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Alkylphenol Contamination in Homarus americanus

Jennifer Renee Urban
University of Connecticut - Storrs, jennifer.urban2010@gmail.com

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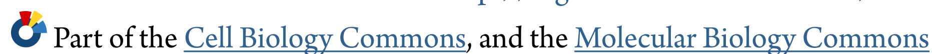
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Alkylphenol Concentrations in *Homarus americanus*

University of Connecticut Honors Thesis

Jennifer Urban

Abstract:

Alkylphenols are pollutants that are present in marine sediments and fishes. In earlier work it has been discovered that alkylphenols are present in the *Homarus americanus*, or the American lobster. Research suggests that alkylphenols could behave as endocrine disruptors as they have been found to affect juvenile hormone activity. It has been hypothesized that lobsters may be able to rid themselves of alkylphenol contamination through secreting these compounds into the environment or sequestering them in their tissues. In this study, I address the question of how lobsters may rid themselves of alkylphenols by analyzing hemolymph, muscle, gill, and shell samples and by looking for the presence of alkylphenols in natural and artificially injected lobsters. A total of thirty lobsters were analyzed. In my first study I found alkylphenols only in the gill tissue samples of natural lobsters after alkylphenols were initially found in the hemolymph, and found none in the muscle and shell samples. The types of alkylphenols found in the gills were often different than the alkylphenols found in the hemolymph. The gills are known as a site for exchange for the lobster. The lobster may not only be excreting alkylphenols from its gill surfaces but these findings suggest that the lobster may also be acquiring alkylphenols in the environment from these surfaces. It is possible that the lobsters may have ingested additional contaminants after the hemolymph samples were taken and before the gill samples were taken. As for the shell and muscle samples, it is possible that by our method the levels were too low to detect since we have a threshold of detection of 1ng/mL. It is also a conclusion that alkylphenols were not sequestered in

these tissues. In the second study, an expanded set of muscles samples from natural lobsters were tested as well as additional lobsters that were artificially injected with one of our alkylphenol compounds of interest, compound three. We found that lobsters injected with peak three showed significantly higher alkylphenol concentrations in all tissues, most notably the gill samples. The non-injected lobsters that died shortly after being in the laboratory, showed mostly peak three but their overall values were much less than those of the injected lobsters.

Introduction:

Alkylphenols are endocrine disrupting chemicals (EDCs) (Laufer et al. 2005) and have been found in marine sediments and samples (Stuart et al. 2005). EDCs can be absorbed by lobsters from water, sediments, and food (Stuart et al. 2005). They have been found in aquatic samples and have been attributed to pollution (Stuart et al. 2005). Alkylphenols have been shown to be toxic to crustaceans at high concentrations and may contribute significantly to lobster mortality (Biggers and Laufer, 2004).

Alkylphenols, as their name implies, arise from the alkylation of phenolic compounds. Alkylphenols have been used as surfactants, detergents, pesticides and plasticizers for the last forty years by industrial corporations. It has been estimated that the synthesis of alkylphenols is on the order of magnitude of 500,000 metric tons per year. Their common use today in industrial parts of the world has raised many concerns among chemists and ecologists in particular. Alkylphenols were first found to be oestrogenic (oestrogen-mimicking) in the 1930s (Dodds and Lawson, 1938). Extensive research on this topic led to the development of more evidence that was published in

1978 (Mueller and Kim, 1978). These findings prove alkylphenols to be endocrine disrupting chemicals. Alkylphenols do not break down in the environment or through the use of traditional water and sewage treatments (Di Corcia et al., 1998). They also tend to remain in oxygen poor environments, which has led to their accumulation in water sediments. This would be an area of prime contact with bottom-feeders such as the American lobster. These recent findings have led to the hypothesis that aquatic animals, particularly detritus feeders, such as the American lobster may bio-accumulate these compounds in their internal organs and tissues. This problem has raised awareness in the European Union were they have banned the use of alkylphenols since the 1980's. Although there is no current ban on the use of these compounds in the United States, future laws will most likely be instated, as the effects of alkylphenols on the environment have been consistent in recent scientific literature. In 2003, the Long Island Sound Lobster Health Symposium drew attention to the fact that lobster populations in Long Island Sound have been declining in part due to pesticides and other chemicals entering the marine environment (Biggers and Laufer, 2004). The issue of alkylphenol contamination of aquatic environments and species is of public health importance to humans because we often consume aquatic animals, like the lobster, which may be sequestering these potentially dangerous compounds. Healthcare information suggests that alkylphenol contamination may relate to human endocrine disorders, problems with fertility, as well as brain and nervous system diseases.

Alkylphenols are thought to have endocrine disrupting effects on the lobster and may even interfere with juvenile hormone (Biggers and Laufer 2004). Juvenile hormones refer to a group of hormones used by many insect and crustacean species that allow for

growth of the larvae stage while preventing metamorphosis. Professor Hans Laufer of the University of Connecticut discovered that methyl farnesoate is the crustacean equivalent of juvenile hormone because it was shown to have juvenile hormone activity (Laufer et al, 2004). In Professor Laufer's research he has found many phenols, including alkylphenols, to have high juvenile hormone activity. The phenolic compounds were tested for juvenile hormone activity through the use of a bioassay, which looked at the effects that these compounds had on the development and metamorphosis of annelid larvae. The significance of these findings is that phenolic compounds showed high juvenile hormone activity in bioassays, which suggests that it is likely that they have serious endocrine disrupting effects (Laufer et al 2004). More recently, Professor Laufer and other researchers found that alkylphenols disrupted the metamorphosis of lobsters that were treated with alkylphenols. After being treated with alkylphenols, lobsters displayed intermediate larvae phenotypes instead of metamorphosing directly into juveniles (Laufer et al 2009). This data serves as direct evidence that alkylphenols interfere with juvenile hormone and more specifically methyl farnesoate.

