Benzo[a]Pyrene Extraction from Crayfish Bioindicator Project

A Major Qualifying Project Report:
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By

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Abstract:

The Bioindicators project continues the work of the previous projects by attempting to isolate the contaminant known as Benzo[a]pyrene from crayfish extract. The end goal is to be able to determine how long each crayfish needs to be exposed to B[a]P to appear in the tissue. Preliminary analysis shows that the specimens may need to be exposed to the contaminants for a longer period of time than the MQP parameters allow.

Background:

Benzo[a]pyrene

Benzo[a]pyrene is an aromatic hydrocarbon (PAHs) with 5 phenol rings (figure 1). Its chemical formula is C20H12 and has a molar mass of 252.31 g/mol. B[a]P has no net charge and can be found in either a liquid or solid form. (pubchem 2015)

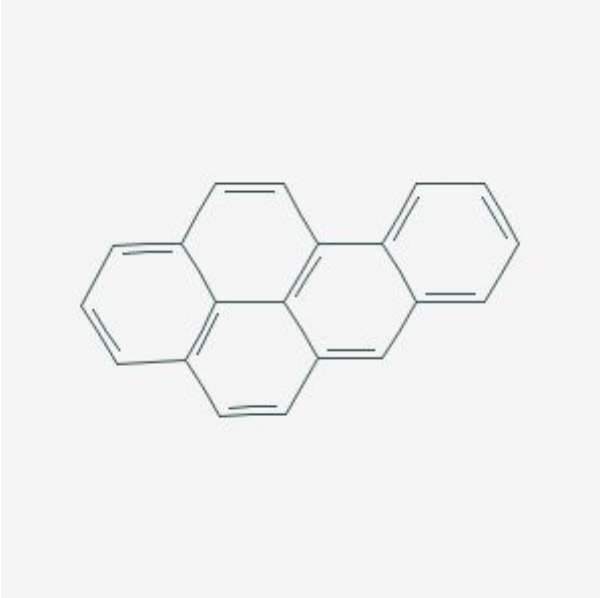

Figure 1: structure of of B[a]P

B[a]P is a byproduct of the incomplete burning of fossil fuels or other organic such as propane or wood. Like other PAHs, B[a]P has a tendency to bond to biological compounds such DNA or lipids. B[a]P is not very soluble in water at 20 degrees C, the solubility was 3.8 μg l−1. (Kot-Wasik 2004) Because of these two factors B[a]P has a tendency to collect at the bottom of lakes and ponds bonded to the organic sediment. It will undergo photo-oxidation after irradiation by either sunlight or fluorescent light while its in organic solvents. (pubchem 2015) It's been known to be cancer causing agent and people and animals. When ingested, it becomes a bioactivated compound benzo[a]pyrene diol oxide (BPDE)(Pubchem 2015) BPDE has the ability to bind with DNA and other organic molecules. It is both a promutagen and a procarcinogen. (Pubchem 2015)

Bioaccumulation of B[a]P

Bioaccumulation occurs when a compound such as B[a]P gets passed up the foodchain from low concentrations in low tier organisms like crayfish into top of the food chain predators such as birds.

It starts when organic material is burned producing B[a]P. B[a]P eventually condenses near the bottom of ponds and streams. The crayfish eat contaminated food contaminating themselves. Other animals feeds off the crayfish causing biomagnification of the contaminant. In such high concentration, it can be harmful and cause cancer to develop. (pubchem 2015)

Previous studies on B[a]P have suggested that it can be stored in the animal or plant tissue was well as sediment. (Niladri 2015) It is expected that this can occur in crayfish as well.

B[a]P has two different pathways it can use to leave the system. The first path is simply being excreted out passing through the digestive system. It also can be degraded either by the enzymes such as P459 1A1 and cytochrome P450 (Aksu, Yidirim & Danabas 2014) which are known to help degrade similar PAHs in crayfish.

B[a]P can also be degraded by sunlight or fluorescent light. However its natural half life is months long. (pubchem 2015) This could happen either before or after it has been absorbed into the tissue as long as crayfish are in a brightly lit environment. However, due to the design of the crayfish habitats and B[a]P's and the time span necessary to degrade B[a]P using sunlight, its impact on this experiment is minimal.

Previous MQP

There have been three previous studies involving Benzo[a]pyrene in crayfish at WPI relevant to this project.

The first experiment (Goscila, LaBue, Lipak 2007) was done in order to investigate the possibility of using crayfish as bioindicators for aromatic hydrocarbons. The crayfish were taken from their habitat. Their findings concluded that there was enough evidence to support the possibility that crayfish can become contaminated with B[a]P. However it was not known how long B[a]P needs to be present in order for the crayfish to become contaminated.

