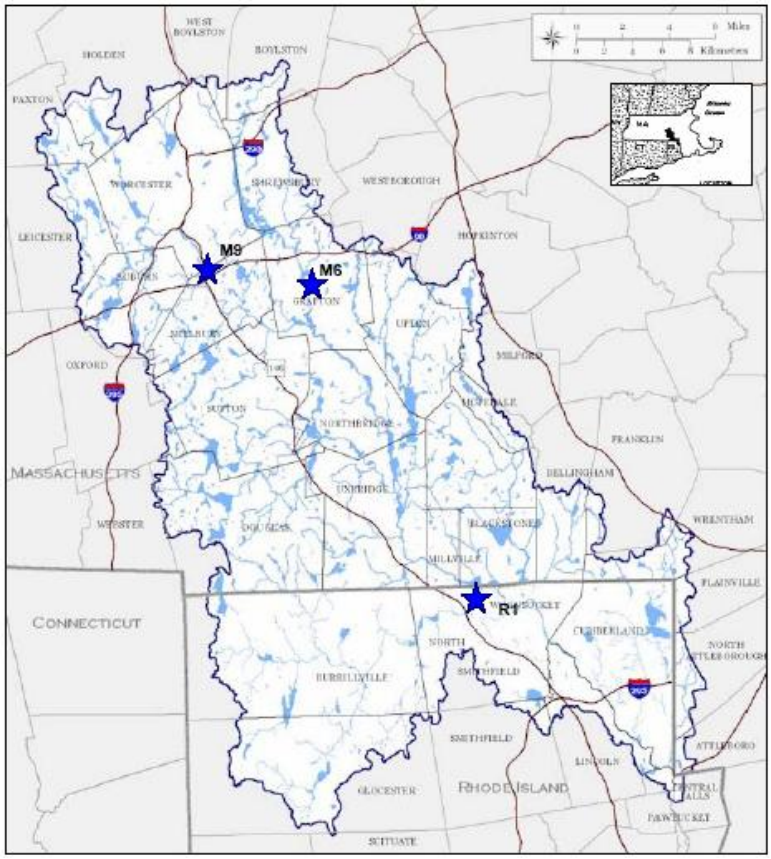

Figure 5: Blackstone River watershed with testing site indicated (M9) (Goscilla et al, 2007)

The section of the stream used for sampling is on the border of Worcester and Millbury, and was chosen for its high potential for B[a]P contamination. The site is beneath the rt. 146 bridge, and has a drain from the highway which pours road runoff into the stream. The site is also in close proximity to an 18-wheel truck gas station, and the Worcester & Providence Rail Road line (Figure 6). These factors, in addition to being located in a fairly industrialized area, make the site ideal for research on B[a]P contamination.

Figure 6: Aerial photograph of the testing site location (adapted from Google Maps)

A 50 meter section of the stream was used for testing (Figure 7). The runoff drain was identified as a point source of B[a]P and five cross-sections of the stream, referred to as transsections for the purposes of this study, were chosen based on relative proximity to the drain. One transsection was taken at the point source, one was taken upstream, and three were selected downstream of the point source (Figure 8). Transsections were tested in order to reveal any gradient of contamination across the site relative to the point source.

Samples were taken from four test points at each transsection. Two test points (1 and 4) were located one foot away from the two banks of the stream. The other two test points (2 and 3) were spaced evenly between the first two. Photographs of each transsection and the line of test points can be found in Appendix A. Test points were meant to reflect any trends in contamination gradient from one bank of a stream to the other.

Figure 7: Sampling site (taken from Transsection 1, looking upstream towards Transsection 5)

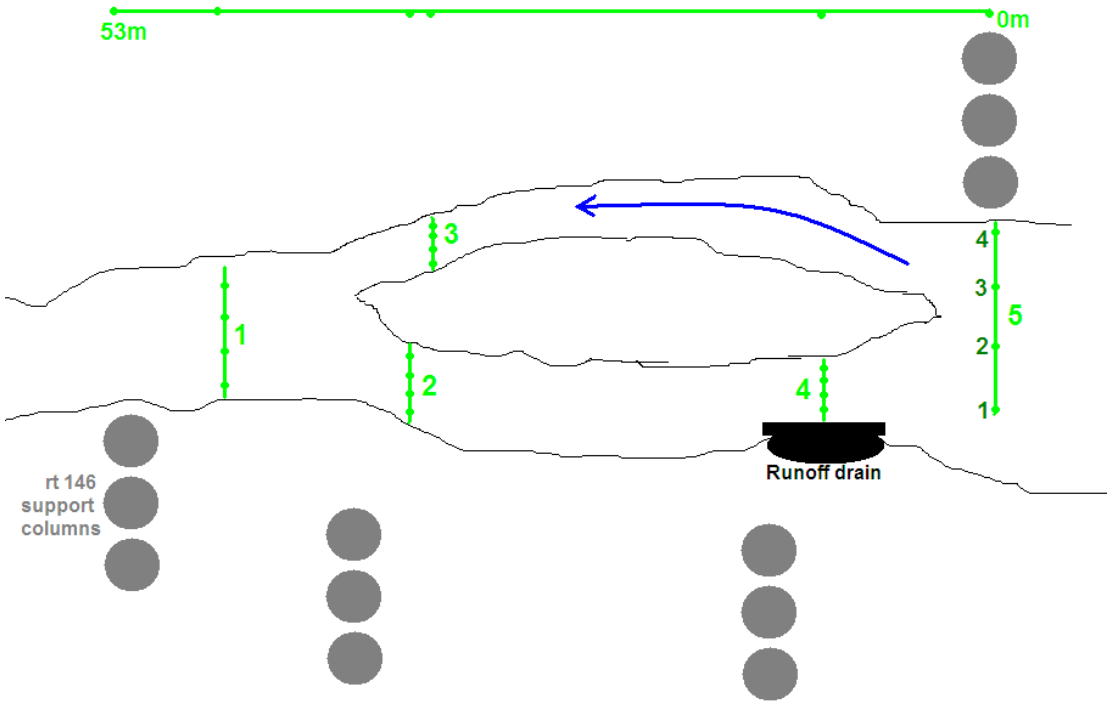

Figure 8: Detailed map of sampling site, with transsections and test points identified

II B. Sample collection and extractions.

A detailed protocol for collecting sediment crayfish, extracting B[a]P, and using gas chromatography to analyze B[a]P concentrations was developed, and is included in Appendix B.

Plastic and rubber instruments and containers were used as rarely as possible during the extraction process so as to prevent leeching of chemicals into the samples. All samples were either frozen or refrigerated when not being processed

Test points at the stream site were established through careful measuring and charting. All sediment samples and crayfish were immediately transported to the lab and frozen to prevent degradation and leeching, as well as to prevent uptake of new chemicals and excretion of B[a]P by crayfish.