In addition to endocrine disrupting activity, alkylphenols may also disrupt shell hardening through interfering with tyrosine cross-linking. Chen et al (2009) showed that alkylphenols compete strongly with tyrosine during shell hardening. Alkylphenols in the shells of lobsters might weaken their normally hard shells. The idea that alkylphenols may lead to shell weakening arises from the chemical structure and similarity to tyro sine and its derivatives. If alkylphenols weaken the shells of lobsters, they may increase the shell's susceptibility to microorganisms, which may ultimately provide a disease projection model for the development of shell disease.

Alkylphenols have been detected in the hemolymph of lobsters from a broad geographic range (Biggers and Laufer, 2004). The hemolymph of 735 lobsters was tested from various regions of New England in 2008. The highest incidences of contamination were found in western Long Island sound, Buzzard Bay, Cape Cod Bay, and offshore areas of the Northeast (Jacobs et al, 2009). Extensive research has been conducted on hemolymph samples of these lobsters while relatively little research has been done in regards to lobster tissues. Laufer et at. (2005) studied alkylphenols in the hemolymph of inshore and offshore lobsters and suggested that if given enough time, lobsters can rid themselves of these contaminants. This was an important observation because it suggested to the fishing industry that they could decontaminate contaminated lobsters by placing them in clean waters. Finding the location of alkylphenols in lobster tissues may help us to discover whether lobsters can in fact rid themselves of alkylphenols by excretion through surfaces such as the gills or prove that they may incorporate alkylphenols into new cuticles for shell formation or tissues.

In this study, I addressed the question of how lobsters respond to alkylphenol contamination. Do they rid themselves of the contamination by excretion through gill surfaces, incorporating them into their shells, or sequestering them into internal organs or muscular tissues? With our techniques we can detect certain alkylphenols in nanogram per milliliter amounts using gas chromatography-mass spectrometry techniques. Because it has been previously shown that alkylphenols are slow to degrade (Biggers and Laufer, 2004), it is very possible that lobsters bio-accumulate them in their tissues in order to remove them from their hemolymph. The location of alkylphenols in the hemolymph, gill, muscles, and shells of lobsters were analyzed in the hopes of finding out more about

how shell disease, lobster's geographical location, and alkylphenol contamination of tissues could be related. In the first study, four lobsters, all with high initial hemolymph contamination, which did not molt were analyzed. The tissues tested in these lobsters were gill, muscle, and shell samples. In the second study, five lobsters were analyzed that had been injected with peak three, one of our known alkylphenols. These lobsters, along with three non-injected lobsters were analyzed for contamination in the following tissues: muscle, hepatopancreas, gonad, gill, epidermis, and new shell. Finally, to expand the data set on muscle tissues, eighteen lobsters were analyzed for the presence of alkylphenol contamination in muscle tissue. These eighteen lobsters were not held long in captivity and most likely model lobsters found in the field.

Methods:

Chemical standards and reagents:

Compounds #1: 2-t-butyl-4-(dimethylbenzyl)phenol, #2: 2,6-bis-(t-butyl)-4-(dimethylbenzyl)phenol, #4: 2,4-bis-(dimethylbenzyl)-6-t-butylphenol were synthesized in the lab by Dr.Bobbitt as described in detail by Stuart (2007). Compound #3: 2,4-bis-(dimethylbenzyl)phenol is available commercially and was purchased from Sigma. I also used methanol, methanol/methylene chloride, and pH2 water. I developed the chemical methodology for this experiment in collaboration with Dr. Molly Jacobs and Dr. James Stuart, and based on published protocols by Dr. Stuart, Dr. Xuejin Pan, and Dr. Bill Biggers (Stuart et al. 2005; Biggers and Laufer, 2004).

Sample collection and animal care:

Lobster maintenance and sample collection was performed by Professor Hans Laufer, Dr. Molly Jacobs, and Ming Chen. Male and female lobsters were collected from Massachusetts, both from Western Cape Cod Bay and offshore areas near Munson Canyon. The first four lobsters with high initial hemolymph contamination arrived in the laboratory between May 29 and 30 of 2008, one from Western Cape Cod Bay and three others from the Munson Canyon. The five lobsters injected with compound three were bleed and injected during the summer of 2008. The three control lobsters with low initial hemolymph contamination died in mid to late July 2008. The remaining eighteen lobsters, which expanded our muscle data set, were all bleed in the early summer months of 2008 and died that same summer. The lobsters were tagged and weighed upon arrival into the laboratory and their carapaces measured. Hemolymph samples were also taken at this time and the lobsters were analyzed for the presence of shell disease. The lobsters were maintained in flow-through seawater at the Marine Biological Laboratory (MBL) at Woods Hole in Massachusetts. When the lobsters died, tissue samples such as gill, muscle, hepatopancreas, and shell were taken and preserved. It should be noted that not all of the lobsters studied had samples of each tissue available. The tissues samples were frozen and the shells were fixed in a 50% acetonitrile solution to preserve any alkylphenolic compounds.