The 2010 MQP was a project that aimed to determine the rate of bioaccumulation in crayfish. However, the group had difficulty keeping their crayfish alive long enough to be studied. They kept them in a high human traffic area, unnaturally lighting, no place for the crayfish to hide and abnormally high temperatures for the season.(Cembrola & Massey 2010) As a result of this, they were unable to run a proper experiment.

Most recently there was a study that was done in 2014 which had a similar goal to to the 2010 MQP. Unlike the previous however, they were successfully able to keep the crayfish in captivity

long enough to attempt to isolate the B[a]P from the crayfish extract. However their extraction method was fundamentally flawed. They used hexane gel column extraction, which only resulted in a yield of 4.16% of all B[a]P added in a controlled experiment. (Briseño, Chadwick & Owusu-Ansah 2014) This made almost all of the data collected useless.

The primary focus of the current 2015 MQP is to continue the work of the previous MQP and to find a way to separate B[a]P in a more efficient manner. Our secondary goal was to determine the rate of bioaccumulation of B[a]P in crayfish.

Extraction Methods:

In preparation for the project, five methods, if including the previously used method, were explored for extracting B[a]P from crayfish extract. This is one of the major focuses of this MQP due to the fact that the previous group had difficulty isolating B[a]P from their extract. (Briseño, Chadwick & Owusu-Ansah 2014)

Methods that were considered, but were decided against were, the light petroleum method, the MASE method and the smeds method (D. Mooibroek 2002) The light petroleum method was the least effective at isolating B[a]P and was similar to the previous MQP's method. The MASE method had potential, but it required specialized microwave equipment. Lastly there was the smeds method, which while it would be effective in theory, required far too much time to run.

The final method settled upon was the dispersive solid phase extraction. (Niladri 2014)(Whitefleet-Smith 1994). It was chosen for it's reliability, speed, and relative availability of the necessary materials. In addition to this, the advisers are somewhat familiar with this method meaning that should trouble arise during the extraction, they may be able to assist easier then if an unfamiliar method was used.

Crayfish:

Crayfish can be found in almost every small pond, ditch or stream in North America. They are useful as bioindicators because of their sensitivity to changes in the environment and tendency to absorb various contaminants such as Benzo[a]pyrene or related chemicals. (Goscila, LaBlue & Banerjee 2007)

Crayfish are omnivores and will eat almost anything fed to them. In addition to this they are bottom-feeding organisms. The combination of these two things makes them extremely prone to coming into contact with Benzo[a]pyrene which sinks to bottom of lakes and attaches to rotting vegetation. The vegetation would then be ingested by the crayfish exposing them to B[a]P. Many animals including bass, birds and even people feed off crayfish. Therefore, it is only natural that crayfish metabolization of B[a]P be studied in order to understand the role it plays in the ecosystem.

Because of their relative abundance and genetic diversity it's important that the crayfish be gathered from one body of water and be of the same species. This way the genetic diversity can be kept under control.

Materials and methods:

Collection of Crayfish

For the sake of keeping the crayfish genetically similar, the crayfish were captured from only two different locations with a majority of them coming from lakes in Charlton and Westborough. The crayfish were captured using modified minnow and crayfish traps. A few different baits were experimented with, but the vast majority of the time liver was bait of choice.

Crayfish Habitats

Learning from the previous MQP studies, the crayfish were kept away from high human traffic areas and abnormally lighting. The WPI greenhouse was selected because of its more natural day/night cycle, lower exposure to humans and more natural seasonal temperatures.

The crayfish are kept in plastic containers with water levels slightly higher than the lengths of their bodies. Containers also contain around an inch of sand and a small plant pot that acts as a hiding place for the crayfish. Each container is roughly 31x19x27 cm. giving a total volume of 15903 cubic centimeters. (figure 2)

Figure 2: Crayfish Habitats

Feeding Groups

Each crayfish was numbered, measured, and put into a group. They were measured from just under the eye stock to the start of the tail. (figure 3) The groups were made first by dividing the females equally and then by size, with each group ending up with a similar average size and total size. Crayfish were fed on Fridays and Mondays.