Sediment samples were centrifuged and the supernatant was removed. These samples were then lyophilized. This ensured that no water-borne B[a]P was present in the samples, and consistent sediment weights could be obtained. Sediment samples were then shaken in petroleum ether for three hours so that the hydrophobic B[a]P could be separated from sediment particles and rock. The petroleum ether was then filtered through Fisherbrand quantitative (Q5) filter paper with medium porosity and medium flowrate. This removed large particles. The sediment sample was then re-shaken with fresh petroleum ether for five minutes, and filtered once again.

Crayfish were allowed to soak in ethanol for at least one day before dissection so that all samples had similar exposure to the chemical. Hepatopancreas and tail muscle tissue were dissected from each individual. Tissue samples were ground to powder using anhydrous sodium sulfate. A filtration column was made using this powder, and hexane was run through the column to extract B[a]P.

Liquid chromatography was then performed by running B[a]P extractions from sediment and tissue samples through a column of silica gel suspended in hexane. The stationary phase in this column was the silica gel. The samples were then dried under nitrogen gas using an evaporation unit made from rubber tubing, glass Pasteur pipettes, and Penn-Plax 5 Gang Valves. The dried containers were rinsed with hexane, then liquid was transferred to a smaller vial and dried once more.

All dried samples were re-suspended in hexane, and gas chromatography was used to separate out and analyze B[a]P. A Perkin-Elmer Sigma 3 Gas Chromatography (GC) machine with a flame ionization detector was used.

The column used was a Supelco SPB-5^{TM} wide bore glass capillary column with the following characteristics:

$$\text{ID} = 0.53\text{mm}, \ \text{Length} = 30\text{m}, \ d_F = 0.50\mu\text{m}, \ \ \text{Beta value} = 265.0$$

The parameters entered in to the GC were as follows:

Oven Temp = 100°C
Inj Temp = 300°C
Det Temp = 300°C

Time 1 = 1 min
Rate = 8°C/min
Time 2 = 15 min

It was found that samples which were relatively pure (low levels of additional contaminants) could be run for a shorter amount of time by decreasing "Time 2" as low as 7 min.

A Hewlett Packard 3395 Integrator was used to analyze the GC results. A "PAH1" method was created with the following parameters:

Run Parameters
Zero = 0
Att2^ = 10
CHT SP = 0.5
AR REJ = 0
THRS = 4
PK WD = 0.04

Timetable events:
0.000 ZERO = 0
0.0000 INTG = 8
6.000 ATT 2^ = 7
20.000 PK WD = 0.20
35.000 STOP

II C. Controls

Three samples were spiked in order to determine the efficiency of the protocol in extracting B[a]P from three different types of sediment. The "Rocky" sample was comprised mostly of gravel (test point 1.1), the "Medium" sample was a mix of gravel and more fine-grain sediment (test point 1.2), and the "Fine-grain" sample was entirely silt (test point 2.1). Ten grams of each type of sediment were measured as per the normal protocol. The samples were then spiked with 40µl of B[a]P diluted in hexane with a concentration of 200ng B[a]P/µl, and shaken at 300 rpm at 30°C for thirty minutes. The sample was then extracted and processed as normal alongside the regular, "non-spiked" samples from the test points.

II D. Analyzing the Data

Integration plots from the GC were used to quantify B[a]P contamination of samples. A series of samples were run through the GC so that B[a]P could be identified on the integration plots and its concentration quantified. The retention times (RTs) of 18 samples of B[a]P at various concentrations were used to ascertain an appropriate range of RT for B[a]P in samples. The average and standard deviation for this group were determined. The standard deviation was doubled, then added to the highest RT obtains and subtracted from the lowest. The resulting values set the RT range to 29.2506 – 30.0103. This RT range helped to identify B[a]P on integration plots. When B[a]P could not be identified with confidence using the RT range, the peaks in the sample integration to known integrations of B[a]P in the standards were compared.

A sample curve was created to determine the concentration of B[a]P in samples. Three dilutions of B[a]P in hexane were used to make the curve: 200 ng B[a]P/µl, 100 ng B[a]P/µl, and 50 ng B[a]P/µl. At least two runs of each concentration were plotted and the "trendline" application in Microsoft Excel was used to determine the equation of the best fit line. The resulting equation was $y = 3001.5x - 2863$, with an R^2 value of 0.96. The concentration of B[a]P in the sample injected was determined using Equation 1.

Equation 1:Concentration of B[a]P in sample injected

$$(\text{Area} + 2863)/3001.5 = \text{Conc (ng/µl)}$$

The concentration of B[a]P in the sediment sample was determined by using the Equation 2.

Equation 2: Concentration of B[a]P in sediment

$$\frac{\text{Conc (ng)}}{1\ \mu\text{l}} \times \frac{40\ \mu\text{l}}{10\ \text{g sediment}} = \text{ng B[a]P/g sediment}$$

A sample calculation using the average area of test point 5.1 is provided below.

Area = 780065.5

(780065.5 + 2863)/3001.5 = 261 ng/µl

$$\frac{260.85\ (\text{ng})}{1\ \mu\text{l}} \times \frac{40\ \mu\text{l}}{10\ \text{g sediment}} = 1040\ \text{ng/g sediment}$$

The percent yield of the control tests was determined through the steps shown in Equation 3. First, the total mass of B[a]P in the spiked samples determined (note: concentration can be calculated using Equation 1). The total mass of B[a]P found in the non-spiked sample was subtracted from this value to determine the total mass of B[a]P which was added in the lab. This number was then divided by the total 8,000 ng originally added to determine the percent yield. These steps are shown in Equation 3.

Equation 3:Percent yield

$$\text{Conc (ng/µl)} \times 40\ \mu\text{l} = \text{Total B[a]P (ng)}$$

$$\frac{\text{Total B[a]P (spiked)} - \text{Ave total B[a]P (normal)}}{8000\ \text{ng}} = \%\ \text{yield}$$

A sample calculation using the Medium spiked sample is shown below.

$$566.04498\text{ng/µl} \times 40\mu\text{l} = 22641.7991\text{ng}$$

$$\frac{22641.7991\text{ng} - 14033.3433}{8000\ \text{ng}} = 107\%\ \text{yield}$$

Concentration of B[a]P in tissue samples was also determined using integration plots. The concentration of B[a]P in the injected sample was determined using Equation 1, then the steps in Equation 4 were used to determine the concentration of B[a]P in the crayfish tissue.

Equation 4: Concentration of B[a]P in tissue

$$\frac{\text{Conc in sample (ng/µl)} \times 40\ \mu\text{l}}{\text{Mass tissue (ng)}} \times \frac{1000\ \text{ng}}{1\ \text{g}} = \text{Conc. in tissue (ng/g tissue)}$$

A sample calculation using integration information for muscle tissue dissection from *O. virilis* specimen 1 is shown below (concentration of B[a]P in the sample was determined using Equation 1).

$$\frac{992.5754\text{ng/µl} \times 40\ \mu\text{l}}{60\text{ng}} \times \frac{1000\ \text{ng}}{1\ \text{g}} = 662000\ \text{ng B[a]P/g tissue}$$

III. Results

GC results and calculations of all samples can be found in Appendix C.

