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Benzo[a]pyrene in River Sediment

James R. Letourneux

Worcester Polytechnic Institute

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Benzo[a]pyrene in River Sediment

Major Qualifying Project

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

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Submitted by:
James R. Letourneux

Submitted to:
Professor JoAnn Whitesheet-Smith

April 30, 2009
Abstract

In continuation of ongoing research exploring the causes, levels and effects of industrial pollutants in local waterways, this project focuses on methods of improving aspects of a bioindicator protocol developed for the detection of benzo[a]pyrene (B[a]P), a procarcinogen identified in a local waterway. A hexane based extraction segment of the protocol was analyzed, and its efficiency determined for a range of sediment types. The effect of exposure time on a range of sediment types to B[a]P-contaminated water was investigated. Extraction efficiencies ranged from 16.5-40.6%, with coarser sediment generally yielding higher recovery. Sediment exposed to B[a]P-contaminated water for 1 week showed decreased yields as compared to 24-hour exposure (8.0-11.9% vs. 32.2-37.1%). Hydrophobic exclusion from water and concurrent association with sediment particles and adsorption into surface grooves was theorized to explain results.
Acknowledgments

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Table of Contents
Abstract .......................................................................................................................... 2
Acknowledgments ........................................................................................................... 3
Table of Figures .............................................................................................................. 4
Table of Tables ............................................................................................................... 5
I. Introduction and Background ...................................................................................... 6
   I A. Bioindicators ........................................................................................................ 6
   I B. Trophic Transfer and Bioaccumulation ............................................................... 6
   I C. Polynuclear Aromatic Hydrocarbons and Benzo[a]pyrene .................................. 6
   I D. Benzo[a]pyrene Bioindicators ........................................................................... 7
   I E. Extraction Protocol Efficiency ........................................................................... 8
   I F. Transfer of B[a]P from Water to Sediment ........................................................ 9
II. Methods ..................................................................................................................... 11
   II A. Gas Chromatography ....................................................................................... 11
   II B. Establishing a Standard ..................................................................................... 12
   II C. Extraction Efficiency ......................................................................................... 12
   II D. Transfer of B[a]P from Water to Sediment ..................................................... 13
III. Results ....................................................................................................................... 14
   III A. Establishing a Standard ................................................................................... 14
   III B. B[a]P Retention Time ..................................................................................... 15
   III C. Extraction Efficiency ....................................................................................... 16
   III D. Transfer of B[a]P from Water to Sediment ..................................................... 17
IV. Discussion .................................................................................................................. 18
   IV A. B[a]P Retention Time ...................................................................................... 18
   IV B. Extraction Efficiency ......................................................................................... 19
   IV C. B[a]P Transfer from Water to Sediment .......................................................... 19
   IV D. Future Work ...................................................................................................... 21
Appendix A – Detailed Methodology ............................................................................ 22
Appendix B – Raw Data Tables .................................................................................... 25
References ..................................................................................................................... 26

Table of Figures
Figure I-1: Bioaccumulation and biomagnification ....................................................... 6
Figure I-2: Benzo[a]pyrene ......................................................................................... 7
Figure I-3: Relationship between surface area and volume ........................................... 8
Figure III-1: Standard curve ........................................................................................ 14
Figure III-2: B[a]P retention times .............................................................................. 16
Figure III-3: Average % recovery from sediments ...................................................... 16
Figure III-4: Average % B[a]P transfer from water to sediment .................................... 17
Figure IV-1: March 31, 2009 retention times ............................................................... 18
Figure IV-2: April 11, 2009 retention times ................................................................. 18
Figure IV-3: April 13, 2009 retention times ................................................................. 18
Figure IV-4: Soxhlet extraction apparatus ................................................................... 21
Figure A-1: Aeration unit ............................................................................................. 24
Table of Tables

Table III-1: Syringe comparison results ................................................................. 14
Table III-2: Extraction efficiency data .................................................................. 17
Table III-3: % Recovery from transfer samples ..................................................... 17
Table B-1: 24-hour transfer data ............................................................... 25
Table B-2: 1-week transfer data ........................................................................ 25
Table B-3: Normalized 24-hour transfer data ...................................................... 26
Table B-4: Normalized 1-week transfer data ....................................................... 26
I. Introduction and Background

In continuation of ongoing research exploring the causes, levels and effects of industrial pollutants in local waterways, this project focused on methods of improving aspects of a bioindicator protocol developed for the detection of benzo[a]pyrene (B[a]P), a polynuclear aromatic hydrocarbon, identified in the Blackstone Valley River.

IA. Bioindicators

A bioindicator is an organism that accumulates a substance of interest, which can be extracted from the organism and analyzed. Certain compounds present in an environment may become incorporated into the bodies of these organisms, primarily by means of feeding, which can then be dissected and the compounds extracted and analyzed in the lab. Such an organism is a suitable bioindicator of that compound. Bioindicator study can yield important information about the quantities, locations and nature of environmental pollutants.

IB. Trophic Transfer and Bioaccumulation

It is possible to use bioindicators to study an environment due to bioaccumulation and biomagnification. Methylmercury (MeHg) contamination in fresh water bodies is a well characterized example of this. Mere trace amounts of MeHg may be present in a water body, but the crux of the issue is that any MeHg that is consumed by members of the ecosystem’s food chain is not expelled from the organisms as waste. Remaining trapped in the organism, MeHg accumulates during its lifespan. Any predators in higher trophic levels that consume contaminated organisms of lower trophic levels will see the same accumulation effect in their own bodies, only magnified (Figure I-1). It was shown that such higher predators, including carnivorous fish and birds, fall victim to higher MeHg exposure than organisms which consume members of lower trophic orders, such as baleen whales, due to this biomagnification. The bioaccumulation effect can be as drastic as a concentration increase of five orders of magnitude. One study showed an increase from 1% to 10% MeHg contamination between water and phytoplankton. Being positioned at the top of most food chains, humans are directly susceptible to these magnification effects.

