Bioaccumulation of Benzo[alpha]Pyrene in Orconectes Limosus Crayfish

Major Qualifying Project

Submitted to the Faculty

of the

WORCESTER POLYTECHNIC INSTITUTE

In partial fulfillment of the requirements for the

Degree of Bachelor of Science

Submitted By:

Taylor Briseño
Michael Chadwick
Benjamin Kwame Owusu-Ansah

_____

Submitted to:

Professor JoAnn Whitefleet-Smith

April 30, 2014

Abstract
Crayfish near industrial developments are commonly exposed to polyaromatic hydrocarbons such as Benzo[alpha]Pyrene which has been shown to bioaccumulate or metabolize. To test this, sample groups of crayfish were fed B[a]P contaminated food followed by clean food at 7 designated time points. Extracted tissue samples were analyzed using HPLC. Increased B[a]P concentrations or possible metabolites correlated with the amount of B[a]P ingested. This process will help to understand how keystone organisms are affected by common carcinogens in their natural environment.

Acknowledgements
We would like to thank everyone who assisted us in the process of completing this project. First, we would especially like to thank our advisor, Professor JoAnn Whitefleet-Smith for her guidance and encouragement through all stages of our project. We would also like to thank Professor Michael Buckholt, Abbie White, Taylor Manning, Glenna Vitello and the Army Corps of Engineers. Finally we would like to thank Sam Petersen for spending an afternoon with us to assist during the collection of crayfish.

Table of Contents

Table of Figures

Table of Tables

Table of Equations

I. Background

I A. Benzo[alpha]Pyrene: The organic compound Benzo[alpha]Pyrene (B[a]P) is a polycyclic aromatic hydrocarbon (PAHs) with five phenol rings (Varanasi, 1980) that has the chemical formula $C_{20}H_{12}$ with a molecular weight of 252.3 grams/mol. It is included in a chemically inert group of carcinogens that exhibits mutagenic, cytotoxic and carcinogenic effects through metabolic activation by mixed function oxygenases (MFO) (Newbold, 1976). PAHs are initially oxygenated by the MFO enzyme system into various organic compounds such as epoxides, phenols and quinones. Epoxides are organic compounds whose molecules are composed of a three-membered ring. This three-membered ring is comprised of an oxygen atom and two carbon atoms. Phenols are groups of chemical compounds that consist of a hydroxyl group (-OH) which directly bonds to an aromatic hydrocarbon. Phenols are mild in acidity and toxicity. They appear as white crystalline solids and are obtained from coal tar. Quinones form a class of organic compounds that come from aromatic compounds. They are achieved through the conversion of an even number of –CH= groups into –C(=O)- groups. Quinones function as electron transport cofactors in photosynthesis and cellular respiration. Further metabolism of these oxygenated products involves the hydration of the epoxide intermediates into dihydrodiols, water-soluble glutathione, glucuronide or sulfate conjugates.

PAHs are derived from both natural and synthetic sources and are commonly found in the environment (U.S. CDC, 1995). In the atmosphere, PAHs occur commonly in by-products resulting from incomplete combustion (U.S. CDC, 1995). Examples of such by-products are industrial processes (such as refinement of crude oil) (Baum, 1978), cigarette smoke (Varanasi, 1980), fossil fuels, and exhaust emissions resulting from gasoline engines, oil-fired heating and burnt coal (U.S. CDC, 1995). PAHs are also found in foods (Lioy, 1988) that are charbroiled or broiled, pickled food items, and refined fats and oils (U.S. CDC, 1995). They can also be found in sludge, drinking water, groundwater, waste water and surface water (U.S. CDC, 1995). Humans can often be exposed to the B[a]P pollutant through food, air and water (Zhu, 1995). As a toxic compound, studies have shown that exposure to B[a]P in mammals can lead to different forms of toxicities such as teratogenicity which causes birth defects, immunotoxicity; affecting the immune system, and hematotoxicity; which destroys red blood cells (Hardin, 1992). In addition, B[a]P has been shown to be one of the causes of aplastic anemia and various forms of cancer including leukemia (Zhu, 1995). In the body, dihydrodiol-epoxide, a derivative of B[a]P, is known to mainly target mitochondrial DNA leading to DNA damage (Backer, 1980).

Particularly, B[a]P is one of the most studied PAH's due to the degree of its mutagenic, cytotoxic and carcinogenic properties. The mechanism of B[a]P-induced toxicity is not really clear. However, studies have shown that some, if not all, of the toxic effects of B[a]P discussed later in this chapter are mediated by the metabolic activation by the MFO system. B[a]P is initially oxidized by MFO enzyme to result in the formation of 6-

hydroxybeno(a)pyrene (6-OH-BP) and BP-7,8-epoxide. The 6-OH-BP can then be oxidized to form quinone metabolites, BP-1,6-,3,6- and 6,12-quinone. BP-7,8-expoxide can also undergo hydration to form BP-7,8-dihydrodiol (BP-7,8-diol) in a reaction catalyzed by microsomal epoxide hydrolase. Further oxidation of BP-7,8-diol by the MFO system results in the formation of the highly reactive compound BP-7,8-dihydrodiol-9,10-epoxide, an ultimate carcinogen (Zhu, 1995). The BP-7,8-diol can be alternatively oxidized by dihydrodiol dehydrogenase to form BP-7,8-quinone (BP-7,8-Q) through an unstable hydroquinone intermediate.

Most PAHs covalently bind to DNA by PAH metabolites. Some PAHs which are weak carcinogens need to go through metabolism to become more potent. Diol epoxides and PAH intermediate metabolites, are mutagenic. They react with DNA to form adducts, which affects the replication of normal cells. The bay theory addresses the variability in regards to the strength of different diol epoxides. This theory predicts that epoxides found in the "bay region" of the PAH molecule will be the most mutagenic and reactive. The bay region is the space the lies between the aromatic rings of the PAH molecules (CDC, 2014). Therefore, the bay region diol epoxide intermediates of PAHs are known to be the most extreme carcinogens for alternate PAHs (U.S CDC, 1995). After this reactive bay region is created, it may then covalently bind to DNA and other cellular macromolecules to trigger mutagenesis and carcinogenesis in mammals (U.S CDC, 1995). The binding of B[a]P to DNA can then hinder DNA replication (U.S CDC, 1995). B[a]P is also a nonpolar compound with a great affinity for organic compounds (U.S.CDC, 1995). This may explain why B[a]P has a strong affinity for DNA and can have harmful effects on it.

Occupational studies have shown that humans most often absorb PAH's through inhalation. Animal studies also reveal that pulmonary absorption of B[a]P takes place and may be influenced by carrier particles and the solubility of the vehicle. However, the degree of absorption is not known. Though it is believed that the absorption of B[a]P following ingestion is low in humans, the oral absorption in animals may vary depending on the lipophilicity of the PAH compounds. PAHs are able to easily penetrate cellular membranes and reside in the body for a long time due to their level of lipophilicity, or ability to dissolve in fats, oils, lipids and non-polar solvents. On the other hand, the metabolism of PAHs, which takes place in all tissues, causes them to be more water-soluble and excretable (U.S. CDC, 1995). The presence of more lipophilic compounds or oils in the gastrointestinal tract increases oral absorption of B[a]P (U.S. CDC, 1995). This suggests that the extent of absorption of PAHs following dermal exposure, inhalation or oral ingestion may be influenced by the vehicle of administration (U.S. CDC, 1995). There is no information in regards to the distribution of PAHs in humans. However, PAH is found to be widely distributed in the tissues of animals after exposure to any PAHs through oral ingestion or inhalation (U.S. CDC, 1995).

While there are no studies on the distribution of PAHs in humans, studies have shown that B[a]P is orally absorbed in human (Buckley, 1992; Hecht, 1979). In a study analyzing the ingestion of foods containing low amounts of B[a]P, the metabolite (1-

hydroxypyrene) was detected in the urine of the volunteers (Buckley, 1992). There were no quantitative data of the excretion of B[a]P provided in the experiment. In a separate study, Hecht *el et.* (1979) examined the concentration of B[a]P in the feces of eight volunteers who ingested meat that contained about 9 μg of B[a]P (Hecht, 1979). In their study, they discovered that the feces of each of the volunteers did not contain detectable amounts of B[a]P. Each volunteers had less than 0.1 μg of B[a]P in their feces. They compared the results to the control experiment in which the same volunteers ingested meat containing undetectable amount of B[a]P. The results for both experiments were similar where undetectable concentration of B[a]P in feces were found. This experiment suggested that most of the ingested B[a]P was absorbed (Hecht, 1979).

Other cytochromes belonging to the family of CYP430, such as CYP2A and CYP2B isoforms, are actively involved in the catalyst of B[a]P oxygenation (James, 1998). However, the major metabolic catalyst in mammals is the NAD(P)H CYP450 also known as CYP1A1 (Mitchelmore, 1998). This type of cytochrome determines the form and position of the B[a]P oxygenation (James, 1998). This pathway also leads to DNA damage in two ways. The first way is the creation of unstable epoxides (trans-7,8-diol-9,10-epoxide) through the catalyzing effect of CYP450 or CYP1A1 and epoxide hydrolase. The trans-7,8-diol-9,10-epoxide produces bulky DNA adducts which may then cause DNA strands to break (Mitchelmore, 1998). The second way is through the formation of cation radicals through the catalytic activity of CYP450 and hydroperoxides. These cation radicals destroy DNA and other macromolecules by binding to them and causing them to be oxidized (Mitchelmore, 1998). When digested, B[a]P may undergo a series of mechanisms as seen in

Figure 1 which illustrates several pathways.

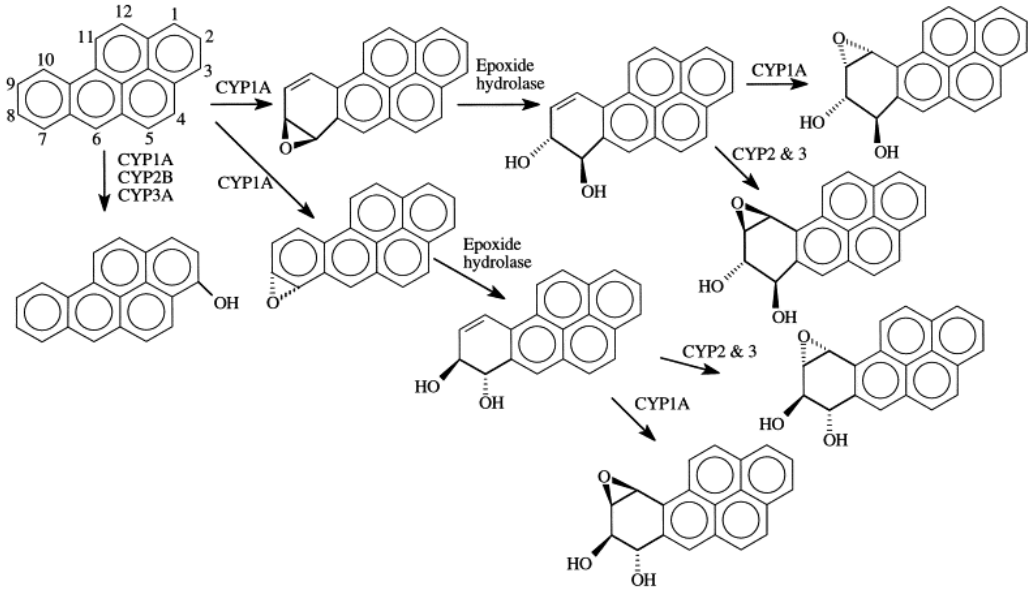

Figure 1 Metabolic pathway of B[a]P (James, 1998)

When crustacean species consume B[a]P, it is metabolized with the aid of Cytochrome P-450 (CYP450), a group of enzymes that catalyze the oxidation of organic compounds. Other common enzymes responsible for the metabolism of B[a]P in crustacean species are Cytochrome P-1 (CYP1), Cytochrome P-2 (CYP2) and Cytochrome P-3 (CYP3); enzyme subgroups of CYP450 (James, 1998). This family of enzymes is found to be dominant in the hepatopancreas; a large gland that performs functions of the liver and pancreas in the crustacean species (James, 1998). This suggests that the majority of synthetic chemicals that are foreign to the body, or xenobiotic substances, including B[a]P may be found in the hepatopancreas (James, 1998). Besides the hepatopancreas, the metabolism activity of CYP450 has been found to also occur in the gill, intestine, stomach and antennal glands of crustacean species, increasing the possibility of B[a]P being present in different organs and tissues (Bielaczyc & Merkisz, 1998; James, 1998).

I B. Bioaccumulation of B[a]P: No published literature on crayfish used as bioindicators of B[a]P were found, however there have been studies done with crayfish as bioindicators for other environmental contaminants. In a study conducted by Anderson (1997), red swamp crayfish were used as bioaccumulation agents of lead nitrate (Anderson, 1997). In the study, some amount of crayfish were exposed to intermediate concentrations of lead nitrates (150 μg^{-1} and 1100 μg^{-1}) for a period of 7 weeks. The clearance of lead was monitored in the third week into the seven-week period. They concluded that lead bioaccumulation was demonstrated to be a time-and dose-dependent factor. They also found out that lead clearance was significant in all the tissues of the crayfish they examined, but not in the hepatopancreas; which is the organ of metal storage and detoxification (Anderson, 1997).