III A. Control tests

Three types of sediment were spiked with B[a]P and processed alongside normal samples to determine the extraction efficiency of the protocol. Analysis of GC integrations (see Table 1, Appendix C) for spiked samples were compared to both the average extractions of other samples from their respective test points, as well as the sample which showed the highest concentration. Both types of analysis showed that rocky sediment had the highest yield of B[a]P, with a 107.61% yield as compared to the average for test point 1.1, and 45.84% as compared to sample 1.1(2). When compared to the average mass of B[a]P extracted from all samples from their respective test points, fine-grain showed the lowest yield, at -36.8%, while the medium sediment had a yield of 30.13%. When comparing yields to the maximum mass extracted from samples at each test point the fine-grain had the greatest yield at 93.55%, followed by the rocky substrate at 45.84%, then the medium with 13.35% yield. It should be noted that the spiked fine-grain sample was not processed simultaneously with all other samples from its test point. The first two non-spiked samples were processed together, then the spiked and a third normal sample were processed about a month later.

Table 1: % yield for spiked samples

Control	Compared to:	% Yield
Rocky	1.1 ave	107.61
	1.1(2)*	45.84
Medium	1.2 ave	30.13
	1.2(1)*	13.35
Fine-grain	2.1 ave†	-36.80
	2.1(3) ‡	93.55

** non-spiked sample with highest yield for test point*
† average for two original samples and one processed later with spiked sample
‡ processed with spiked sample

III B. Sediment samples

Five transsections were selected at the stream site, and stream sediment was collected from four test points at each transsection. Duplicate samples from each test point were processed in the lab and analyzed (Table 2, Appendix C). Analysis showed that transsection 4 (at the point source) had the highest concentration, followed by transsection 3 (directly downstream), and transsection 2 (indirectly downstream) (Figure 9). Transsection 1 had the least contamination of all sections downstream of the point source. Transsection 5, which was upstream of the runoff drain, had the least amount of contamination.

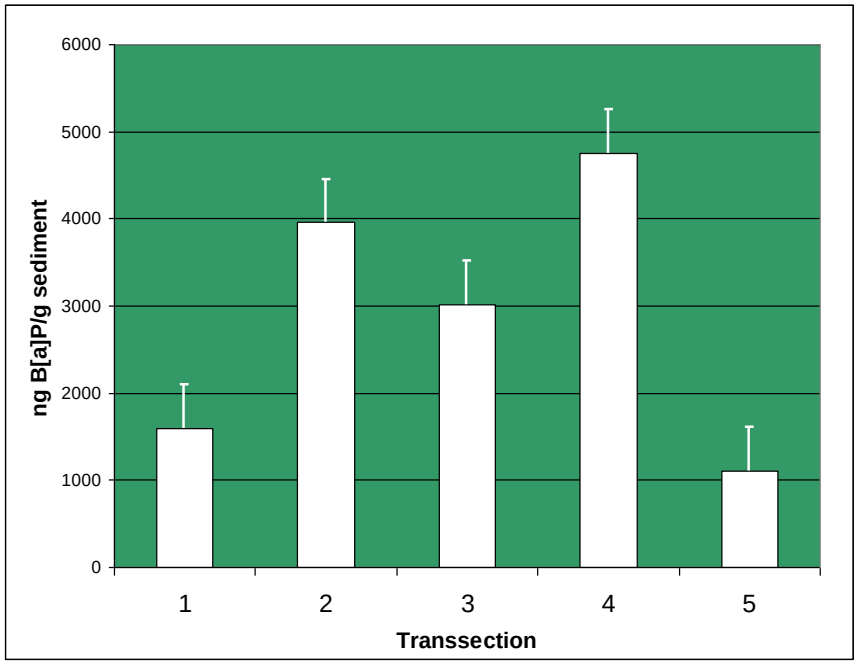

Figure 9: Average B[a]P concentration (ng/g sediment) of all test points from each transsection

Data was also analyzed to identify trends in concentration gradients across transsections (Figure 10). The only transsections were found to have similar contamination patterns were 1 and 5. These transsections had both the lowest concentrations of B[a]P and the most even contamination levels across the test points. Transsection 4 showed much higher B[a]P concentrations towards the near bank, which is directly in front of the runoff drain. Transsections 2 and 3 both had the highest concentrations on the banks which ran along the "island" in the middle of the stream (the far bank of 2 and the near bank of 3).

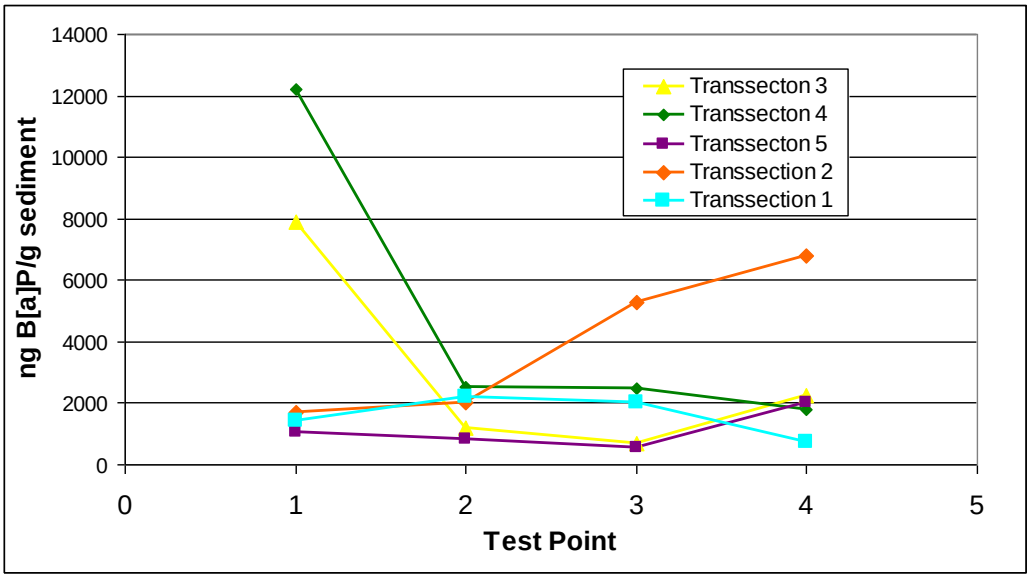

Figure 10: B[a]P concentrations (ng/g sediment) at all test points within each transsection

III C. Crayfish

Gut analysis was performed on three crayfish to determine whether or not *O. virilis* at the stream site ingest B[a]P through sediment. Qualitative comparisons of sediment and gut content under magnification (Figure 11) showed that sediment-like particles were present in the stomachs of the crayfish.