IC. Polynuclear Aromatic Hydrocarbons and Benzo[a]pyrene

In addition to mercury contamination, many waterways and water supplies fall victim to pollution from industrial run-off and automobile waste products, some of the major constituents
of which are polynuclear aromatic hydrocarbons (PAHs). PAHs compose a large family of over 10,000 molecules, all of which are hydrophobic hydrocarbons with at least two aromatic ring structures. Various PAHs are commonly used in asphalt, oils, mothballs, and many other useful products, and most are not known to cause ailments such as cancer, though relatively few are well characterized.

The particular PAH of interest to this project is benzo[a]pyrene (B[a]P), shown in Figure 1-2. B[a]P is produced when organic matter is incompletely combusted, be it through industry, automobile use, or any other combustion process, including the burning of cigarettes. Unlike many other PAHs, B[a]P is a well characterized procarcinogen, its metabolites shown to cause lung and skin cancer in laboratory animals and to enhance human papillomavirus synthesis in humans.

B[a]P’s mechanism of carcinogenicity involves enzymatic conversion of B[a]P to benzo[a]pyrene diol epoxide followed by intercalation and covalent adduction of the planar molecule between nucleotide bases in DNA. During their normal processes, DNA replication and repair enzymes may encounter one of these intercalated molecules and mistake it for a nucleotide base, adding or neglecting to add the appropriate base and resulting in a point mutation in the genetic code. Over time, these mutations may occur in key locations along the genome of an organism, causing certain genes to become overly expressed or repressed and leading to constituent cell proliferation, i.e. cancer. B[a]P has been strongly linked to mutations in the p53 gene, a tumor suppressor gene which regulates cell death. It has also been shown that treating cultured human bronchial epithelial cells with metabolites of B[a]P (such as B[a]P diol epoxide) will cause the cells to show the same types of mutations on the p53 gene as cells extracted from lung tumors. In addition to its carcinogenic properties, B[a]P has been shown to enhance the onset of abdominal aortic aneurysms in mice, a condition long known to be associated with cigarette smoking in the elderly.

1D. Benzo[a]pyrene Bioindicators

The presence of B[a]P has been detected in the Blackstone Valley River, along which many industries have developed during the past two hundred years. Previous work has tied areas of high run-off into the river to increased contamination by B[a]P. In the same way that
certain organisms may be used to monitor MeHg movement through an ecosystem, an organism that accumulates B[a]P may be used as an indicator of contamination in the Blackstone Valley River. The crayfish *Orconectes virilis* has been targeted for B[a]P bioindicator studies at Worcester Polytechnic Institute. As a bottom feeder of the Blackstone River, *O. virilis* maintains a constant exposure to water and sediment, much of which is contaminated, and has been shown to accumulate B[a]P in the hepatopancreas and tail muscle tissue.\(^9\)

### I E. Extraction Protocol Efficiency

In line with this research, protocols for B[a]P extraction from *O. virilis* tissues and from riverbed sediment were designed. Though the extraction protocols were successful in isolating B[a]P from either environmental source, little information was gained about the efficiency of the protocol. It thus became one of this project’s goals to analyze the extraction’s efficacy by “spiking” a mass of PAH-free sediment with B[a]P, carrying out the extraction, and comparing the protocol’s percent yield to the original amount of B[a]P introduced to the sediment. Though the procedure in this project was carried out in the previous study, the sediment that was spiked was not first cleared of B[a]P or other PAHs, and final results relied on subtraction and estimation. Addressing this question required the selection of variables to be studied. Possible variables included sediment exposure time to B[a]P, amount of B[a]P introduced to sediment, amount of sediment, and sediment texture, though others surely exist. The variable whose effect on extraction efficiency was least intuitive was chosen to be analyzed: sediment texture. Three classes of texture, fine-grain, medium-grain and coarse-grain, were decided upon for the experiment. Other variables such as sediment amount and amount of B[a]P added to sediment were kept constant. Since B[a]P is a hydrophobic molecule, it associates with sediment rather than water, and should therefore be localized to the surfaces of sediment particulates in the spiked samples. Hypothesizing which sediment type would yield the best recovery can be aided by some simple mathematics. Given two sets of spherical objects with the same total surface area (\(SA = 4\pi r^2\)), where each sphere in one set (set A) has radius \(r\) and the other set (set B) \(r/2\), set A

![Figure I-3: Relationship between volume and surface area of two sets of differently sized spheres with radius differing by a factor of two.](image-url)
will occupy twice the volume of set B. Expressed differently, two such sets of spheres with equal volume will differ in surface area by a factor of 2. Thus, a sample of coarse-grain sediment will possess less surface area than a sample of fine-grain sediment of the same volume, and will therefore have less area available for B[a]P adsorption.

