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CRAYFISH (*ORCONECTES VIRILIS*) AS BIOINDICATORS OF POLYCYCLIC AROMATIC HYDROCARBON CONTAMINATION IN THE BLACKSTONE RIVER VALLEY

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Abstract

The use of crayfish as bioindicators of polycyclic aromatic hydrocarbons (PAHs), specifically benzo[a]pyrene, was investigated. These contaminants have been identified by the EPA as potential human carcinogens, and are common in industrial areas like the Blackstone region of Massachusetts. Crayfish blood, hepatopancreas, and muscle, along with water, and sediment samples from the Blackstone River were analyzed using gas chromatography to determine benzo[a]pyrene concentrations. Results show the potential of crayfish to act as bioindicators of PAHs, while creating a base for future research.

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1 **Introduction**

We investigated the use of crayfish as bioindicators of polycyclic aromatic hydrocarbon (PAH) contamination. Environmental contamination by the PAH benzo[a]pyrene (B[a]P), identified by the USEPA as a potential human carcinogen, is a common consequence of industrialization. The Blackstone region of Massachusetts has been heavily industrialized since the early 1800s, and therefore is ideal for testing possible levels of PAH contamination. Crayfish blood, hepatopancreas, and muscle, along with water, and sediment samples from the Blackstone River were analyzed using gas chromatography to determine PAH concentrations.

1.1 *Polycyclic Aromatic Hydrocarbons*

Polycyclic aromatic hydrocarbons, referred to as PAHs, are a group of over 100 structurally similar chemical compounds formed during incomplete incineration of organic substances. There are three primary types of hydrocarbons: 1) *biogenic*, or produced through biologic processes such as the early stages of diagenesis (changes in chemistry of rocks that have been subject to physical, biological, or chemical alterations at low temperatures and pressures), 2) *petrogenic*, or those derived from petroleum inputs, and 3) *pyrogenic* – created from combustion of petroleum and wood (Boehm *et al*., 1998). Although PAHs are naturally present in the environment – the results of volcanic eruptions, natural geologic processes and forest fires – anthropogenic sources have become more common contributors to total environmental PAH concentration (AMAP, 1998; Freeman & Cattell, 1990). Petrogenic and pyrogenic sources are the primary contributor to PAH concentrations present in the environment today. The use of fossil fuels in industry, transportation, and heating has increased over the past 100 – 150 years, directly correlating with an increase of PAH concentration in the environment (Jones *et al*., 1989). This correlation will be discussed further in the following sections.

PAHs are most commonly found as mixtures of two or more compounds and therefore are typically studied as a group of compounds (Jongeneelen, 1997). Furthermore, their similar structures tend to result in the same behavioral characteristics, and therefore allow scientists to focus on a small number that are more easily studied and to generalize results to other PAHs. Seven PAHs have been categorized by the International Agency for Research on Cancer (IARC) as probable human carcinogens; however the remaining PAHs have not been studied as extensively and therefore should not be assumed to be “noncarcinogens” (Nisbet & LaGoy, 1992). The majority of studies that have been conducted focus on inhalation as the primary means of human exposure to PAHs. These studies demonstrate an increased mortality of lung cancer patients due to sources such as oven emissions, roofing tar emissions, and cigarette smoke (Wynder & Hoffmann, 1967; Lloyd, 1971). Few studies investigate human death by systemic effects from oral exposure to PAHs (US DHHS, 1995). However, studies of oral exposure in lab mice have been conducted. In one study, mice given 120 mg/kg/day of B[a]P were observed to have a decrease in life span. The primary cause of death for these mice was toxic depression of bone marrow which led to hemorrhaging or infections (Robinson, 1975; as cited in US DHHS, 1995).

The United States Environmental Protection Agency (US EPA) Persistent Bioaccumulative and Toxic (PBT) Pollutants Strategy identifies and attempts to reduce negative

effects of PBT pollutants on human health and the environment. These chemicals have unique characteristics allowing them to spread easily through air, water, and soils. Due to these qualities, as well as their potentially carcinogenic properties, 16 PAHs have also been identified as priority pollutants. Benzo[a]pyrene (B[a]P), a level one substance described by the EPA as teratogenic, toxic, and highly carcinogenic, is both a PBT and priority pollutant (Juhasz & Naidu, 2000). In an effort to regulate these chemicals, the EPA, as well as individual states, have identified the most likely human sources of PBT/PAH contamination and imposed regulations upon these activities.

During the 1980s, scientists believed that approximately 36% of the PAHs found in the atmosphere were contributed by motor vehicles, 17% by forest fires and aluminum production, 12% by residential wood burning, 11% by coke manufacturing, 7% by power generation, and 3% by garbage incineration (Benner & Gordon, 1989). Many of these sources are now subject to regulations by federal and state governments. For example, there are regulations for the proper storage and removal of oil wastes as well as how to deal with possible oil spills (United States Government, 1994). Since many of these production processes are non-point pollution sources (pollutants indirectly delivered to a body of water through land runoff, precipitation, drainage, etc.: Environmental Protection Agency, 2002), it is difficult to enforce these regulations. For example, run-off from parking lots most often includes oil from cars, which is a large contributor to PAH concentrations in streams and rivers (van Metre, *et al*., 2000). Efforts to manage such non-point sources include Section 319 of the Clean Water Act, which requires states to identify such sources and contaminated waters. States are then eligible for federal and state grants designated for implementation of plans to clean up contaminated sites and sources (United States Government, 1994). Drinking water sources are the easiest to regulate for the protection of human health from PAHs since they are already controlled. The EPA periodically updates these standards according to cancer classifications and toxicological reviews. As of 2006, eleven PAHs were regulated through the EPA's Drinking Water Standards and Health Advisories (US EPA, 2006).

Air is the main carrier of PAHs, and has been measured to contain an average of several nanograms per cubic meter. PAHs from these sources enter the atmosphere mainly via smoke outputs. They are then distributed between gas and particle phases, and are removed by oxidative and photolytic reactions as well as wet and dry deposition (Garban *et al*., 2002). Wet deposition is the process through which gaseous and aerosol forms of PAHs can be extracted from the atmosphere by precipitation (Dickhut & Gustafson, 1995). Most PAHs however are found as hydrophobic particles and are therefore not likely to be washed out by precipitation (Golomb *et al*., 1997; Hillery *et al*., 1998). Dry deposition, or the process of settling, impaction, and absorption of atmospheric chemicals, has consequently been identified as the principal form of atmospheric loading. This atmospheric deposition or fallout has been identified as a primary source of PAH loading in many water sources (US EPA, 2000).

Atmospheric PAHs are deposited into soils and bodies of water (Dorr, 1995). Those deposited on land can be incorporated into vegetation and surface run-off, or mobilized by air currents to eventually resettle on land or water (Lawrence & Weber, 1984; Walker *et al*., 1999). This transport can cause PAHs to travel large distances to regions where anthropogenic sources are uncommon. For example, PAHs have been found in cores of the ice sheet in Greenland,

snow samples in European mountains, and remote areas of agricultural Bavaria (Martens *et al*., 1997; Masclet *et al*., 2000; Carrera *et al*., 2001).

As mentioned previously, an increase in PAHs was observed in the past 100 – 150 years, correlating with an increase in use of fossil fuels in industry. A decrease in PAH concentrations however was reported in the 1970s and 1980s, and was attributed to a decrease in the use of coal in power plants, residential heating, and industrial emission control (Christensen & Zhang, 1993). This reduction has been countered by an increase in urban sprawl, decentralized employment, and an increased use of private vehicles for transportation. As part of a United States Geological Survey (USGS) National Water Quality Assessment program, sediment cores were taken from seven reservoirs and three lakes – sites with a range of land uses and percent urbanization. The cores were analyzed for several elements including PAHs. The results showed a change in the source of PAHs during the last 40 years from un-combusted to combusted fossil fuels, coinciding with increased urbanization and traffic (van Metre *et al*., 2000).

1.2 *Blackstone River Valley*

These national trends can be observed in the rich industrial history of the Worcester, Massachusetts area. The Blackstone River Valley, which begins in Worcester, is known as the "Birthplace of the American Industrial Revolution," and was designated a National Heritage Corridor in 1986 (National Park Service, 2006). Although the river may be most famous for powering Slater Mill, the first successful cotton-spinning factory, the majority of the river's 74.03 km (46 miles) contains a rich manufacturing history dating back to the early industrial revolution. The headwaters of the Blackstone in Worcester include Mill Brook, the Middle River, and Tatnuck Brook. These bodies of water once flowed in and around the city and provided water and power to a diverse array of industries including blacksmiths, printers and manufacturers of potash, lead pipes, and paper. During this period Worcester was also known for its cotton, wool, and wire mills (Washburn, 1917). Each of these industries had the potential to discharge PAHs not only in the air, but directly into the water sources which were fueling the urban industry. Many of these industries have since disappeared from Worcester, following technological advances; however they have left behind hazardous pollutants that still persist in the Blackstone River and its tributaries (Massachusetts DEP, 1998).

Historical industries are not the only potential contributors to PAH contamination in the Blackstone Valley. As discussed above, PAHs are distributed through common heating and current industrial emissions as well as traffic sources. One study completed in Roxbury, MA assessed the levels of PAHs around a bus terminal and found substantially elevated levels around the terminal and along routes (Levy *et al*., 2001). The Worcester region is still very much an industrial, urban area, and therefore subject to all of these urban sources. Studies have also shown that atmospheric deposition can take place thousands of miles away from the actual source of pollutants. It is therefore possible that contaminants found in both urban and more rural parts of Massachusetts may be the result of emissions from other states with heavy industrial activity, such as Ohio or Michigan (Golomb *et al*., 2001).

1.3 *Biological Indicators: theory and policy*

Environmental monitoring is an important process which involves constant or periodic testing of environmental properties to identify and track pollutants in air, soil, and water which may affect plant animal, or human health; i.e. PAHs (US EPA, 2007a). Water monitoring strategies employed by scientists can be either abiotic or biotic. Chemical and physical water quality assessments have been most commonly used due to ease of measurement. These abiotic assessments have included such properties as temperature, salinity, dissolved oxygen, and nutrient levels.

The process of establishing acceptable levels for each of these characteristics typically includes three general steps. First, scientists select organisms for testing according to several parameters including biological, methodological, and societal relevance (Burger & Gochfeld, 2001). Such criteria call for bioindicators that will reflect changes in the environment quickly, and with a measurable response. Indicators should also be common and easily monitored, potentially on a long time-scale. Once an organism has been selected, the second step is to carry out laboratory tests to determine its tolerance levels of chemical and physical water quality parameters. The data generated by these experiments are then used to establish acceptable levels of salinity, dissolved oxygen, chemical concentration etc. The final goal of this process is for officials to develop and implement an appropriate water quality protection or restoration program (Yoder, 1998).

Once established, water quality monitoring programs focus on testing the chemical and physical properties of water bodies and comparing quantitative results with those generated by the *in vivo* organism study. This approach, while a step towards water quality improvement, has the potential to do more harm than good. For example, to protect streams from sewage pollution, it was common to install sanitary sewers in stream beds to protect prevent contamination of the water source from effluent. This action resulted in miles of habitat destruction, degrading the streams it was meant to protect (Yoder, 1998). This leads to the conclusion that chemicals cannot be the sole focus of monitoring, but rather, the environment must be assessed as a whole – both biotic and abiotic factors included.