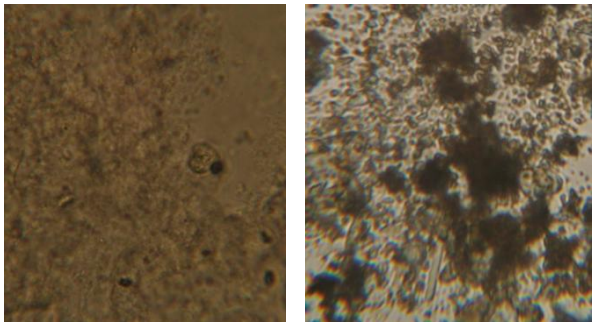

Figure 11: Contents of crayfish gut (left) compared to stream sediment (right)

Samples of tail muscle tissue and hepatopancreas tissue were dissected from four crayfish which were caught at the stream site. Analysis of the samples (Tables 3-4, Appendix C) indicate that B[a]P was more concentrated in the muscle tissue of the crayfish than in the hepatopancreas (Table 2). Qualitative observations of the relative sizes of the crayfish correlate with the results, as the two largest crayfish (1 and 3) had higher concentrations of B[a]P than 2, which was smaller.

Table 2: B[a]P concentrations in hepatopancreas (HP) and muscle tissue of O.virilis

O.virilis	**B[a]P (ng/g tissue)**	
	HP	**Muscle**
1	0	661716.9
2	N/A	548110.8
3	9204.731	77291.13
4	0	0

IV. Discussion

IV A. Controls

Processing of three types of sediment (rocky, medium, and fine-grain) using the prescribed protocol showed that the protocol was successful in extracting and detecting B[a]P from stream sediment. Contrary to expectations, the protocol was most efficient at extracting B[a]P from rocky sediment, with 45-107% recovery. The medium substrate showed a range 0f 13-30%, and the fine grain had the range with the lowest bound at -36-94%. This is due to the fact that B[a]P is hydrophobic and not very volatile – factors which cause it to associate with stream sediment rather than the water. In the stream environment this could lead to much higher deposition of B[a]P in fine-grain sediment than rocky sediment, which would explain the difference between yield in rocky, medium and fine-grain sediment reflected in the results of this study. However, this strength of association actually leads to lower extraction rates in spiked samples. This is because the B[a]P is so strongly attached to the sediment, the extraction process cannot fully separate it from porous, fine-grained samples as it can from hard, rocky surfaces. Other studies have suggested that fine-grain sediment will always retain a certain portion of B[a]P, thereby preventing any extraction process from ever providing 100% yield (Jonker & Koelmans, 2002). Another group of researchers found that B[a]P extraction efficiency increases as sediment grain sizes increases up to 300μm, and that even grains as large as 500μm will give higher yields than sediment with grains that are 65μm or finer (Letellier & Budzinski, 1999).

IV B. Sediment Samples

Comparisons of average contamination levels for five transsections at varying distances from the runoff point source showed that sediment directly upstream of the source had lower contamination than any point at or downstream of the source (1100 ng/g sediment). The transsection at the point source had the highest levels of B[a]P at 4750 ng/g sediment. The first transsection directly downstream of the point source had the second highest concentration (3960 ng/g sediment), followed by a transsection indirectly downstream of the source (3010 ng/g sediment). The transsection furthest downstream of the point source had the lowest concentration of any transsections downstream of the source, at 1600 ng/g sediment. These results confirm the hypothesis that contamination at or beyond a point source will be higher than any point upstream, and that concentration decreases as the water travels further from the point source.

Contrary to the hypothesis that concentration levels would be highest at test points in the center of the streambed, comparisons of average contamination of test point samples within each transsection provide no evidence of any trend in concentration across streambeds. Gradients across transsections can be explained somewhat by observing water flow across the site. Transsection 5 shows relatively stable concentrations across the stream, reflecting the uniform pattern of water flow over the sediment. The high concentration of test point 1 of transsection 4 (4.1) is undoubtedly due to its location directly under the runoff drain. The sharp decrease in concentration in points 4.2-4 to a

point only slightly higher than those found in 5.1 and 5.2, which flow towards transsection 4, suggests that little B[a]P from the drain reaches the far bank at that transsection. This is a reasonable conclusion as the water at this point flows "vertically" downstream from the test point, not "horizontally" across the stream. Any horizontal movement of B[a]P is most likely due to irregular conditions, for example, high water levels after periods of rainfall. Higher concentration towards the far bank of transsection 2 rather than the near bank (as with the transsection 4, which is directly upstream) may be due to eddies and small rapids between transsections 4 and 2 which could be redirecting water flow. The high concentration at test point 3.1 could also be reflective of water flow patterns in which water flowing directly from transsection 4 never reaches further than one meter into transsection 3. Not surprisingly, all other points in transsection 3 are almost identical to those in transsection 5, which is directly upstream of 3. The similarity in B[a]P levels at transsections 1 and 5 show that the majority of B[a]P which enters the stream through the runoff drain is deposited into sediment in the distance between transsections 4 and 1. This reflects a fairly quick deposition rate of B[a]P, especially considering the speed at which the water flows over the streambed. It is estimated that in more slow-flowing streams this distance would be even shorter, since B[a]P would have more time to deposit in the bed of the stream in a shorter distance.

The distribution gradient across transsection 1 is the only one found to follow the hypothesized gradient, with higher concentrations found in the center of the stream. Uneven distribution patterns across the transsections could be related to the varied flow patterns at different sections of the test site. Water flow is fairly even and uninterrupted for at least 50 m downstream of the test site, and it is possible that horizontal distribution of B[a]P in that portion of the stream would be similar to that observed in transsection 1.

Several suggestions for future testing methods of stream sediment can be made based on the findings in this study. It is suggested that studies designed to track B[a]P contamination in relation to point sources such as highway runoff drains collect samples from test points within 50 m of the source, as levels were found to return to pre-point source levels within that distance (see Figure 9 in the Methods section of this paper for distances between point sources). Research on more widespread contamination of stream sediment due to a variety of local sources of pollution should collect samples from points which are further than 50 m from point sources. A test of the time it takes for water to travel to the test point from a direct point source is also suggested, with points being established at least 50 seconds in flow time from the point source. This number is derived from the fact that objects floating in the water at transsection 4 require about 45 seconds to reach transsection one, at which time most of the runoff-induced B[a]P has been deposited. Because the water between these points is moving fairly quickly, this flow-time provides a legitimate guideline for appropriate deposition time. Conformance to these two requirements will ensure that point sources do not skew sediment contamination and cause abnormally high B[a]P concentrations, since B[a]P originating from point sources will have had sufficient time to be deposited. B[a]P levels from such test points will be reflective of the stream in general.

The number of test points necessary for accurate representation of horizontal distribution patterns appears to depend on the geography of the stream and resultant flow patterns. Distribution at the transsections in this study suggests that highly varied geography and flow in a stream bed leads to inconsistent horizontal distribution patterns while more even flow of water leads to more uniform concentrations across transsections, and may result in higher deposition at the center of stream beds. However, more research on distribution patterns in areas of even flow is required before such conclusions can be drawn.