However, the samples were measured out by mass, not volume, so density must be taken into account as well. Ignoring differences in the chemical density of each constituent particle of the sediments, and dealing strictly with packing density (i.e. treating each sediment particle as the smallest relevant particle), set B will be denser than set A (Figure I-3). This conclusion can be reached intuitively in two ways. First, approximately 72 spheres from set B occupy the same volume as 9 spheres from set A. Alternatively, exclusion area can be considered. A theoretical sphere set (arranged as those in Figure I-3) with zero exclusion volume would require infinitesimally small spheres to comprise it, while a theoretical sphere set with infinite exclusion volume would require infinitely large spheres to comprise it. Again, ignoring individual particle density, the former would have a greater packing density than the latter. Given these considerations, it was expected that extraction from coarser-grained sediments would yield more B[a]P than from finer-grained sediments, which have more surface area on which to hold B[a]P, and therefore more surface area from which to attempt to, and possibly fail to, recover B[a]P.

I F. Transfer of B[a]P from Water to Sediment

When considering the real-life situation of B[a]P contamination in any given river, a major aspect in understanding the amount of B[a]P in any given sediment sample concerns the transfer of B[a]P from contaminated water to the sediment itself. Two major variables theorized to significantly affect the amount of B[a]P deposited to sediment were chosen to be tested: sediment texture and exposure time to contaminated water. Since B[a]P is a hydrophobic molecule, it will preferentially associate with other hydrophobic particles, and segregate apart from hydrophilic ones. It is thermodynamically more favorable for B[a]P to associate with sediment particles than with water. By the same argument established in section D above, finer-grained sediments have more available surface onto which B[a]P may be adsorbed than does coarser sediment. Thus, it was anticipated that finer-grained sediment would adsorb more B[a]P than coarse sediment. Since it of course takes time for such adsorption to carry through to completion, sediment exposed to B[a]P-contaminated water for a long time period would be expected to adsorb more B[a]P than sediment exposed to water for only a short time.

The sediment classes already discussed were chosen for use in this experiment, with 10 g of each sediment apportioned for the study (in duplicate). In determining the lengths of time the sediments should be exposed to B[a]P-contaminated water, real-life scenarios were considered. In a setting such at that from where the sediment samples were originally collected (the Blackstone River), a short exposure time would correspond to a passing rain shower that might wash B[a]P from roadways and industrial sites into the river, and upon ending, the influx of B[a]P would cease. A long exposure time would arise if multiple rain storms passed through the area over about a week, or if a very large rainstorm persisted in the area. B[a]P deposited into the water of a river would be washed quickly downstream, and adsorbed over a wide area of sediment. In a laboratory situation this scenario is very difficult to recreate, as a continuous flow of water and influx of new B[a]P would feasibly require some manner of large, complicated pumping apparatus and system of keeping sediment localized and separated from the pumping apparatus.
machinery. Thus, the experimental setup consisted of hydrating sediments with ~100 mL rdH$_2$O in beakers and adding B[a]P drop-wise to the surface of the water. The samples were then affixed to a rocking platform to attempt to simulate water flow. Short exposure-time samples were allowed to rock for 24 hours, and long exposure-time samples were left to rock for 1 week. Samples were processed and analyzed using the same methodology applied to sediment samples taken for testing.
II. Methods

II A. Gas Chromatography

It was necessary to address several important issues in devising a gas chromatography (GC) protocol to be used in assaying B[a]P amounts, including several modifications to previously conceived GC and integrator run parameters, choice of injection syringe, establishment of a satisfactory standard curve, and an issue involving the addition of, or lack thereof, an air bubble to the injection syringe after uptake of sample.

A Perkin-Elmer Sigma 3 Gas Chromatography machine with a flame ionization detector was used for separation, and a Hewlett Packard 3395 Integrator was used for data analysis. The column used was a Supelco SPB-5™ wide bore glass capillary column with the following characteristics:

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

A GC and Integrator protocol for detection of B[a]P was constructed by Penny Gikas in 2008, and was used with little modification in this project. It is included in Appendix A. Changes made to this protocol include adjustment of integrator threshold (THRSH) from 4 to 0 (decrease increases sensitivity to peaks) and addition of integrator function (INTG) 2 at Time 0.000 minutes (command resets baseline at all valley points). Thus, the GC and integrator run parameters, respectively, used for establishing a B[a]P standard curve and mediating syringe issues were as follows:

**GC**
- Oven Temp = 100°C
- Inj Temp = 300°C
- Det Temp = 300°C
- Time 1 = 1 min
- Rate = 8°C/min
- Time 2 = 15 min

**Integrator**
- ZERO = 0
- ATT 2^ = 10
- CHT SP = 0.5
- AR REJ = 0
- THRSH = 0
- PK WD = 0.04

Timetable events:
- 0.000 ZERO = 0
- 0.000 INTG = 8
- 0.000 INTG = 2
- 6.000 ATT 2^ = 6
- 20.000 PK WD = 0.20
- 35.000 STOP
II B. Establishing a Standard

In order to use the GC to quantify B[a]P, a trendline of areas reported from standardized B[a]P solutions injected into the instrument must first be constructed. A B[a]P standard (1 mL × 1046 µg/mL in acetone) was purchased from Supelco™ Analytical, and was used to create a dilution series of the following concentrations, in hexane:

1046.0 ng/µL, 523.00 ng/µL, 261.5 ng/µL, 130.75 ng/µL, 65.375 ng/µL, 32.688 ng/µL

Samples (1 µL) were drawn up into a Hamilton 10-µL Gas-Tight syringe #1701, followed with ~1 µL air; doing so prevented loss of sample at the tip of the syringe, and resulted in more accurate and reproducible area outputs as compared to no bubble (data not shown). Samples were injected into the GC, and areas and retention times of peaks identified as B[a]P were recorded and used to create the standard curve shown in Figure III-1.