The lobsters chosen for the first study were non-molters with high levels of alkylphenols in their initial hemolymph samples. Lobster 838M was collected on 5/29/08 and lobsters 840M, 841M, and 842M in 5/30/08 and they were bleed on the same dates. Lobster 838M died on 6/26/08, 840M on 6/19/08, 841M on 6/27/08, and 842M on

6/30/08; these were also the dates that the tissue samples were collected. More information on these lobsters can be found in Table 1.2. In the second study the five lobsters injected with compound three all died shortly after their injections. Lobster 901M was bleed on 07/16/08, injected on 07/24/08, and died during molting on 07/29/08. Lobster 848M was bleed on 05/30/08, injected on 06/01/08, 06/05/08, 06/12/08, and 06/24/08, it then died on 06/24/08. Lobster 1036C was bleed on 06/11/08, injected on 06/11/08, 06/18/08, 06/24/08, and later died during molting on 07/04/08. Lobster 1038C was bleed on 06/11/08, injected on 06/11/08, 06/18/08, and 06/24/08, and died on 07/03/08. Lobster 1049R was bleed on 08/19/08, and also bleed, injected, and molted on 08/27/08, and died on 09/03/08. More information on the initial condition of the injected lobsters can be found in Table 2. The three control lobsters included in the study were lobsters 896M, 888M, and 883M. Lobster 896M was bleed on 07/21/08, and died during molting on 07/21/08. Lobster 888M was bleed on 07/18/08 and died during molting on 07/18/08. Lobster 883M was bleed on 07/22/08, and died during molting on 07/22/08. The information for the control lobsters can be found in Table 3. The remaining eighteen lobsters were chosen because of their representation of the realistic lobster population. These lobsters all died within one to three weeks of being in captivity so they most likely reflect contamination levels in the current lobster population. Background information on these eighteen lobsters can be found in Table 4.

Sample preparations:

Except where described below, alkylphenols were extracted from the hemolymph and tissues of *H. americanus* as described by Biggers and Laufer (2004).

Initial extraction of solid samples

Hemolymph samples were poured into Teflon® microwave liners by using 20mL of methanol/methylene chloride to transfer the sample. The gill, muscle, hepatopancreas, gonad, epidermis and new shell samples all were dried out first and weighed to record a dry weight. We ground up the gill, gonad, hepatopancreas, and muscle samples with a mortar and pestle and used 20mL of hexanes to transfer them into the microwave tubes. The new shell and epidermis samples were ground with the mortar and pestle but did not turn into a ground up mixture instead they remained very fibrous and tough. We microwaved the samples in a CEM microwave (Mars™) Extractor at power of 30% of 1600 W for 2.00 min.

The shell samples were dried and weighed and were approximately 2.0 grams. They were placed into beakers and extracted with 100mL of a 10% concentrated glacial acetic acid/water solution for 24-48 hours; with a watch glass placed on top of the beaker to catch any splattering. After 24-48 hours, when no more bubbles of carbon dioxide were forming, the acetic acid extract was decanted and poured into a 200mL volumetric flask and neutralized by adding drops of 5 Molar sodium hydroxide until the resulting solution indicated a pH of about 6. The shells were rinsed with distilled water and then extracted for 24-48 hours in 100mL of 6 Molar urea, to which 0.1% trifluoracetic acid had been added. The next day the urea solution was poured off and added to the 100mL volumetric flask and stirred with 0.5g of sodium chloride before bringing the volume to 100mL with de-ionized water. These samples were then stored at 4˚C until solid-phase extraction.

Filtering of Gill, Gonad, Hemolymph, Hepatopancreas, and Muscle Samples

The gill, gonad, hemolymph, and muscle samples were removed from the microwave and then filtered to separate out the solids. Funnels were lined with 12.5 cm Whatman filtering paper #5 (porosity: medium, flow rate: slow) and placed above clean 40mL vials. The contents of the microwave tubes were then transferred into the funnels and the samples were rinsed through the filter with no more than 20mL methylene chloride/methanol for the hemolymph samples and 20mL of hexanes for the gill, gonad, hepatopancreas, and muscle samples. Once the filtering was completed, 5mL of aqueous 0.9% KCl was added to the 40mL vials containing the hemolymph samples. The phase separation step was not done for the tissue samples because the phase separation was often not clear and we did not want to risk losing any alkylphenols during the separation. Each 40mL hemolymph vial was then capped, vortexed for 1 minute, and centrifuged for 10 minutes in an International Equipment Company, IEC Clinical Centrifuge on level 2. After centrifuging the hemolymph samples, the contents separated into two layers; an aqueous layer and a methanol/methylene chloride layer. I used a pipette to transfer the top aqueous water layer into a 100mL volumetric flask. The methanol/methylene chloride layer was then blown down with a steady stream of nitrogen gas for a couple hours or overnight. The tissues samples, containing hexanes, did not go through this phase separation step but were rather put directly into the nitrogen dehydrator after the filtering step. After the samples were dry, the remaining residues were transferred into their corresponding aqueous volumetric flasks by rinsing them three times with 0.50mL of methanol. The transfer was completed using the smallest amount of methanol possible. Then I added 50mL of pH 2 water and 0.5g of NaCl to each 100mL volumetric flask and

stirred them. The volumetric flasks were then diluted to 100mL with pH 2 water. The samples were stored at 4˚C until solid phase extraction.