IV C. Crayfish

Gut analysis of *O.virilis* caught at the testing site confirm that ingestion of sediment is a possible pathway for B[a]P entry into crayfish tissue. Analysis of B[a]P concentrations in hepatopancreas tissue did not reinforce crayfish as bioindicators for B[a]P, as only one of three crayfish tested positive for B[a]P. This could be due to inaccurate dissection of tissue, as the hepatopancreas is a fairly small organ which is embedded in the thoracic cavity. Because B[a]P was presumably present in the sediment found in the digestive systems of the crayfish but was only detected in the hepatopancreas tissue of one individual, the results of this experiment do not provide conclusive evidence that B[a]P which is bioconcentrated in crayfish at the test site enters the body through contaminated sediment. Neither does this information disprove introduction of B[a]P through the digestive system. It is also possible that the hepatopancreas tissue of the crayfish tested was not efficiently filtering out the B[a]P, allowing B[a]P to enter the intestine and diffuse into the bloodstream from whence it would eventually be deposited into cells (e.g. muscle tissue). This hypothesis is supported by the gut analysis and muscle contamination levels produced by this study.

Muscle tissue analysis was a much stronger indication of crayfishes' potential as bioindicators for B[a]P, as three of four crayfish tested positive for B[a]P with levels in the individual with the greatest contamination (OV1) found to be 167% the levels found in transsection 4. This data provides strong support for the use of crayfish as indicators of contamination in a stream environment. The effect of size, and presumably age, was also observed in the individuals, as the two larger crayfish (OV1 and 3) showed higher levels of muscle contamination than OV2, which was smaller.

While the results of this study do confirm the validity of crayfish as bioindicators, the small amount of data regarding B[a]P concentrations in crayfish tissues limits the inferences which can be made regarding the pathway of bioaccumulation and possibility of biomagnification. Thus, few suggestions can be made about ways to use this information to learn about B[a]P pollution. More research must be done on means and levels of bioaccumulation in *O.virilis* at the site.

IV D. Sources of error

There were several opportunities for error which were inherent in the experimental design of this study. First, small sampling sizes limited the number of inferences derived from

the results, and inconsistency within results for test points and transsections affected the confidence with which those inferences could be made. While some strong conclusions were made, larger sampling sizes would have been beneficial, especially when investigating concentrations in crayfish tissues. Further doubt was cast on the validity of hepatopancreas studies because of difficulty in isolating the tissue during dissections (no doubt is present on the identity of the tail muscle tissue, which was easy to locate).

Additional error could have been derived from changes made to the protocol partway through the research process (see Appendix C). First, samples were temporally separated. Sediment samples were collected over the course of six months (November, 2007 – April, 2008). Sediments were collected in reverse numerical order, with all test points for each transsection sampled on the same day. Studies have shown that PAHs are present at higher concentrations in cold, winter temperatures (Valerio & Pala, 1991), which is a possible cause for skewing of results. However, the fact that concentration actually decreased in transsections 1-4 despite their separation by cold, winter months means that while temporal separation could skew results somewhat, valid conclusions could still be drawn with confidence.

The changes to the final extraction methods listed in Appendix C were a cause for additional error. However, the fact that most of the changes which presumably led to more efficient extraction were made after the processing of test point 4.1, which yielded more B[a]P than any other test point, indicates that error due to protocol changes was not enough to irreparably skew results.

IV E. Future work

Because any environmental phenomenon can have countless unknown contributing factors, a great amount of additional research can always be suggested. In the case of this study, several different approaches can be taken as a "next step" in researching B[a]P contamination of streams and use of crayfish as bioindicators of sediment contamination, as well as that of the ecosystem at large.

Future research on sediment contamination levels could include the sampling of test points across transsections in evenly flowing water (as opposed to the "rapids" of the site tested in this study), monitoring of contamination in stream site tested in this study over the course of several years, and the application of the protocol developed in this study to other stream sites. Research on the effects of water flow patterns across areas of varied geography could also provide valuable insight into contamination levels of stream sediment. Additional development of the protocol could be focused on creating specific protocols for different types of sediment, and more "natural" controls could be created by exposing the controls to water-born B[a]P and allowing longer exposure time before processing. Additional extraction methods could also be explored. Other studies have employed a wide range of extraction techniques which includes microwave pressurization, sonification, and pressurized hot water extractions (Bangkedphol et al, 2006) (Kronholm et al, 2004).

Much research must also be done on crayfish contamination before they can be employed as bioindicators. Sample sizes should be greatly increased, with factors such as size (which presumably reflects age) controlled. Crayfish could also be tracked to reveal any error due to migration rates. Also, tissues reflective of B[a]P contamination through sources besides ingestion of B[a]P by crayfish should be studies. The gills of crayfish could be analyzed to reflect contamination through water, or algae which might be eaten by crayfish could be analyzed for bioaccumulation of B[a]P.

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Appendix A

Photographs of each transsection are provided below as a supplement to Figure 9 in the Methods section of this paper so that each test point can be located in future studies. The "near bank" in these photographs is the bank on the side of the walking path.

A B

Figure 12: a) Transsection 1 (view from near bank to far bank), b) view from far bank to near bank. Note that straight line is from tree to the column closest to the water

Figure 13: Transsection 2 (view from far bank to near bank). Note that test points are all in line with three columns

Figure 14: Transsection 3 (view from near bank to far bank)

Figure 15: Transsections 2 and 3 (view from far bank of 3 to near bank of 2). Note that all test points for both transsections are in line with the three columns

Figure 16: View of Transsection 4 from a) far bank to near bank, and b) near bank to far bank. Note that the test points are in a straight line from the runoff drain to the left-most (when viewing from the near bank) column across the stream