Table 1: Groups

Feeding Groups					
Control	**Size**	**Short Feed**	**Size**	**Long feed**	**Size**
5(F)*	**29.01**	**2(F)**	**30.44**	**19 (F)**	**25.88**
22	**33.55**	**6**	**36.20**	**32 (F)**	**32.88**
14	**34.65**	**33**	**34.29**	**14**	**34.65**
17	**30.80**	**26**	**30.10**	**23**	**31.95**
25	**29.50**	**20**	**29.00**	**27**	**30.00**
24	**28.54**	**31**	**28.54**	**30**	**28.77**
8	**27.95**	**16**	**27.66**	**18**	**27.87**
15	**27.32**	**28**	**26.78**	**9**	**27.44**
12	**25.44**	**7**	**25.32**	**21**	**26.65**
1	**24.27**	**13**	**23.10**	**29**	**24.05**
11	**20.00**	**3**	**19.30**	**4**	**20.88**

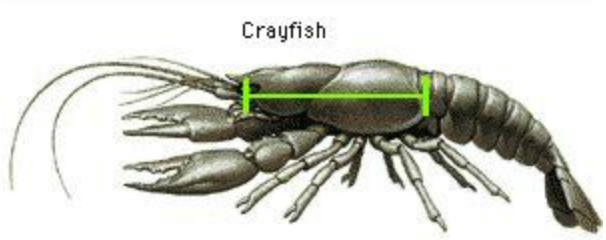

Figure 3: Crayfish are measured from eyestock to start of tail. (*Mackers)*

Crayfish Harvest

Feeding for all three groups began on 10/02/15. The short feed and control crayfish were harvested on 1/25/16. They were taken out of their containers and frozen in liquid nitrogen. They were left in liquid nitrogen until they stopped bubbling, were labeled and put into plastic bags. The bags were then transported to the lab and were put into the freezer.

Sample Preparation:

The crayfish were ground into mush, filtered through two layers of sieves, and dried into a powder by a lyophilization. The dried crayfish were kept in separate labeled glass containers. The samples where then put into a refrigerator until they are ready.

HPLC Chromatography Method:

HPLC samples were run in a similar way as the previous MQP (Briseño, Chadwick & Owusu-Ansah 2014) The instrement we are using is a HPLC-5A280-178. It runs with a PepMap C18 4.6X250mm Silica C18 (5µL, 300A) column. The Temperature the column is set to 38 C. The flow rate was 1 µL/min. Jar A had 40% acetonitrile 40% water and jar B had 100% acetonitrile with a linear gradient from 100% A to 100% B. However, instead of the 30 mins the previous MQP used we stretched the gradient time to 35 mins.

Extraction methods

Dispersive solid phase extraction was used to extract B[a]P from dried crayfish samples. The basic protocol is as follows. 0.2 g of dried crayfish was added to 2mL Eppy tubes. 1 mL of acetonitrile were added to the tubes and was vortexed on a high setting for 1~ minute or until the powdered crayfish was completely dissolved. After this the tube was left to incubate at room temperature for 1 minute. After this the tube was centrifuged at 10,000g for 5 min. This should cause the solids to settle to the bottom into a pellet.

The liquid was micro pipetted from the eppy tube and transferred to a labeled 16 x 150 test tube without disturbing the pellet. Added 1 mL of acetonitrile to the pellet, vortexed for a minute and then removed the remainder of the liquid into the test tube, then repeated those steps.
From there the liquid was dried under liquid nitrogen and stored in tubes under wrap.

In order to determine the best possible way of extracting B[a]P from crayfish we experimented with several different methods of extraction. Each one has one or perhaps several variations made in them in an attempt to maximize the yield of the extraction.

Method 0.0

0.0 was a test which involved adding 0.2 g within (0.001 g) of crayfish was added into an eppy tube with 0.8 mL of water vortexed for a minute. After vortexing 2 mL Acetonitrile (AcCN) was added to to the eppy tubes. After this, 0.4 mgSO_4 and 0.1 NaCl was added to each tube, the tube was then vortexed for 1 minute or until completely saturated.

From there the extract was centrifuged for five minutes until pelleted, and then liquid was removed from the pellet into a bottle which was dried under nitrogen. The resulting dry residue was dissolved in 0.1 ml acetonitrile vortexed until all solids were dissolved, 2 ml hexane was added and vortexed for one minute. The hexane solution was filtered through a silica gel column,

dried a second time, and dissolved in 1 ml of acetonitrile. The samples were then analyzed using HPLC protocol.

Method 1.0
Method 1.0-3.0 were carried out at the same time and used the same method as seen above with a few adjustments.

For 1.0 acted as a control for these groups. It was 0.0 done at the same time as the rest of these experiments for consistency.

Method 2.0
After the water and acetonitrile was added to the tube The extract was sonicated for 10 mins and hand mixed every 1-2 mins while sonicating. We then preceded to continue method by spinning the remaining extract into a pellet.

Method 3.0
Instead of using acetonitrile as a solvent, method 3 used 2ml hexane as a solvent for creating a pellet. We didn't manage to isolate any B[a]P with this method.

Method 4.0
Method 4.0 was an experiment that switched out the former plastic tubes for a glass one and let the tubes stand overnight for 3 days while in a shaker. 5ml of water and 2 ml acetonitrile was added and sonicated for 10 minutes, before being centrifuged and having the liquid removed to go on to the next steps. The water was an accident due to the sonicator.