II C. Extraction Efficiency

In order to assess the efficiency of the previously established extraction protocol, the procedure was applied to three sediment types – fine grain, medium grain and coarse grain – spiked with a known quantity of B[a]P, and run in duplicate.

Half of each of the remaining sediment samples gathered by Penny Gikas from the Blackstone Valley Riverbed were pooled together based on texture (estimated by eye) into the aforementioned categories. Each sediment mixture was then individually sifted through a mesh (pore dimensions ~2 mm²) and collected in separate 500-mL glass Teflon-capped jars. This removed any very large particles (>2 mm diameter), and resulted in a more uniform texture in each sample. To assure that no ambient B[a]P was present, each jar was filled with about 200 mL hexane and was placed in a floor shaker for two days (300 rpm, 40°C). Following the wash, the sediment in each jar was allowed to settle, and the hexane was carefully poured off into a separate, labeled waste container, through Q5 quantitative filter paper fitted in a glass funnel. Sediment remaining in the filter paper was transferred to a new, labeled 500-mL glass Teflon-capped jar. Jars were left uncapped in a fume hood overnight, covered lightly with a Kimwipe®, to allow sediments to dry completely.

Ten grams of each sediment type (Fine, Medium and Coarse) were weighed out and transferred to clean, labeled 100-mL glass jars with Teflon-lined phenolic caps, in duplicate. To each jar was then added 10 µL of 1046 ng/µL stock B[a]P solution in acetone, resulting in 10,460 ng B[a]P introduced to each sediment. Samples were capped tightly and shaken vigorously, then arranged on a rocking platform at ~1.6°C. Jars were affixed to the rocker surface using duct tape, girdling each jar completely, such that the rocking motion caused each jar to shift slightly upon each rocking cycle. This assured constant mixing of sediment and B[a]P. The apparatus was allowed to run at least 24 hours.

A more detailed protocol for the B[a]P extraction and GC analysis from this point onward may be found in Appendix A.
Following the addition of B[a]P to sediments, the hexane-based extraction being analyzed began with adding hexane to each 100-mL sample bottle (to ~150% of sediment volume) and shaking at 300 rpm at 40°C for at least 3 hours \(^\text{10}\). The samples were then filtered through Fischer Q5 quantitative filter paper and the filtrate collected. The filtrates of each sample were passed over a 4.5-cm silica gel column in order to remove hydrophilic species. Columns were constructed from 5-inch glass Pasteur pipettes modified by inserting a small amount of glass wool into the neck, and packing 1 part silica : 3 parts hexane to the 4.5-cm mark. Eluents were collected and dried under nitrogen, using the apparatus depicted in Figure 1 of Appendix A. Dried sample vials were rinsed with 250 μL hexane using a 250-μL syringe, and the hexane collected using a separate 250-μL syringe and emptied into 2-mL amber vials with septum caps. The rinse was repeated at least 5 times. Samples were once again dried completely under nitrogen gas and stored at 4°C.

II D. Transfer of B[a]P from Water to Sediment

Ten grams of each class of sediment (fine, medium, coarse) were weighed and transferred to 250-mL beakers in quadruplicate. The beakers were assembled on a rocking platform in a 3 × 4 grid and secured to the platform with duct tape. Water was added to each beaker (100 mL), and the contents of each stirred vigorously. B[a]P standard was added via Hamilton 10-μL syringe (10 μL × 1046 ng/μL stock), and NOT stirred subsequently. Each beaker was covered with parafilm, and the apparatus set in a 1.7°C refrigeration unit rocking at maximum speed and maximum tilt. Two beakers from each set of four of the same sediment type were left to rock for one week, and the rest began B[a]P extraction after 24 hours.

Once samples had been exposed to B[a]P-contaminated water for the appropriate amount of time, they were removed from the apparatus and the contents of each beaker transferred to 250-mL centrifuge bottles, duplicates balanced with one another using a double-pan balance. The six samples were loaded into a JLA-16.2500 rotor and centrifuged in a Beckman-Coulter J2-HS floor centrifuge at 10,500×g for 10 minutes. Samples were carefully removed from the rotor, and supernatant removed gently, so as not to disturb sediment, using a serological pipette and autopipetter.

Sediments were then transferred completely to 300-mL lyophilizer jars with fitted rubber tops, using as little rdH\textsubscript{2}O as possible to rinse the contents of each centrifuge bottle. The contents of each jar were shell-frozen by dipping and rotating the jar in a liquid nitrogen bath. Once frozen, jars were attached to the lyophilizer and dried for 24 hours. The previously described protocol for hexane based B[a]P extraction followed the lyophilizing process.
III. Results

At the onset of the project, there was some inquiry over which of two types of syringe would perform more reliably and accurately, especially for use in GC injections. The two syringes were the Hamilton™ 10-µL Gas-Tight #1701 and the Hamilton™ 1-µL Gas-Tight #7101.

III A. Establishing a Standard

The standard curve generated as described in section II-B is shown in Figure 1. A trendline generated from the data is defined by the following equation and R² value:

\[ y = 4389.6x - 55456 \]  
\[ R² = 0.9963 \]

Difficulties were encountered while attempting to establish a standard curve. Prior to using the Hamilton 10-µL syringe, a Hamilton 1-µL syringe was being used for GC injections. It was noticed, however, that the areas reported by the integrator were erratic and not consistent with the amount injected, and a suitable standard curve could not be generated. Upon suspicion of the syringe’s fault, the two were tested side by side, injecting 1 µL and 0.5 µL of stock standard with each syringe and comparing results, shown in Table 1.