A

B

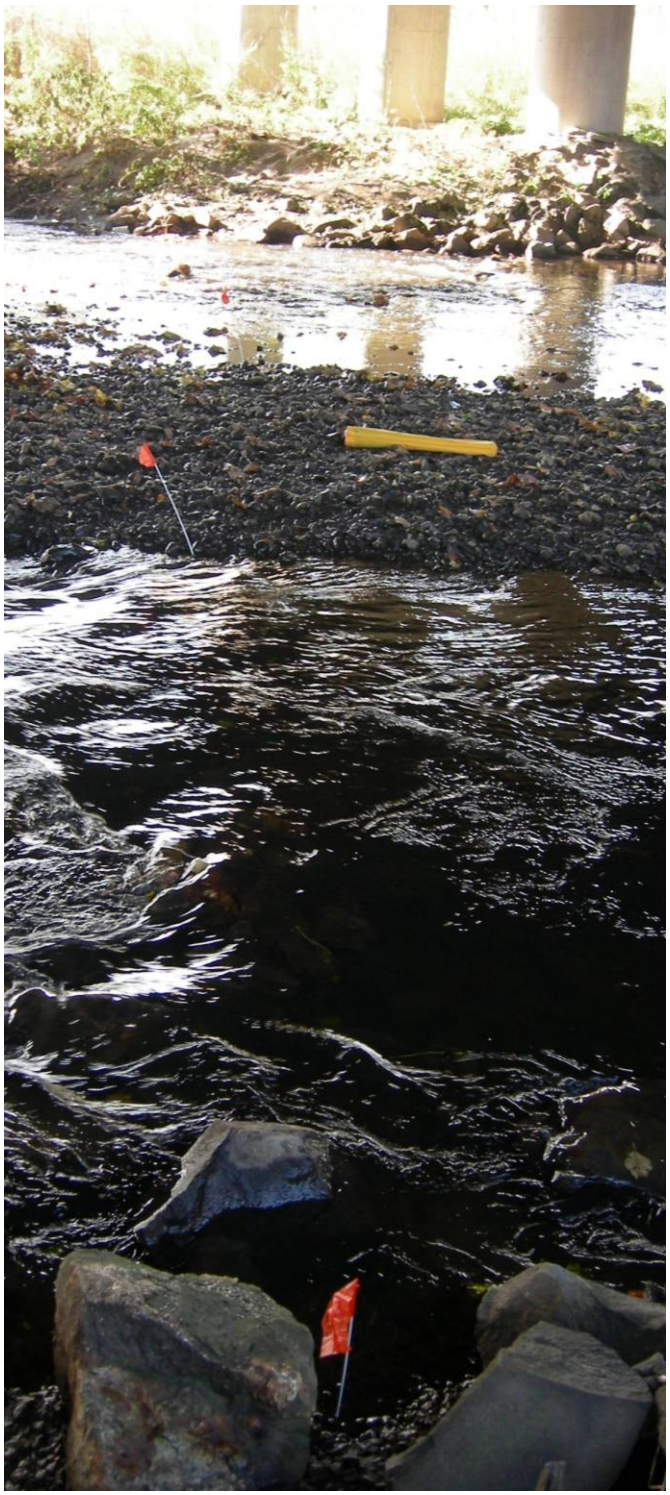

Figure 17: Transsection 5 (view from near bank to far bank).
Note that all test points are in line with the three column.

Appendix B

The following provides detailed instructions for the protocol listed in Methods sections IIB-E of this paper.

1. Obtaining Samples

1.1 Stream Sediment

Selecting Test Points:

Find five suitable sections in the stream, making sure that the water level and current are safe for wading. Make sure that there is a landmark to identify the section of the stream. For each cross-section of the stream, identify four points from which samples will be collected. To do this, measure the width of the stream. Collect samples from a foot into the stream on each side, and then from two more evenly spaced points in the stream.

---1 ft---**Pt 1**-------3 ft-------**Pt 2**-------3 ft-------**Pt 3**-------3 ft-------**Pt 4**---1 ft---

Collecting Samples:

A T-corer should be used for points with ample sediment. To use the corer, insert the shaft into sediment so that the tip reaches 3-4 inches into the soil. Rotate the corer and pull it out. If the point is too rocky for T-corer collection, use a metal spade to dig down to a point where the T-corer can be filled.

Place the samples in a clean 250 mL centrifuge tube, and cap immediately. In no more that 3 hours, bring the samples to the lab and store at -21°C

1.2 Crayfish

Crayfish may be caught at any point within the bounds of the sampling area. Nets are recommended, but any means may be used. Crayfish should be transported back to the lab live in a bucket of stream water. Upon arrival at the lab, remove the water from the bucket and euthanize the crayfish by freezing them at -21°C until they are no longer moving. Preserve crayfish by storing them in 70% ethanol.

2. Extracting B[a]P from Stream Sediment Samples

2.1 Initial Processing of Samples:

If the stream samples are frozen, thaw them in a water bath at 37°C. Centrifuge the samples in their collection tubes at 4200 rpm for 15 minutes at 21°C. If the samples are not adequately separated, centrifuge them again. The contents of the tubes should then be recorded, including descriptions of the different types of material in the tube and the approximate volume of each layer.

Next, draw off as much supernatant as possible without taking any sediment. Some liquid may be left in the tube if it is full of particles (if the sample is especially fine-grain, any remaining liquid will actually make shell-freezing the sample easier).

2.2 Lyophalizing Samples:

The stream samples should be shell frozen before lyophilizing. Shake the contents of the tube beforehand so that they are evenly mixed. Hold the tube at a sharp angle and spin it while freezing so that a thin layer of the sample covers the inside walls of the tube and as little of the sample as possible is left at the bottom of the tube. Liquid nitrogen works well for this procedure (note: protective gloves should be worn when working with liquid nitrogen).

After the sample is frozen, remove the cap of the centrifuge tube and warm the neck of the tube. Securely cover the top of the bottle with Parafilm, and poke a few small holes in the plastic. Place all samples in the freezer until they are about to be lyophilized.

Stream samples should be lyophilized overnight. This process will produce a sample of completely dried stream sediment and debris.

2.3 Petroleum (pet) Ether Extraction

Shake the dried stream sample to create a uniform mixture. Measure 10g of each mixed dry stream sample and place it in a clean 100 mL glass bottle. All glassware for this procedure should be thoroughly cleaned with detergent in a dishwasher, and should be free of any "water spots." All glass bottles should have plastic caps with Teflon liners intact.

Using a glass pipette and pipetaid, add 10 mL of pet ether to each sample (fine-grain samples may need more than 10mL to become saturated). All work using pet ether should be done under a fume hood, and goggles and gloves should be worn. Shake the stream samples at 300 rpm at 30°C for at least 3 hours. Once this is done, place the bottles under the hood, allowing time for the contents to settle.

Set a filtration apparatus up by placing a folded sheet of filter paper in a glass funnel, which should in turn be placed in a clean 100mL glass bottle. Tape the paper funnel to the glass funnel. All funnels should be washed with detergent and hot water prior to filtering. Follow washing with tap and distilled water rinses, and allow them to drain dry.

Using a glass pipette, moisten the filter paper with enough pet ether to completely saturate the paper and leave some unabsorbed hexane remaining at the base of the filter paper funnel. Use the glass pipette to remove the pet ether from the beaker and filter it into the new glass bottle. Pet ether evaporates quickly, so beakers of extract should be capped when not in use. Each stream sample should have its own filtration apparatus.

Using a glass pipette, add another 10 mL pet ether to the sample (once again, more can be used when needed), and re-shake at 300 rpm at 30°C for 5 min. Once again, use a glass pipette to extract the top layer of ether and filter it into the glass bottle. Once this is done, rinse the filter paper funnel with 2-3 mL of pet ether and allow this to filter into the glass bottle. Store the extract at 4°C until needed.

2.4 Silica Gel Column

Setting up the column:

A fresh column should be prepared for each new sample to be filtered.

Make a slurry composed of 1 part silica gel:3 parts hexane (note: goggles and gloves should be worn when working with hexane). Place a small amount of glass wool into the neck of a glass Pasteur pipette (remove any cotton swab which may already be in the tube first). Measure 4.5 cm up from the top of the glass wool and mark the length with a permanent marker. Attach the pipette to a ring stand, and place a glass bottle labeled "waste hexane" under the pipette.