Because theses sample had so much liquid it was divided into two separate layers to dry.

Method 5.0
The amount of water was doubled to 1.6 ml and 2x the amount of mgSO and salt were also doubled in order to absorb the extra water.

Method 6.0
The sample was left in a glass test tube for 7 days. It was taken out and sonicated every three days during this time period and vortexed. This was to test what method 4 was trying to test for without water leaking into the tube. The tube was sonicated for 10 mins before it was centrifuged and going through the remaining steps. In addition to these changes, I accidently bought this particular tube home where it was left in a refrigerator.

Method 7.0
This method was the last chance we had to test a method, so we tried several diffrent things. 2.4 ml of water were added and sonicated for 50 mins. After this 5 ml of acetonitrile was added to the water. The method then continues as normal.

Results:

In order to determine what concentrations of B[a]P we were detecting we constructed a standard curve using several dilutions of B[a]P in acetonitrile. The y-axis is the area under the curve and the x-axis is the total concentration of B[a]P ng per l.

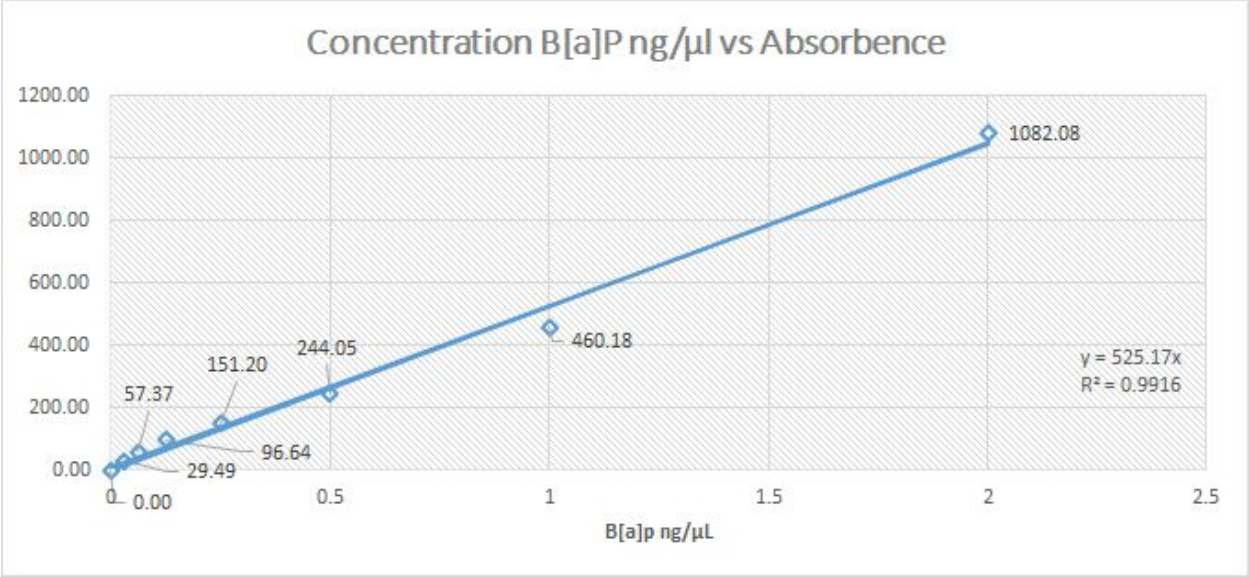

Figure 4: B[a]P standard curve

We found that the average retention time was variable for each batch of acetonitrile 40% acetonitrile. For half the the tests we had a retention time of 31.60 for batch 1, 28.51 for batch 2 and 28.86 for batch number 3.

To determine the total amount of crayfish B[a]P in a crayfish we'd take the area under the curve and apply the formula:

$$B[a]P \ \ mg/g \ crayfish = (((525.17/X) * 1000) * 0.2)/1800$$

Unfortunately, neither the long term crayfish or the short term crayfish yielded any significant yields. None of them had any level of B[a]P in them. HPLC readings showed no spike at 28.5 minutes where the B[a]P would be expected.

Figure 6 shows examples of peaks that are examples of things that are not B[a]P. B[a]P is only found within a specific time frame.