There have been major qualifying projects (MQP) done at Worcester Polytechnic Institute (WPI) in which crayfish were used as bioindicators for B[a]P (Gikas, 2008; Goscila, 2007). Goscila, *et al.* (2007) in their study concluded that crayfish can be used to detect B[a]P in an environment, hence possess the qualities of acting as bioindicators of PAH contamination (Goscila, 2007). The results achieved from the study conducted by Gikas (2008) supported her hypothesis that B[a]P was indeed a contaminant in the Blackstone Valley stream (Gikas, 2008). Again, Gikas' results revealed that the concentration of B[a]P accumulated in the muscle tissue of the crayfish was much higher than that in the hepatopancreas (Gikas, 2008). Finally, there were evidence in Gikas' results suggesting that crayfish did ingest B[a]P. There where B[a]P present in the stomach of the crayfish after a gut analysis was performed (Gikas, 2008). These findings support the fact that crayfish can be used as bioindicator of B[a]P through their bioaccumulation of the toxic.

In her project, Ashley Sutton (2009) tested her first hypothesis, which stated that the runoff from route 146 serves as a point source for B[a]P contamination in the area of interest in the Blackstone Valley stream (Sutton, 2009). Sutton's result, which supported her hypothesis showed that out of the five sampling sites chosen, sampling site 4 located

directly below the suspected point source had the highest level of B[a]P contamination per sediment (577.84 ng B[a]P/g sediment). This particular point source was two feet from the outer shore, where the drain is located (Sutton, 2009). The further away from the area of interest she was (the drain), the lower the concentration of B[a]P and vice-versa. This result supports the results of the previous MQP discussed above. Sutton's second hypothesis tested was that crayfish did serve as bioindicators of B[a]P levels in their habitats (Sutton, 2009). Sutton hypothesized that the higher the concentration of B[a]P in a point source, the greater the bioaccumulation of B[a]P in crayfish found in that point source in question. Therefore, it was predicted that crayfish located in point source or sampling site 4 would have the highest concentration of B[a]P. Sutton argues that the age of crayfish should be one of the essential factors determining the concentration of B[a]P bioaccumulated by crayfish (Sutton, 2009). In other words, all things being equal, the concentration of B[a]P bioaccumulated by crayfish is directly proportional to how old the crayfish is. The age of crayfish in B[a]P contaminated waters has a direct correlation with the degree (based on years) of exposure, bioaccumulation and ingestion of B[a]P. The lengths of crayfish tails were used as direct proportion to their ages respectively (Sutton, 2009). Using the concept of the tail length, the second hypothesis was supported since crayfish in sampling site 4 had the highest average B[a]P concentration of 0.017 ng B[a]P/mg tissue/mm tail length. Crayfish in sampling site 5 (the site with the lowest B[a]P level) had the lowest average B[a]P concentration of 0.0094 ng B[a]P/mg tissue/mm tail length (Sutton, 2009). Using crayfish as bioindicators and bioaccumulation keystone animals, Sutton concludes that there is a correlation between the levels of B[a]P contamination at different sites and the length of the crayfish tail and tail muscle tissue (Sutton, 2009). It is salient not to notice that the concentration of B[a]P is a specific location of the stream will go a long way to determine the concentration of B[a]P bioaccumulated by the crayfish in that location. This means that the factors needed to be considered in this case are age and positional factors.

I C. Blackstone River Watershed: The Blackstone River begins in Worcester, Massachusetts and runs over the Slater Mill falls into the Seekonk River at the head of the Narragansett Bay. Construction began in 1823 with the goal of creating a 45 mile long canal that measured 35 feet in width at the top, 18 feet at the bottom, and a uniform depth of 4-6 feet. The canal was proposed because of an influx in industry developments in the area during the American industrial revolution (Blackstone River Watershed, 2014). The canal was intended to improve transportation efficiency between industrial producers and their markets. A wide array of mills opened along the river including blacksmiths, printers, as well as wool, rubber, wire, and cotton mills. Perhaps the most recognized mill was the Slater mill, which was the first to use mechanically spinning looms to improve production rates (Blackstone River Watershed, 2014). Each of these industries produced byproducts containing PAHs as discharged dyes, leather byproducts, and heavy metals that flowed directly into the canal. The pollution accelerated at an alarming rate, to the point where the Massachusetts Department of Health considered the river to be "offensive in its course, from Worcester to the state line at Blackstone" in the early 1900s (Kerr, 1990). The passage of the Clean Water Act in 1972 initiated a relatively weak effort to improve the

water quality throughout the river system. Despite the effort, the Blackstone River Valley was considered the "most polluted river in the country with respect to toxic sediments" in 1990 (Kerr, 1990). The cleanup effort continued with minimal success. In 2010, the EPA concluded that the water quality "[was] not sufficient to meet state water quality standards". Recent water quality tests have shown that these aromatic pollutants can still be found in the river today, over 100 years after they were released (Byeong-Kyu et al., 2010).

The Quinebaug River originates at the bottom of the East Brimfield dam. This river is considered part of the Blackstone River Watershed, but it does contain the same high levels of pollution. The crayfish were pulled from this river just below the dam in an effort to establish a sample population with relatively low levels of toxicity (Blackstone River Watershed, 2014).

II. Introduction

This study was conducted with several goals in mind. The primary objective was to investigate the correlation between crayfish and B[a]P, and to support or refute the idea that they function as bioindicators. Similar experiments have been run in the past, but none of them could produce any reliable, quantified data to support their hypothesis. Several steps were taken in order to avoid potential downfalls seen in previous studies. Our experiment was conducted by collecting orconectes limosus crayfish from a specific aquatic environment where the ecosystem was subject to relatively little pollution. This allowed us to establish a relatively clean baseline when testing the crayfish for B[a]P accumulation.

II A. Crayfish as BioIndicators: A bioindicator species is used as a representative signal of the ecological health of a certain area. Bioindicators are generally a strong representative of the health of an ecosystem, physical or chemical data pulled from a bioindicator can be extrapolated across the entire ecosystem. The presence of a particular substance in a bioindicator indicates the presence and movement of that substance through the food chain. Crayfish are a large source of food for many freshwater organisms that cannot uptake and accumulate carcinogens or toxins in their environment due to different metabolic pathways. The fact that crayfish can accumulate these toxins, however, means that every animal above the crayfish in the food chain will be exposed to toxins that the crayfish uptake. Our bio indicator, the orconectes limosus crayfish, is a primary consumer which means it rests near the bottom of the food chain where there is the most room for upward movement, like most bioindicator species.

The use of orconectes limosus crayfish as a bioindicator, specifically with respect to B[a]P, seems logical. Crayfish are solitary bottom dwellers, and feed off of small animals and plant matter which puts them in close contact with any trace metals or hydrocarbons in their ecosystem. Similarly, native crayfish species are sensitive to pollutants and changes in their environmental conditions. They are, however resilient and can usually adapt fairly quickly to these changing conditions in order to live in moderately polluted environments (Adams, 2010). This resiliency allows the limosus species to be used in any tests involving toxins or carcinogens (Adam, 2010).

II B. Past Projects: Several projects have been conducted on the relationship between B[a]P and the Blackstone River Watershed. In 2009, James Letourneux conducted an investigation into the protocol for detecting carcinogens in local waterways. He used gas chromatography to test the extraction efficiency of hexane on samples with known amounts of B[a]P. The samples were split three main categories; fine, medium, and coarse, based on the particle size of the sample. There was a positive correlation between hexane retention efficiency and the increasing grit of the samples, but the retention data showed significantly lower numbers than expected. The highest observed yield was 42.5%, which was seen in coarse sediment after a 24 hour exposure period. The significant results seen in James' project support the scientific basis for this study. The presence of B[a]P throughout different sediment means that the subject crayfish will remain in contact with

the PAH regardless of what their immediate surroundings are like in the river (Letourneux, 2009).

Another project, conducted in 2007, used the same basic principles to test the PAH accumulation in crayfish. There were several problems mentioned in the report that helped to shape the methodology of this experiment. Firstly, the project members were not able to establish the presence of naturally occurring B[a]P, which meant that they were not able to accurately determine which crayfish were accumulating high percentages from different areas within the stream. Adding controlled amounts of B[a]P to our crayfish over a known amount of time, allowed for a better idea of how much B[a]P was expected out of each crayfish for a 100% retention rate. This removed several variables from the project and created a narrower focus on whether crayfish are able to retain PAHs , such as B[a]P, in any significant concentrations (Goscilla et al, 2007).

The most recent study involving the bioaccumulation of B[a]P was conducted in 2009 by Matthew Cembrola and James Massey. The study aimed to test the relationship between the bioaccumulation of B[a]P and the amount ingested over time. The group predicted that the amount of B[a]P found in the crayfish tissue would correlate with how much contaminated food the crayfish were fed. During this study, a significant amount of sample crayfish died prematurely, affecting the conclusions that could be made. There were several reasons why their results may have been compromised. When the crayfish were brought into the lab, they were caged in groups of 3 or 4. The shelving units with the crayfish were in a busy area of the lab and potentially led to a high stress environment. Similarly, their tanks lacked any sediment or structure which could have contributed to early death. The contaminated food that was prepared was set at a concentration of 2000ng/g, based on the MQP in 2008 (Gikas). Although this concentration was used based on the assumed maximum exposure of B[a]P in the sediment in the crayfish habitat, it may have been too high. This study also limited their study to only test the tail tissue of each crayfish (Cembrola et al, 2009). This limitation may have caused B[a]P levels to appear lower than they were. In order to successfully test for the relationship between the bioaccumulation of B[a]P over time our project had several modifications to the methodology to ensure that the whole sample group of crayfish could be tested.

III. Methodology

III A. Crayfish Collection: Each of the crayfish used in this study were collected over the course of three separate trips from the East Brimfield Dam in Sturbridge, MA, a part of the Blackstone River Watershed. This dam was specifically chosen as the collection site for our study based on its known presence of crayfish populations and due to the assumed cleanliness of the sample site. The sample crayfish were obtained through hand collection and by using fishing nets. Once crayfish were caught, they were placed in buckets filled with dam water and some plants. Over the course of these three trips, a total of 102 Orconectes Limosus crayfish along with several quinebaugensis crayfish were collected and brought back to our lab to be sexed and individually caged. Only the limosus were used for the remainder of the study. The cages were stored on two shelving units in an isolated microscopy lab in Goddard Hall at WPI and there was one window that the crayfish could receive natural sunlight from. Each of the plastic tanks (cages) was filled with tap water as well as 2 cups of medium grit sand and one medium sized ceramic flowerpot. Water levels were maintained to be about 4 inches high and each cage had two holes for air. There were a total of 61 males and 41 females. Ten crayfish were designated as the control group while eighty one crayfish were designated to act as the seven time points for testing. The remaining eleven crayfish acted as a group of long-term feeds who were fed B[a]P contaminated food for the entirety of the study. These eleven were caught in the last collection trip and begun receiving contaminated food several weeks following the main testing group. Two crayfish were not included in the study due to one death and one sample that was dropped.

III B. Food Preparation: Once the crayfish were caged, specially prepared food was given to each crayfish every 3-4 days. The food was specifically prepared to estimate the highest yield of B[a]P that could be accumulated in the tissue of the crayfish at each designated time-point. When preparing the food, it was estimated that in the wild, crayfish consume food that has an approximate concentration of 1000 ng B[a]P/g (Sutton, 2009). The first step in preparing the contaminated food begun by mixing 33 µL of 910ng/uL B[a]P to 100mL of distilled water. This combination was mixed completely using a glass mortar and pestle. Next, 30 g of Fluval Tropical Fish Flakes was slowly added to the mixture while grinding and stirring the mixture until it was an evenly distributed consistency of distilled water, B[a]P and fish food. Approximately 10mL of distilled water was then slowly added to thin the mixture until it was a wet solution but still avoiding a completely liquefied paste consistency. The wet food was then carefully pipetted onto Pyrex baking sheets using a 2mL serological pipet and left to dry for 24 hours in a 37°F incubator. The food was pipetted in rows about 3-5 mm in diameter. After the food was thoroughly dried, it was broken up into smaller pieces, placed in a 50 mL amber glass bottle and stored in a 2°C refrigerator. This was done by scraping the dried food off the baking sheet using a spatula and breaking them into small pieces that were about 0.02g in size. The food was carefully scooped up and placed in a labeled amber bottle for storage. Non-contaminated was prepared using

similar methodology except that B[a]P was not added to the mixture. Instead, 33 µL of pure acetone was used in the place of B[a]P. This non-contaminated food was left to dry for 24 hours, similar to the contaminated food. It was later broken up and scooped into a 50mL amber glass bottle. This bottle was also stored in a 2°C refrigerator.

III C. Freezing and Lyophilization: While the crayfish were being fed, there were seven designated time points at which groups of ten-fifteen crayfish would be frozen in liquid nitrogen and stored in individual Ziploc bags. Table 1 lists the dates at which each set of crayfish were pulled and frozen.

Table 1: Dates of extraction of each set of crayfish

Name of Group	Date	Number of Crayfish	Number of Males	Number of Females
Controls	November 1, 2013	10	8	2
Time Point 1	November 14, 2013	10	6	4
Time Point 2	December 12, 2013	10	6	4
Time Point 3	January 10, 2014	10	6	4
Time Point 4*	February 7, 2014	10	6	4
Time Point 5*	March 6, 2014	14	8	6
Time Point 6*	March 20, 2014	12	7	5
Time Point 7*	April 3, 2014	13	8	5
Long Term Feed**	April 10, 2014	11	5	7

*: Crayfish were switched from contaminated food to non-contaminated food on January 16, 2014 (13 weeks)
**: Fed contaminated food for 24 weeks

All the Ziploc bags containing the frozen crayfish were put into a Ziploc Tupperware container and stored in a -80°C freezer. When a group of crayfish was frozen, the crayfish were individually ground up using a ceramic pestle and mortar in order to break the shell and separate the tissues. Distilled water was used to homogenize the ground crayfish. Using three different sieves simultaneously, the tissue of the ground crayfish was separated

from the shell and other solid materials by sifting it through the sieves. The sieves were composed of a course size (on top), a medium size (between) and a very fine size (bottom). Between each grind, the sieves, mortar and pestle being used were washed with micro 90 soap solution and distilled water. Each of the filtered samples (in liquid form) was transferred into 300 mL lyophilizer jars with lids and then shell frozen using liquid nitrogen. The frozen crayfish samples in the 300 mL jars were then put on a lyophilizer in sets of 5 to 7 and left overnight. The following day, the samples were taken off the lyophilizer. The dry samples were individually collected into pre-weighed 20 mL glass vials and weighed again to determine the weight of each sample. The weight of each sample was recorded along with the date lyophilized, the sex, and what sample group they were part of.