<table>
<thead>
<tr>
<th>Volume Injected (1046 ng/µL)</th>
<th>10-µL Syringe</th>
<th>1-µL Syringe</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 µL</td>
<td>4,619,485</td>
<td>890,370</td>
</tr>
<tr>
<td>0.5 µL</td>
<td>2,592,104</td>
<td>72,217</td>
</tr>
<tr>
<td>Ratio:</td>
<td>0.5611</td>
<td>0.0811</td>
</tr>
</tbody>
</table>

Table III-1: Results from a simple syringe comparison experiment. The 10-µL syringe was chosen for subsequent injections as its ratio of the reported areas from solutions differing in concentration by 1/2 conforms more closely to the expected 2:1 than does the 1-µL.

Subsequent injections were performed using the 10-µL syringe, based on the closer conformity of its results to the expected 2:1 ratio than those of the 1-µL syringe.
III B. B[a]P Retention Time

An average B[a]P retention time (RT) was calculated using standard curve data and data from daily standards and spiked samples, including those standard injections whose area results were considered erroneous, since the RT should be independent of quantity. B[a]P peaks were identified as having RT between 27.598 and 28.875 minutes, with an average RT of 28.402. Figure III-2 compiles these results, and indicates region of 1 standard deviation. When attempting to identify BaP in experimental samples, peaks with RT in this range were suspected to correspond to B[a]P. In some cases, experimental samples that were not spiked could be compared to those that were, if of the same sediment texture. Although sediments had been washed thoroughly with hexane, experimental traces contained many more peaks than just that due to B[a]P. This may be due to the effects of hexane on sediment; subsequent exposures to hexane may have released small molecules from sediment particles. Alternatively, there may simply have been species present in the sediments that were very insoluble in hexane. The unidentified contaminants lead to traces with many peaks, some approaching 100. Adding a known amount of B[a]P standard to an aliquot of these samples resulted in a trace with a much stronger B[a]P peak. Comparison of the pure standard, the experimental sample and the spiked sample allowed for easy discrimination of B[a]P in the experimental sample. However, B[a]P RT in most experimental samples were more difficult to discriminate, since it was not economically practical to spike every sample. In these cases, neighboring peaks to that deduced to be B[a]P, and seen on duplicate traces, were compared with one another. Those that were ubiquitous and did not vary in area or RT from trace to trace were ruled out as not being due to B[a]P. Recurrent peaks such as these could actually be used as “landmarks,” proximal to which B[a]P was expected to be found. Nonetheless, the first region of traces examined was always the region bounded by the RTs mentioned, and those suggested by spiked samples.

It was noticed over the course of studies that RTs varied from day to day as much as ±1.0 min, but would not fluctuate significantly within a single day’s use. Thus, it ultimately proved most reliable to check the B[a]P RT each day that samples were run than to use an average RT, and results were analyzed against a standard that was run the same day as samples. Retention times used to analyze data are listed at the bottom of their corresponding data tables (Appendix B).
Concentrated extracts from spiked sediment samples were analyzed via gas chromatography. Reported areas and the B[a]P standard curve were used to calculate the amount of B[a]P present in each sample. These quantities are listed in Table 2, and average percent recovery for each sediment type is diagrammed in Figure 2. B[a]P amounts were calculated using Equation 1. The following calculation uses fine-grain sample 1 as an example:

\[
\text{Area} = 314736 = 4389.6x - 55456 \quad [3]
\]
\[
x = (314736 - 55456)/4389.6 = 84.334 \text{ ng/µL} \quad [4]
\]
\[
84.334 \text{ ng/µL} \times 40 \text{ µL} = 3373.4 \text{ ng B[a]P} \quad [5]
\]
III D. Transfer of B[a]P from Water to Sediment

Concentrated extracts were analyzed via gas chromatography as in III B above. Areas were correlated to B[a]P standard curve and amount of B[a]P in each sample was calculated as described above (Appendix B lists these results). Recovery percentages were based on the original 10,460 ng B[a]P added to each sample, and are shown in Table III-3. A side-by-side comparison of the amounts of B[a]P calculated to have been transferred from water to sediment for each sediment type is depicted in Figure III-4. Transfer percentages were calculated by dividing the amount extracted by the average extraction efficiency (determined in III C) for each sediment type, and comparing this value to the original amount of B[a]P added to samples. The following is fine-grain sample 1 as an example:

\[
\frac{4269.46 \text{ ng}}{0.245} = 17451.31 \text{ ng} \quad [6]
\]

\[
\frac{17453.31 \text{ ng}}{10460 \text{ ng}} = 166.8\% \quad [7]
\]

Transfer data was the result of averaged duplicate runs.

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Sediment Type</th>
<th>Area</th>
<th>RT</th>
<th>B[a]P Extracted (ng)</th>
<th>% Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S</em> 5</td>
<td>200 ng Standard</td>
<td>674333</td>
<td>27.947</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>1</td>
<td>Fine</td>
<td>314736</td>
<td>27.901</td>
<td>3373.36</td>
<td>32.25</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>136012</td>
<td>27.885</td>
<td>1744.74</td>
<td>16.68</td>
</tr>
<tr>
<td>3</td>
<td>Medium</td>
<td>134153</td>
<td>27.424</td>
<td>1727.80</td>
<td>16.52</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>283786</td>
<td>27.942</td>
<td>3091.32</td>
<td>29.55</td>
</tr>
<tr>
<td>5</td>
<td>Coarse</td>
<td>237657</td>
<td>27.826</td>
<td>2670.98</td>
<td>25.54</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>411038</td>
<td>27.949</td>
<td>4250.90</td>
<td>40.64</td>
</tr>
</tbody>
</table>

Table III-2: B[a]P content in final extracts from fine, medium and coarse-grained samples. Each sediment sample was spiked with 10,460 ng B[a]P prior to extraction. Final values for extracted B[a]P calculated as in Equations 3-5 above. Extraction efficiency is displayed as percentage of original B[a]P recovered.