Solid-phase extraction

A Supelco ENVI Chrom P cartridge was set up and rinsed three times with 2mL of methanol followed by three times with 2 mL of pH 2 water using the Supelco Visiprep at a flow rate of about 1mL/min. Each 100mL sample was then passed through a labeled SPE cartridge at a flow rate of 3mL/min. After the samples had been completely passed through the columns, the alkylphenols were captured into the SPE cartridges, which were then rinsed three times with 2mL of pH 2 water. Each cartridge was then dried under a gentle stream of nitrogen gas for 30 minutes and then eluted with methanol. For the elution, each tube was rinsed four times with 2mL of methanol and the samples were collected into clean centrifuge tubes and dried overnight under a gentle stream of nitrogen gas.

The shell samples began as 300-400mL samples. Because of this greater volume we used larger Envi Chrom SPE cartridge and we used a greater transfer volume of 40mL of methanol instead of 8mL. In all other respects solid phase extraction of these samples was carried out as described above. The shell samples were very messy after the initial solid phase extraction, so we re-suspended them in methanol and performed a second solid phase extraction then drying the samples overnight under a nitrogen dehydrator. This helped us to clean up the samples so that we could cleanly transfer them during the GC-MS step.

GC-MS

After the samples had been blown down overnight, the remaining residue was transferred into the GC-MS tubes. First 100µL of Internal Standard Solution (5.0 ng/mL of phenanthrene and biphenyl) was added to each centrifuge tube. This solution was transferred into labeled glass GC-MS tubes using a 50µL pipette. The GC-MS tubes used were 1.5mL glass vials with 100µL inserts with polymer feet made by Agilent. We then rinsed twice with 50µL of methanol, and used the same transfer syringe to transfer the sample into the GC-MS vials. When the samples were messy and it was hard to get the liquid to separate out from the solids, we centrifuged the samples for 10 min in our International Equipment Company, IEC Clinical Centrifuge at speed six. Centrifuging between each rinsing was sometimes necessary to ensure clean samples for the GC-MS machine.

The GC/MS instrument was a Hewlett-Packard (HP now Agilent) 5890 Series II GC with a 5970 Mass Selective Detector (MSD) operating under HP Standard ChemStation, vs. A 0.300, 1986-1996. The GC/MS was equipped with an automated split/splitless injector (Model 7673). The injection was set in the splitless mode, with the purge gas open at 1.0 min. A 1.00 microliter auto-injection was performed that was set for 6 solvent (methanol) rinses, then 6 sample rinses and then 6 sample syringe pumps before a rapid automatic injection was performed. We used a Capillary Column GC with methyl silicone liquid phase, 30 meters long, 0.25 mm internal diameter, 0.25 micron film thickness (Description: TR-1/Ms supplied by Thermo/Fischer). The Column Temperature Program was set at: Initial Temp. 50 °C, hold 2 min., temperature ramp at 15 °C/min. to 250 °C, then a second temperature ramp from 250 °C at 5 °C/min. to 270

°C followed by a 5 min. final temp hold at 270 °C to bake the column. This column temperature program took 24.3 min. The injector was set at 240 °C with the M.S. interface set at 280 °C. The Mass Selective Detector was set for the SIM (or Selected Ion Monitoring) Mode to only scan for 10 msec each of the following: Peaks 154.0 (biphenyl), 178.0 (phenanthrene), 253.0 and 268.0 (compound 1), 309.0 and 324.0 (compound 2), 315.0 and 330.0 (compound 3), and 371.0 and 386.0 (compound 4). The Mass Selective Detector was auto tuned with perfluorotributylamine (PFTBA) at masses: 69.0, 219.0 and 502.0.

Mass Spectrometric detection was using the selective ion monitoring mode and quantification were performed as described by Laufer et al (2005) but with phenanthrene (M.S. 178) as the internal standard. The detection limit of the method was ≤ 1 ng for each Compound, and we re-calibrated every 2-3 months using known standards to ensure that results from different years and different GC/MS equipment were comparable**.** Percent recovery ± standard deviation for the entire method (extraction + purification + GC/MS) based on positive controls with known standards was 21% ± 16% for Compound 1, 1% ± 1% for Compound 2, 27% ± 4% for Compound 3, and 29% ± 15% for Compound 4 (Jacobs et al 2009). Recoveries from biological samples are typically lower than recoveries from water or sediment samples (e.g., Mouatassim-Souali *et al.*, 2003) because of the additional purification steps required (Gadzala-Kopciuch *et al.*, 2008).

Quantitative analysis

Gas chromatography and mass spectrometry were used to identify peaks for biphenyl, phenanthrene, and our alkylphenols of interest; compounds 1, 2, 3, and 4. After

running a range of known concentrations of biphenyl, phenanthrene, and Compounds 1, 2, 3, and 4, through the GC-MS, Dr. Stuart made multiple calibration curves for each compound in question. These calibration curves were then used to calculate the concentrations of alkylphenols in our samples based upon the dry weight of the sample and the area under the corresponding peak in the gas chromatograph.