Yoder (1998) agrees with this conclusion, stating that “biological integrity is influenced and determined by *multiple* chemical, physical, and biological factors” (author’s emphasis). Abiotic parameters only measure the amount of contaminants present in water or sediments, not the effects these chemicals have on individual species or the ecosystem as a whole. Bioindicators, on the other hand, can function as indicators of “exposure” to contaminants as well as the “effect” of said contaminants (Mayer *et al*., 1992). “Exposure” and “effect” may be evaluated in two ways: chemical analysis of individuals, and observation of population dynamics. The latter has been added to many state water quality management processes and is now recommended (NH DES, 2007 & US EPA, 2006). Chemical analysis of individuals in these populations can supplement observations by providing data and justification for observed community structures. Such analysis can also identify additional risks of the studied pollutant(s), most importantly bioaccumulation and biomagnification (defined below).

Several examples of state water quality monitoring programs follow this policy of focusing water quality assessment on the basis of biologic parameters. The New Hampshire Evaluation of Sediment Quality Policy uses several sequential components to evaluate environmental risk. As part of the Sediment Quality Triad Approach for Sediment-Dwelling Organisms, chemical analysis, sediment toxicity bioassays, and community assessments are conducted (NH DES, 2005). These steps confirm the presence of pollutants and identify the effects of those pollutants on representative organisms such as *Hyalella azteca* and *Chironomus dilutis*. The third step in the process determines how the identified pollutant acts in a natural community. This practice not only recognizes the presence of pollutants, but also whether or not the presence of these pollutants is detrimental to the natural population.

New Hampshire also focuses on bioaccumulation and the potential effects of resulting biomagnification. Bioaccumulation is defined herein as the uptake of a chemical by an organism from food, water, or sediment (Thomann, 1989). Many of these chemicals are heavy metals or hydrophobic organics which are difficult to detect in water samples because they exist in low concentrations that are hard to analyze using common methods (Zimmerman *et al*., 2000). These pollutants are often taken up by organisms at the bottom of the food chain – those closest to the source – and are transferred from prey to predator, increasing the body burden of each organism as they proceed up the food chain. This process is referred to as biomagnification, and can potentially affect humans as contaminants are processed up the food chain to consumables such as fish or waterfowl. Common examples of chemicals that bioaccumulate and subsequently biomagnifiy are DDT (dichlorodiphenyltrichloroethane) which has greatly affected bird populations, and mercury, which is taken up by fish and transferred to humans via consumption (Atwell *et al*., 1998; Nakamaru *et al*., 2001). In light of these consequences, the NH DEP has developed a process for determining contaminant tissue concentrations for aquatic organisms that uses field-collected specimens (selected as described above) to study bioaccumulation and determine risk.

Ohio employs similar strategies when monitoring water quality. Most pertinent to this project is their use of toxicity tests, tissue residues, and biomarkers. These parameters, which fall into Ohio's exposure indicator category of water quality status, are used along with stressor and response indicators. These indicators identify pollution sources and resulting effects on populations in their natural environment and are used in concert with toxicological data to manage water (OhioEPA, 2007).

1.3.1 Biological Indicators: Empirical examples

In recent years, studies on bioindicators have increased in an effort to identify optimum species for the identification and tracking of specific chemicals. Organisms such as mussels, fish, serviceberry, lichens, ants, and protozoa have been identified as viable sources of environmental information for water, air, and soils.

Marine Water Quality Indicators: Mussels

One such example is the International Mussel Watch program, sponsored by the National Oceanographic and Atmospheric Administration (NOAA), the United Nations Environmental Program, and United Nations Educational, Scientific and Cultural Organization (UNESCO) Intergovernmental Oceanographic Commission. The purpose of this project is to “quantify the sources and rates of input of [societal] wastes so that the current status of environmental health can be assessed and that trends may be determined” (Jernelov, 1996). It evolved from research done in 1975 by Professor Edward Goldberg of Scripps Institution of Oceanography, who introduced the idea of an organismal global monitoring program which would prove valuable in detecting global trends (Jernelov, 1996). Bivalve species were first evaluated as potential indicators to be used for this program due to their sedentary nature and ability to accumulate target pollutants. These species have been widely used, including studies in Europe, Canada, China, Latin America, South America, and the United States (Zhao *et al.,* 2005). The final decision to use mussels in the Mussel Watch was based on the prevalence of these organisms as well as their diversity in both marine and freshwater ecosystems.

In the Initial Implementation Phase, 76 sites in South America, Central America, the Caribbean, and Mexico, were sampled for chlorinated pesticides, PAHs and PCBs. Specific analytes included common pesticide chemicals including: the DDT family, methoxychlor, beta-hexachlorocyclohexane, aldrin, and chlordanes. The resulting data not only identified certain regions (mostly urban areas) above the limits for chlorinated pesticides and PAHs, but also built a database for area scientists and a foundation for the extension of the program to other nations (International Mussel Watch Committee, 1995). Since the initial study, the International Mussel Watch program has been extended to both the United States and the Mediterranean. In 2002, the International Commission for the Scientific Exploration of the Mediterranean Sea approved the Mediterranean Mussel (*Mytilus galloprovincialis*) as the primary quantitative bioindicator of radionucleotides and trace metals (Nakhle, 2006).

Air Quality Indicators

Much like canaries used in mines as indicators of methane and carbon monoxide, certain plant species have been used as indicators of poor air quality. Lichens, which are a symbiotic relationship between a fungus and an alga, were used as early as 1866 to evaluate the quality of air (Conti *et al*, 2001). Lichens are dependent on their surroundings for nutrients, do not shed parts as they grow, and are able to absorb contaminants over their whole surface area. They have therefore been identified as “permanent control systems” for the evaluation of air pollution. The effects of these air pollutants on lichens are evaluated by changes in photosynthesis, chlorophyll, respiration, ATP production, membrane damage, and chemical production (Conti, 2001). The potential of evaluating elemental composition of lichens was studied, however due to high variability and difficulty in stabilization it was determined the procedure to be implausible. (Stolte *et al*., 1993). Pollutants targeted in lichen studies include SO_2 and nitrogen- and sulfur-based pollutants resulting from pesticide use (Showman, 1975; McCune, 2000).

Similar to lichens, Saskatoon serviceberry (*Amelanchier alnifolia*), referred to as Saskatoon, has been used in studies of air quality. Sulfur dioxide (SO_2), nitrogen oxides (NO_x), and hydrocarbons (R-H), among others are chemicals negatively affect the heath of this plant as well as the environment as a whole. The foliage of the Saskatoon has been identified as comparatively sensitive to sulfur dioxide. Injuries to the leaves of this plant are visibly evident, making them an easily identifiable indicator. A study subjecting Saskatoon plants to sources of SO_2, NO_x, and R-Hs identified the foliar injury symptoms to be very useful as biological indicators of acute or chronic ambient SO_2 exposures (Krupa, 2001).

These indicators are readily available in the environments being studied, and have shown reliability in displaying pollutant levels and their environmental effects. Rivers in the New England area have similar pollution problems; however few organisms are prevalent enough to be a sustainable resource for recognition of pollutants. As will be discussed in the following section, crayfish are not only abundant but also a highly adaptable species that has potential for use in the field of bioindicators.

1.4 *Crayfish Ecology and Mechanisms of Contamination*

Crayfish, which are decapod crustaceans, and the largest freshwater invertebrate, have been suggested as potential bioindicators of certain contaminants. They are a keystone species because as omnivorous feeders, they eat living and dead plant matter, potentially impacting the biomass of aquatic macrophytes (macroscopic plant life) and macroinvertebrates (animals without a backbone that large enough to be seen with the naked eye) (Harm, 2002). This study focused on the species *Orconectes virilis,* which range throughout much of North America. This species of crayfish is invasive in the New England area, introduced around 1963 from the Midwest extending to the Mississippi river (Aiken, 1965). *O. virilis* are found in rivers, on rocky substrates, and in lakes, where the sandy, weedy bottom provides an optimal habitat. They typically live beneath rocks, but are also capable of constructing networks of burrows in riverbanks (Hazlett, 1974). Crayfish are resilient and in the case of a food shortage, larger ones may be cannibalistic, feeding on the weak. Crayfish lie at the bottom of the food chain, providing food to many predators, including: invertebrates, fish, amphibians, reptiles, birds (herons), and mammals (otters and mink) (Hogger, 1988).

In temperate regions, crayfish are most active between May and September. During the winter months, they survive by moving to deeper water or further under the substrate. Both water temperature and the presence of predatory fish impact the location of crayfish populations. For *O. virilis*, the mating season lasts from July to September with females laying eggs from late May to early June. With an average life span of three years, in their first summer, juvenile *O. virilis* molt a total of five times, followed by another three to four times during the second summer. Maturity is reached within the first or second year, at which time adults range from 23 to 27 mm in size, reaching a maximum of around 55 mm in their third year (Harm, 2002).

1.4.1 Mode of PAH Contamination

Crayfish feed at the bottom of the river, sifting through sediment and soil. As previously stated, high weight PAHs such as B[a]P, accumulate in sediment and soil and therefore would be expected to contaminate the plant material which is an essential part of the crayfish diet. A study conducted by Meuduc (2005) transplanted *Salicornia fraglis*, or glasswort (a plant that can be used a herb), from salt marshes in Finistere, France, into soil contaminated with heavy fuel oil. They concluded that PAHs, including B[a]P and Pyrene, bio-accumulated in glasswort after the plants were transplanted into the contaminated soil. Over the first four weeks of the study, the rate of bioaccumulation of B[a]P increased until it suddenly dropped, while pyrene continued to accumulate for an additional week. Pyrene, phenanthrene, and chrysene were the major three PAHs that bio-accumulated in the glasswort (Meudec, 2005).

Another study investigated PAH contamination of water, sediment, macrophytes and clams, six months after the *Estrella Pampeana* oil spill off the Coast of Rio de la Plata, Argentina (Colombo, 2004). It was determined that 2.4 +/- 2.1 μg/g of PAHs existed in macrophytes – five times higher than the baseline (determined from measurements prior to the spill) of 0.5 μg/g. Contamination levels of macrophytes with aromatic hydrocarbons were consistent with levels in contaminated water and sediment samples (Colombo, 2004). Both of the above mentioned studies indicate that plants can accumulate PAHs, suggesting the potential contamination of crayfish through their diet.

1.4.2 Anatomy

The hepatopancreas of crayfish is responsible for the production of digestive enzymes, storage of most nutrients, and energy reserves, and the detoxification of xenobiotics, such as B[a]P (Harm, 2002). During the period of premoult, lipid storage in the hepatopancreas is at its highest, while during postmoult it is at its lowest (Santos, 1997). Located in the thoracic cavity, the hepatopancreas is composed of two halves connected by the pyloric stomach. Each half consists of hundreds of blind-ended tubes, which are bathed in haemolymph. These tubes fuse to one another forming a collecting duct that leads into the pyloric stomach and midgut.

Detoxification of xenobiotics occurs not only in the hepatopancreas but also in the antennal gland, where heavy metals and organic contaminants collect. The antennal gland uses cytochrome P450-dependent mixed function oxygenases for the biotransformation of xenobiotics and is also involved in metabolism of fatty acids and steroids (Harm, 2002). Biochemically, the antennal gland differs from the hepatopancreas; first, because it uses nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor for electron transfer to cytochrome P450 while the hepatopancreas uses nicotinamide adenine dinucleotide (NADH), and second, because they produce different metabolites. Therefore, the hepatopancreas and antennal gland are responsible for different types of detoxification (Harm, 2002). A study conducted by Jewel (1997) supported this investigation by showing B[a]P to have a much higher catabolic activity in the antennal gland than in the hepatopancreas.