III D. Dry Crayfish Sample Extraction: When preparing samples, hydrophobic extracts were isolated using a specific set of steps using a silica gel column. First, Borosilicate glass disposable Pasteur pipette, size 5 ¾, were packed with about 1.5 mm of glass wool into the tip. A mixture of silica gel and hexane was added to the pipette until it reached a height of 6.02 cm high (Sutton, 2009). The column was then washed with 2 mL of pure hexane using a 2 mL glass pipette to ensure that the column was settled and that the silica did not dry out. This was done using the 5 ¾ sized pipette. In an individual disposable culture tube, size 16x150 mm, 0.2 g of each individual sample was weighed and placed in the test tube. The crayfish sample was then homogenized for 3-5 minutes using a glass pestle in the test tube with 3mL of pure hexane until it was a consistent mixture. The mixture was left to sit for three minutes so that excess tissue would settle once the hexane was fully saturated. To collect the column flow-through, a 20 mL clean glass bottle was placed beneath the column and was labeled with the date, sex, and group the sample was part of. The saturated hexane from the test tube was then transferred, using a Borosilicate glass disposable Pasteur pipette, size 9 with a rubber bulb, to the top of its respective column. The extraction and transfer process was repeated two more times by adding 1 mL aliquots of pure hexane to the test tube and homogenizing the sample with the glass pestle. In between each homogenization of the remaining tissue in hexane, the glass pestle was rinsed with micro 90 soap and distilled water. There were a total of three runs of saturated sample through the column. Finally, 2 mL of pure hexane was run through each column to ensure that the samples were fully washed through the silica gel. The collection vials with the flow through sample were then placed in a gas hood under individual nitrogen gas tubes to ensure the hexane evaporated. This process isolated non-volatile extracts in the vials. The vials were left for about 30 minutes until the hexane was fully evaporated. The vials were then capped and stored in a cardboard box in a -25°C freezer.

III E. Preparation of Sample: Samples were individually prepared so that they could run on the high-performance liquid chromatography (HPLC) instrument. Each dried sample in the 20 mL glass bottle was taken out of the -25°C freezer and placed on the bench top. 250 µL of 100% acetonitrile was quickly inserted into each sample bottle using a 250 µL Hamilton air tight syringe. The solvent was swirled careful along the bottom of the bottle for 1 minute to have the solvent run over the sides of the bottle and the bottom. Individual

2 mL amber glass bottles were all labeled with the date of preparation and group name of each sample. Using a Hamilton 500 µL air tight syringe, 250 µL of the sample in the 20 mL bottle was transferred to a 500 µL luer-locked syringe with 13 mm Nylon membrane filter attached to it that would eject the volume into the 2 mL amber glass vial. This sample preparation was done for each sample that was loaded onto the instrument. Samples were then immediately placed on the HPLC instrument to run. When samples were not running on the instrument, they were covered with parafilm and stored in a -25°C freezer.

III F. HPLC Methodology: When calibrating the HPLC to detect B[a]P in samples, the instrument's methodology was specifically structured for our project. The instrument is a HPLC-5A280-178 and runs with a PepMap C18 4.6X250mm Silica C18 (5µL, 300A) column. The column is set at 38°C and the flow rate is set at 1 mL/min. When injecting samples, 50 µL of sample are injected at a speed of 200 µL/min. There were two mobile phases used when running the samples. Mobile Phase A: 40% Acetonitrile in DIH_2O and Mobile Phase B: 100% Acetonitrile. During each run, the solvent gradients were specific for the samples and the timetable for mobile phase A and B can be seen in Table 2 below. The samples ran with a Stop-Time of 32 minutes and then ran for a Post-Time of 10 min. A needle wash of 100% acetonitrile was injected after every individual sample run and was located in Vial 100. At the conclusion of a sample run, a method called "Control Wash Bottom" was run where 100% acetonitrile was injected at a rate of 1 mL/min for 10-15 min.

Table 2: Timetable of Solvent Gradients for HPLC Sample Runs

Time (Min)	A [%]	B [%]	Flow (mL/min)	Max Pressure Limit [bar]
0.00	100.0	0.0	1.000	400.00
30.00	0.0	100.0	-	-
30.10	100.0	0.0	-	-

III G. Control Silica Gel Column and Filter Experiments: In order to ascertain the % yield of B[a]P from the hexane extraction method (refer to "Dry Crayfish Sample Extraction") and sample preparation using a filter, two control experiments were conducted to test the effectiveness of this project's techniques. The first test was the hexane yield control experiment. The excess control sample tissue that did not contain B[a]P according to the HPLC data were all combined together. It was found that control sample 4 and 9 contained traces of B[a]P and were not included in this test. The entire sample of dried crayfish was placed in a glass pestle and mixed with a total of 45 mL distilled water and 2.1 µL pure B[a]P (at 910 ng/µl) to ensure that the sample was a wet consistency. When homogenizing, the distilled water was added initially, then the B[a]P and finally the dried sample. The mixture was homogenized with a glass mortar and pestle then transferred into a

lyophilizer jar and left to run on the lyophilizer overnight. The total weight of the remaining tissue was 1.1094 g and five separate vials of 0.2 g sample were extracted once the sample was completely dried. Silica gel columns were run with each sample following the exact protocol outlined in dry crayfish sample extraction. Excess hexane was evaporated using nitrogen gas. The samples were then prepared for the HPLC by adding 250 µL acetonitrile to each vial. Each sample was diluted to a concentration of 0.425 ng/mL using acetonitrile, assuming 100% of the B[a]P was retained through the columns. The vials were run on the HPLC, and the resulting readouts produced the hexane yield information. While the five samples were running, the Mobile Phase B began to run low and only samples 1-3 generated results. The results from this test can be seen in Table 3. An average of the area under the curve from these three samples in comparison to the known area under the curve of a pure B[a]P sample that ran with it was taken. The yield of B[a]P was 4.16% and was found by calculating (5.13616 mAU/123.42970)*100. The chromatograms from these tests can also be seen in Figure 12, Figure 14, Figure 15 and Figure 16 in the Appendix.

The second test that was ran was a filter test to ensure that B[a]P was not impeded at all by the pipet filters during the dry sample preparation. First, two samples of pure B[a]P were diluted to 0.2 ng/µL in acetonitrile using a Hamilton 500 µL air tight syringe. The first sample was then transferred into an amber vial using a pipet nylon filter, as used in the "Preparation of Samples" protocol. Both of the syringes were then cleaned using pure acetonitrile. The second sample was transferred to an amber vial, but was not run through a filter during the process. Both samples were loaded on the HPLC, and the resulting data in Table 3 showed whether the filter had an effect of B[a]P concentration. It was found that there was an 82.8% yield of B[a]P when using a filter, calculated using the equation: (102.16253/123.42970)*100. The chromatograms from these tests can also be seen in Figure 12 and Figure 13 in the Appendix.

Table 3: Control Hexane Test and Filter Test Raw Data

Type of Test	Area Under the Curve (mAU)
Pure B[a]P Sample (Unfiltered)	123.42970
Control Hexane Test 1	4.44232
Control Hexane Test 2	5.64120
Control Hexane Test 3	5.32493
Filtered (0.2ng B[a]P)	102.16253

III H. Analysis of B[a]P Samples and Standard Curve: In order to compare the concentrations of B[a]P in each of the crayfish samples, a standard or calibration curve was calculated. To begin to derive the standard curve, a trial run of sample with 1000 B[a]P/µL was loaded onto the HPLC for testing of the concentration of analyte. The results showed that 1000 ng B[a]P/µL gave an area of about 5400 mAU when 0.5 µL was injected. Once it was known that the sample is 5400 mAU/500 ng B[a]P, the concentration could be

simplified to 11 mAU/ng B[a]P. It was first estimated that the crayfish with the highest B[a]P concentration would contain approximately 600 ng B[a]P/animal. This was found by estimating the highest amount of the analyte that would be consumed by the long term feed within this experiment. Each of the samples that were prepared for the curve contained 250 µL of sample in case there is a need for a second run. Each run on the HPLC injects 50 µL. In order to guarantee that the samples can be compared to a standard curve, the highest point of the curve was originally set at 2000ng/500 µL of 100% ACN. This first STD-1 was created using a stock solution of 910ng B[a]P/µL on February 6, 2014. The first standard contained 500 µL of solution and the equation used to determine how much B[a]P was used was done using the calculation below:

$$C_1V_1=C_2V_2$$

$$(910\text{ng}/\mu\text{L B[a]P})(V_1)= (500\mu\text{L})(4\text{ng B[a]P})$$

$$V_1= 2.2\ \mu\text{L of } 910\text{ ng}/\mu\text{L B[a]P in ACN}$$

Equation 1: Determination of Appropriate B[a]P Concentration

To create the STD-2 through STD-10, a 1:2 serial dilution was done. Each of the remaining STD vials contained 250 µL of pure ACN except for STD-10 that had 500 µL. Two separate standard curves were prepared on February 6, 2014 and March 2, 2014. The curve prepared on February 6, 2014 can be seen in the Appendix in Table 10 and the curve from March 2, 2014 can be seen in Table 11 of the Appendix. Before running samples on March 6th, 2014 the methods used to prepare samples for the HPLC changed slightly. A gas tight syringe was now being used to pull up samples before running them through the filter to increase the accuracy of the dilution. On March 5, 2014, an additional run of STD 1 through STD-4 from March 2, 2014 were run and the data can be seen in Table 12 in the Appendix. Using this information, the results of the final standard curve can be seen in Table 4 as well as in a line graph in Figure 2. The points of the curve have an R-value of close to 1 which shows that the data is valid and significant. An additional data point of 0 was added at the bottom of the curve to adjust the curve. This was done to ensure that the B value fell as close to 0 as possible because the pure solvent run did not show a peak in the correct B[a]P retention time range. Some of the concentration of B[a]P may be lower than the curve and this helped to ensure that the standard curve contained the sample's data. The equation of the line in Figure 2 is $y = 301.65x + 0.3719$ and the R-Value is 0.9982 to show that the line is almost completely linear.

Table 4: Calibration Curve of Pure B[a]P on HPLC from March 2 and March 3

Concentration (ng/µL)	Area Under the Curve (mAU)
0.2	62.9745
0.2	59.00197
0.1	29.74286
0.1	30.26791
0.05	14.93573
0.05	15.62386
0.025	7.70431
0.025	8.17016
0.0125	4.57073
0.00625	2.56402
0.003125	1.52638
0.0015625	1.06165
0	0

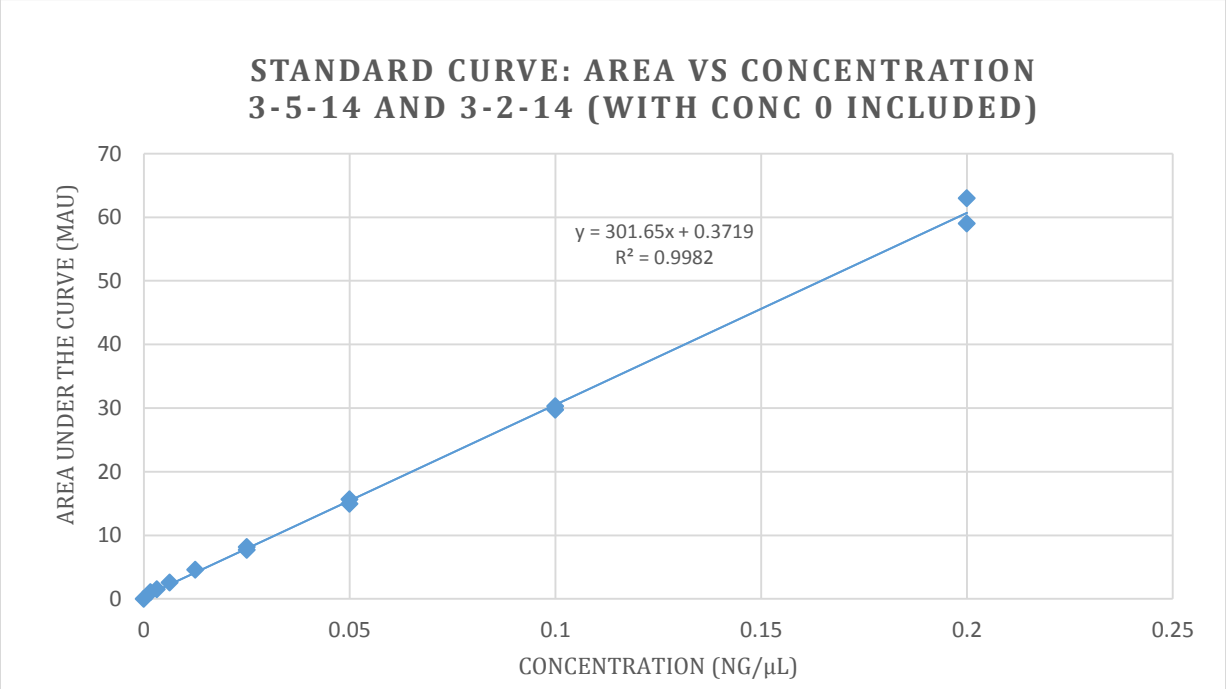

Figure 2: Graph of Standard Curve Concentrations Vs Area on HPLC

III I. Identifying B[a]P on Chromatograms: Once samples ran through the HPLC, chromatograms were collected for every sample and analyzed for the presence of B[a]P. In order to determine the presence or absence of the analyte, a range of retention times was determined using the retention times of pure B[a]P samples that ran on the HPLC for the

standard curves. Throughout the experiment, there were two different ranges of retention times that were identified for B[a]P based on the solvent batch of the Mobile Phase A. The Mobile Phase was switched out and refilled three times throughout the entire runs on the HPLC and this caused a slight drift in the retention time of B[a]P. The standard curves that were run on February 6, 2014 and March 2, 2014 were run using solvent batch 2. The standard curve on March 5, 2014 was run using solvent batch 3. Table 5 lists the range of retention times from the two solvent batches as well as which sample groups were run at that time. To collect additional retention times of pure B[a]P when using solvent batch 3, a sample vial of 0.2 ng/µL was injected within each sample run beginning with Time Point 4. These ranges for the retention times were absolute cut-offs when analyzing the chromatograms.