At the beginning of each GC-MS run, we loaded a standard solution consisting of phenanthrene, biphenyl, and compounds 1, 2, 3, and 4 in order to locate the peaks for the alkylphenols in the sample based on their relative time of appearance and abundance. I ran each sample in the GC-MS machine and manually integrated the area under the curve for phenanthrene and compounds 1, 2, 3, and 4. I used the calibration curve in conjunction with the dry weights of the samples to calculate the concentration of any alkylphenols present. Phenanthrene was used as an internal standard to control for handling and injection of the samples. The phenanthrene peak was used to scale the areas under all peaks.

Statistical analysis:

In the first study, I used a Chi Square test to test the correlation between the gill and hemolymph tissue samples and their levels of alkylphenol concentrations. I added nine additional lobsters to the chi square test from a previous study due to a low amount of initial data. Information on these lobsters can be seen in Table 1.4. No other statistical analysis was performed on the rest of the data set.

Results:

Presence and total alkylphenol concentrations found in lobsters

All initial hemolymph samples, taken from the lobsters upon arrival into the lab, were analyzed by Dr. Stuart. The first four lobsters I studied were lobsters 838M, 840M, 841M, and 842M. Lobster 838M had alkylphenols 1, 3, and 4 in its hemolymph sample from May 29, 2008 (Table 1.1). Lobster 838M also had a total hemolymph alkylphenol concentration of 1507.30 ng/mL. Alkylphenol peaks 1 and 2 were found in the gill samples of lobster 838M taken from June 26, 2008 and had a total gill alkylphenol concentration of 108.07 ng/mL. Lobster 840M had alkylphenols 1, 3, and 4 in its hemolymph sample from May 30, 2008, with a total hemolymph alkylphenol concentration of 543.58 ng/mL. Alkylphenol peaks 1 and 3 were found in 840M's gill samples taken from June 16, 2008 with a total gill alkylphenol concentration of 1345.09 ng/mL. Lobster 841M had alkylphenols 1, 3 and 4 in its hemolymph sample from May 30, 2008, with a total hemolymph alkylphenol concentration of 405.85 ng/mL. Alkylphenol peak 3 in its gill samples taken from June 27, 2008 had a total gill alkylphenol concentration of 441.85 ng/mL. Lobster 842M had alkylphenols 1, 3, and 4 in its hemolymph sample from May 30, 2008 with a total hemolymph alkylphenol concentration of 60.32 ng/mL. Alkylphenol peak 3 was found in gill samples collected on June 6, 2008, with a total gill alkylphenol concentration of 490.85 ng/mL (see Table 1.1). The first study was done on four lobsters which all had some level of peaks 1, 3 and 4 initially in their hemolymph. All four lobsters showed at least one type of alkylphenol present in their gill samples. The peak present did not directly correspond to alkylphenols present in the hemolymph as one lobster showed peak 2 in its gill and peak 2 was not

found in it’s hemolymph. Three out of the four lobsters showed peak 3 in their gill samples, which also had the highest ng/mL amounts. The results for these four lobsters can be found in Table 1.1.

In the second study, five lobsters were studied after being injected with compound 3, or peak 3. The five lobsters I studied were out of thirty-five injected lobsters from April 2009. Of the 35 injected lobsters, 16 (46%) were initially contaminated with alkylphenols and 19 (54%) were uncontaminated. Initial contamination, particularly in the controls, is a confounding factor for our injection experiments. A conservative approach would be to eliminate all initially contaminated lobsters from any analysis of the injection experiment (Jacobs 2009). These lobsters were 901M, 848M, 1036C, 1038C, 1049R. In addition three control lobsters, 896M, 888M, and 883M were added because of their low initial hemolymph contamination levels. Background information on the injected lobsters can be found in Table 2 and information on the control lobsters can be found in Table 3. After being injected, lobster 901M showed peak 3 in muscle, hepatopancreas, and gill and there were no gonad, epidermis, or new shell samples for 901M. No other alkylphenols were found in lobster 901M. Lobster 848M showed peak 3 in muscle, hepatopancreas, gonad, and gill. 848M also showed peak 1 and 4 in the gonad samples and had no epidermis or new shell samples to test. Lobster 1036C had peak 3 in muscle, hepatopancreas, gonad, and new and old gill. This lobster had two gill samples taken. Lobster 1036C also had peaks 2 and 4 in the new gills and had no epidermis or new shell samples to be studied. Lobster 1038C, which showed peak 3 in muscle, hepatopancreas, gonad, and gill. This lobster had no other peaks present and also did not have epidermis or new shell samples to be tested. The final injected lobster studied was

1049R which showed peak 3 in it's muscle although there was no hepatopancreas, gonad, gill, epidermis, or new shell samples for this lobster. Lobster 896M was the first control lobster to be studied. It showed no alkylphenols in it's muscle, hepatopancreas, gonad, or gill samples. However, 896M did show 1.6 ng/mL of peak 1 in it's epidermis and 0.5 ng/mL of peak 1 in it's new shell samples. Lobster 888M had no peaks in it's muscle, hepatopancreas, or gill samples. Lobster 888M did show 0.3 ng/mL of peak 1 in the gonad sample, 0.6 ng/mL of peak 1 in the epidermis, and 0.1 ng/mL of peak 1 and 2.9 ng/mL of peak 3 in the new shell sample, although all of these values are very close to our detection threshold and are very small. The last control lobster is 883M which had 8.3 ng/mL of peak 3 in it's muscle and 0.2ng/mL of peak 1 in it's new shell but no alkylphenols in it's hepatopancreas, gill, or epidermis samples.