Crayfish have partially closed circulatory system in which the heart pumps haemolymph into seven main arteries that branch into smaller vessels that then lead to the body’s organs and

appendages. Twenty-seven percent of a crayfish's body volume is occupied by haemolymph which consists of haemocytes and plasma. Within the haemolymph, where glucose is the main blood sugar, lipids are transported as high-density lipoproteins (LDL) (Harm, 2002). Produced in the hepaopancreas, haemocyanin is the most dominate protein in the haemolymph, where it uses copper to transport oxygen (Harm, 2002).

1.5 *Chemistry and Analysis of PAHs and Benzo[a]pyrene*

The chemical structures of all PAHs are built from a base consisting of at least two benzene rings fused together via a pair of carbon atoms (US EPA, 2003). These rings can exist in either a linear, angled, or clustered fashion, all of which form a structure in which all carbon and hydrogen atoms can be found in a single plane (Sims & Overcash, 1993; US EPA, 2003). As the number of fused benzene rings increases, the magnitude of several chemical and physical properties also increases. High-molecular weight PAHs, or those with a large number of benzene rings, result in a greater aromaticity of the molecule, therefore making them more hydrophobic than lesser weight PAHs. Because of this, high weight PAHs are less likely to occur in water (Wilson & Jones, 1993). High weight PAHs exhibit more acute properties than low weight PAHs; such as having a lower vapor pressure and a higher octanol-to-water partition coefficient. Molecules with high octanol-water partition coefficients are more resistant to microbial degradation, which is employed to remove low weight PAHs from the environment. This is a result of both the decreased water solubility and increased resonance energy and toxicity (Cerniglia, 1992; Wilson & Jones, 1993). High weight PAHs are more difficult to degrade than low weight PAHs in part because of their lower volatility (Wilson & Jones, 1993; US EPA, 2003).

One common characteristic of PAHs is that they are naturally found in complex mixtures (US EPA, 2003). This fact makes it difficult to detect specific PAHs, especially those that are high weight. The need to develop an easy procedure for the detection of PAHs is paramount, due to properties such as acute toxicity presented by low weight PAHs (Sims & Overcash, 1993), and genotoxicity (toxic effects imposed on an organism resulting in damaged DNA, responsible for mutations and cancer) exhibited by high weight PAHs (Lijinksky, 1991; Mersch-Sundermann *et al.*, 1992; Nylund *et al.,* 1992; Phillips, 1983). Benzo-[a]-pyrene (B[a]P) is a high weight PAH of concern and has been shown to be genotoxic to both prokaryotes and mammalian cells, resulting in DNA binding, sister chromatid exchange, chromosomal aberrations, point mutations and transformations (de Serres & Ashby, 1981; Hollstein *et al.,* 1979; Jushasz & Naidu, 2000).

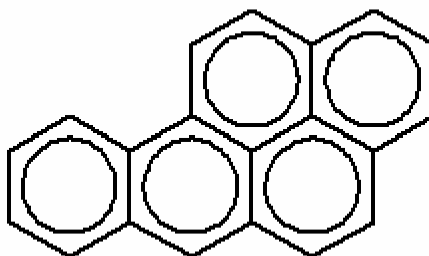

Figure 1: Chemical structure of B[a]P.

Considered a high priority pollutant by the EPA, B[a]P was selected as the focus PAH for our investigation of crayfish as bioindicators for several reasons, including the chemical properties of the molecule. B[a]P consists of five clustered benzene rings (Figure 1) and shares characteristics with other high weight PAHs (Cerniglia, 1992; Erickson *et al.,* 1993). Poor water solubility results in the tendency of B[a]P to adhere to surfaces, such as sediment or soil (US EPA, 2003). This makes crayfish prime candidates for the detection of such PAHs since they live and feed on the river bottom, where the highest concentrations of B[a]P should exist.

1.5.1 Isolation and Gas Chromatography Analysis of PAHs

Isolations of B[a]P involved a combination of extraction, liquid (LC) and gas chromatography (GC). In both forms of chromatography, the solutes partition due to the component's hydrophobic nature, leaving the hydrophobic compounds dissolved in the mobile phase which can then be separated and collected. Chromatography is a widely used separation technique that depends on the solubility, adsorption and volatility properties of the substances. A mobile phase, or the solute, moves past a stationary phase and depending upon the above mentioned properties: solubility, absorption and volatility, partitioning of the compounds between the solute and stationary phase will occur. This results an effluent containing PAHs along with any other hydrophobic compounds in the solute (Adams *et al*., 1970).

The final extracted samples then underwent GC, also known as Vapor Phase Chromatography due to the vaporization of the solute by high temperatures. The sample is swept through a high temperature glass column, inside of which is a collection of beads onto which a high boiling liquid is absorbed; this is also known as the stationary phase. The mobile phase for GC is a carrier gas, in this case N_2. The entire column is heated in the oven, facilitating the separation of the components depending upon the differences in vapor pressure of the molecules and their solubility in the coating on the stationary phase (Bobbitt et al., 1968). This results in the separation of the mixture into parts, which are then detected and printed as a series of peaks and times which correspond to the concentrations and retention times of the components within the mixture (Adams et al., 1970). A GC set up with consistent temperature, column and gas flow will result in components of the mixture exhibiting a characteristic retention time. This allows for the GC to be programmed to detect specific compounds (B[a]P) and for the recognition of these compounds based on a control run that provides it's unique retention time. The integrator prints the series of peaks, along with the area underneath each peak. While the retention time is used for recognition, the area is used to determine the concentration of the desired compound. Analysis of the printed peaks was conducted using, EPA method 610 for PAH GC analysis.

1.6 *Goals of Study and Predictions*

The main goal of this study was to determine if crayfish could be used as bioindicators of PAHs. To this end, crayfish blood, hepatopancreas, and muscle, along with water, and sediment samples from the Blackstone River were analyzed using gas chromatography to determine PAH concentrations. This research serves as a pilot study for identifying crayfish as bioindicators, as well as assessing the availability of B[a]P in the Blackstone River. It was predicted that there would be a positive correlation between B[a]P concentrations in sediment and tissue. It was also

predicted that there would be a higher concentration of B[a]P in hepatopancreas of crayfish because toxins are known to accumulate in the digestive system of crustaceans (Lee et al., 1976, as cited in Varanasi, 1989; Little et al., 1985, as cited in Varanasi, 1989). Positive results in this tissue, as well as muscle, would have important implications for consumption of these organisms by higher order animals such as birds or humans, which consume these tissues.

2 **Methods**

2.1 *Sampling Locations*

Crayfish (*Orconectes virilis*), sediment, and water samples were collected from three sites in the Upper Blackstone River Watershed (Figure 2).

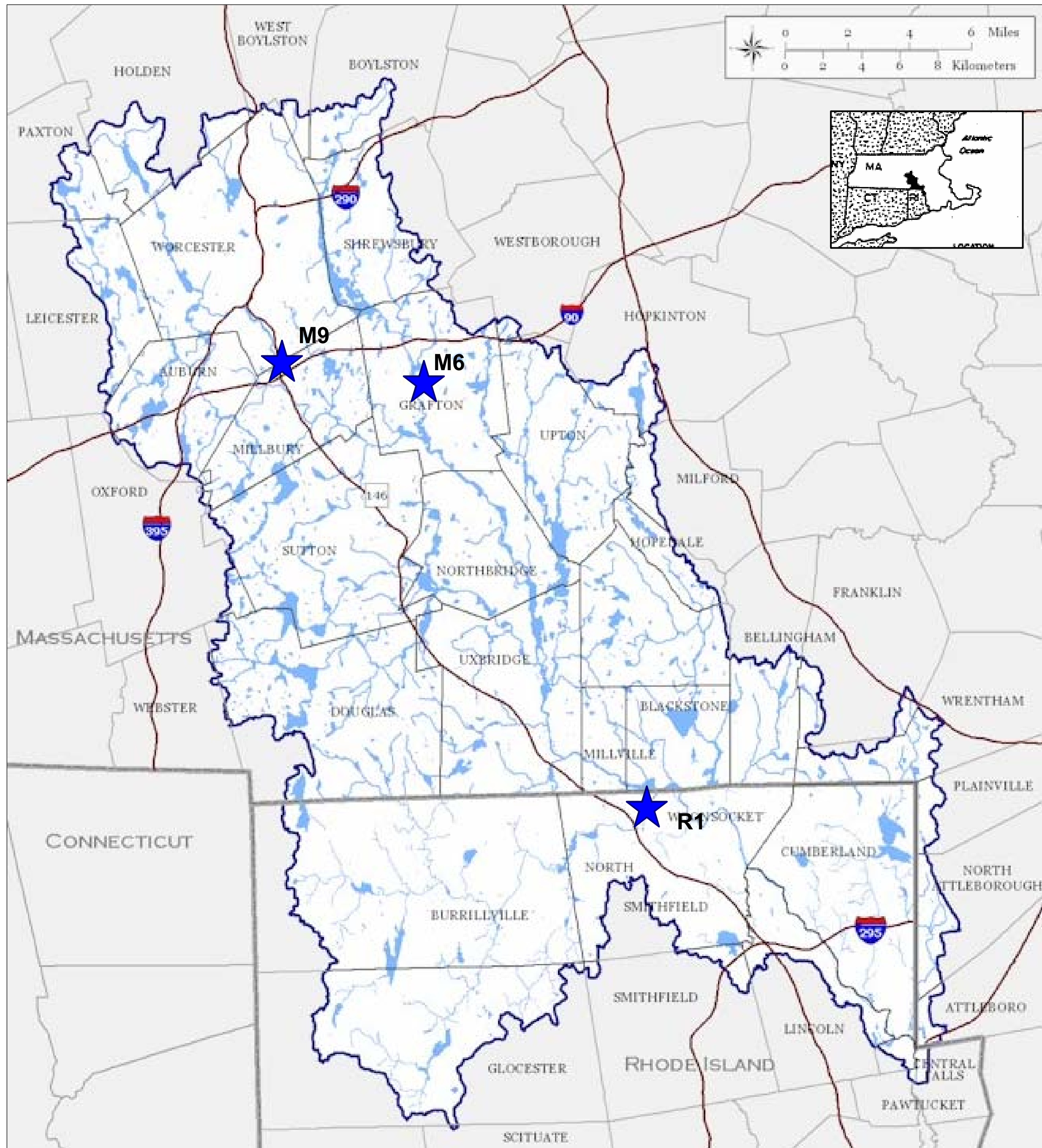

Figure 2: Blackstone River Watershed with sample locations denoted.
See below for site maps and pictures; text for site descriptions (Blackstone River Watershed Association, 2007).