* A purified B[a]P standard was run consecutively to samples in order to establish RT.

Figure III-4: Percent of added B[a]P transferred from water to sediment for each sediment type over two time frames, with one standard deviation.

<table>
<thead>
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<th>1-week % Yield</th>
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<td>Coarse 2</td>
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</tbody>
</table>

Table III-3: Percent recoveries of B[a]P from sediments exposed to contaminated water (10,460 ng B[a]P) for 24 hours and 1 week.
IV. Discussion

IV A. B[a]P Retention Time

Though sediments were thoroughly washed with hexane prior to experimentation, the wash was not successful in completely removing extraneous substances, which resulted in integrator traces with many peaks not due to B[a]P. This complicated the identification and analyzing of B[a]P. Thus, a strategy for identifying B[a]P was developed. Although the traces were littered with extraneous peaks and the retention time for B[a]P varied from one day to another (as seen from B[a]P standard runs, Appendix B), a general consensus was reached on B[a]P’s identity on the integrator traces. Figure III-2 displays known B[a]P retention times for all standard and spiked runs. Retention times deviate from the mean (28.402) by as much as ±0.80 min. As mentioned earlier, however, it was more reliable to identify B[a]P based on a standard run on the day of analyses than on this averaged value. This can be seen when the data sets are broken apart, and 1 SD applied to each (Figures IV-1 through IV-3).

Average RTs in Figures IV-1 through IV-3 are 27.839, 27.653 and 27.863, respectively. The RT could vary as much as ±1.0 min from day to day, but rarely exceeded a deviation of ±0.3 min within a day’s runs. This tendency is illustrated in the above figures.

Even in spite of these averages, assigning a peak as B[a]P was accomplished using a combination of supporting evidence. In addition to RT tendencies, peaks neighboring those suspected to be B[a]P were compared alongside duplicates, and in the case of the transfer study, alongside the alternate time-frame samples of the same sediment type, giving four samples
across which to determine B[a]P’s peak – or more accurately, across which to eliminate peaks that were seen constitutively on other traces. These “landmark” peaks showed even less RT variability than B[a]P, and less variability in area, as well, making them easily identifiable. The final piece of evidence in deciding which peak corresponded to B[a]P was by spiking select samples, and comparing the spiked GC output with the trace obtained from B[a]P standard and the unspiked sample. This gave conclusive results about the location of B[a]P for the spiked sample, as a dose-response trend was seen. Taken along with the relative invariability of B[a]P’s RT within a given day, these clues made for solid conclusions as to the B[a]P RT in each trace.

IV B. Extraction Efficiency

The most important fact to consider when assessing the extraction efficiency data is that the results are based on only two runs, making the significance of any results statistically questionable. Nonetheless, the difference in average extraction efficiency (Figure III-3) between fine sediment and coarse sediment may be real. By the line of reasoning put forth in the Introduction, coarse sediment possesses less total surface area than does fine sediment, by volume, providing less sites onto which B[a]P may adsorb. Less surface area on which to adsorb means less surface area from which to attempt to extract B[a]P – and vice versa for finer sediment. Considering that sediment particles are not perfect spheres, and in fact contain many nooks and cracks on their surfaces, a larger total surface area would also correspond to a greater number of such nooks. B[a]P, a small hydrophobic molecule, could easily be adsorbed onto sediment in these cracks, making “entrenched” populations of B[a]P more difficult to extract than molecules adsorbed onto the outer surface. The data supports this hypothesis, though many more sample sets would need to be run in order to conclude definitively that coarse sediment yields more B[a]P than fine.

An aspect of the transfer study results also lends support to this hypothesis. As seen in Tables 1 & 2 of Appendix B, samples exposed to B[a]P-contaminated water for 24 hours yielded much higher levels of B[a]P than samples exposed for 1 week. The difference in these yields is so great, it is very unlikely due to chance. Given that B[a]P is hydrophobic and separates from water, a longer time frame would allow B[a]P to associate more completely with sediment, becoming more deeply entrenched in surface grooves, and resulting in more difficult extraction; greater surface area and more grooves would be expected to amplify this effect, leading to lower yields from finer sediment.

IV C. B[a]P Transfer from Water to Sediment

As stated previously, extraction efficiency data is based on only two sets, calling into question the statistical significance of the results. Because transfer amounts were calculated using extraction efficiency results (as in Equation III-6), the statistical significance of these results are also thrown into question. The most profound characteristics of the data, however, are independent of statistical analysis, or are clearly of real significance despite lack of such analysis.

Figure III-4 diagrams the amount of B[a]P transferred from water to sediment for each sediment set over two time frames. The most striking aspect of the data is the clear difference in the amount of B[a]P transferred between 24-hour and 1-week sets. These results seem counterintuitive – how could over 100% of the B[a]P be transferred to sediment in 1 day, yet
only 30-45% be transferred in a week? The results are an artifact of the method used to calculate these values. As shown in Equation III-6, raw extraction amounts were divided by the extraction efficiency determined for each sediment type, and this value compared to the original B[a]P added. Thus, the more important data when considering the effect of time on the transfer of B[a]P from water to sediment, especially in the case of 1-week samples, is the raw extraction data, shown in Table III-3.