The final eighteen lobsters were only tested for the presence of alkylphenols in their muscle samples. Initial hemolymph values for these lobsters can be found in Table 4. The results from the muscle samples can be seen in Table 5. Twelve out of the eighteen lobsters, or 67%, came out positive for at least one alkylphenol in their muscle tissues.

Comparing types of alkylphenols found in each lobster

All of the four lobsters tested with initially high hemolymph levels showed at least one common alkylphenol peak in both their hemolymph and gill. In some cases, alkylphenols were seen in the gills that were not present in the hemolymph and vice versa. For example, lobster 838M had alkylphenol # 1 in both its hemolymph and gill, but only had alkylphenol #2 in the gill and not in the hemolymph and alkylphenol #3 and #4 in its hemolymph but not in its gill.

Alkylphenol #3 was found in both lobsters 841M and 842M. All lobsters had alkylphenols 1, 3, and 4 in their hemolymph. Lobster 841M had 405.8 ng/mL in its hemolymph, 840M had 543.58 ng/mL in its hemolymph, 838M had 1507.30 in its hemolymph, and lobster 842M had 60.32 in its hemolymph. Lobsters 841M and 842M only had alkylphenol peak three in their gills. Lobster 842M had 490.85 ng/mL of alkylphenol in its gill, 841M had 441.98 ng/mL in its gill. These were the only alkylphenols detected in these two lobsters gill samples.

Alkylphenols present in muscle and shell tissues

No alkylphenols were found in any of the muscle or shell samples in the first study. During the second study, all of the five injected lobsters showed peak three contamination in their muscle samples. The epidermis and new shell samples studied for the control lobsters yielded a very small presence of peak one in the epidermis samples of 888M and 896M as well as in the new shell samples of 888M, 896M, and 883M; 888M also showed a small amount of peak three. We also had a strong presence of alkylphenol contamination in the eighteen additional lobsters studied. Roughly 67% of those lobsters showed one or more alkylphenols present in their muscle tissue.

Tested statistical correlation between hemolymph and gill contamination

A chi square test was conducted to analyze the relationship among concentration of alkylphenol in hemolymph and gill samples of our lobsters. I included nine more lobsters from previous studies conducted by Dr. Laufer and colleagues (Laufer et al. unpublished data) i.e. Table 3, for a total of 13 lobsters. Five of the lobsters had hemolymph and gill contamination, one lobster had hemolymph but no gill contamination, one lobster had no hemolymph and just gill contamination, and six

lobsters had neither hemolymph nor gill contamination. I found a significant correlation between hemolymph and gill contamination. This test was conducted and a p value of 0.0127 was found. This is significant as a p value < 0.05 shows a significant correlation. This further suggests that there is a significant correlation between hemolymph and gill alkylphenol contamination.

Discussion:

The four lobsters included in the primary study were of particular interest because they all had relatively high initial hemolymph alkylphenol contamination and they all had not molted after these hemolymph samples were taken. This was an important parameter, as it is not yet well understood whether or not lobsters can rid themselves of alkylphenols by storing them in their shells and then shedding them during molting. This allowed us to test where the alkylphenol contamination in their hemolymph would reside. It is still surprising to find no alkylphenols in the shell samples as we could hypothesis that lobsters do rid themselves of alkylphenols through incorporating them into their shells. However, in the expanded second study, small amounts of peak one and three were found in epidermis and new shell samples, which may suggest that lobsters may be incorporating these compounds into their shells as these tissues eventually develop into hard shells. The levels are very low which also may suggest that they are at or around our detection threshold level, which may suggest why none were found in the preliminary study. If any alkylphenols are found to be in the shell samples, we could hypothesize that these alkylphenols were from previous contamination in the hemolymph and there would be no correlation between the alkylphenol types and concentrations found in the lobster's

tissues with those found in the shell samples. The method of treatment for these shells is currently under investigation, as changes to our procedure may need to be made.

None of the four lobsters in the first study had alkylphenols in the muscle samples. Our sample size was small, but this would be an important association to look at as lobster meat is something that is consumed and alkylphenols could have endocrine effects on our bodies as well. It is important to mention however that alkylphenol contamination has been found in muscle tissues from previous experiments as well as muscle samples from my secondary study. Both injected and non-injected lobsters were found to have levels of alkylphenols present in their muscle tissues upon further investigation. Because of these findings, muscle tissues have been proposed to have a storage role in the lobster (X. Pan et al. 2005).

No alkylphenols were found in our shell samples. The shell samples were difficult in this experiment because it was often difficult to extract a pure liquid during the transfer step into the GC-MS tubes. This led to an increase in the amount of transfer solvent and therefore a more dilute sample to run through the GC-MS machine. Because of this possibility we cannot conclude entirely that alkylphenols were not present in the shell samples but that they just were not present at substantial levels. It is also important to note that these lobsters were non-molters, meaning that there may not have been an opportunity for the lobsters to incorporate the alkylphenols in to their shells if they were not contaminated in the past. If the lobsters were going to sequester the alkylphenols into their shells, they would do so during a molt when a new shell is secreted. It may be an important new experiment to look at molting lobsters. It would be very informative to look at the new epidermis and shells formed from contaminated lobsters to see if these

concentrated their alkylphenol contaminants there. It is also important to make a point that we are not positive that our current procedure for treating our shell samples does not chemically alter or leave some alkylphenols behind. Postdoctoral fellow Molly Jacobs, who questions whether or not some alkylphenols may be left behind in the acetonitrile fixative, proposes that we test these solutions as well for the presence of any alkylphenols. The acetonitrile that is used to fix the compounds in the shell samples can also be used as an extraction solvent and this leads to the hypothesis that not all of the alkylphenol compounds in these shell samples are being accounted for. We are currently testing this hypothesis by analyzing the acetonitrile fixative solution to look for the presence of alkylphenols that may not have been accounted for in the previous method.