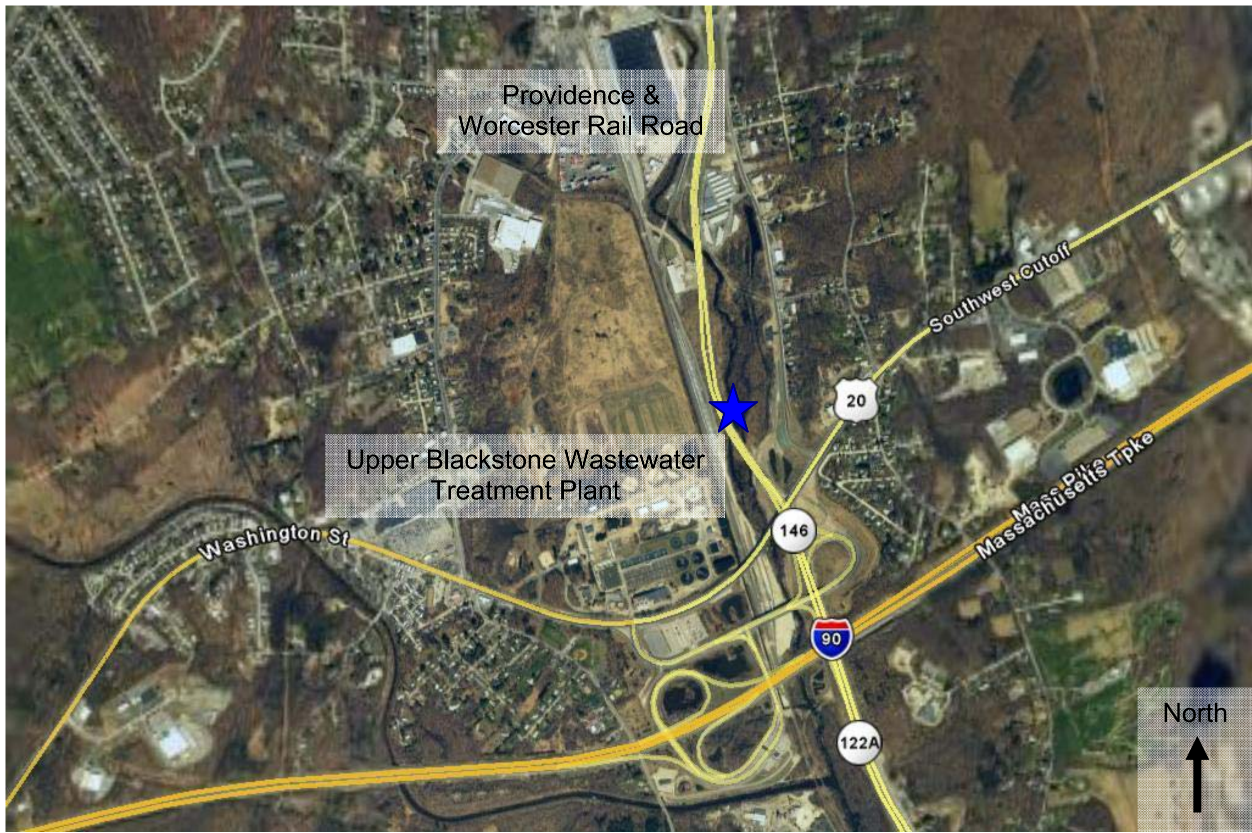

Figure 3: M9 – Blackstone River under Route 146, Worcester/Millbury, MA line.
Collection site is indicated by the blue star.

Figure 4: Blackstone River Collection Site M9.

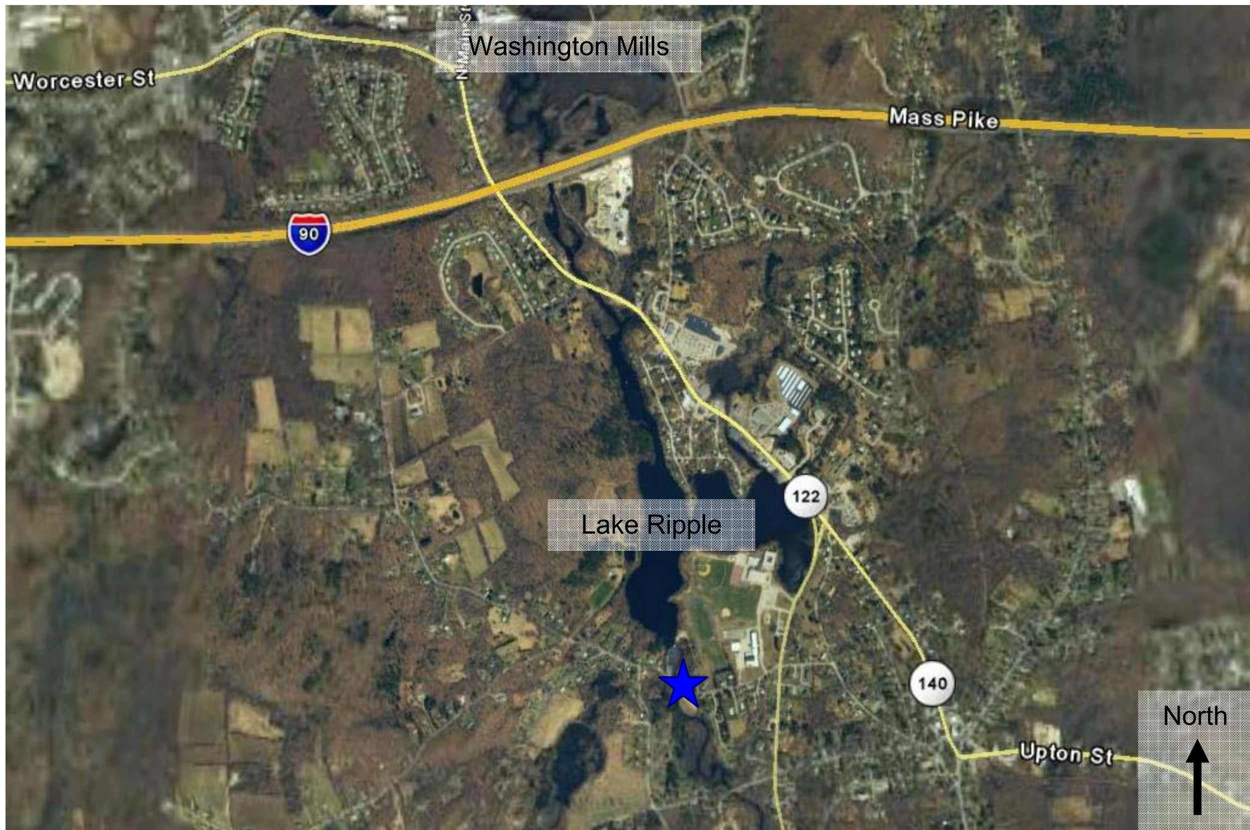

Figure 5: M6 – Below Lake Ripple, Grafton, MA.
Collection site is indicated by the blue star.

Figure 6: Quinsigamond River Collection Site M6.

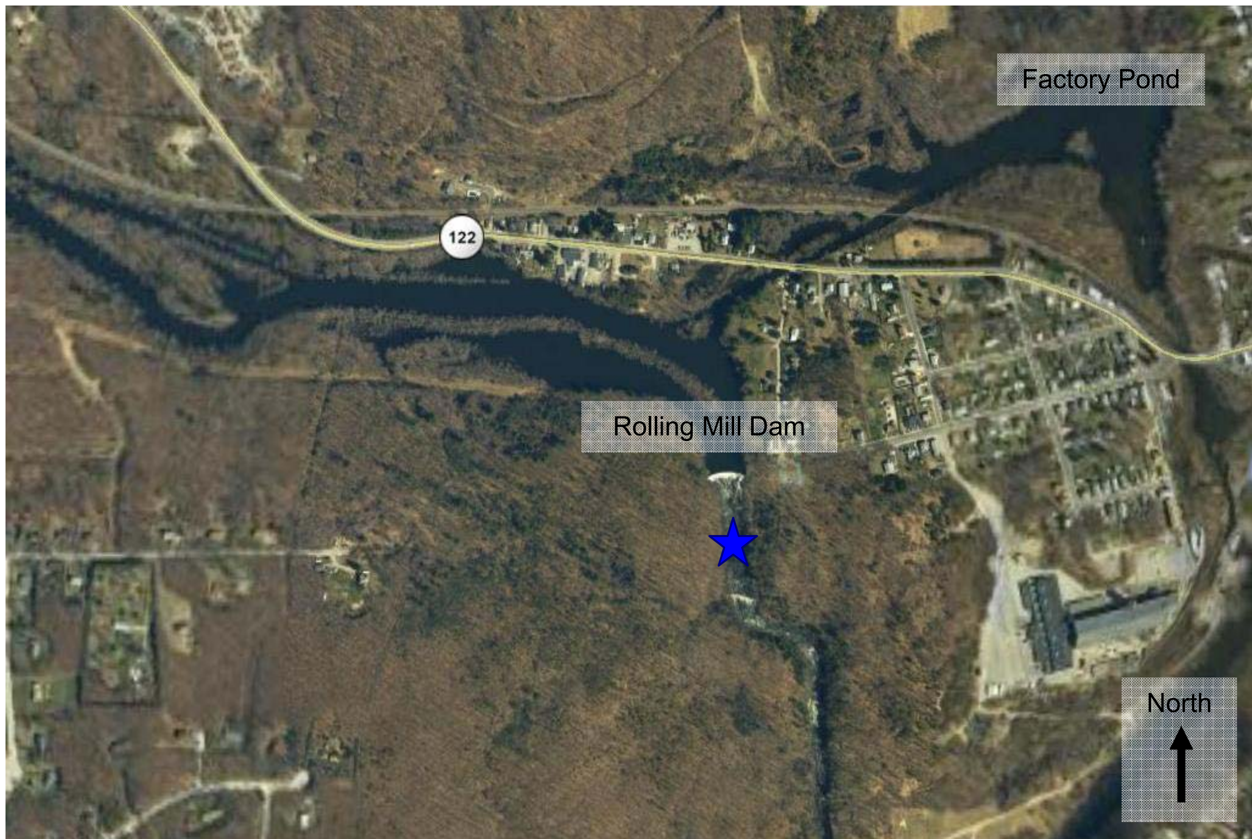

Figure 7: R1 – Blackstone River at Blackstone River Gorge, North Smithfield, RI.
Collection site is indicated by the blue star.

Figure 8: Blackstone River Collection Site R1.

These locations were chosen on the basis that each differed in factors which could contribute to varying levels of pollution, along with existing large crayfish populations. Fifteen crayfish were hand and seine collected from Grafton, Massachusetts (M6, Figure 5); sixteen from a location in

Worcester, Massachusetts (M9, Figure 3); and ten from North Smithfield, Rhode Island (R1, Figure 7). Site M6 is located directly below the dam at Lake Ripple, site M9 under a Route 146 bridge, and site R1 just south of the Massachusetts border below the Rolling Mill Dam in the Blackstone River Gorge.

Live crayfish were transported in water back to the laboratory at Worcester Polytechnic Institute (WPI) in ~20L buckets. Two water samples were taken from each site by filling 50mL conical tubes under water. Two sediment samples were also taken by filling 50mL conical tubes with silt, sand and gravel. All samples were returned to the lab within a few hours for processing. Water and soil samples were immediately frozen and held at -20 °C until processing.