Average recovery from 24-hour samples ranged from 32.2% to 37.1%, comparable with extraction efficiency data, whereas average 1-week sample recovery ranged from 8.0% to 11.9%, a drastic decrease. According to these results, B[a]P is less extractable from sediments when they are exposed for a longer time. In an environmental scenario, a short exposure time would correspond to a rainy day, the precipitation washing B[a]P off of roadways and industrial sites into the ground and rivers, where it would become associated with sediment particles, but be “washed” with the subsequent flow of cleaner water. A long exposure scenario would probably not be found in a rapidly flowing river, as fresh water would continuously disperse contaminants. Rather, ponds, lakes, and more stagnant rivers would be susceptible to longer B[a]P exposure after a series of days’ rain washed the contaminant into these water bodies, where it could be expected to sit for some time. Subsequent precipitation may dilute the contaminant, or introduce more, depending on factors such as local industrial activity and automobile use. Such a scenario puts stagnant bodies of water at greater risk of contamination than bodies with high flow volume.

When Figures III-3 and III-4 are compared, an inverse relationship is seen within sediment types, between amounts extracted and amounts transferred. Though the trend seen in Figure III-4 (that less B[a]P is transferred to coarser sediment) is of a small degree and thus statistically questionable, the trend is seen over both time frames, and is well in line with the argument that finer sediment can adsorb more B[a]P than coarse, and yield less. Again, more data sets would be required to definitively address this issue.

There was much speculation about the cause of the drastic decrease in B[a]P yields seen between 24-hour samples and 1-week samples. The first inclination was to ascribe the decrease to decomposition of B[a]P. The EPA describes B[a]P as being susceptible to photodegradation at the surface of waters, due to delocalized electron excitation by UV light, but gives B[a]P a half-life of 43 days in such conditions\textsuperscript{11}. However, degradation is significantly retarded upon adsorption onto sediment particles\textsuperscript{11}. The EPA has also found that B[a]P does not undergo hydrolysis in aqueous environments, and so rules out a second theory for the lower yields involving mineral-based catalytic hydrolysis of B[a]P\textsuperscript{11}. Taken together, these characteristics strongly refute decomposition as the reason for the lowered yields in 1-week samples. A theory that more feasibly explains the decreased recovery in 1-week samples is that as a hydrophobic molecule, B[a]P associates very strongly with sediment rather than water, and given a longer time frame over which to do so, will be driven entropically to associate with sediment to an ever greater degree, becoming more deeply entrenched in grooves and cracks on particle surfaces and more difficult to extract by means of the extraction protocol examined herein.

In analyzing the results, it would have been useful to have information about the amount of B[a]P left in water after sediment exposure. This would have allowed for more definitive assessment of the levels of B[a]P transferred to sediment, as a “bottom-up” and a “top-down” approach could be taken: data from sediment extraction and water extraction could be compared, and would be expected to sum to the original amount of B[a]P added. B[a]P could have been
extracted from water by means of hydrophobic interaction chromatography (HIC). In this type of separation, B[a]P-contaminated water is run over a hydrophobic stationary phase, and salt added to the solution in order to induce a stronger interaction between the stationary phase and hydrophobics, i.e. B[a]P\textsuperscript{14}. A special type of HIC, known as reverse-phase chromatography, uses a stationary phase consisting of long chain hydrocarbons (C\textsubscript{8} or C\textsubscript{18}) linked to an inert silica-based support, though for the purposes of B[a]P an aromatic-based stationary phase may be more effective. As the aqueous solution is eluted, hydrophobic solvent is added in increasing amounts, allowing for collection of fractions in order of increasing hydrophobicity\textsuperscript{14}. The hydrophobic solvent would have to be relatively volatile, in order for the separation to be integrated into the existing extraction protocol, specifically drying under nitrogen.

**IV D. Future Work**

Subsequent research on benzo[a]pyrene contamination in waterways will have to address the issue of statistical significance, by obtaining more data sets and forming a clear, definitive trend about B[a]P transfer from water to sediment and the effect of sediment texture on extraction yields. Construction of a texture-independent extraction protocol should also be considered. Possibilities for improved extraction protocols include introduction of a sonication step, concurrent with hexane washing. This may prove useful in releasing entrenched B[a]P molecules from grooves in particle surfaces, and result in higher yields. Another option for extractions is the soxhlet extractor, shown in Figure IV-4. The apparatus continuously circulates clean solvent through the sediment, and the B[a]P-containing solvent is then condensed by cooling. The solvent flows back down into the boiling flask (some escapes into the air), and B[a]P is collected inside the flask. Whether or not the soxhlet extractor would eliminate the effects of sediment texture on yields would have to be determined in future experiments. Finally, a different organic solvent may dissolve B[a]P more efficiently than hexane. Benzene or toluene share aromatic structures with B[a]P that would lead to a stronger interaction between the two; this may also help draw B[a]P out of hard to reach crevices in sediment particles.