Table 5: Retention Time Data for Solvent Batch 2 and 3 when Determining B[a]P

Solvent Batch	Dates of Standard Curve	Maximum Retention Time	Minimum Retention Time	Samples Run
Solvent Batch 2	February 6, 2014 and March 2, 2014	24.142	23.951	Controls, Time Point 1, and 2
Solvent Batch 3	March 5, 2014 and pure B[a]P samples during sample runs	24.035	23.701	Time Points 3, 4, 5, 6, 7 and long term feed

IV. Results and Discussion

IV A. Bioaccumulation of B[a]P: At the conclusion of the study, chromatograms had been collected for samples that were analyzed on the HPLC. Due to issues with the HPLC, the results from Time Point (TP) 7 could not be used. When the Long Term Feed ran on the instrument, sample number 88 from TP7 was saved so that results could be gathered from that TP. These results could be seen in Figure 17. There were also two crayfish that were not included in the study because they died. Another sample wasn't included because it was dropped on the floor. To assess the concentrations of B[a]P found in each sample group, each of the samples was analyzed by looking at the retention time (minute) of the peaks as well as the area under the curve (mAu). This information was then included in Table 6 in order to prepare a bar graph quantifying how much B[a]P could be seen in samples over time as shown in Figure 3. A total of 38 samples out of 100 contained B[a]P so 38% of the samples contained the metabolite.

Table 6: Concentration and Area Under the Curve for all Crayfish Samples Having B[a]P

Sample Group	Sex	Size (g)	Sample #	Concentration (ng/mL)	Area Under the Curve (mAU)
Control	M	0.222	4	3.62878	0.329889091
	M	0.5	9	17.19962	1.563601818
TP 2	F	2.261	24	23.2061973	255.26817
	M	0.431	26	0.16898455	1.85883
	M	0.650	27	0.14277	1.57047
	M	0.478	28	0.11042455	1.21467
	M	0.794	29	0.78613818	8.64752
TP 3	M	0.528	31	0.95018818	10.45207
	F	0.448	37	0.054055	0.594605
	F	0.417	39	0.19609273	2.15702
	F	0.31	40	0.08528582	0.938144

Table 6: Continued

Sample Group	Sex	Size (g)	Sample #	Concentration (ng/mL)	Area Under the Curve (mAU)
TP4	F	0.5817	41	16.26434	1.478576364
	F	0.289	42	0.84164364	9.25808
	M	0.3628	43	0.36823	4.05053
	F	0.362	44	0.91656909	10.08226
	M	0.490	45	9.25394273	101.79337
	M	0.871	48	0.47213455	5.19348
	F	0.1615	49	2.75844364	30.34288
	F	0.236	50	8.10077364	89.10851
TP 5	M	0.363	52	0.06725555	0.739811
	F	0.292	56	0.756461	0.068769182
	M	0.690	58	0.05950227	0.654525
	M	0.701	59	0.39615091	4.35766
	M	0.487	62	0.10819909	1.19019
	M	0.521	63	0.29593364	3.25527
TP 6	M	0.331	70	0.059282	0.652102
	F	0.719	74	2.09481	23.04291
TP 7	M	0.309	77	13.79596	1.254178182
	M	0.380	78	1.26517	0.115015455
	M	0.1939	83	1.27841	0.116219091
Long Term Feed	F	0.628	93	12.21779	1.110708182
	F	0.547	94	10.89676	0.990614545
	F	0.723	95	6.24505	0.567731818
	F	0.631	96	6.21139	0.564671818
	M	0.409	97	4.11607	0.374188182
	M	0.168	98	0.985193	0.089563
	M	0.450	99	3.94726	0.358841818
	M	0.317	100	6.24638	0.567852727

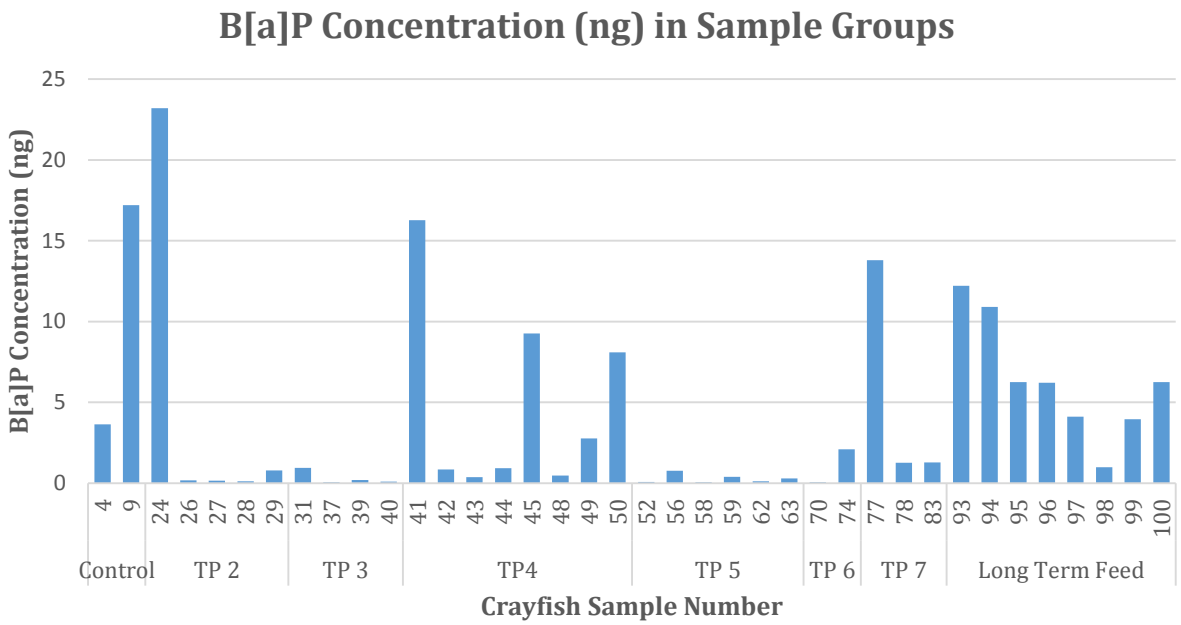

Figure 3: Concentration of B[a]P in Crayfish Sample Groups

When looking at the results from Table 6, there was not enough statistical evidence to conclude that the amount of B[a]P fed to crayfish correlated to the bioaccumulation in their tissue. Similarly, there was no correlation between sex and size of the crayfish that contained B[a]P. It had been previously hypothesized that there would be a linear relationship between the concentration of B[a]P in sample crayfish and the amount of B[a]P ingested. From the results in Table 6 and Figure 3, this linear relationship could not be seen.

As seen in Figure 3 there were two control samples that contained B[a]P. When choosing a collection site for the crayfish, it was assumed that the East Brimfield Dam was relatively free of contaminants in the water and sediment. This was done so that the crayfish participating in the study would begin at baseline before being fed contaminated food. The presence of B[a]P and other particulates in these control samples suggests that the East Brimfield Dam is a contaminated site. While sample crayfish were chosen at random, it was difficult to discern whether these crayfish were older than the other controls possibly affecting how long they were exposed to various pollutants. When samples were collected, they were not chosen based on projected age so there is not enough information to find a correlation between sex, size and the presence of B[a]P in the crayfish. As concluded in Sutton's MQP, tail size in crayfish may be indicative of age and future studies could seek information that could conclude if there is a relationship between age and the amount of B[a]P found in tissue. Figure 4 shows the chromatogram for Control number 9 which contained a significant amount of B[a]P. The peak representing B[a]P is highlighted with a red box. It can be seen that the crayfish had many additional peaks at

various times, showing that there were possibly several other particulates in the sample tissue.

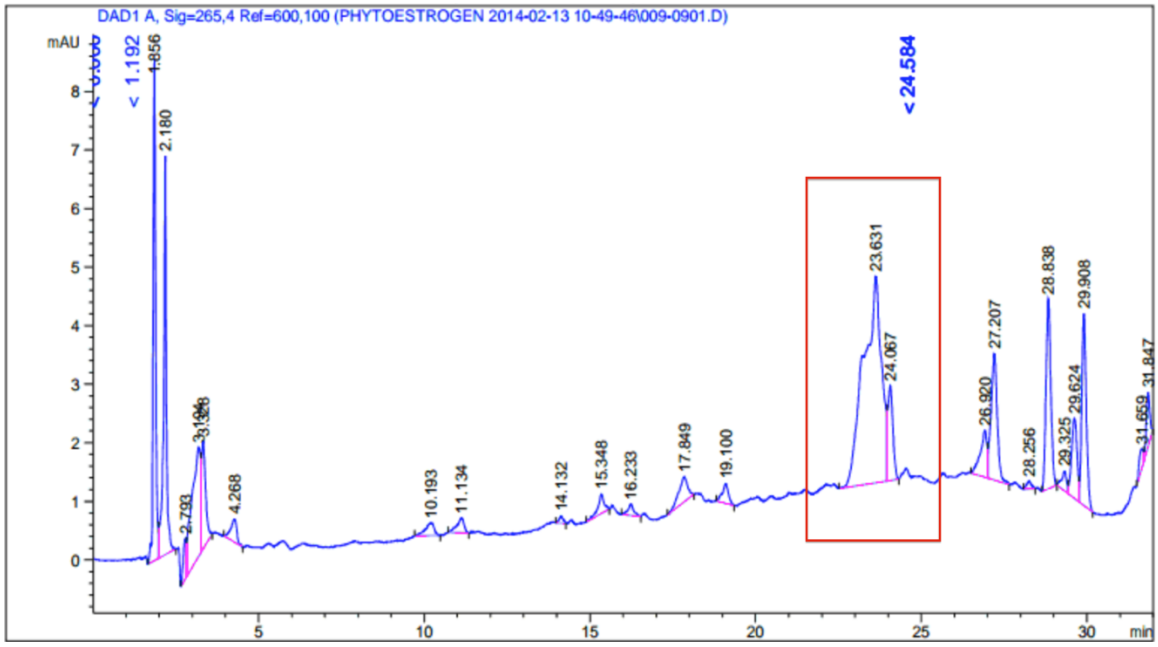

Figure 4: Chromatogram from Control # 9 with B[a]P Peak Highlighted

It could also be seen from Figure 3 that TP2 and TP3 had very little presence of B[a]P. It was expected that increasing levels of B[a]P would be seen over time as the crayfish were being fed contaminated food. TP3 had a smaller amount of B[a]P. On January 16, 2014 the sample groups were switched to non-contaminated food and it was expected that the presence of B[a]P would begin to decrease. By looking at samples in TP4, there was a sudden influx of samples containing significant amounts of B[a]P. More than half of the samples in TP4 contained B[a]P when there were fewer samples in TP2 and TP3 that contained B[a]P. In TP5, the presence of B[a]P decreased rapidly although there were still six samples that contained B[a]P. In TP6 and TP7, there was a decrease in the presence of the metabolite, as expected. In the long term feed (LTF), the results were as expected. There was a presence of B[a]P in almost all of the samples. The concentrations were smaller than expected as the concentrations in the long term feeds were still less than that in control sample 9 and sample 24 in TP2.

Table 7 shows a summary of how many crayfish were analyzed in each group as well as how many contained B[a]P. It also highlights the % of crayfish that contained B[a]P relative to each sample group as well as the total number of crayfish in the study. This Table contains columns that also have the average retention time and area under the curve for samples that contained peaks at 7.1-7.4 minutes. As seen in Table 7, the control, TP1 and TP2 did not contain any peaks at this retention time. Furthermore, there was only one sample in the Long Term Feed that contained a peak at this time.

Table 7: Summary of the Presence of B[a]P in Sample Groups and Average Retention time and Area Under the Curve for Peaks at 7.1-7.4 minutes

Sample Group	Number of Crayfish (That ran on HPLC)	Number of Crayfish with B[a]P per Sample Group	% Crayfish B[a]P Positive per each Sample Group	% Crayfish Positive per Total Sample Group (Total: 94)	Number of Crayfish per sample group with peak at 7.1-7.4 min	Average Area per Time Point in peaks at 7.1-7.4 (mAU)	Average Retention Time in 7.1-7.4 Range (Min)
Control	10	2	20	2.13	0	0	0
TP1	10	0	0	0	0	0	0
TP2	10	5	50	5.32	0	0	0
TP3	10	4	40	4.26	10	24.4	7.2
TP4	9	8	80	8.51	9	27.1	7.2
TP5	14	6	42.9	6.38	14	39.4	7.4
TP6	12	2	16.7	2.13	12	55.3	7.4
TP7	8	3	37.5	3.19	8	31.0	7.4
LTF	11	8	72.7	8.51	1	22.1	7.4
Total	94	38		40.4	54		

IV B. Contaminated Crayfish Samples: Following this study, one of the primary concerns was that there were sample crayfish that were previously contaminated prior to the study. A contamination in the crayfish could affect the results by causing chromatograms to be skewed, affecting how much area under the curve is found for different peaks. It could also cause early death in our samples. It could be seen in several chromatograms that there was a possibility of contamination. As seen in Figure 5 below, representing sample number 41 from TP4, the baseline is severely skewed. The B[a]P peak is also skewed on the baseline as well as the peak at 7.2 minutes. There are also many additional peaks that could represent different pollutants and contaminants that the crayfish had been previously exposed to.