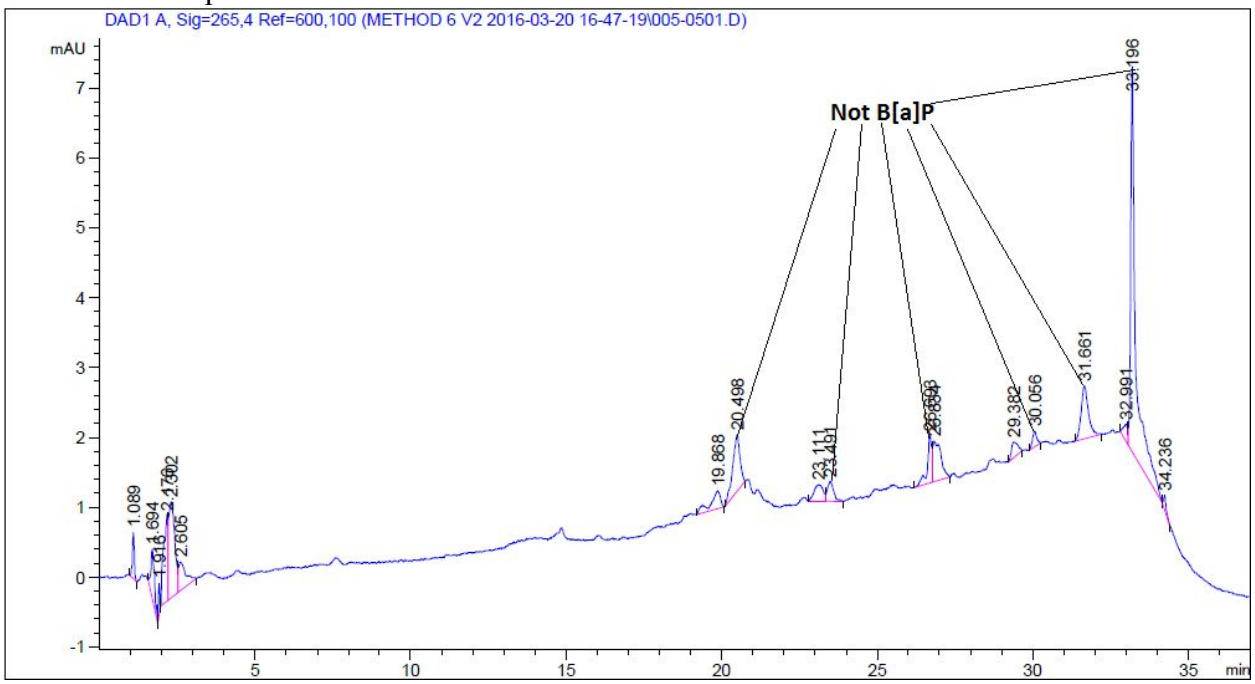

Figure 6: Long feed Crayfish number 9

Figure 8 shows the HPLC trace of 1ng/uL B[a]P as an example of what a B[a]P should look like. It it should have a retention of around 28.5 minutes retention time, however the retention time will shift every time the acetonitrile solvent is changed.