Another aspect of environmental B[a]P contamination is that the molecule rarely exists freely in the atmosphere. Rather, it quickly adsorbs onto ambient dust particles, pollen, etc., and settles into water and sediments in this form. In this research, B[a]P was added directly to water in the transfer study, in un-adsorbed molecular form. Future work may analyze B[a]P transfer from air to water, or water to sediment, with this consideration in mind.
Appendix A – Detailed Methodology

Production of Original Dried Sediment

Centrifugation:

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). 9

Lyophilizing 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. 9

Hexane Extraction of B[a]P from Dried Sediment

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

When the shaking was complete, the sediment samples were filtered. The filtration apparatus consisted of a sheet of Q5 filter paper that had been folded and inserted into a clean glass funnel. The filter paper was saturated with hexane, and then the apparatus was suspended using a ring stand so that it filtered into a 40 mL vial. Individually, the contents of each shaken bottle were poured into the filtration apparatus, allowing the liquid to drain into an appropriately labeled 40-mL vial. 5 mL of hexane was used to thoroughly rinse the inside of the 100 mL shaking bottle, and then this hexane was poured over the sediment remaining in the filter paper, as a means of rinsing the sediment. This rinse was performed two more times, using 5 mL of hexane for each rinse. The filtered extracts were stored in labeled vials at 4°C until needed. 10
Silica Gel Chromatography

Setting up the column:

A fresh column was prepared for each new sample to be filtered. A slurry composed of 1 part silica gel:3 parts hexane was made, and a small amount was transferred to a 120-mL Erlenmeyer flask for working (note: goggles and gloves should be worn when working with hexane). A 5-inch glass Pasteur pipette was modified by inserting a small amount of glass wool into the neck (any cotton swab already in the tube was first removed). A mark was made 4.5 cm up from the top of the glass wool with a permanent marker. The pipette was then attached to a ring stand, and a glass bottle labeled “waste hexane” was placed under the pipette. The Erlenmeyer flask was swirled vigorously to thoroughly mix the silica gel slurry and, using a glass pipette and pipette-aid, slurry was quickly removed and was slowly and evenly added to the modified pipette until the pipette was filled to the 4.5-cm mark with silica gel. Once all of the hexane had dripped out, the bottle of waste hexane was removed and a 40-mL collecting vial was placed under the column.

Filtering samples:

Using a glass pipette, hexane extract was slowly applied to the column. Once the entire sample had been drawn from the 100-mL glass bottle, 5 mL hexane was added to the bottle; the bottle was capped and shaken lightly. The hexane was then applied to the column, and the entire sample was allowed to drip from the column. Finally, an additional 2-5 mL hexane was added to the column to elute any extract remaining in the silica. The final extract was colorless and contained no particles. Separated extracts were stored at 4°C until needed.

Evaporation under Nitrogen

Drying apparatus:

To create the drying apparatus (Figure A-1), pieces of rubber tubing were attached (length does not matter so long as all pieces on each aeration unit are equal) 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. A Pasteur pipette was melted and stretched using a Bunsen burner just beyond the point where the tube broadens after the neck. The stretched portion of the pipette was broken and inserted into the end of each tube (the neck should be in the tubing). The aeration units were then attached to a ring stand, and connected to each other with rubber tubing. The entire unit was then connected to a tank of nitrogen gas.
Drying samples:

The pipette ends were cleaned with ethanol and Kim-wipes before and after each use. Vials were placed 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 were open. Once the gas was on, tubes were carefully inserted into the vials, and individual valves were closed until air was coming out of the tubes at a desired rate. This prevented overflow of air from splashing liquid from the vials. The 40-mL collecting vials containing the extracts from the silica gel column were dried to completion with nitrogen gas. 

Two syringes (250—500-μL) were cleaned with hexane. One syringe was loaded with 250 μL of hexane and was used to rinse the dried 40-mL collecting vials, allowing the hexane to run down the sides of the vial. After capping the vials and swirling the contents, the second syringe was used to draw up the contents. The solutions were then transferred into 2-mL amber vials with septum caps. The rinsing process was repeated at least 5 times, cleaning syringes with hexane between each sample. Once the 40-mL vials had been rinsed, the 2-mL amber vials were dried completely under the nitrogen gas. Extracts were stored at 4°C.

Gas Chromatography

A Hamilton 10-μl Gas-Tight #1701 syringe was used to load the machine. The syringe was cleaned with hexane (hexane drawn up to full volume and ejected at least three times) before use. Hexane (40 μl) was added to the amber collection vial to be assayed, piercing through the septum.
cap to do so (never opening the cap). The vial was swirled and finger vortexed until contents were well dissolved (if contents will not go into solution, vials may be incubated in 37°C water bath for ~5 min.), then drawn up with syringe and ejected out of the syringe several times to further mix the sample. Sample to be tested was loaded into the syringe (1 μL), making sure that no air bubbles are present in the tube. An additional ~1.0 μL of air was drawn into the needle, the needle was inserted into the GC loading site, and the sample was injected into the GC. The integrator was started simultaneously with the injection, followed immediately by the GC temperature program. 

Appendix B – Raw Data Tables

<table>
<thead>
<tr>
<th>Sediment Type</th>
<th>Area</th>
<th>RT</th>
<th>Total B[a]P (ng)</th>
<th>% Yield</th>
<th>Ave B[a]P Recovered</th>
<th>Ave % Yield</th>
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**Average RT:** 27.647

*From standard run on 4/11/09 (RT = 27.598)
†From standard run on 4/13/09 (RT = 27.847)

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<th>Sediment Type</th>
<th>Area</th>
<th>RT</th>
<th>Total B[a]P (ng)</th>
<th>% Yield</th>
<th>Ave B[a]P Recovered</th>
<th>Ave % Yield</th>
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**Average RT:** 27.943

†From standard run on 4/13/09 (RT = 27.847)
‡From standard run on 4/14/09 (RT = 27.838)
### 24-hour Exposure

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### 1-week Exposure

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### References


8. Blackstone River Watershed Team; Massachusetts Environmental Trust **2000**.