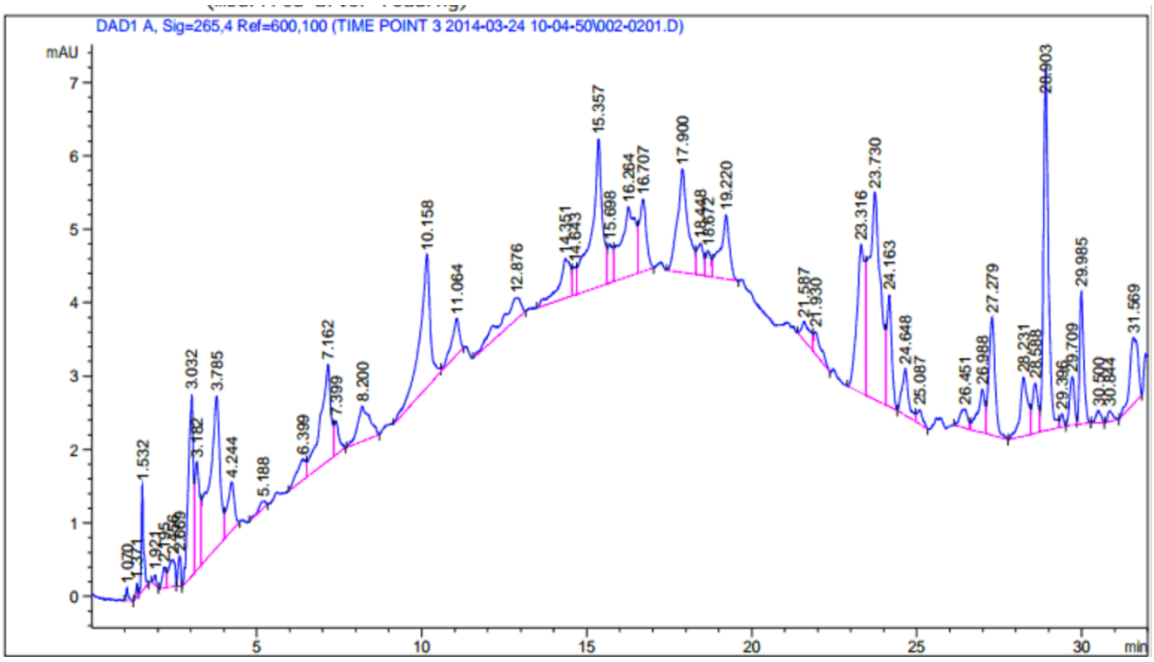

Figure 5: Chromatogram from Time Point 4 # 41

To address the problem of contaminated samples, the collection site of the crayfish should be considered. The crayfish were all picked from the East Brimfield Dam but the radius of the collection site was large. Depending on the location of where the crayfish were collected, there may have been a higher amount of run-off water that was polluted. Age might also be a factor. Older crayfish could have been exposed to different pollutants longer. To help gather stronger results, the collection of sample crayfish should be done early and carefully. The radius of the collection site should be studied to be able to identify if there are areas of the dam that are more polluted than others. Size should also be considered when collecting sample groups. This could help to identify if there is a correlation between size and the amount of B[a]P found in their tissue.

IV C. Peaks of B[a]P Byproducts at 7.1-7.4 Minutes: After observing the chromatograms, it was also noted in all samples from TP3 through TP7, there were recurring at 7.1-7.4 minutes. The average area under the curve can be seen in Table 7 above for each of the time points as well as the average retention time for these peaks at each time point. It can also be seen from Table 7 that 100% of the samples in TP3 through TP7 had a peak at this time in the samples. The average size of these peaks was relatively consistent throughout the samples.

By studying the B[a]P pathway, as seen in the background chapter in

Figure 1, it can be reasonably assumed that this peak was the end product of the B[a]P pathway. The B[a]P metabolic pathways leads to (+)benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide, a hydroxylated form of B[a]P. This compound, because of its hydroxide groups, is more polar than the B[a]P molecule, which shifts its retention time to the left. It

was assumed this will have a relatively strong presence in samples that had ingested B[a]P, which would have been metabolized, resulting in this end product. As it could be seen in Time Points 3-7, this end of the pathway remains in their tissue over time. Figure 6 below shows a chromatogram from sample number 39 from Time Point 3 highlighting this peak.

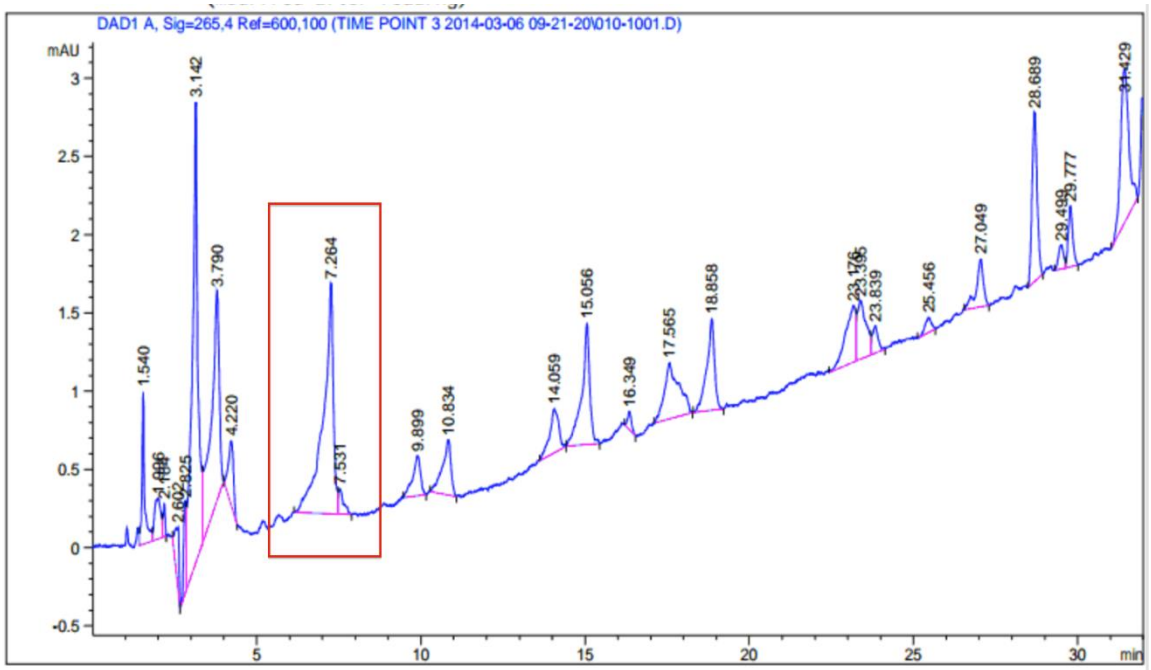

Figure 6: Chromatogram from Time Point 3 # 39

It is also important to note that although long term feeds were exposed to B[a]P, only one of the eleven samples showed this peak. This could be the results of the PAH being fully metabolized by the time it was analyzed. It could also be a result of the long term crayfish having enough time to flush out excess pollutants from their system, decreasing their bodies reaction to B[a]P and its byproducts. As seen in Figure 7, the long term feed does have a peak for B[a]P at 23 minutes but not one at 7 minutes.

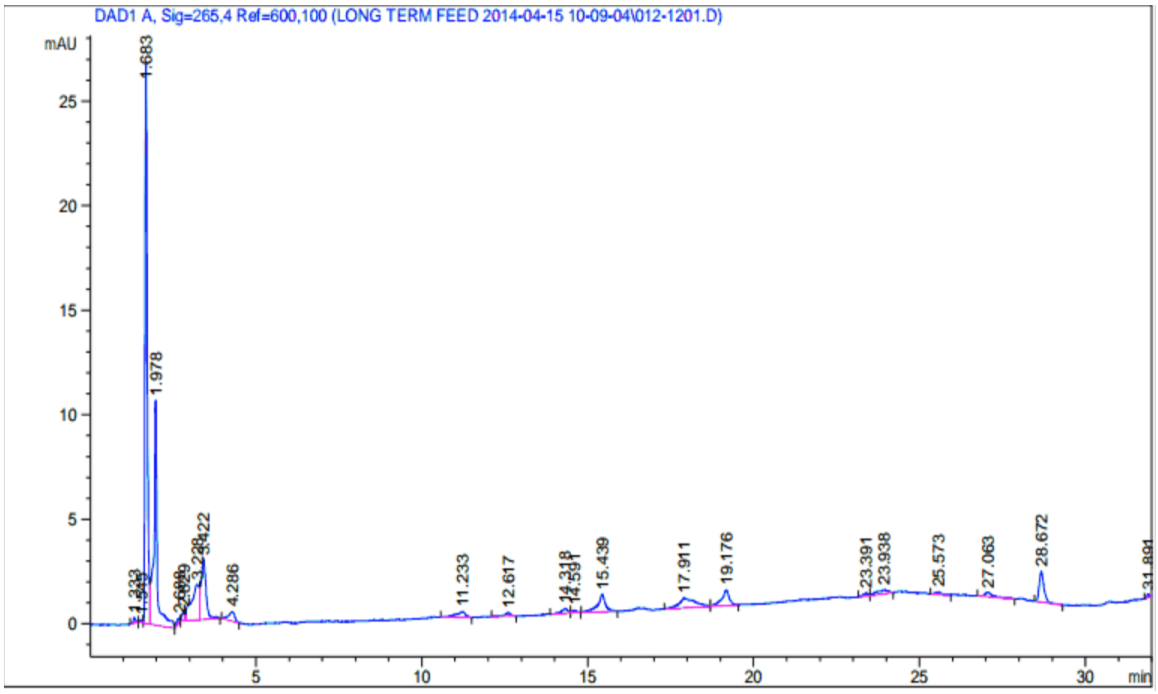

Figure 7: Chromatogram from Long Term Feed # 97

IV D. Comparison of Past MQP: At the beginning of this project, when discussing the methodology, there were several changes made to the protocol that different from the MQP by Cembrola and Massey (2009). While some of the methods helped to gather stronger data there were still adjustments that should be made for future projects to ensure that the most significant data is collected. One of the first changes that we made was the storage of crayfish during the study. In the 2009 MQP, crayfish were placed in tanks of 1-3 crayfish in a high stress environment with no sediment in their tanks. It is believed that this was a leading factor in the number of deaths experienced throughout their project. To address this, we individually caged each of our crayfish and left them in an isolated room that had continuous direct sunlight. We only experienced one death during our study, of an unknown cause, and may have experienced more if we had not adjusted our methodology. We also modified the concentration of the contaminated food that was prepared so that it was a lower concentration than that of Cembrola and Massey's MQP. We used information from Sutton's MQP (2009) to find the best techniques necessary to prepare contaminated food that would help us gather significant results and avoid premature death.

IV E. Alternative Methodology: At the conclusion of this project, several areas of the methodology that could have been modified to help gather stronger data were found. The first observation was that the methods of extracting B[a]P from sample tissue using hexane silica gel column was less efficient than initially expected. When a control test was conducted to test the percent yield of B[a]P through this column, the yield was 4.16%,. This was very low and could have been a large indicator of why there was an absence or very low concentrations of B[a]P in early time point samples. This could have also caused a smaller amount of B[a]P to be observed in each of the samples throughout the study. In

order to change how B[a]P was extracted from the dried tissues samples, it is highly recommend that future groups explore alternative solvents that can be used to separate B[a]P from the sample. Also, control tests to find the % yield of B[a]P should be conducted early in the study to ensure that the strongest extraction techniques are being used. When conducting our control hexane tests, we only had three samples run on the HPLC and this is not enough information to be certain that our extraction techniques always gave a low yield of B[a]P, but based on the % yield that we saw, alternative extraction methods should be explored in future projects.

Another issue that was found while running samples on the HPLC was the preparation of different solvent batches or Mobile Phase A when they ran out. Every time there was a new batch made of this solvent, it caused a slight drift in the retention time. Although we ran pure samples at the start of the running of a new solvent batch to find drifts in the retention time, this change could have affected some of our results.

V. Conclusion

Once each of the samples were analyzed for the presence of B[a]P in their tissue, it was concluded that there was not enough statistical evidence to support the bioaccumulation of B[a]P in Limosus Orconetes crayfish. It is also unclear whether crayfish are ideal indicators when studying the bioaccumulation of a PAH although there is a strong potential that they are based on the significant presence of B[a]P in the tissue of two control samples. The hypothesis that the presence of B[a]P in tissues would correlate with the amount of B[a]P digested was not supported from the results. Future experiments should be done with alternative methods to support whether crayfish bioaccumualte B[a]P in their tissue.

References

Adams, S., Schuster, G.A. & Taylor, C.A. 2010. *Orconectes limosus*. In: IUCN 2013. IUCN Red List of Threatened Species. Version 2013.2. www.iucnredlist.org

Anderson, M. B. (1997). Bioaccumulation of lead nitrate in red swamp crayfish (Procambarus clarkii). *Journal of hazardous materials, 54*(1-2), 15-29.