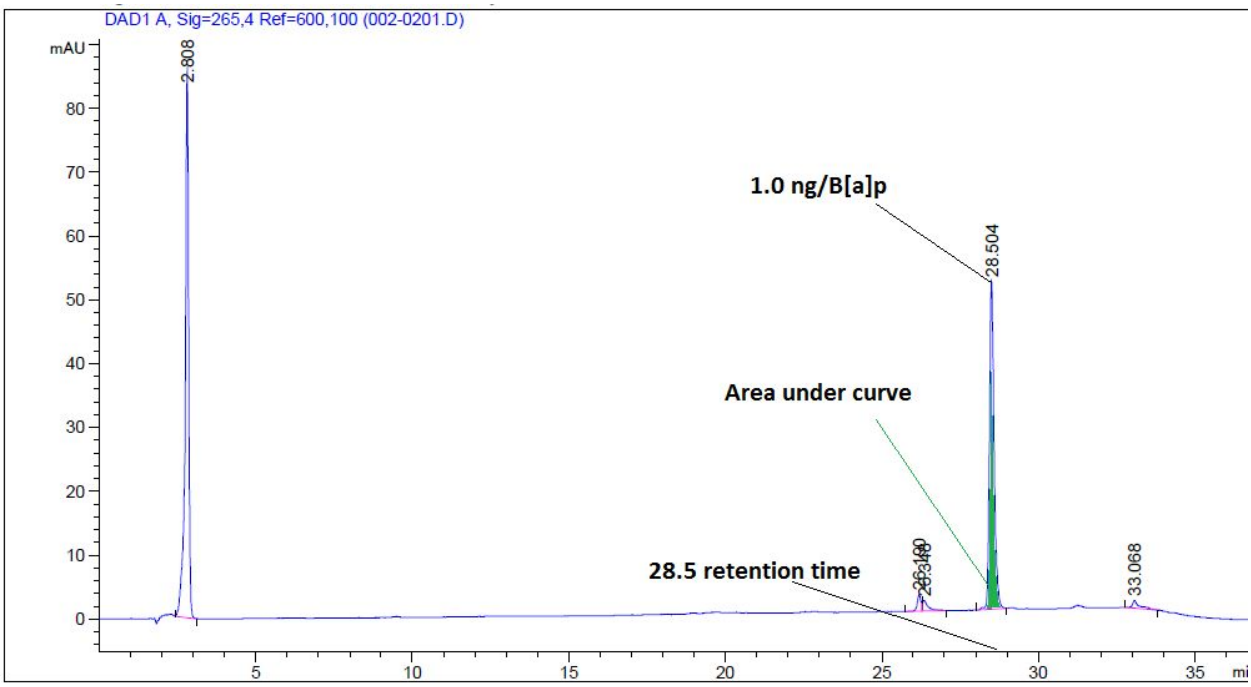

Figure 8: HPLC trace of the 1ng/uL B[a]P standard

Figure 5 is an example of a typical B[a]P extract. There are a couple of spikes in the data, but the B[a]P spike is at 28.5 minutes.

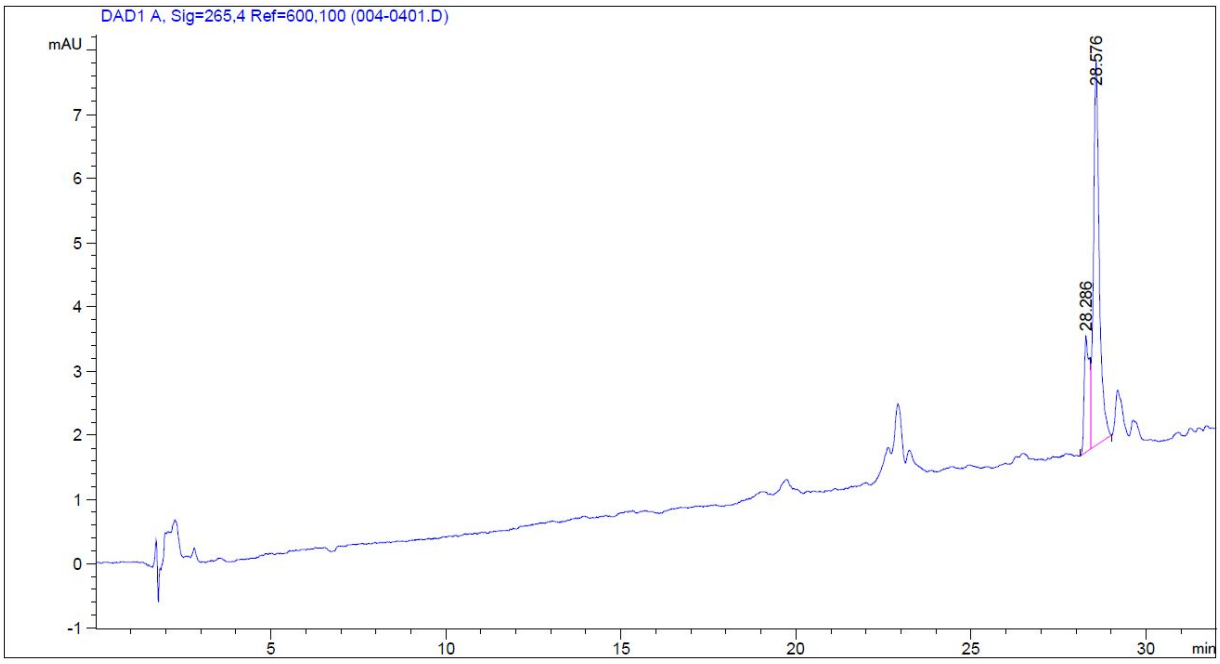

Figure 5: Method 1.0 B[a]P

Our best result was using method 1.0. The Area for 1.1 was 68.95 which results in a concentration of 164.1 ng/g which gives a total yield of 9.11%

An unquantifiable piece of information is that only the female Crayfish that had been infected with B[a]P had red colored tissue instead of the normal brownish color. We are unsure of what this means, but it could be some form of protein related to the reproduction organs.

Extraction Method Results:

Table 2 is the culmination of seven test methods conducted over a period of several weeks.

Table 2: Area, Concentration, and yield of various methods

Methods	Area	Concentration L	Concentration ng/g	yield
*0.0	68.95	0.131	164.12	9.12
1.1	25.98	0.049	247.31	13.74
1.2	17.62	0.034	167.79	9.32
2.1	12.43	0.024	118.30	6.57
2.2	15.26	0.029	145.24	8.07
3.1	17.85	0.034	169.90	9.44
3.2	14.66	0.028	139.53	7.75
4.1 first	0.00	0.000	0.00	0.00
4.1 second	5.35	0.010	50.95	2.83
4.2 first	6.46	0.012	61.51	3.42
4.2 second	9.22	0.018	87.80	4.88
5.1	0.00	0.000	0.00	0.00
6.1*	0.00	0.000	0.00	0.00
7.1	18.06	0.034	171.95	9.55

For samples 0.0-3.2 we used a retention time near 28.25 minutes. From 4.1-7.1 we used time frames around 28.55.