2.2 *Blood and Tissue Sampling*

Hemolymph samples between 0.3-0.5g were taken from the base of the walking leg of live crayfish using a needle and syringe (Figure 9A). Crayfish were euthanized by freezing at -20 ^{o}C. After freezing, crayfish were dissected to remove samples of muscle and hepatopancreas. Between 0.022g and 0.454g of muscle were collected from the largest claw of each specimen (Figure 9B). The carapace was cracked and between 0.046g and 0.522g of hepatopancreas removed (Figure 9C). All samples were stored individually at -80°C in 1.2mL eppendorf tubes.

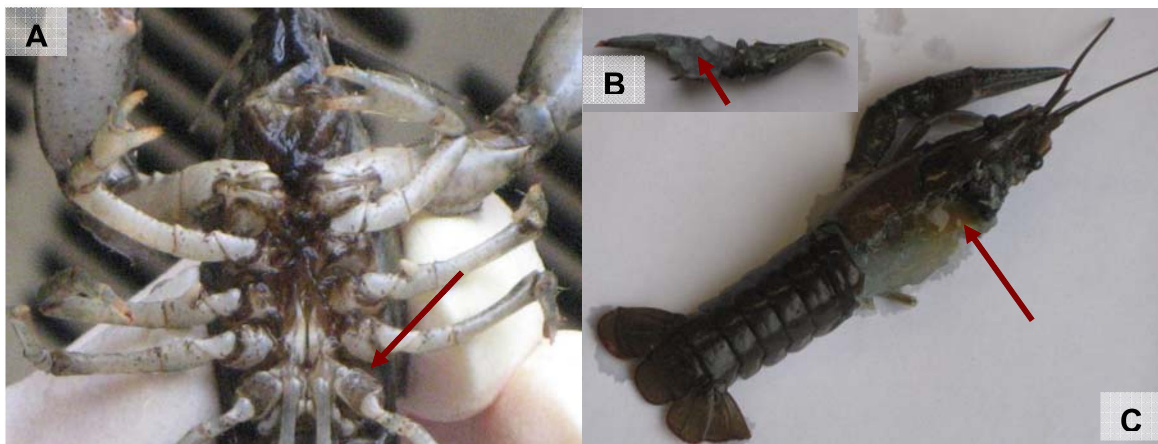

Figure 9: Crayfish dissection for tissue samples.

2.3 *Extraction of PAHs from Blood and Tissue Samples*

Extractions of PAHs were done using a glass column made from a five inch Pasteur pipet, by removing the cotton plug and then inserting a small amount of glass wool into the tip. The column was clamped onto a ring stand above a glass 10 mL collection vial. Using a mortar and pestle, samples (30 – 60 mg haemolymph, hepatopancreas and muscle) were ground with 1.0 g of sodium sulfate (anhydrous) into a free flowing powder. The powder was then transferred to the glass column and rinsed four times with 2 mL of petroleum ether. The collected eluents were then dried down with N_2.

A stock of "slurry" solution was made with one part silica gel and three parts hexane. Liquid chromatography was conducted using another glass column set up as explained above and clamped to a ring stand above a collection vial. One and a half mL of re-suspended slurry served

as the stationary phase. The vial containing the N_2 dried sample was rinsed with 250 µL of hexane and run through the column a total of four times. Finally, the column was eluted with 9 mL of hexane.

The samples were again dried with N_2, and then, using a glass syringe, were rinsed with 250 µL of hexane four times, transferred to a 2 ml air-tight amber vial, and once again dried under N_2. Twenty µL of hexane were added to each sample contained in the amber vial before injection.

2.4 *Water and Sediment Extractions*

B[a]P was extracted from 50 mL water samples with a series of three rinses with petroleum ether in a glass separatory funnel. Three mL of petroleum ether were added to the separatory funnel for each rinse, for a ratio of distilled water to petroleum ether of 50:3 (v:v). Water/petroleum ether mixtures were swirled, as shaking will result in emulsion (Adams *et al.*, 1970), for one min and allowed to separate. The settled and separated petroleum ether from each rinse was combined while the water was reused for any following rinses. For each sample, the combined petroleum ether was then run through a Pasteur pipet packed with 1.0 g sodium sulfate and blocked with glass wool. Collected eluents were then prepared for liquid chromatography by drying under N_2.

B[a]P was extracted from 10 g (wet weight) of sediment via a pair of rinses each with 50 mL of petroleum ether in a 100 mL glass bottle. Sediment/petroleum ether mixtures were shaken for ten minutes and then allowed to separate. The settled and separated petroleum ether from each rinse was combined while the water was reused for any following rinses. For each sample, the combined petroleum ether was then run through a Pasteur pipet packed with 1.0 g sodium sulfate and blocked with glass wool. Collected eluents were then prepared for liquid chromatography by drying under N_2.

2.4.1 Liquid Chromatography of Water and Sediment Samples

A stock of “slurry” solution was made with one part silica gel and three parts hexane. Liquid chromatography was conducted using another glass column set up as explained above and clamped to a ring stand above a collection vial. One and a half mL of re-suspended slurry served as the stationary phase. The vial containing the N_2 dried sample was rinsed with 250 µL of hexane and run through the column a total of four times. Finally, the column was eluted with 9 mL of hexane.

The samples were again dried with N_2, and then, using a glass syringe, were rinsed with 250 µL of hexane four times, transferred to a 2 ml airtight amber vial, and once again dried under N_2. Twenty µL of hexane were added to each sample contained in the amber vial before injection.

2.5 *Controls*

A set of controls (referred to herein as individual controls) were arranged using water spiked with B[a]P. Three beakers were set up with 1 L of water: a negative control (no added B[a]P), a low positive (2 ppb B[a]P), and a high positive (20 ppb B[a]P). All three beakers were allowed to aerate for 1 day after the addition of B[a]P before a crayfish (collected from a site in the Quinebaug River in Sturbridge, MA) was added to each. Once the crayfish were added, they remained in the beakers for 1 week with only the addition of food. A food control was run through the extraction process in order to detect any PAHs the control crayfish may have been receiving from this source. After a week crayfish were removed from the beakers; water and tissue samples were taken and run through the extraction process before GC analysis.

A second set of spiked controls (referred to herein as isolation controls) was carried out with a field collected specimen from the Quinebaug River in Sturbridge, MA. Hemolymph, hepatopancreas, and muscle samples were taken and divided into positive and negative controls. Negative controls underwent the same extraction process as all other samples, as did positives, with the exception of the addition of 4,000 ng of B[a]P applied directly to the tissue before grinding with sodium sulfate.

2.6 *Gas Chromatography*

Using a Supelco SP-2250™ glass column (2 m x 2 mm), and a Perkin-Elmer Sigma 3 Gas Chromatography (GC) machine with a flame ionization detector each dried sample was analyzed via an Hewlett Packard 3395 Integrator. GC was set up with a zone temperature of 280º C; an initial temperature of 100º C; a final temperature of 280º C; an initial time of 4 minutes; a ramp rate of 8 minutes; a final time of 30 minutes; and a carrier gas flow rate of 56 mL/min. The regulator operating pressures for air, HiPure nitrogen (N_2), and hydrogen (H_2) gases were set to 48-50 psi, 60 psi and 30 psi, respectively. Each dried sample was dissolved in 20 µL hexane. Using a Gas-Tight syringe, 1 µL of dissolved sample was extracted from the vial followed by 1 µL of air, before being injected into the injection port. Signal from the GC was sent to the HP 3395 integrator. A report was received from the integrator for each sample displaying a series of peaks and times, representing the area and detection time of separated components in the mixture. The list below details the integrator program implemented.

Integrator program "PAH"
Run parameters
Zero = 0
ATT2^ = 7 (Y scaling factor)
CHT sp = 0.5 (chart speed)
AR REJ = 0 (minimum area required for peak to be reported)
THRS = 4 (Y-axis peak detection minimum)
PK WD = 0.04 (peak width for integration fit)

Timetable events
0.0000 INTG = 8
0.1000 zero = 0
0.2000 INTG = 2
30.00 PK WD = 0.20
50.000 PK WD = 2.00
56.000 STOP

2.7 *Data Analysis*

Final retention times for B[a]P and the remaining fifteen EPA PAHs were gathered and used for calibration of the GC (Table 1).

Table 1: Retention times of B[a]P.

Run Number	Retention time (minutes)
1	36.560
2	35.869
3	36.017
4	36.441
5	36.804
6	37.045
7	36.930
8	37.010
9	36.765
10	36.839
11	36.852
12	36.875
13	36.764
14	36.771
15	36.787
16	36.802
17	36.795
18	36.773

Using these retention times, the mean and standard deviation were calculated. An acceptable range of retention times for B[a]P in experimental samples and controls was defined as any peak with a retention time within two standard deviations of this mean. All integrations for tissue, sediment, and water samples were sorted according to this range to identify presence of B[a]P.

Integration plots were also assessed for peak height. This allowed a partition in results between negative (no retention times in range; Figure 10A), trace (retention time in range, the integrator recorded a small area but there was no visible peak; Figure 10B), and positive (retention time in range with visible peak; Figure 10C). Trace and positive results were analyzed according to the equations below. Mean B[a]P concentrations for samples were calculated with positive samples; trace samples were grouped as negatives.

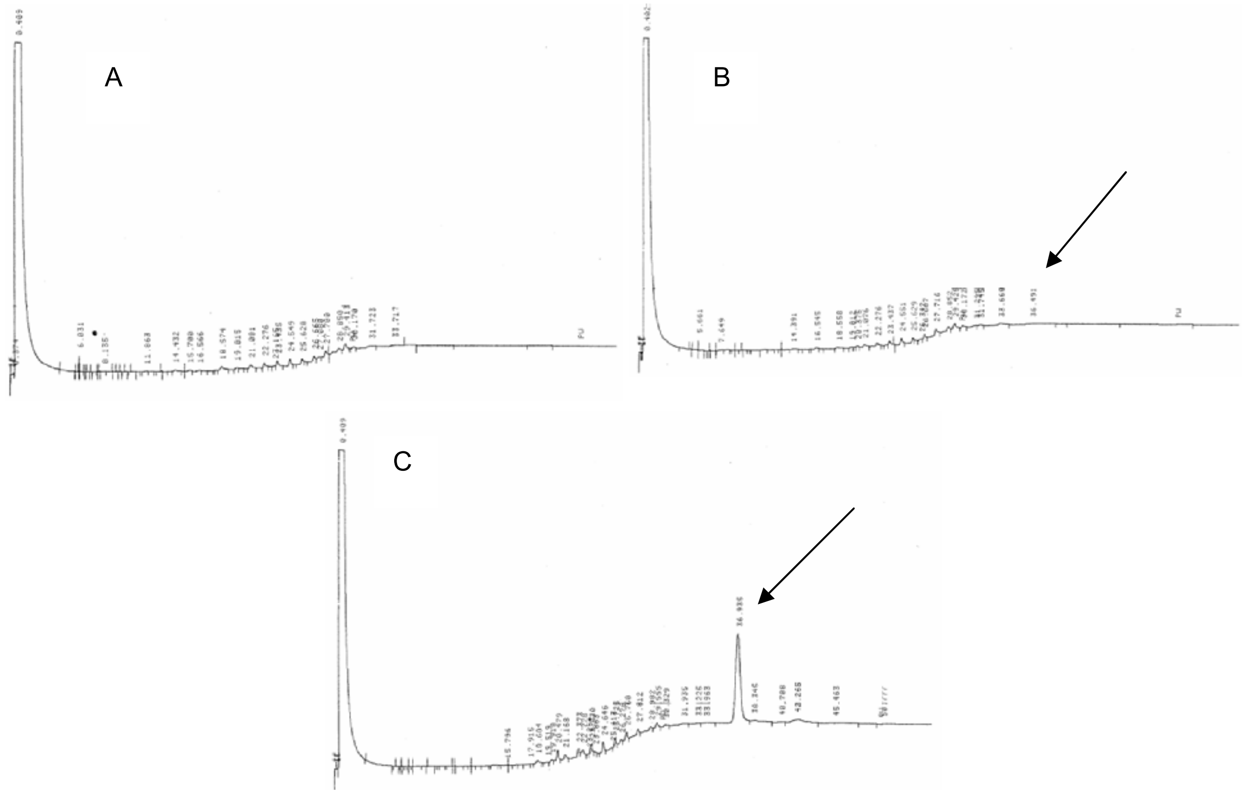

Figure 10: Examples of negative, positive, and trace integration plots.