Backer, J. (1980). Mitochondrial DNA is a major cellular target for a dihydrodiol-epoxide derivative of benzo[a]pyrene. *Science (New York, N.Y.), 209*(4453), 297-299.

Baum, E. (1978). Occurrence and surveillance of polycyclic aromatic hydrocarbons. *Polycyclic hydrocarbons and cancer, 1*, 45-70.

Bielaczyc, P., & Merkisz, J. (1998). *Cold start emissions investigation at different ambient temperature conditions*: Society of Automotive Engineers.

Blackstone River Watershed (2014) http://www.ririvers.org/wsp/Watersheds/BlackstoneRiverWatershed.htm

Buckley, T. J. (1992). An examination of the time course from human dietary exposure to polycyclic aromatic hydrocarbons to urinary elimination of 1-hydroxypyrene. *British journal of industrial medicine, 49*(2), 113-124.

Byeong-Kyu L. & Van T. V (2010). Sources, Distribution and Toxicityof Polycyclic Aromatic Hydrocarbons(PAHs) in Particulate Matter. *Department of Civil and Environmental Engineering, University of Ulsan, Korea* http://www.intechopen.com/download/get/type/pdfs/id/11767

CDC (2014). Polycyclic Aromatic Hydrocarbons (PAHs) How Do PAHs Induce Pathogenic Changes? http://www.atsdr.cdc.gov/csem/csem.asp?csem=13&po=10

Cembrola, M & Massey, P. (2010). *Bioaccumulation and Degradation Rates of Benzo [a] pyrene in Crayfish.* WORCESTER POLYTECHNIC INSTITUTE.

Gikas, P. E. (2008). *Benzo [a] pyrene contamination of sediment in a Blackstone Valley streambed.* WORCESTER POLYTECHNIC INSTITUTE.

Goscila, J., LaBue, J. & Trahan-Liptak, J. (2007). *Crayfish (Orconectes virilis) as bioindicators of polycyclic aromatic hydrocarbon contamination in the Blackstone River Valley.* WORCESTER POLYTECHNIC INSTITUTE.

Hardin, J. A. (1992). Mechanisms by which benzo[a]pyrene, an environmental carcinogen, suppresses B cell lymphopoiesis. *Toxicology and applied pharmacology, 117*(2), 155-164.

Hecht, S. S. (1979). Analysis of faeces for benzo[a]pyrene after consumption of charcoal-broiled beef by rats and humans. *Food and cosmetics toxicology, 17*(3), 223-227.

James, M. O. (1998). Cytochromes P450 in crustacea. *Comparative biochemistry and physiology. C, Comparative pharmacology and toxicology, 121*(1-3), 157-172.

Kerr M. (1990). Rhode Island Sea Grant Fact Sheet. The Blackstone River http://seagrant.gso.uri.edu/factsheets/blackstone_river.html

Letourneux, J. (2009). *Benzo [a] Pyrene in River Sediment*. WORCESTER POLYTECHNIC INSTITUTE

Lioy, P. L. (1988). The Total Human Environmental Exposure Study (THEES) to Benzo(a)pyrene: Comparison of the Inhalation and Food Pathways. *Archives of environmental health, 43*(4), 304-312.

Mitchelmore, C. L. (1998). Evidence for cytochrome P-450 catalysis and free radical involvement in the production of DNA strand breaks by benzo[a]pyrene and nitroaromatics in mussel (Mytilus edulis L.) digestive gland cells. *Aquatic toxicology, 41*(3), 193-212.

Newbold, R. F. (1976). Exceptional mutagenicity of a benzo[a]pyrene diol epoxide in cultured mammalian cells. *Nature (London), 261*(5555), 52-54.

Sutton, A. (2009). *Contamination of Sediment and Crayfish with Benzo [a] pyrene in a Blackstone Valley Stream.* WORCESTER POLYTECHNIC INSTITUTE.

U.S. Centers for Disease Control Agency for Toxic Substances and Disease Registry (ATSDR).
1995. "Toxicological Profile for Polyaromatic Hydrocarbons (PAHs)." http:// www.atsdr.cdc.gov/toxprofiles/tp69.html

Varanasi, U. (1980). Metabolic activation and covalent binding of benzo[a]pyrene to deoxyribonucleic acid catalyzed by liver enzymes of marine fish. *Biochemical pharmacology, 29*(5), 753-761.

Zhu, H. (1995). Characterization of Benzo[a]pyrene Quinone-Induced Toxicity to Primary Cultured Bone Marrow Stromal Cells from DBA/2 Mice: Potential Role of Mitochondrial Dysfunction. *Toxicology and applied pharmacology, 130*(1), 108-120.

Appendix

Table 8: Collection of all Raw Crayfish Sample Data

Number	Group	Sex	Dry Weight (g)	Retention Time for B[a]P (min)	Area Under the Curve in Samples with B[a]P (mAU)	Conc. (ng B[a]P)	Retention Time of Additional 7 Min Metabolite	Area Under the Curve for Samples with 7 Min metabolite (mAU)
1	Controls	F	0.144	None			None	0
2	Controls	F	0.41	None			None	0
3	Controls	M	0.349	None			None	0
4	Controls	M	0.222	23.982	3.62878	0.32988909	None	0
5	Controls	M	0.51	None		0	None	0
6	Controls	M	0.6	None		0	None	0
7	Controls	M	0.7	None		0	None	0
8	Controls	M	0.3	None		0	None	0
9	Controls	M	0.5	24.067	17.19962	1.56360182	None	0
10	Controls	M	0.4	None		0	None	0
11	TP 1	F	0.7	None		0	None	0
12	TP 1	F	0.6	None		0	None	0
13	TP 1	F	0.6	None		0	None	0
14	TP 1	M	0.3	None		0	None	0
15	TP 1	F	0.6	None		0	None	0
16	TP 1	M	0.7	None		0	None	0
17	TP 1	M	0.5	None		0	None	0
18	TP 1	M	0.6	None		0	None	0
19	TP 1	M	0.5	None		0	None	0
20	TP 1	M	0.5	None		0	None	0
21	TP2	M	0.625	None		0	None	0
22	TP 2	F	0.151 (But 0.2 was weighed out)	None		0	None	0
23	TP 2	F	2.415	None		0	None	0

Table 8: Continued

Number	Group	Sex	Dry Weight (g)	Retention Time for B[a]P (min)	Area Under the Curve in Samples with B[a]P (mAU)	Conc. (ng B[a]P)	Retention Time of Additional 7 Min Metabolite	Area Under the Curve for Samples with 7 Min metabolite (mAU)
24	TP 2	F	2.261	24.12	255.26817	23.2061973	None	0
25	TP 2	F	2.372	None		0	None	0
26	TP2	M	0.431	23.947	1.85883	0.16898455	None	0
27	TP 2	M	0.65	23.958	1.57047	0.14277	None	0
28	TP 2	M	0.478	24.143	1.21467	0.11042455	None	0
29	TP 2	M	0.794	24.142	8.64752	0.78613818	None	0
30	TP 2	M	0.694	None		0	None	0
31	TP 3	M	0.528	23.819	10.45207	0.95018818	7.244	42.61341
32	TP 3	M	0.601	None		0	7.206	12.23602
33	TP 3	M	0.326	None		0	7.215	27.44379
34	TP 3	M	0.66	None		0	7.225	21.26617
35	TP3	M	0.465	None		0	7.225	16.22543
36	TP 3	M	0.201	None		0	7.242	18.02959
37	TP 3	F	0.448	23.749*	5.95E-01	0.054055	7.245	20.82236
38	TP 3	F	0.342	None		0	7.242	37.37383
39	TP 3	F	0.417	23.839	2.15702	0.19609273	7.264	31.74903
40	TP 3	F	0.31	23.825	9.38E-01	0.08528582	7.279	15.74496
41	TP 4	F	0.5817	24.163	16.26434	1.47857636	7.162	29.15506
42	TP 4	F	0.289	24.031	9.25808	0.84164364	7.075	43.24675
43	TP 4	M	0.3628	23.816	4.05053	0.36823	7.111	24.98455
44	TP 4	F	0.362	24.031	10.08226	0.91656909	7.194	23.54214
45	TP 4	M	0.49	24.008	101.7934	9.25394273	7.238	21.54779
46	TP 4	Sample Was Dropped				0		
47	TP 4	M	0.3286	None		0	7.301	39.98837
48	TP 4	M	0.871	23.868	5.19348	0.47213455	7.332	20.4517

Table 8: Continued

Number	Group	Sex	Dry Weight (g)	Retention Time for B[a]P (min)	Area Under the Curve in Samples with B[a]P (mAU)	Conc. (ng B[a]P)	Retention Time of Additional 7 Min Metabolite	Area Under the Curve for Samples with 7 Min metabolite (mAU)
49	TP 4	F	0.1615	23.971	30.34288	2.75844364	7.334	23.95372
50	TP 4	F	0.236	24.021	89.10851	8.10077364	7.366	16.82078
51	TP 5	F	0.301	None		0	7.349	21.74931
52	TP 5	M	0.363	24.001	7.40E-01	0.06725555	7.346	40.62347
53	TP 5	M	0.282	None		0	7.361	41.76569
54	TP 5	F	0.25	None		0	7.355	41.19835
55	TP 5	F	0.33	None		0	7.361	41.97452
56	TP 5	F	0.292	23.948	7.56E-01	0.06876918	7.358	27.69386
57	TP 5	F	0.2	None		0	7.363	42.24989
58	TP 5	M	0.69	23.785	6.55E-01	0.05950227	7.37	53.78532
59	TP 5	M	0.701	23.989	4.35766	0.39615091	7.366	57.0079
60	TP 5	M	0.531	None		0	7.372	49.82681
61	TP 5	M	0.715	None		0	7.353	39.01157
62	TP 5	M	0.487	23.985	1.19019	0.10819909	7.353	38.73777
63	TP 5	M	0.521	23.867	3.25527	0.29593364	7.358	27.15838
64	TP 5	M	0.572	None		0	7.35	28.80688
65	TP 6	F	0.477	None		0	7.345	87.9509
66	TP 6	M	0.329	None		0	7.363	86.81517
67	TP 6	M	0.304	None		0	7.348	50.52395
68	TP 6	M	0.355	None		0	7.366	47.44394
69	TP 6	M	0.453	None		0	7.354	41.63172
70	TP 6	M	0.331	24.014	6.52E-01	0.059282	7.348	28.06587
71	TP 6	M	0.506	None		0	7.361	36.89657
72	TP 6	F	0.831	None		0	7.363	48.82069
73	TP 6	F	0.74	None		0	7.373	56.05846
74	TP 6	F	0.719	23.986	23.04291	2.09481	7.386	61.1042
75	TP 6	M	0.251	None		0	7.375	57.6489

Table 8: Continued

Number	Group	Sex	Dry Weight (g)	Retention Time for B[a]P (min)	Area Under the Curve in Samples with B[a]P (mAU)	Conc. (ng B[a]P)	Retention Time of Additional 7 Min Metabolite	Area Under the Curve for Samples with 7 Min metabolite (mAU)
76	TP 6	F	0.43	None		0	7.375	60.2111
77	TP 7	M	0.309	24.001	13.79596	1.25417818	7.363	51.13347
78	TP 7	M	0.38	24.01	1.26517	0.11501546	7.374	57.49874
79	TP 7	M	0.376	None		0	7.361	27.8718
80	TP 7	M	0.362	None		0	7.342	17.95521
81	TP 7	M	0.317	None		0	7.353	45.047
82	TP 7	M	0.236	None		0	7.339	2.43139
83	TP 7	M	0.1939	23.831	1.27841	0.11621909	7.353	53.63543
84	TP 7	F	0.372	None		0	None	0
85	TP 7	M	0.2476	None		0	None	0
86	TP 7	F	0.232	None		0	None	0
87	TP 7	F	0.538	None		0	None	0
88	TP 7	F	0.563	None		0	7.39	30.97045
89	TP 7	F	0.377	None		0	None	0
90	LTF	F	1.417	None		0	7.392	0.07962
91	LTF	F	0.551	None		0	None	0
92	LTF	F	0.956	None		0	None	0
93	LTF	F	0.628	23.887	12.21779	1.11070818	None	0
94	LTF	F	0.547	23.895	10.89676	0.99061455	None	0
95	LTF	F	0.723	23.924	6.24505	0.56773182	None	0
96	LTF	F	0.631	23.897	6.21139	0.56467182	None	0
97	LTF	M	0.409	23.938	4.11607	0.37418818	None	0
98	LTF	M	0.168	23.919	9.85E-01	0.089563	None	0
99	LTF	M	0.45	23.939	3.94726	0.35884182	None	0
100	LTF	M	0.317	23.912	6.24638	0.56785273	None	0

Table 9: Keys Explaining Feature in Table 8

	Small	<0.3g
	Medium	0-3-0.7g
	Large	>0.7g
	Samples that Ran in Solvent Batch 2	
	Solvents that Ran in Solvent Batch 3	

Table 10: Standard Curve from February 6, 2014

	Concentration (ng/µL)	Area Under the Curve (mAU)
STD-1	4	1841.22559
STD-2	2	952.30804
STD-3	1	485.8768
STD-4	0.5	253.57309
STD-5	0.25	135.10429
STD-6	0.125	73.77847
STD-7	0.0625	42.39774
STD-8	0.03125	26.56377
STD-9	0.015625	19.17291
STD-10	0.0078125	14.87625

Table 11: Standard Curve from March 2, 2014

	Concentration (ng/µL)	Area Under the Curve (mAU)
STD-1	0.2	59.00197
STD-2	0.1	30.26791
STD-3	0.05	15.62386
STD-4	0.025	8.17016
STD-5	0.0125	4.57073
STD-6	0.00625	2.56402
STD-7	0.003125	1.52638
STD-8	0.0015625	1.06165