For Figure 6, the table from table 2 in the form of a bar graph so that they may be compared to each other.

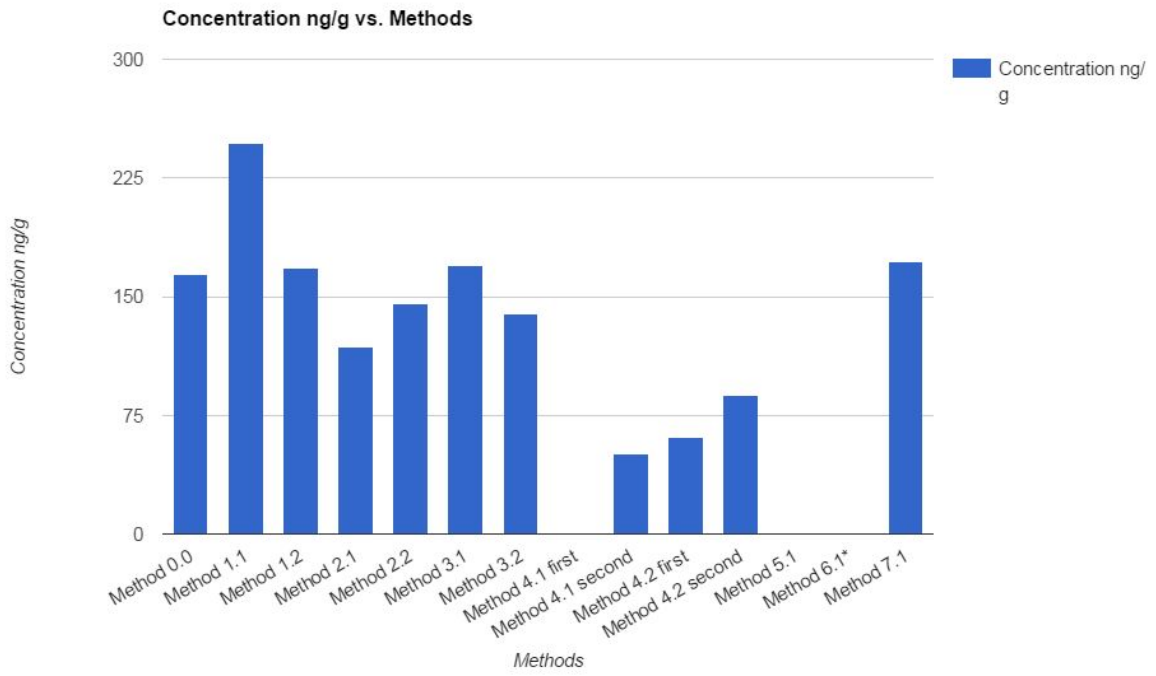

Figure 6: Concentration of experimental method experiments

Discussion:

Even though we ultimately failed to removed any B[a]P from the crayfish, we were able to learn several valuable things about the extraction of B[a]P and the keeping of crayfish themselves. We came up with several ideas for the next MQP and improved on the previou extraction method.. Hopefully, a future MQP can learn where to focus their research on.

We were able to improve the B[a]P yield to from the previous MQP's (Briseño, Chadwick & Owusu-Ansah 2014) extraction up from the previous years MQP. The 2013 MQP had an average yield of 4.16%, and using method 1.1, we were able to get a 9.11% yield. While this is an an improvement, 9.11% is abysmal for our purpose. If the amount of B[a]P is already low, and we are getting a 9.11% yield, there is a possibility that B[a]P will be missed when looking into the short term, or longer term food.

For the sake of being able to efficiently determine if any B[a]P is building up in the crayfish over the course of the term, a higher yield is needed. As far as methods for extraction went method 1.1 and 6.1 had the highest total yields. However, it's not known for sure that 6.1 is actually B[a]P. 6.1 took exactly a minute longer than normal to for a large spike was found. It's very strange as most reading were falling within 0.01 A from each other. It's possible that since this extraction was left over the course of a week it may have have changed chemically.

Between the other methods there wasn't much of a difference in yield. All of the methods, besides for the ones that used water as a solvent, had roughly the same 8-13% yield. For comparison, last years MQP had a 4% yield.