Interestingly, all the lobsters with the initially high hemolymph contamination showed at least one or two alkylphenols present in their gill tissues. In addition, the second study of the injected lobsters showed that all of the injected lobsters showed the injected peak three in their gills while none of the control lobsters showed any alkylphenol contamination in their gills (Table 3.4). This supports the hypothesis that lobsters may be able to excrete these contaminants through their gills (Laufer et al. 2005). The gills of the lobster serve as an exchange surface for the lobster and are often used for both excretion and absorption of important nutrients. This place of exchange may be a place where lobsters can acquire or release alkylphenols to the environment.

The three control lobsters studied further showed the impact that the injections of peak three had on the other lobsters. The control lobsters showed no hepatopancreas or gill contamination. In the muscle samples only lobster 883M showed a small peak three and in the gonad samples only 888M had a small peak one. The control lobsters also had

very low values of alkylphenols in the epidermis and new shell samples. Overall, the level of alkylphenol contamination was significantly lower in the non-injected lobsters.

Lobsters 841M and 842M both had peak #3 present in their gill and hemolymph samples and were both from the Munson Canyon area of Massachusetts. Neither of these lobsters suffered from shell disease.

One important facet of this study was the results for the new and old gill samples for lobster 1036C, as seen in Table 3.4. The old gill showed only peak three, while the new gill showed peaks two and four in addition to peak three. This suggests that the new gill may be incorporating peak one from the hemolymph into its growing new gill tissue. This was very interesting to see because of its implication for how these animals may be dealing with alkylphenol contamination.

There seemed to be no correlation between which alkylphenols were found in the gills and in the hemolymph of the same lobsters. Some of the lobsters had alkylphenols in their gills that were not present in their hemolymph initially suggesting that the lobsters may be able to absorb alkylphenols through the intimate contacts that their gills make with the environment. It is also possible that the lobsters may be using internal organs as a means of storage for these alkylphenol compounds. This is a possibility as the lobsters may have picked up the alkylphenol contamination from their tanks or their food supply. Lobster food can also have alkylphenol contamination and it has been an important experiment to analyze food samples, such as muscle and fish samples, and see if the lobsters could be acquiring this contamination from their food. With this experiment, timing would be an important factor to see if alkylphenol concentrations in the hemolymph were highest immediately after consumption of alkylphenol-contaminated

food. One caveat to this experiment is that there may not be a correlation between alkylphenols present in the lobsters hemolymph and tissues because of deposits of alkylphenols from previous hemolymph contamination. It becomes increasingly difficult to track the lobsters exposure and means of acquiring and storing these compounds. The experiment of analyzing lobster food samples was conducted by Rumla Rizvi in the spring of 2009. In her experiment she tested five Rhode Island sediment samples, of which two had peak 3 contamination and one had peak one contamination so 60% of the Rhode Island sediment samples showed some form of alkylphenol contamination. She also tested two Connecticut sediment samples, one from Pfizer and one from New London. The New London sample showed 32 ng/g of peak one contamination. Out of the fish samples she studied, 50% of them showed the presence of at least one alkylphenol. Her invertebrate samples showed even more contamination; 55% of these samples showed the presence of at least one alkylphenolic compound. The most striking samples and the ones Rumla found the highest levels of contamination were the mussel and scallop samples. The mussel sample had 211 ng/g of Peak one and 2,162 ng/g of peak 3. The scallop sample had 6.660 ng/g of peak 3. More about these results can be found in her thesis report: (Rumla 2009).

I used a chi square test to test the hypothesis that hemolymph and gill contamination should be correlated. We found there to be a significant correlation among the lobsters that had and did not have hemolymph contamination with gill contamination. This indicated that there might be a relationship between hemolymph and gill contamination (p value of 0.0127). This is significant as a p value < 0.05 shows a significant correlation.

Since there was found to be a correlation between alkylphenols in the hemolymph and gills but there was no correlation found between the types of alkylphenols found in each of these tissues, it is suggested that lobsters may both release and absorb alkylphenols from the environment through their gills.

Geographic patterns may also be linked to shell disease. Although my sample size was small, I can draw some conclusions. Of the four lobsters I studied, only one, lobster 838M, was from Western Cape Cod Bay. This was the only lobster that had shell disease. Another interesting fact about lobster 838M was that it was the only lobster that had peak #2. This lobster showed peak #2 in its gills, and it was not seen in any other hemolymph or gill samples.

Future experiments can be proposed from these findings. It would be important to look at the shells of contaminated lobsters after they have molted to see if they sequester alkylphenol contamination in their shells or epidermis. It would also be beneficial to look at the food supply of the lobsters and see if they are acquiring alkylphenols from their food supply. To further establish that gills are a way that lobsters can secrete alkylphenols, you could inject a labeled alkylphenol to see if it shows up in their gills or the tank. In addition you can see if other lobsters in the same tank pick up that traced alkylphenol. If one lobster that was not injected with the traced alkylphenol but then shows it in its tissue or hemolymph analysis then you can assume it acquired it from its environment.