The following equation was used to calculate the percent yield for the three types of isolated control tissues. This includes the area-to-mass correlation of 1ng B[a]P per 73,000 units of area which was determined by running B[a]P standards of known concentrations.

$$\frac{\text{area}}{1\ \mu\text{L sample injected}} \Bigg| \frac{1\text{ ng B[a]P}}{73{,}000\text{ units of area}} \Bigg| \frac{20\ \mu\text{L sample}}{4{,}000\text{ ng B[a]P}} \Bigg| \frac{100\%}{} = \%\text{ yield}$$

The following equation was used to calculate the approximate amount of B[a]P in positive and trace tissue and sediment samples for each site (M6, M9, and R1).

$$\frac{\text{area}}{1\ \mu\text{L sample injected}} \Bigg| \frac{1\text{ ng B[a]P}}{73{,}000\text{ units of area}} \Bigg| \frac{20\ \mu\text{L sample}}{\text{g sample}} = \frac{\text{ng}}{\text{g}} = \text{ppb}$$

Once all concentration data was calculated, a statistical analysis was carried out using SPSS (Statistical Package for the Social Sciences) to generate box-and-whisker plots to visualize variability in the data.

3 Results

3.1 *Controls*

The laboratory experiment which subjected three individuals to varying levels of B[a]P returned only trace amounts of B[a]P, in both tissue and water samples assessed from each beaker.

The analysis of spiked controls demonstrated that extraction of B[a]P from each crayfish tissue type was possible. There was a 70.5%, 55%, and 57.5% yield from blood, hepatopancreas, and muscle, respectively.

3.2 *River Samples*

Each of the three field sites were assessed for water, sediment, and tissue B[a]P concentrations. All sites returned negative results for B[a]P in water, while for all there were either trace or positive concentrations for sediments from each site (Figure 11). Although only one of the two sediment samples assessed for M6 showed B[a]P, this site had the highest average sediment concentration, at 392 ppb. M9 had the second greatest B[a]P concentration, with little difference between each sample.

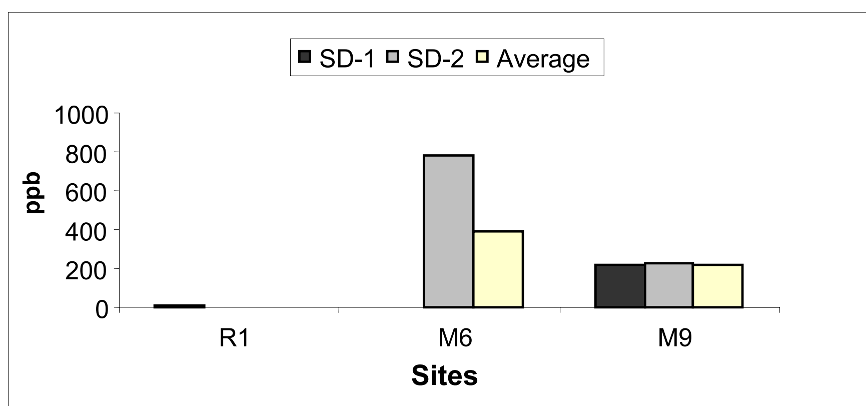

Figure 11: B[a]P concentration (ng B[a]P per gram of sample) in 2 sediment samples (SD-1, SD-2) from each field site.

Three tissue types were analyzed for B[a]P concentrations at each site. The total number of samples analyzed can be found in Table 2. On average, tissue samples from M6 showed a substantially higher concentration of B[a]P, as was also seen in sediment (above). No B[a]P was detected in hepatopancreas or muscle samples from R1, however there was one positive result for blood at this site. M9, on the other hand, showed low levels of positives for hepatopancreas and muscle; no B[a]P was detected in blood samples (Table 3).

Table 2: Number of samples for each site and tissue type.

	Blood	Hepatopancreas	Muscle
R1	10	9	10
M6	12	10	11
M9	9	9	9

Table 3: Average amount of B[a]P in ppb.

	Blood	Hepatopancreas	Muscle	Sediment
R1	222	0	0	4.41
M6	9150	9610	9450	392
M9	0	170	249	221

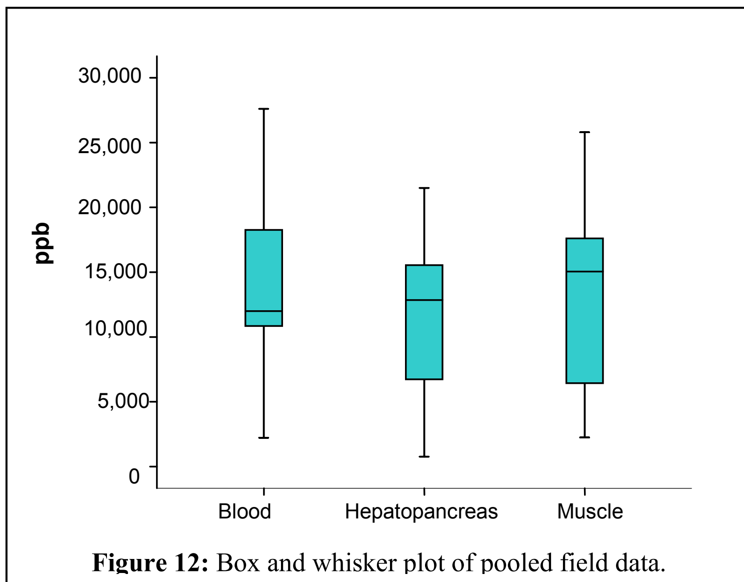

Figure 12: Box and whisker plot of pooled field data.

A box and whisker plot was formed by pooling data from all samples that gave a positive B[a]P signal from all sites (Figure 12). This analysis displayed no outliers, and little variation between tissue types.

Overall, when comparing tissue types, hepatopancreas had the greatest number of positive/trace samples for each field site. Muscle and blood were similar in numbers of positive/trace results (Figure 13).

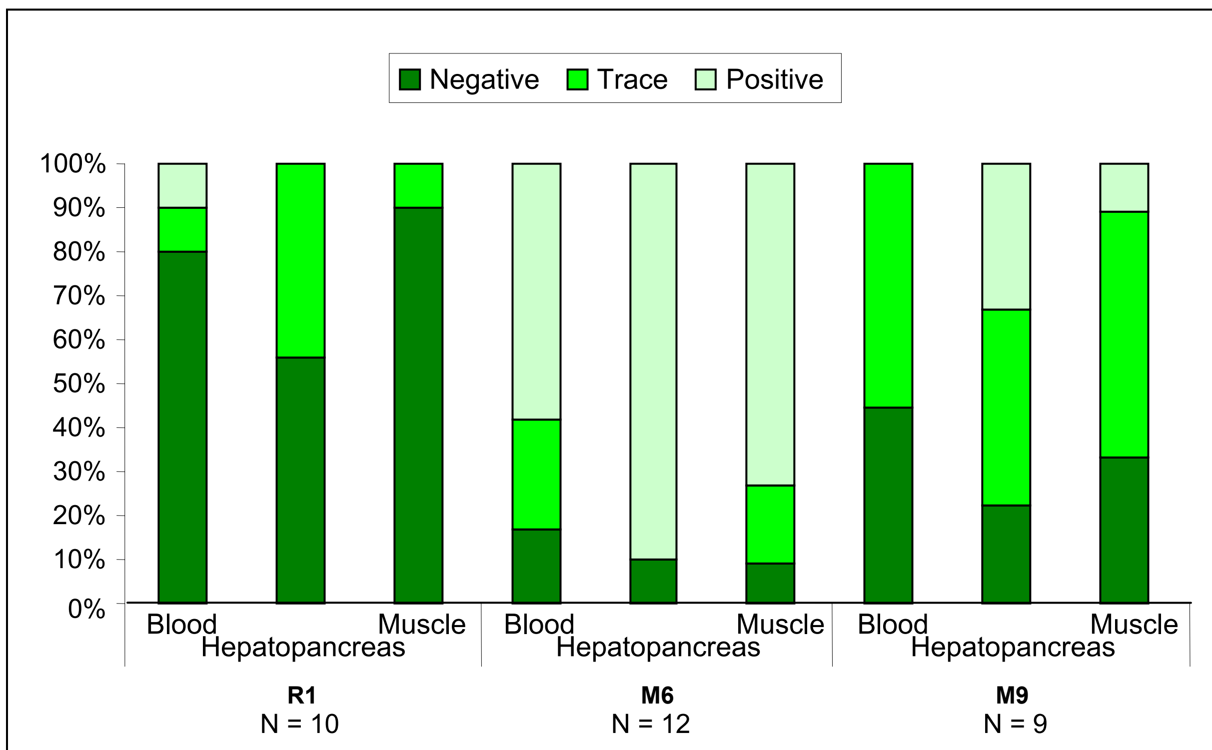

Figure 13: Percentage of positive, negative and trace results by site and tissue type.

4 **Discussion**

4.1 *Controls*

The procedure used for the extraction and analysis of B[a]P from crayfish tissue samples was shown to work by the positive results obtained from the isolation controls. PAHs recovered from fish tissue have been shown to range from seven to 76%, with a median recovery of 64% (Ozretich *et al.*, 1986). The procedure used here for extraction of PAHs from crayfish tissue was shown to extract no more than 70.5% of the actual B[a]P concentration, within the range of the Ozretich *et al*. study (1986). This would imply that actual B[a]P concentrations in samples may be at least 30% greater than B[a]P concentrations detected through gas chromatography.

Water samples from each of the individual controls, in which crayfish were subjected to one of three different concentrations of B[a]P (negative, 2 ppb B[a]P, 20 ppb B[a]P) detected no B[a]P. This is expected, as with all water samples, given that B[a]P is a hydrophobic, high molecular weight substance and therefore not likely to be present in detectable concentrations, in water (Wilson & Jones, 1993). It is characteristic of hydrophobic molecules to adhere to more hydrophobic surfaces, such as the side of the beakers, or crayfish excrements. This is probably one reason why B[a]P was not detected in any of the tissue samples isolated from the control crayfish. The primary source of PAHs in crayfish is likely to come from their diet, as they are detritus feeders, feeding in, or in close proximity to, sediment, where the hydrophobic PAHs accumulate. The control set ups contained only water and crayfish, and the absence of food and sediment from the environment neglected to provide the primary route of PAH exposure. The lack of detectable B[a]P in all types of tissue samples taken from the control crayfish suggests that contaminated water alone is not sufficient to result in accumulation of B[a]P. An alternative design for such a control could be to provide a more realistic artificial environment that includes both sediment and food. Captive-born crayfish populations could be reared in environments with varying concentrations of B[a]P and used for a variety of research projects.