Table 12: Standard Curve from March 5, 2014

	Concentration (ng/µL)	Area Under the Curve (mAU)
STD-1	0.2	62.9745
STD-2	0.1	29.74286
STD-3	0.05	14.93573
STD-4	0.025	7.70431

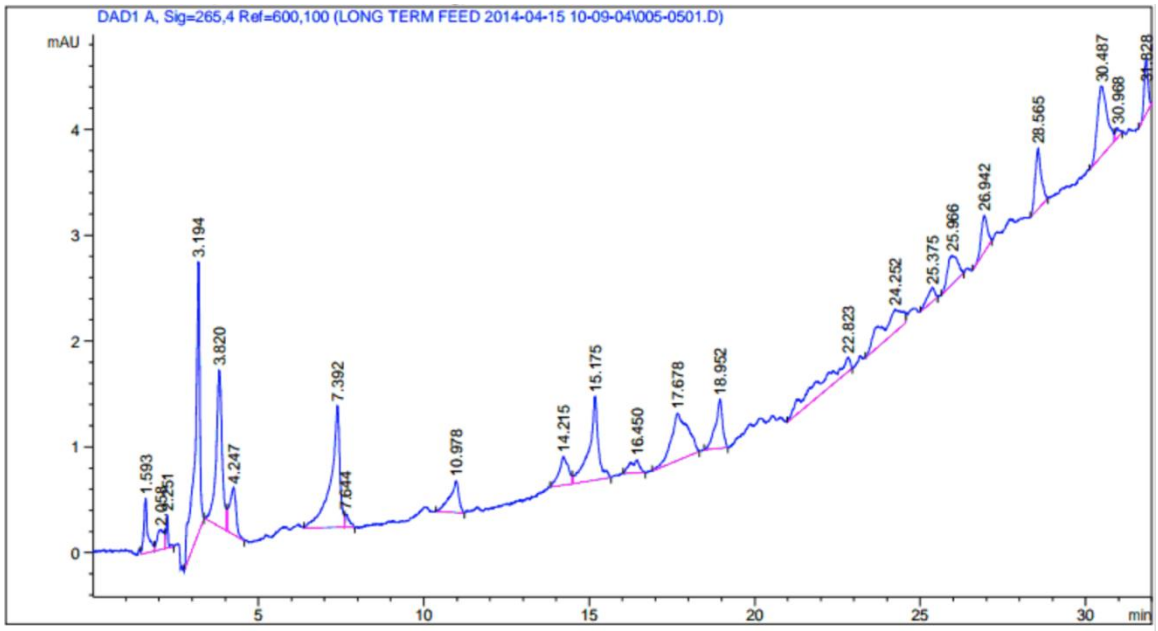

Figure 8: Chromatogram from Long Term Feed # 90

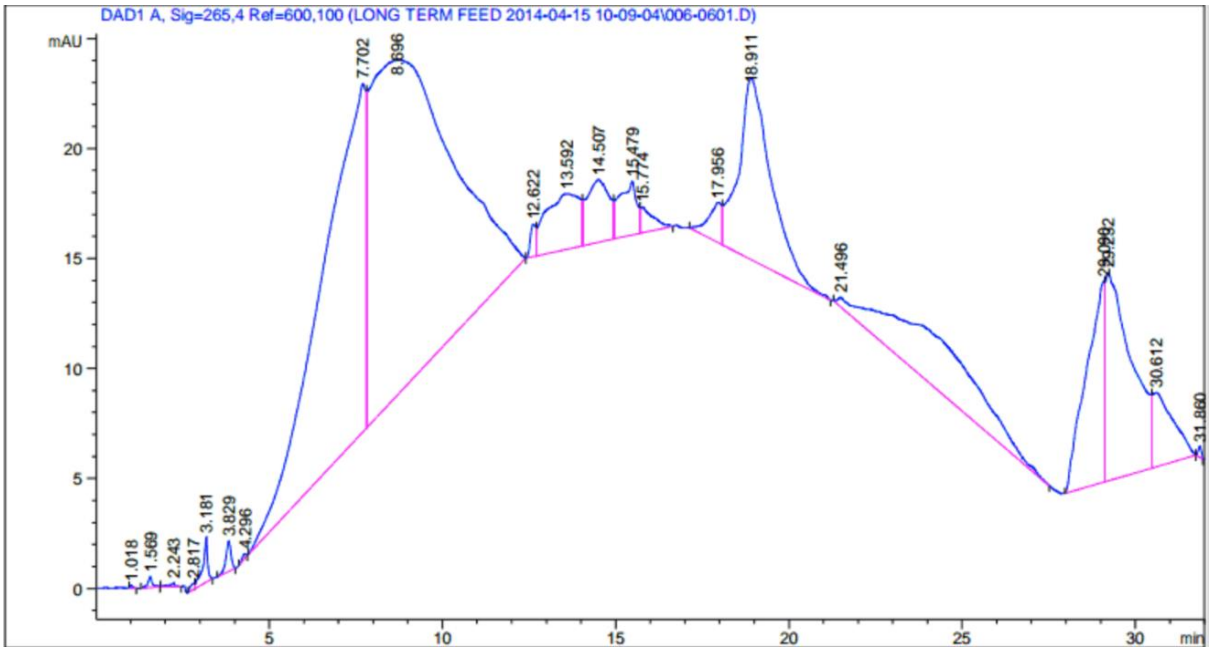

Figure 9: Chromatogram from Long Term Feed #91

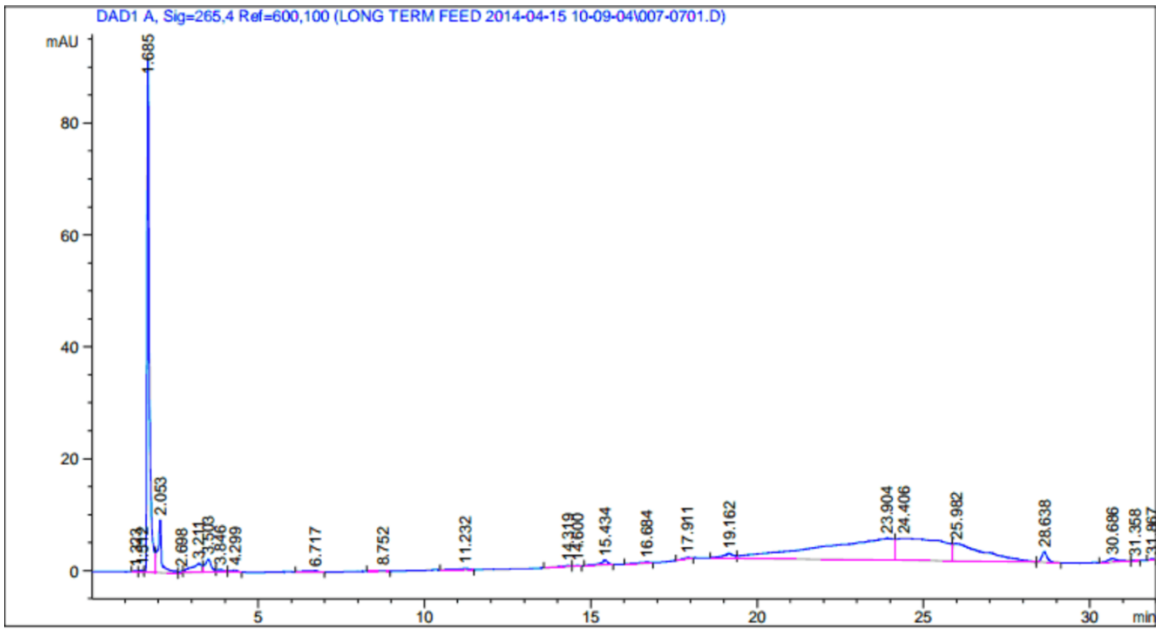

Figure 10: Chromatogram from Long Term Feed # 92

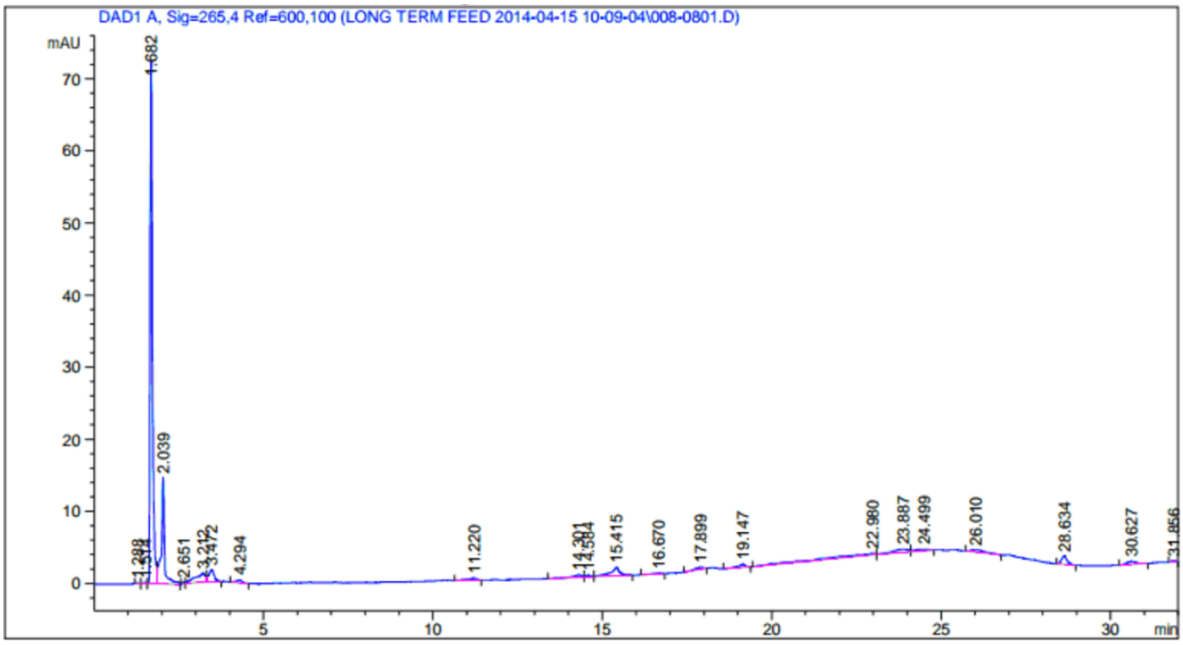

Figure 11: Chromatogram from Long Term Feed # 93