Thoroughly mix the silica gel slurry. Using a glass pipette and pipetaid, quickly remove some of the slurry and add it slowly and evenly to the pipette until it reaches the 4.5 cm mark. Once all of the hexane has dripped out, remove the bottle of waste hexane and place a 20 mL collecting vial under the column.

Filtering samples:

Using a glass pipette, slowly apply the pet ether extract to the column. Once the entire sample has been drawn from the 100 mL glass bottle, add 5mL hexane to the bottle, cap, and shake lightly. Apply this hexane to the column, and allow the entire sample to drip from the column. This final extract should be colorless and contain no particles. It should be stored at 4°C until needed.

2.5 Drying Samples

Drying apparatus:

To create the drying apparatus (see Fig. 6), attach pieces (length does not matter so long as all pieces on each aeration unit are equal) of rubber tubing to the outlets of an aquarium aeration system. Each vent in the system must have a valve by which it can be turned on and off. Melt and stretch a Pasteur pipette just beyond the point where the tube broadens after the neck. Break the stretched portion of the pipette and insert it into the end of each tube (the neck should be in the tubing). Attach the aeration units to a ring stand, and connect them to each other with rubber tubing. The entire unit should then be connected to a tank of nitrogen gas.

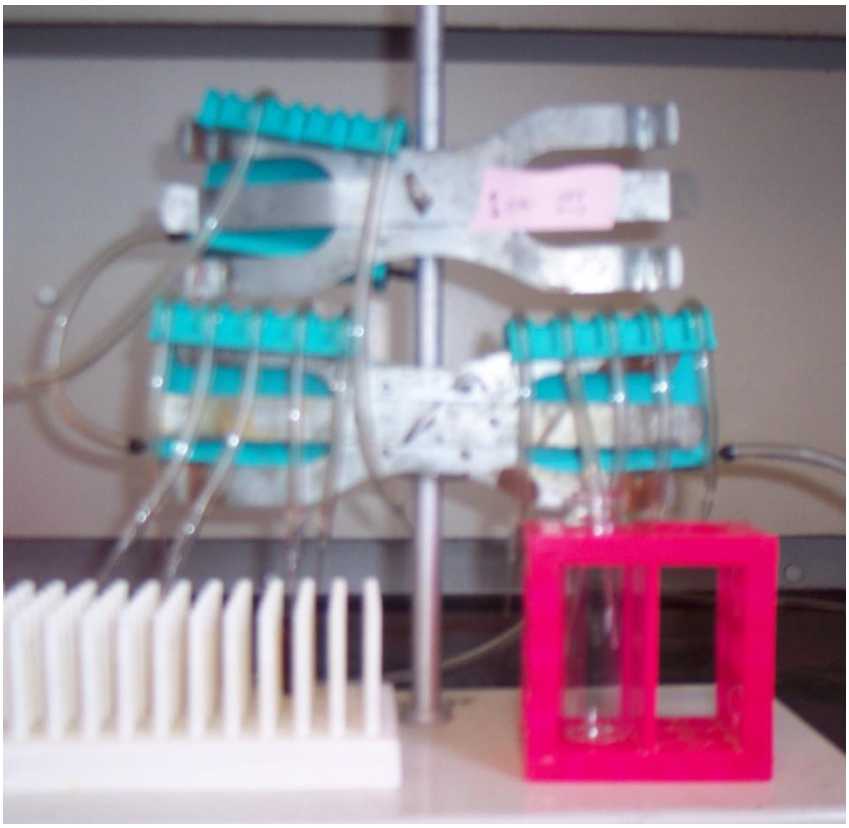

Figure 1: Aeration unit (made with Penn-Plax 5 Gang Valve units)

Drying samples:

The pipette ends should be cleaned with ethanol and Kim-wipes before and after each use. Placed vials in a test tube rack to hold them still when being dried. When turning the gas on no tubes should be inserted into the vials, and all vent valves should be open. Once the gas is on, tubes should be carefully inserted into the vials, and individual valves can be closed until air is coming out of the tubes at a desired rate. This will prevent overflow of air from splashing liquid from the vials.

Dry the 20mL collecting vial containing the extract from the silica gel column to completion with nitrogen gas.

Clean two syringes (250-500µl) with hexane. Load one syringe with 250 µl of hexane and use it to rinse the dried 20mL collecting vial, allowing the hexane to run down the sides of the vial. Cap the vial and swirl the contents, then use the second syringe to draw up the contents. Deposit the hexane into a 2mL amber vial with a septum cap. Repeat the rinsing process at least 5 more times. Once the 20mL vial has been rinsed, completely dry the 2mL amber vial under the nitrogen gas.

Any extractions may be stored at 21°C if necessary.

3. Extracting B[a]P from Crayfish Tissue

3.1 Dissecting tissue

Dissect out the hepatopancreas tissue of a crayfish, and the tail muscle. Be sure that the intestine and nerve cord are not extracted out with the tail muscle. Extracted tissues should be stored in 70% ethanol in Eppendorf tubes and frozen when not being used for extractions.

3.2 Tissue Extraction

Glass column:

Remove the cotton plug from a 5" Pasteur pipette and pack enough glass wool to block the neck. Connect the pipette to a small funnel (washed and rinsed with hexane first) with rubber tubing. As little tubing as possible should be used. Attach the column to a ring stand with the tip in a 20-40mL glass collection vial.

Extracting tissue:

Place 1.0g of sodium sulfate (anhydrous) and 30-60g tissue in a glass mortar (wash and rinse with hexane first), and grind them together until a free-flowing powder is formed. Pour the powder into the funnel, and tap it until the bulk of the powder falls into the pipette. Rinse the mortar and pestle with 2mL of hexane and transfer this to the funnel. Repeat this rinse 4 more times.

3.3 Final Processing

Run the elute from the sodium sulfate column through a silica gel column, dry it with nitrogen gas, then rinse the container with hexane and dry the liquid once again under nitrogen gas (as described in Methods sections 2.4-2.5)

4. Gas Chromatography

Use a 10µl Gas-Tight syringe to load the machine. The syringe should be cleaned with hexane (draw the hexane up to full volume and eject at least three times) before use. Add 40µl of hexane to the amber collection vials, piercing through the septum cap to do so (never opening the cap). Swirl and finger vortex the vial, then draw it up and eject it out of the syringe several times to further mix the sample. Load the syringe with 1µl of the sample to be tested, making sure that no air bubbles are present in the tube. Draw an additional ~0.5µl of air into the needle, and insert the sample into the GC. Start integrator simultaneously with the injection, followed immediately by the GC temperature program.