4.2 *Field samples*

All water samples analyzed – even those known to have B[a]P concentrations such as in the individual controls – were negative. As explained in the previous paragraph, the hydrophobic nature of B[a]P accounts for the lack of B[a]P detected in water samples. A positive control involving the direct addition of B[a]P to sediment or water samples immediately before extraction (as in the isolated tissue controls) was not carried out.

Analysis of sediment samples demonstrated either positive or trace concentrations for each of the three sites assessed. This again relates to the hydrophobic properties of B[a]P and its affinity for surfaces, including sediments (Tolosa *et al*., 2004). The positive results from M6 and M9 were expected, as these sites may have been subjected to heavy pollution. As previously discussed, M9 is located directly under a highway bridge and adjacent to a rail road. These settings have been shown as sources of PAHs, B[a]P especially, and therefore it is reasonable to conclude that the M9 location would show the levels of B[a]P discovered in this study (Harrison *et al*., 1996). The M6 site, directly below Lake Ripple in Grafton, is further out of the city than

M9 and has fewer signs of any local industrialization – historical or current. The lake, however, is directly downstream of an industrial area (for example, Washington Mills), and has been shown to hold contaminated sediments (CMG Environmental Inc., 2005). The recent dredging of this lake may have disturbed those pollutants which were not taken with the dredged sediment, and allowed them into the water column, free to move downstream and eventually settle back into the substrate (Thibodeaux & Duckworth, 2001). Dredging is a legitimate concern as a source of pollutants – studies have shown pollutants which had been disturbed and released, having been detected further downstream days to months later (Rice & White, 1987). An abnormal 'dirty' background was also observed in M6 samples. Many of the samples taken from the refrigerator appeared solid, but 'melted' in less than a minute. Most of these samples correspond with an offscale background where individual peaks could not be seen, as demonstrated in Figure 14. This background may be the result of another pollutant not studied here, however should be considered when analyzing samples from M6.

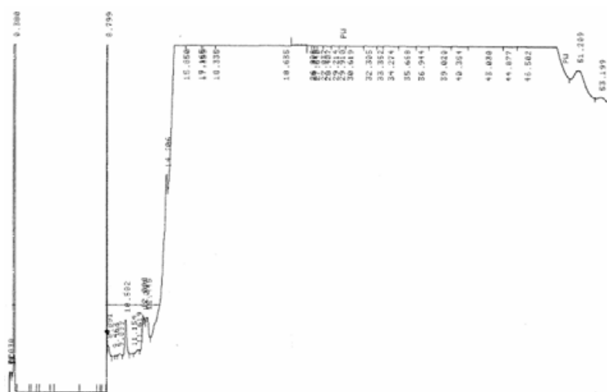

Figure 14: Example of an offscale/dirty background from M6.

Determining where in the body of a crayfish B[a]P tends to accumulate is valuable not only to develop a more accurate procedure, but also for assessing the potential danger of the bioaccumulation of PAHs. For example, concern should be raised if results were to suggest that substantial concentrations of B[a]P were detected in crayfish digestive tissues– one tissue used for human consumption (Briggs-Reed et al., 1997). All positive field samples were pooled according to tissue type, as shown in Figure 12. The median B[a]P concentration was similar in all tissues; although further comparison of our individual crayfish tissue data is likely to show inconsistent correlations between tissue samples in the same individual. This cannot be commented on quantitatively here, as there were not enough individuals that had all positive tissue types. However, our data indicate that all tissue types are capable of accumulating similar concentrations of B[a]P. Figure 13 shows the percent of samples from each tissue type that displayed either a negative, trace or positive amount of B[a]P. Hepatopancreas samples generated the most positive results overall followed by muscle and blood. This may be a consequence of the role of this tissue plays in detoxification, much like a human liver. Xenobiotics such as B[a]P are likely to accumulate in the hepatopancreas as it takes time for them to break down. Unmetabolized B[a]P has been found in the hepatopancreas of blue crabs and lobsters as long as 6.5 weeks after removal of the contaminated source (Lee et al., 1976; Little et al., 1985). This demonstrates that B[a]P is stored for extended times in the hepatopancreas, as determined in this study.

However, though more positive results, and higher concentrations of B[a]P were detected in hepatopancreas samples, it is equally important to investigate the detection of B[a]P in muscle. It was expected that out of the three tissues sampled, the hepatopancreas would be most like to contain detectable PAHs. This is due to its direct correlation to the digestive system, which is the primary route of PAH uptake in crayfish. Along with its use for human consumption, muscle tissue is not related to the digestive system and primary route of PAHs, as the hepatopancreas is. Therefore, the fact that B[a]P has accumulated in muscle, however small the concentrations, suggests another exposure route such as long-term bioaccumulation.

4.3 *Possible Errors*

There are several areas of this research that could be improved and may have contributed to errors. First, not enough sediment samples were collected to determine an accurate estimation of B[a]P concentration. This was apparent in the M6 site which showed high concentrations of B[a]P for one sediment sample taken, but no evidence of any B[a]P in the second. In further research it may be beneficial to note exactly which part of the river each sample was collected from, and collect from several areas – shore, middle, surface, and several inches deeper, for example. A second possible area of error was in the small sample sizes for R1 and M9. These samples were too small for any significant statistical analysis with only one to four positive samples. Thirdly, the M6 site may have produced unreliable data based on the offscale/dirty background mentioned previously. This background may have skewed results and not allowed the full concentration of B[a]P to be determined. Finally, the integrator program was changed slightly because of build-up on the column. Although the differences in programs were small, this change may have influenced data generated after this change (old and current integrator programs are denoted in Appendix A).

4.4 *Final Conclusions and Additional Research*

Along with verifying the procedures used to extract and analyze PAHs, our research has shown that crayfish can be used to detect B[a]P in an environment, and therefore have the potential for acting as bioindicators of PAH contamination. This provides a good base for an array of future research projects, such as looking further into which tissue type accumulates the majority of PAHs. Higher sample sizes from the sites could provide for better statistical analysis when comparing environments with carrying levels of PAH contamination. The analysis of a greater number of sediment samples would also assist in better determining the level of PAH contamination within the river and how that concentration correlates to crayfish tissue samples. A true positive control of sediment and water, similar to those carried out in the isolated tissue controls, would allow for a determination of percent yield in these substances, and a possible confirmation of procedures. Future laboratory research could involve rearing a series of populations of captive-born crayfish in different amounts of PAH contamination; such an experimental design would allow for a controlled environment to determine the primary route of PAH uptake in crayfish as well as a study of bioaccumulation. A qualitative observation of the samples studied here shows a possible connection between crayfish size and B[a]P concentration in tissue. Since it has also been shown that crayfish size and age correlate (Harm, 2002), it is

possible that those crayfish collected in M9, which were comparatively small, were young-of-the-year and therefore did not have the opportunity to accumulate as much B[a]P as those collected at M6, which were substantially larger, and may have included crayfish in their second year. A quantitative study assessing at the size and/or age of crayfish and B[a]P concentrations would therefore be useful.

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Appendix A: Raw Data

Table A1: Raw data for tissue, sediment, and water; from all sites and controls, samples not listed here were negative.

Sample[1]				Retention Time	Area	Rounded Retention Time[2]	Rounded Area[2]	ng B[a]P	ng B[a]P, rounded[2]	Integrator Program[3]	Notes[4]		
R1	OV	4	B	36.848	600,000	36.80	600,000	8.219	8.220	Old	Clean		
R1	OV	6	H	36.482	101,947	36.50	102,000	1.397	1.400	Old	Clean	Trace	
R1	OV	8	H	36.442	7,868,160	36.40	7,870,000	107.808	108.000	Old	Clean	Trace	
R1	OV	8	M	37.183	200,000	37.20	200,000	2.740	2.740	Old	Clean	Trace	
R1	OV	9	B	37.165	100,000	37.20	100,000	1.370	1.370	Old	Clean	Trace	
R1	OV	9	H	37.225	21,621,760	37.20	21,600,000	295.890	296.000	Old	Clean	Trace	
R1	OV	10	H	37.329	40,989,568	37.30	41,000,000	561.644	562.000	Old	Clean	Trace	
M6	OV	1	B	37.278	200000	37.30	200,000	2.740	2.740	Old	Clean	Trace	
M6	OV	1	H	36.033	300000	36.00	300,000	4.110	4.110	Old	Off scale		
M6	OV	1	M	36.624	200000	36.60	200,000	2.740	2.740	Old	Clean	Trace	
M6	OV	2	B	37.257	200000	37.30	200,000	2.740	2.740	Old	Clean	Trace	
M6	OV	2	H	36.848	3513138	36.80	3,510,000	48.082	48.100	Old	Off scale		
M6	OV	3	B	36.765	2478558	36.80	2,480,000	33.973	34.000	Old	Off scale	Trace	
M6	OV	3	H	37.327	200000	37.30	200,000	2.740	2.740	Old	Clean		
M6	OV	3	M	37.149	200000	37.10	200,000	2.740	2.740	Old	Clean	Trace	
M6	OV	4	H	36.916	3213142	36.90	3,210,000	43.973	44.000	Old	Off scale		'solid'
M6	OV	4	M	36.865	2867848	36.90	2,870,000	39.315	39.300	Old	Off scale		'solid'
M6	OV	5	B	36.825	3475133	36.80	3,480,000	47.671	47.700	Old	Off scale		'solid'
M6	OV	5	H	36.956	2778165	37.00	2,780,000	38.082	38.100	Old	Off scale		'solid'
M6	OV	5	M	36.950	3494536	37.00	3,490,000	47.808	47.800	Old	Off scale		'solid'
M6	OV	6	B	36.981	2669125	37.00	2,670,000	36.575	36.600	Old	Off scale		'solid'
M6	OV	6	H	36.860	86100608	36.90	86,100,000	1,179.452	1180.000	Old	Off scale		'solid'

[1] Samples labeled as: Site, Species, Specimen Number, Tissue Type, or Site, Sediment/Water, Sample Number
[2] Rounded to three significant figures.
[3] Denotes which of the two programs were used for specified sample.
[4] Clean/Off scale – 'Dirty Background' ; Trace – No visible peak on integration plots ; 'solid' – these samples appeared solid when removed from refrigerator.