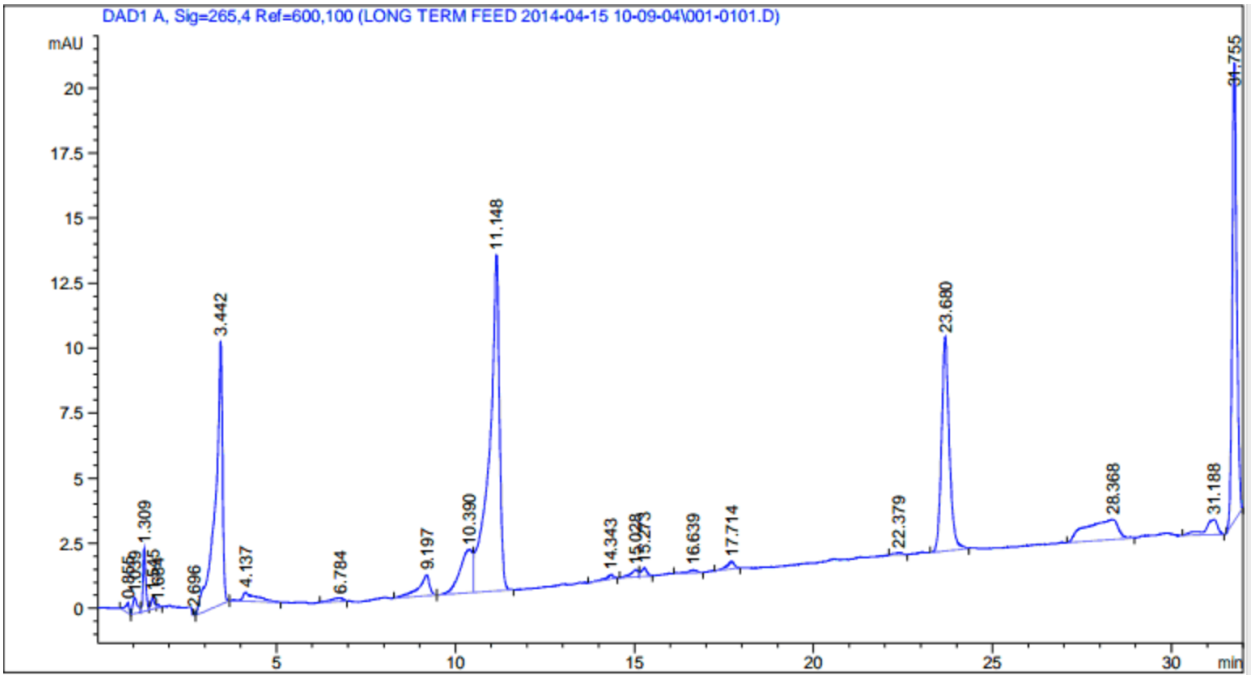

Figure 12: Unfiltered Pure B[a]P 0.2ng Control Test

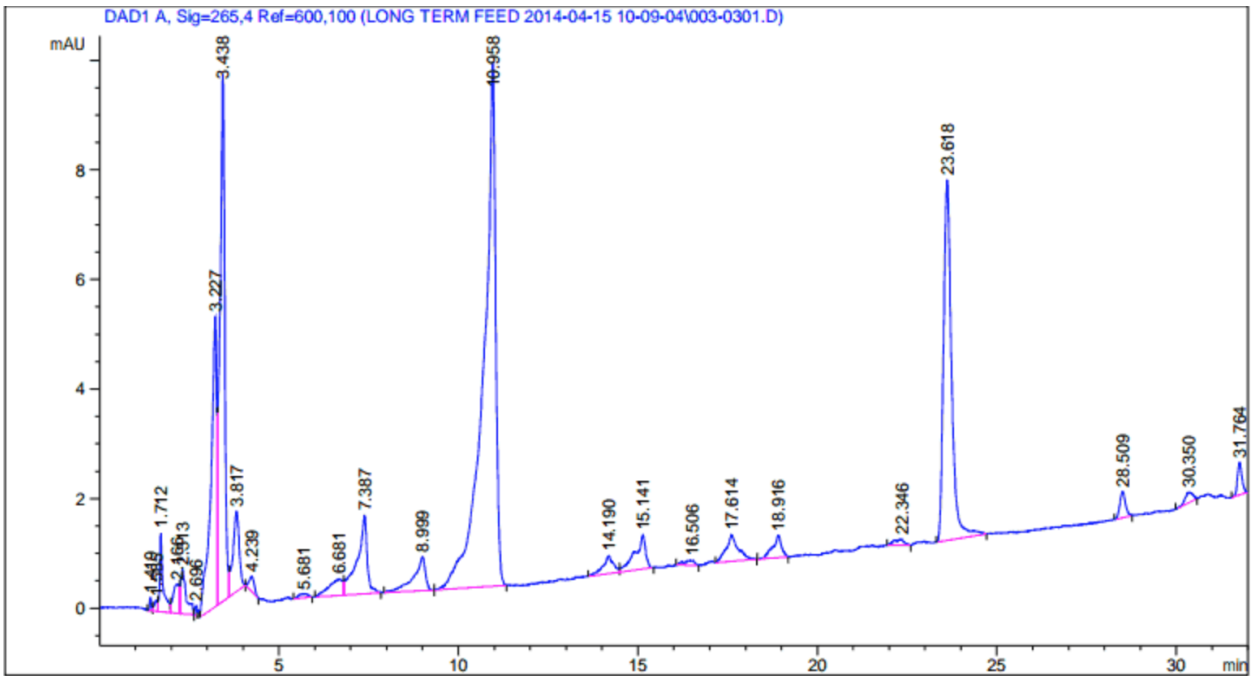

Figure 13: Filtered Pure B[a]P 0.2ng Control Test

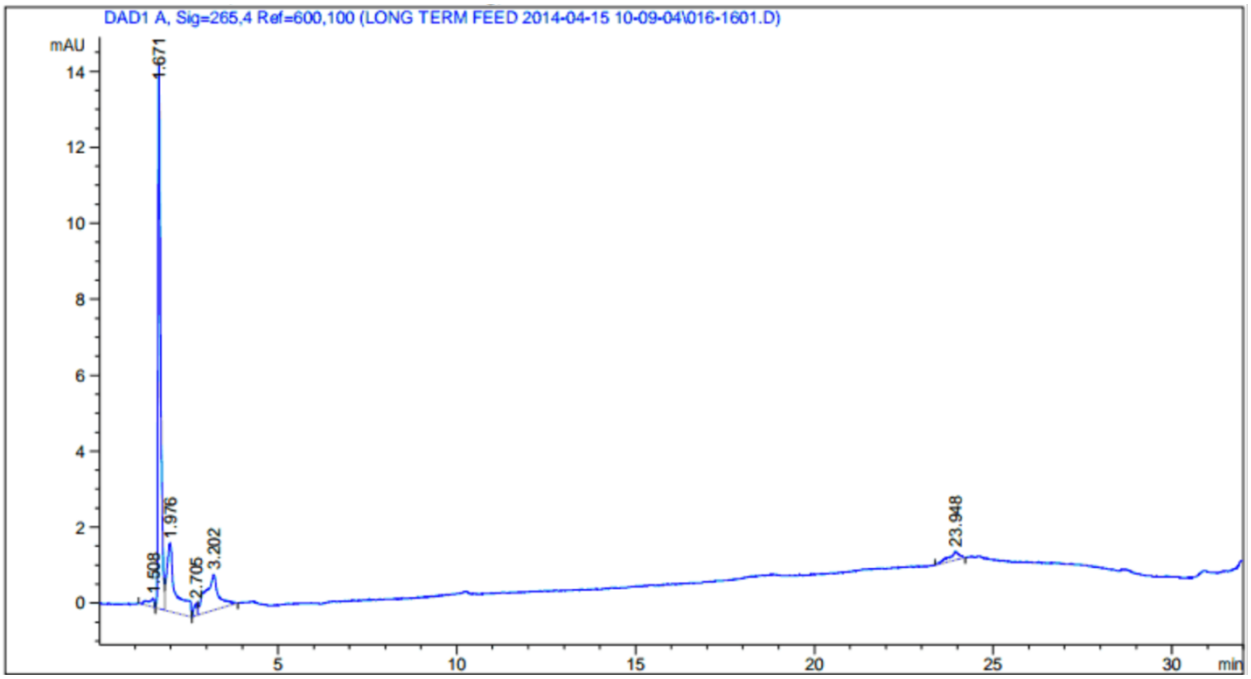

Figure 14: Control Hexane Test 1

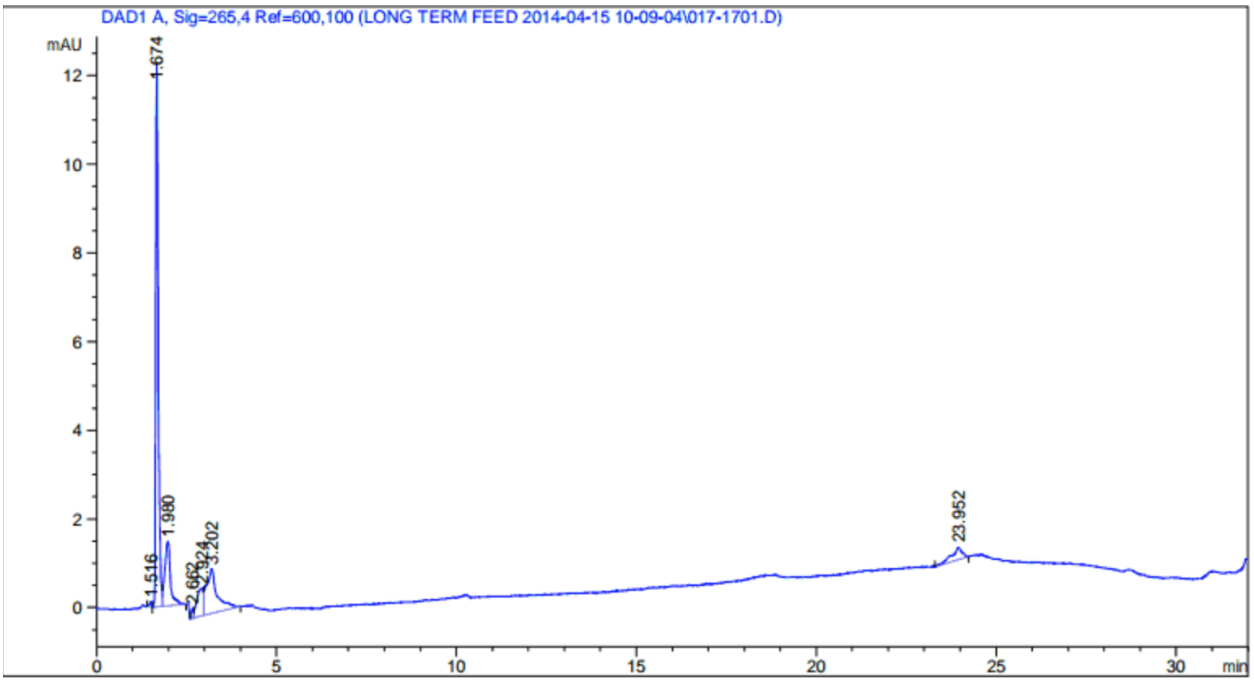

Figure 15: Control Hexane Test 2

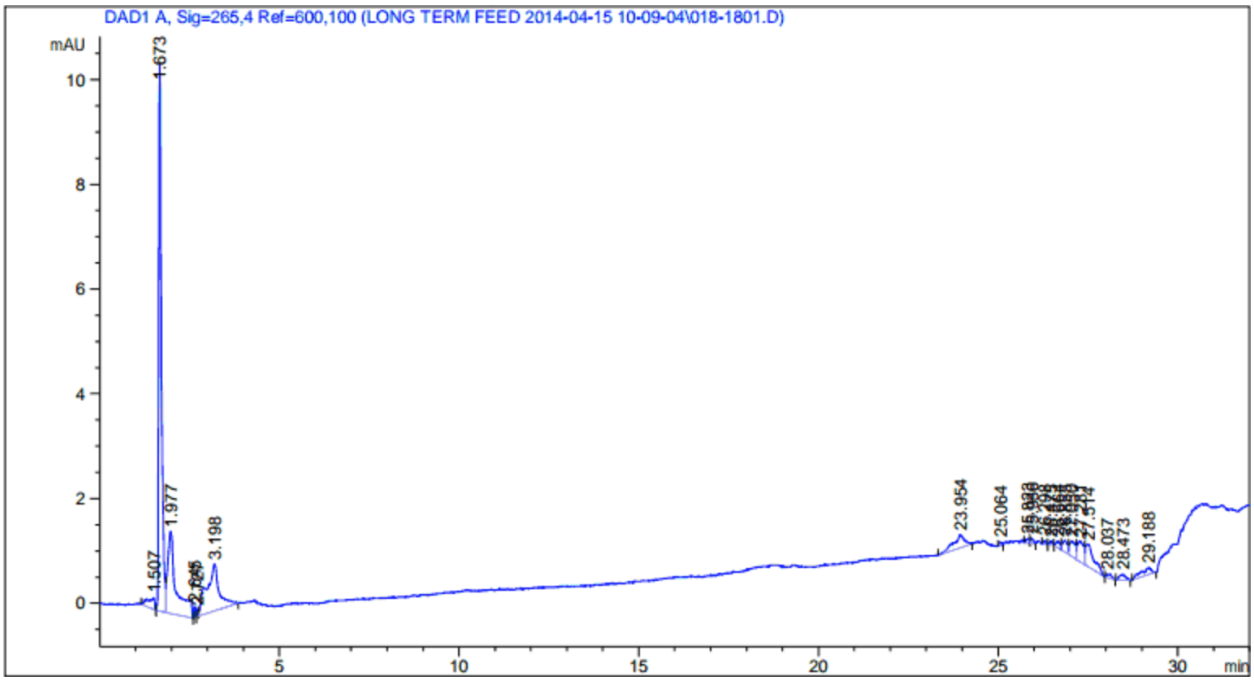

Figure 16: Control Hexane Test 3

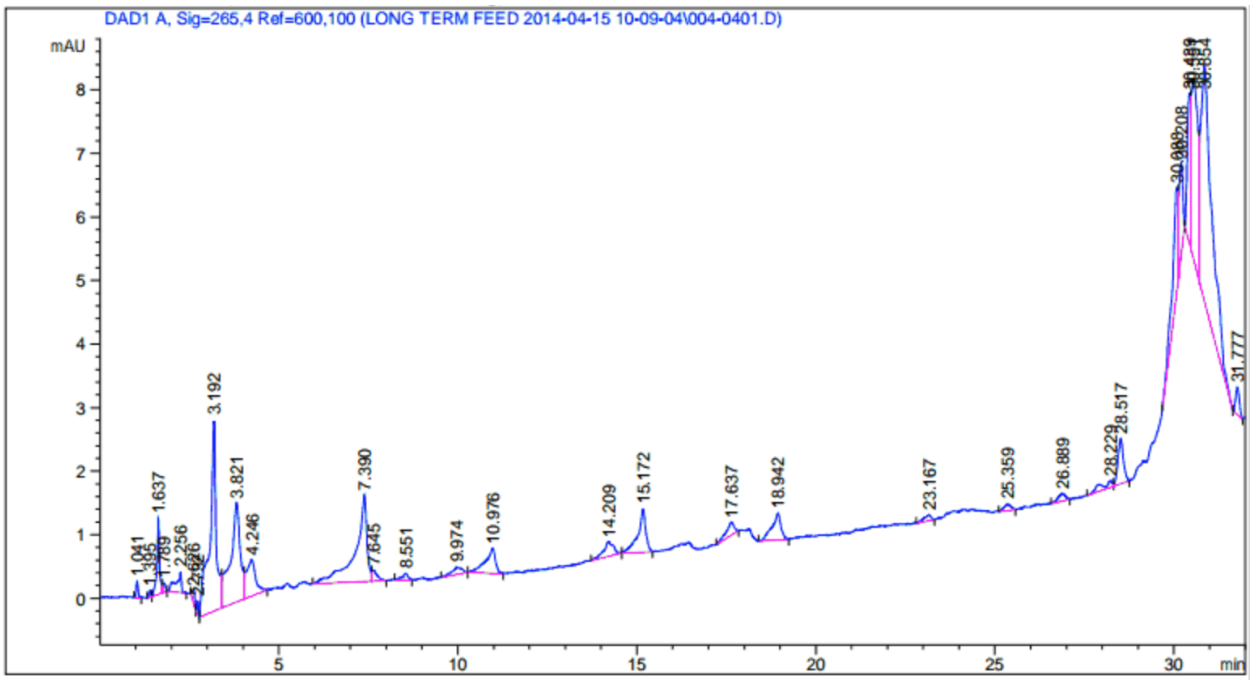

Figure 17: Chromatogram from Time Point 7 #88