Appendix C

Alterations made during the development of the protocol:

2. Extracting B[a]P from Stream Sediment Samples
2.3 Petroleum (pet) Ether Extraction

The protocol at the outset of this research called for the use of glass wool packed into the neck of a glass funnel as a filter for separating pet ether and dissolved solutes from stream sediment particles. The wool was found to be inefficient, as particulate matter was not being filtered out. A new filtration system was made by placing a folded piece of filter paper into a glass funnel, but flow rate through this filter was too slow, and the pet ether always evaporated before it could be completely filtered and collected in the glass bottle. Fisherbrand quantitative (Q5) filter paper with medium porosity and medium flow rate finally proved to provide the adequate filtration levels and flow rate, so long as it is pre-moistened with pet ether before extracts are filtered, to prevent evaporation of the extraction.

The original protocol used 10 mL pet ether added to the dried sediment samples, which was not enough to saturate the highly absorbent fine-grain samples enough for a supernatant to be drawn off for filtration. Thus, all fine-grain samples were shaken with up to 15 mL of pet ether.

2.4 Silica Gel Column

Setting up the column:

Original setup of silica gel columns called for 1.5 mL of "slurry" (1 part silica gel: 3 parts hexane) to be added to all columns. Because of silica gel's density as compared to hexane it was very difficult to draw up uniform concentrations of the gel to add to each pipette, and so the volumes of the columns were very varied. It was decided that slurry in all columns should be added until the column measured 4.5 cm from the top of the glass wool packed into the tip of the pipette to the top of the silica gel. This controlled the volume of the columns used for all samples.

Appendix D

Table 2A: Data for spiked samples

Control	Normal Point	RT	Area	ng/ul	B[a]P recovered	% yield
Rocky		29.625	1696121	566.044978		
	1.1 ave			350.833583	215.2113943	107.61%
	1.1(2)			474.37015	91.67482751	45.84%
Medium		29.655	1844366	615.435282		
	1.2 ave			555.167916	60.26736632	30.13%
	1.2(1)			588.742296	26.69298684	13.35%
Fine-grain		29.661	667280	223.269365		
	2.1 ave			296.864012	-73.59464668	-36.80%
	2.1(3)			36.179244	187.0901213	93.55%

Table 3A: Concentrations of B[a]P in hepatopancreas tissue

O.virilis	RT	Area	ng/ul	Total ng	Mass tissue (mg)	ng B[a]P/mg tissue	ng B[a]P/g tissue
1		0	0	0	60	0	**0**
2			0.953856	38.15426	0	N/A	**N/A**
3	29.54	31672	11.50591	460.2365	50	9.204730968	**9204.730968**
4		0	0	0	50	0	**0**

Table 4A: Concentrations of B[a]P in muscle tissue

O.virilis	RT	Area	ng/ul	Total ng	Mass tissue (mg)	ng B[a]P/mg tissue	ng B[a]P/g tissue
1	29.806	2976352	992.5754	39703.02	60	661.7169193	661716.9193
2	29.941	2464869	822.1663	32886.65	60	548.1108335	548110.8335
3	29.93	345121	115.9367	4637.468	60	77.29113221	77291.13221
4	N/A	0	0	0	60	0	0

Table 5A: Data for all sediment samples

Transsection	Pt	RT (min)	Area (0.125 µV-sec)	ng/µl	total ng	ng/g sediment	AVE ng B[a]P/g sediment	Transsection
1	1(1)	29.252	679369	227.29702	9091.88073	909.1880726		1595.158421
	1(2)	29.611	1420959	474.37015	18974.8059	1897.480593	1403.334333	
	2(1)	29.475	1764247	588.7423	23549.6918	2354.969182		
	2(2)	29.638	1562700	521.59354	20863.7415	2086.374146	2220.671664	
	3(1)	29.333	1427031	476.39314	19055.7255	1905.572547		
	3(2)	29.405	1576505	526.1929	21047.7161	2104.771614	2005.172081	
	4(1)	29.927	119452	40.751291	1630.05164	163.0051641		
	4(2)	29.803	1002569	334.97651	13399.0605	1339.906047	751.4556055	
2	1(1)	29.853	1772210	591.3953	23655.8121	2365.581209		3954.668999
	1(2)	29.977	786584	263.01749	10520.6997	1052.069965	1708.825587	
	2(1)	29.713	3009525	1003.6275	40145.1008	4014.510078		
	2(2)	29.952	49769	17.535232	701.409295	70.14092954	2042.325504	
	3(1)	29.47	3523933	1175.0112	47000.4464	4700.044644		
	3(2)	29.291	4374147	1458.2742	58330.9678	5833.096785	5266.570715	
	4(1)	29.468	1981366	661.07913	52886.3302*	5288.633017		
	4(2)	29.55	3116174	1039.1594	83132.7536*	8313.275362	6800.95419	
3	1(1)	29.943	235375	214.62036	8584.81426	858.481426		3011.949192
	1(2)		11182418	3726.5637	149062.549	14906.25487	7882.368149	
	2(1)		860229	287.55356	11502.1423	1150.214226		
	2(2)		945949	316.11261	12644.5044	1264.450441	1207.332334	
	3(1)		641320	214.62036	8584.81426	858.481426		
	3(2)		395962	132.87523	5315.00916	531.5009162	694.9911711	
	4(1)	29.873	3022014	1007.7884	40311.5376	4031.153756		
	4(2)	29.25	368615	123.76412	4950.56472	495.0564718	2263.105114	
4	1(1)	29.705	1547565	516.55106	20662.0423	2066.204231		4750.133267
	1(2)	29.212	16760104	5584.8632	223394.529	22339.45294	12202.82859	
	2(1)	29.67	513576	172.0603	13764.8243*	1376.482425		
	2(2)	29.429	1371800	457.992	36639.3603*	3663.936032	2520.209229	
	3(1)	29.406	981912	328.09429	13123.7714	1312.377145		
	3(2)	29.419	2753968	918.48442	36739.377	3673.937698	2493.157421	
	4(1)	29.36	1485302	495.8071	19832.2839	1983.228386		
	4(2)	29.494	1186817	396.36182	15854.4728	1585.447276	1784.337831	

* indicates data was adjusted for samples diluted in 80 µl hexane before loading into GC

Table 5A (cont.)

Transsection	Pt	RT (min)	Area (0.125 µV-sec)	ng/µl	total ng	ng/g sediment	AVE ng B[a]P/g sediment	Transsection
5	1(1)	29.443	756205	252.89622	10115.8487	1011.584874	1043.382975	1101.838248
	1(2)	29.385	803926	268.79527	10751.8108	1075.181076		
	2(1)	29.78	1030427	344.25787	13770.3148	1377.031484	827.5222389	
	2(2)	29.261	205751	69.503248	2780.12994	278.0129935		
	3(1)	29.224	719264	240.58871	9623.54823	962.3548226	532.4611028	
	3(2)	29.284	74101	25.641846	1025.67383	102.567383		
	4(1)	29.679	2143621	715.1371	28605.4839	2860.548392	2003.986673	
	4(2)	29.282	858136	286.85624	11474.2495	1147.424954		

* indicates data was adjusted for samples diluted in 80 µl hexane before loading into Gc