Method one, which was our basic method seems to be the best method. Occasionally it gave yields higher than 10%, but sonication doesn't seem like it made much of a difference to the overall yield. Part of the problem could be the lyophilization process. The MQP from 2009 suggests that finer extract material is harder to remove B[a]P from. (Jame R. Letourneux, 2009) The lyophilization process makes the crayfish into very fine powder. This may be the reason the yield was so low. Either an even more efficient extraction method is needed, or the wet extract from the 2007 MQP may be used (Jennifer Goscila, 2007)

The fact that none of the B[a]P was absorbed by the crayfish opens up several lines of questions. Was the yield simply too low to detect any B[a]P? Where the crayfish not eating their food? We know that B[a]P can be present in crayfish (Jennifer Goscila 2007) so why won't the captive crayfish absorb the B[a]P when directly fed B[a]P food pellets? Could it be possible that the crayfish take in B[a]P from the water directly rather than through contaminated food?

One possible explanation for this phenomenon is that crayfish were not fed enough B[a]P infused pellets for a long enough time. Afterall, they have a chemical pathway in which they can remove it slowly. However, increasing the time the crayfish are fed for is impossible with a one term unless perhaps if the feeding schedule was made 3 times a week or if the B[a]P levels in the food were doubled we could start to see results in the integration plot.

Another possibility is that Crayfish were eating algae instead of the food they were given. Unlike the previous MQP, we had an algae bloom in the habitats. This was due to the crayfish habitats being kept in the sunlight in the greenhouse instead of in the basement of the lab. Since crayfish are omnivores, they are able to access an alternative source of food that isn't contaminated with B[a]P. Eating from a food source that isn't B[a]P contaminated would reduce the total amount of B[a]P that they absorb hypothetically.

Lastly it's possible that B[a]P is not absorbed through the food as we hypothesized. Instead, it may be being breathed directly through their gills. This would mean that it's bypassing the digestive system and the intestinal tract and getting directly into the blood. This would make the food pellets completely unnecessary and saves time. The process may happen quicker than this MQPs period. B[a]P doesn't degrade quickly and could be directly added to the habitats. Instead of testing the time they are fed, we could create groups based on the concentration of B[a]P /ml in the water. This experiment would serve to both counter the algae problem, since over time, the algae will become contaminated with B[a]P.

In summary:

- We found no B[a]P in the long, short, or control Crayfish.
- We increased the B[a]P concentration from ~4% to ~9%.
- 9% is not high enough to warrant the continued use of the lyophilizer.

Going forward:

- Look for a new drying method that does not involve such fine particles associated with lyophilization.
- Test whether or not B[a]P is absorbed through the digestion system or through the gills.

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Appendix:

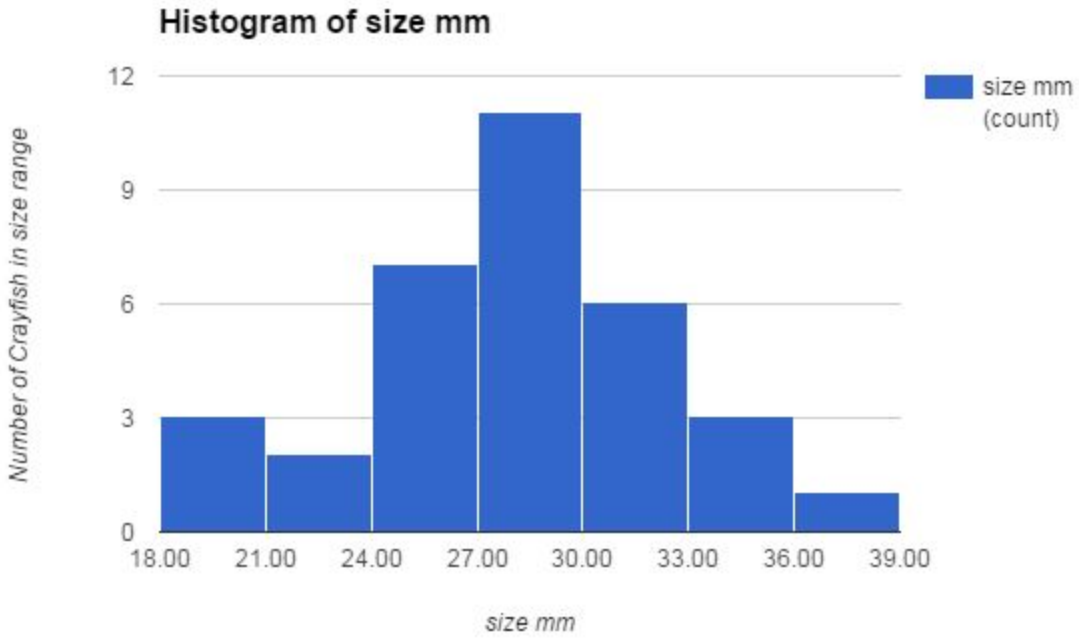

Figure 7: Size of each crayfish arranged in a histogram.