Sample				Retention Time	Area	Rounded Retention Time	Rounded Area	ng B[a]P	ng B[a]P, rounded	Integrator Program	Notes		
M6	OV	6	M	36.959	3097925	37.00	3,100,000	42.466	42.500	Old	Off scale		‘solid’
M6	OV	7	B	36.980	2844502	37.00	2,840,000	38.904	38.900	Old	Off scale		‘solid’
M6	OV	7	H	36.973	5816458	37.00	5,820,000	79.726	79.700	Current	Off scale		‘solid’
M6	OV	7	M	36.997	3447997	37.00	3,450,000	47.260	47.300	Old	Off scale		‘solid’
M6	OV	8	B	36.934	4920403	36.90	4,920,000	67.397	67.400	Current	Off scale		‘solid’
M6	OV	8	H	36.944	3019862	36.90	3,020,000	41.370	41.400	Current	Off scale		‘solid’
M6	OV	8	M	37.025	6025354	37.00	6,030,000	82.603	82.600	Old	Off scale		
M6	OV	9	B	37.094	5645139	37.10	5,650,000	77.397	77.400	Current	Off scale		
M6	OV	9	H	36.972	3949294	37.00	3,950,000	54.110	54.100	Current	Off scale		‘solid’
M6	OV	10	B	36.825	2398934	36.80	2,400,000	32.877	32.900	Current	Off scale		‘solid’
M6	OV	10	H	36.585	1196749	36.60	1,200,000	16.438	16.400	Current	Off scale		‘solid’
M6	OV	10a	M	37.032	5705728	37.00	5,710,000	78.219	78.200	Current	Off scale		
M6	OV	10b	M	36.650	2385638	36.70	2,390,000	32.740	32.700	Current	Off scale		‘solid’
M6	OV	11	B	36.818	2883064	36.80	2,880,000	39.452	39.500	Current	Off scale		‘solid’
M6	OV	11	M	36.732	1802931	36.70	1,800,000	24.658	24.700	Current	Off scale		‘solid’
M9	OV	3	H	36.963	80768	37.00	80,800	1.107	1.110	Current	Clean	Trace	
M9	OV	3	M	36.476	88593	36.50	88,600	1.214	1.210	Current	Clean	Trace	
M9	OV	4	H	36.33	293017	36.30	293,000	4.014	4.010	Current	Clean		
M9	OV	4	M	36.511	172697	36.50	173,000	2.370	2.370	Current	Clean	Trace	
M9	OV	5	B	36.305	130103	36.30	130,000	1.781	1.780	Current	Clean	Trace	
M9	OV	5	H	36.343	44210	36.30	44,200	0.605	0.610	Current	Clean		
M9	OV	5	M	36.588	207150	36.60	207,000	2.836	2.840	Current	Clean	Trace	
M9	OV	6	B	36.318	248989	36.30	249,000	3.411	3.410	Current	Clean	Trace	
M9	OV	6	H	36.307	40955	36.30	41,000	0.562	0.560	Current	Clean		
M9	OV	6	M	36.328	262065	36.30	262,000	3.589	3.590	Current	Clean		
M9	OV	8	B	36.31	200942	36.30	201,000	2.753	2.750	Current	Clean	Trace	
M9	OV	8	H	36.317	31433	36.30	31,400	0.430	0.430	Current	Clean	Trace	
M9	OV	8	M	36.348	70867	36.30	70,900	0.971	0.970	Current	Clean	Trace	
M9	OV	9	B	36.091	61216	36.10	61,200	0.838	0.840	Current	Clean	Trace	
M9	OV	9	M	36.041	66762	36.00	66,800	0.915	0.920	Current	Clean	Trace	

Sample				Retention Time	Area	Rounded Retention Time	Rounded Area	ng B[a]P	ng B[a]P, rounded	Integrator Program	Notes		
M9	OV	10	B	36.168	170103	36.20	170,000	2.329	2.330	Current	Clean	Trace	
M9	OV	11	H	36.793	54246	36.80	54,200	0.742	0.740	Current	Clean	Trace	
M9	OV	12	H	36.491	193088	36.50	193,000	2.644	2.640	Current	Clean	Trace	
R1	SD	1		36.375	323268	36.40	323,000	4.425	4.420	Current	Clean		
R1	SD	2		36.374	90973	36.40	91,000	1.247	1.250				
M6	SD	1		36.189	195242	36.20	195,000	2.671	2.670	Current	Clean		
M6	SD	2		36.563	30205568	36.60	30,200,000	413.699	414.000	Current	Off scale		
M9	SD	1		36.726	8662022	36.70	8,660,000	118.630	119.000	Current	Clean		
M9	SD	2		36.686	5249034	36.70	5,250,000	71.918	71.900	Current	Clean		
2ppb			B	36.712	49540	36.70	49,500	0.678	0.680	Current	Clean		
2ppb			M	36.643	104576	36.60	105,000	1.438	1.440	Current	Clean		
spike			B	36.978	10254400	37.00	10,300,000	141.096	141.000	Current	Clean		
spike			H	36.935	8051824	36.90	8,050,000	110.274	110.000	Current	Clean		
spike			M	36.939	8407654	36.90	8,410,000	115.205	115.000	Current	Clean		
neg			M	36.514	75891	36.50	75,900	1.040	1.040	Current	Clean		
neg	Water			36.397	84381	36.40	84,400	1.156	1.160	Current	Clean		
2ppb	Water			36.354	124226	36.40	124,000	1.699	1.700	Current	Clean		

Table A2: Raw data and calculations of ppb for all clean, positive samples.

Sample				Retention Time	Area	Rounded Retention Time	Rounded Area	ng B[a]P	ng B[a]P, rounded	ng*20microL	Original Sample (g)	ngBAP/g of Sample	PPB (Rounded)
R1	OV	4	B	36.848	600,000	36.80	600,000	8.219	8.2	164	0.074	2,221.622	2,220
M6	OV	1	H	36.033	300,000	36.00	300,000	4.110	4.1	82	0.060	1,370.000	1,370
M6	OV	2	H	36.848	3,513,138	36.80	3,510,000	48.082	48.1	962	0.064	15,031.250	15,000
M6	OV	3	H	37.327	200,000	37.30	200,000	2.740	2.7	55	0.071	771.831	772
M6	OV	4	H	36.916	3,213,142	36.90	3,210,000	43.973	44.0	880	0.069	12,753.623	12,800
M6	OV	4	M	36.865	2,867,848	36.90	2,870,000	39.315	39.3	786	0.08	9,825.000	9,830
M6	OV	5	B	36.825	3,475,133	36.80	3,480,000	47.671	47.7	954	0.062	15,387.097	15,400
M6	OV	5	H	36.956	2,778,165	37.00	2,780,000	38.082	38.1	762	0.063	12,095.238	12,100
M6	OV	5	M	36.950	3,494,536	37.00	3,490,000	47.808	47.8	956	0.062	15,419.355	15,400
M6	OV	6	B	36.981	2,669,125	37.00	2,670,000	36.575	36.6	732	0.065	11,261.538	11,300
M6	OV	6	H	36.860	86,100,608	36.90	86,100,000	1,179.452	1180.0	23,600	0.066	357,575.758	358,000
M6	OV	6	M	36.959	3,097,925	37.00	3,100,000	42.466	42.5	850	0.058	14,655.172	14,700
M6	OV	7	B	36.980	2,844,502	37.00	2,840,000	38.904	38.9	778	0.073	10,657.534	10,700
M6	OV	7	H	36.973	5,816,458	37.00	5,820,000	79.726	79.7	1,594	0.074	21,540.541	21,500
M6	OV	7	M	36.997	3,447,997	37.00	3,450,000	47.260	47.3	946	0.059	16,033.898	16,000
M6	OV	8	B	36.934	4,920,403	36.90	4,920,000	67.397	67.4	1,348	0.064	21,062.500	21,100
M6	OV	8	H	36.944	3,019,862	36.90	3,020,000	41.370	41.4	828	0.064	12,937.500	12,900
M6	OV	8	M	37.025	6,025,354	37.00	6,030,000	82.603	82.6	1,652	0.064	25,812.500	25,800
M6	OV	9	B	37.094	5,645,139	37.10	5,650,000	77.397	77.4	1,548	0.056	27,642.857	27,600
M6	OV	9	H	36.972	3,949,294	37.00	3,950,000	54.110	54.1	1,082	0.067	16,149.254	16,100
M6	OV	10	B	36.825	2,398,934	36.80	2,400,000	32.877	32.9	658	0.06	10,966.667	11,000
M6	OV	10	H	36.585	1,196,749	36.60	1,200,000	16.438	16.4	328	0.091	3,604.396	3,600
M6	OV	10b	M	36.650	2,385,638	36.70	2,390,000	32.740	32.7	654	0.034	19,235.294	19,200
M6	OV	11	B	36.818	2,883,064	36.80	2,880,000	39.452	39.5	790	0.062	12,741.935	12,700
M6	OV	11	M	36.732	1,802,931	36.70	1,800,000	24.658	24.7	494	0.162	3,049.383	3,050
M9	OV	4	H	36.330	293,017	36.30	293,000	4.014	4.0	80	0.07	1,145.714	1,150
M9	OV	5	H	36.343	44,210	36.30	44,200	0.605	0.6	12	0.057	214.035	214
M9	OV	6	H	36.307	40,955	36.30	41,000	0.562	0.6	11	0.065	172.308	172

Sample				Retention Time	Area	Rounded Retention Time	Rounded Area	ng B[a]P	ng B[a]P, rounded	ng*20microL	Original Sample (g)	ngBAP/g of Sample	PPB (Rounded)
M9	OV	6	M	36.328	262,065	36.30	262,000	3.589	3.6	72	0.032	2,243.750	2,240
R1	SD	1		36.375	323268	36.40	323000	4.425	4.42	88	10.028	8.815317112	8.82
M9	SD	1		36.935	8051824	36.90	8050000	110.274	110	2,200	10.06	218.6878728	219
M9	SD	2		36.939	8407654	36.90	8410000	115.205	115	2,300	10.312	223.0411171	223
M6	SD	2		36.563	30205568	36.60	30200000	413.699	414.0	8,280	10.557	784.3137255	784
isolation	OV		B	36.978	10254400	36.80	10300000	141.096	141.0	2,820	0.063000	44761.90476	44,800
isolation	OV		H	36.935	8051824	36.30	8050000	110.274	110.0	2,200	0.069000	31884.05797	31,900
isolation	OV		M	36.939	8407654	36.70	8410000	115.205	115.0	2,300	0.070000	32857.14286	32,900

Table A3: Table of Retention times for the 16 EPA priority PAHs.

Compound	Retention Times										
Napthalene	6.714	6.194	5.847	6.043	6.212	6.702	6.739	6.288			
Acenapthylene	13.175	12.903	12.892	12.97	13.015	13.162	13.167	13.08			
Acenapthene	13.705	13.45	13.519	13.56	13.607	13.739	13.733	13.641			
Fluorene	15.474	15.253	15.36	15.457	15.485	15.57	15.566	15.465			
Phenanthrene + Anthracene	19.123	18.884	19.061	19.065	19.162	19.09	19.047	19.141	19.135	19.18	19.19
	19.225	19.195	19.173								
Fluoranthene	23.15	22.956	23.14	23.249	23.251	23.273	23.235				
Pyrene	24.005	23.81	24.005	24.206	24.175	24.205	24.112	24.054	24.143	24.15	24.108
	24.11	24.129									
Benz[a]antracene	28.212	27.983	28.216	28.328	28.33	28.344	28.349	28.32			
Chrysene	28.455	28.05	28.4	28.57	28.573	28.586	28.562				
Benzo[b]fluoranthene + benzo[k]fluoranthene	33.865	33.318	33.781	34.042	34.056	34.059	34.075	34.035			
Benzo[a]pyrene	36.56	35.869	36.017	36.441	36.804	37.045	36.93	37.01	36.765	36.839	36.852
	36.875	36.764	36.771	36.787	36.802	36.795	36.773				
Dibenz[a,h]anthracene + Indeno[1,2,3-c,d]pyrene	49.741	47.774	48.84	49.535	49.569	49.539	49.613	49.724			
Benzo[ghi]perylene	53.568	51.791	53.099	53.915	53.935	53.925	53.991				