Tables and Figures:

Table 1.1—Table of alkylphenol concentrations in tissues of non-injected lobsters. The concentrations of alkylphenols are in units of ng/mL for hemolymph samples and ng/g for gill, muscle, and shell samples.

Sample	Tissue Type	Dates of sample collection	Peak #1	Peak #2	Peak #3	Peak #4	Total
838M	Gill	06/26/08	18.86	89.20	0	0	108.07
	Hemolymph	05/29/08	269.39	0	853.87	384.03	1507.30
	Muscle	06/26/08	0	0	0	0	0
	Shell	06/26/08	0	0	0	0	0
840M	Gill	06/19/08	10.21	0	1334.87	0	1345.09
	Hemolymph	05/30/08	138.61	0	279.17	125.80	543.58
	Muscle	06/19/08	0	0	0	0	0
	Shell	06/19/08	0	0	0	0	0
841M	Gill	06/27/08	0	0	441.98	0	441.98
	Hemolymph	05/30/08	45.48	0	241.56	118.49	405.85
	Muscle	06/27/08	0	0	0	0	0
	Shell	06/27/08	0	0	0	0	0
842M	Gill	06/30/08	0	0	490.85	0	490.85
	Hemolymph	05/30/08	30.68	0	21.68	7.95	60.32
	Muscle	06/30/08	0	0	0	0	0
	Shell	06/30/08	0	0	0	0	0

Table 1.2—Background information on lobsters.

Sample	State	Sex	Carapace Length (cm)	Location	Shell Disease	Collection and Bleed Dates	Date of Death
838M	MA	F	8.9	Western Cape Cod Bay	Y	05/29/08	06/26/08
840M	MA	M	7.8	Munson Canyon	N	05/30/08	06/19/08
841M	MA	F	8.6	Munson Canyon	N	05/30/08	06/27/08
842M	MA	M	8.3	Munson Canyon	N	05/30/08	06/30/08

Table 1.3—Background information on lobsters added from a previous study, to add to chi square test.

Sample	State	Location	Shell disease	Hemolymph contamination	Gill contamination
797M	MA	South	Y, moderate	No	No
805M	MA	Offshore	N	No	No
808M	MA	Offshore	N	No	No
826M	MA	Offshore	N	No	No
751C	CT	LIS South	N	No	No
1001C	CT	East	N	No	No
858M	MA	South	N	Yes	No
997C	CT	East	Y	No	Yes
448C	CT	-----------	------------	Yes	Yes

Table 1.4—Results of a chi square test comparing the relationship between alkylphenols in the hemolymph and in the gills.

Chi Square Test	Gill Alkyl +	Gill Alkyl –	Total
Hemolymph Alkyl +	5	1	6
Hemolymph Alkyl –	1	6	7
Total	6	7	13

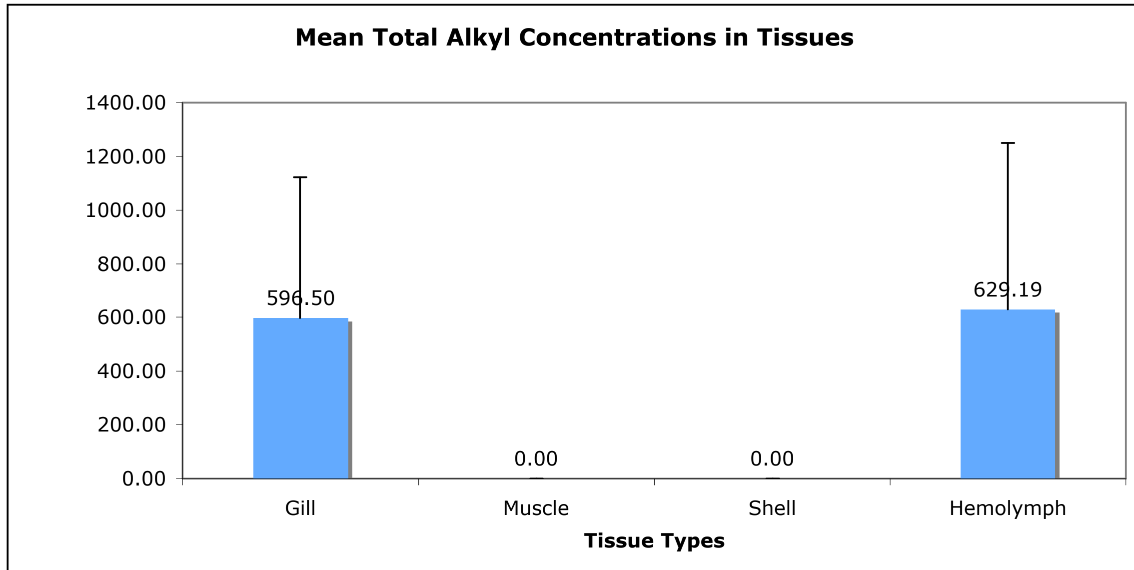

Figure 1—Mean total alkylphenol concentrations in gill, muscle, shell, and hemolymph samples of non-injected lobsters: 838M, 840M, 841M, and 842M. The error bars here represent the standard deviation

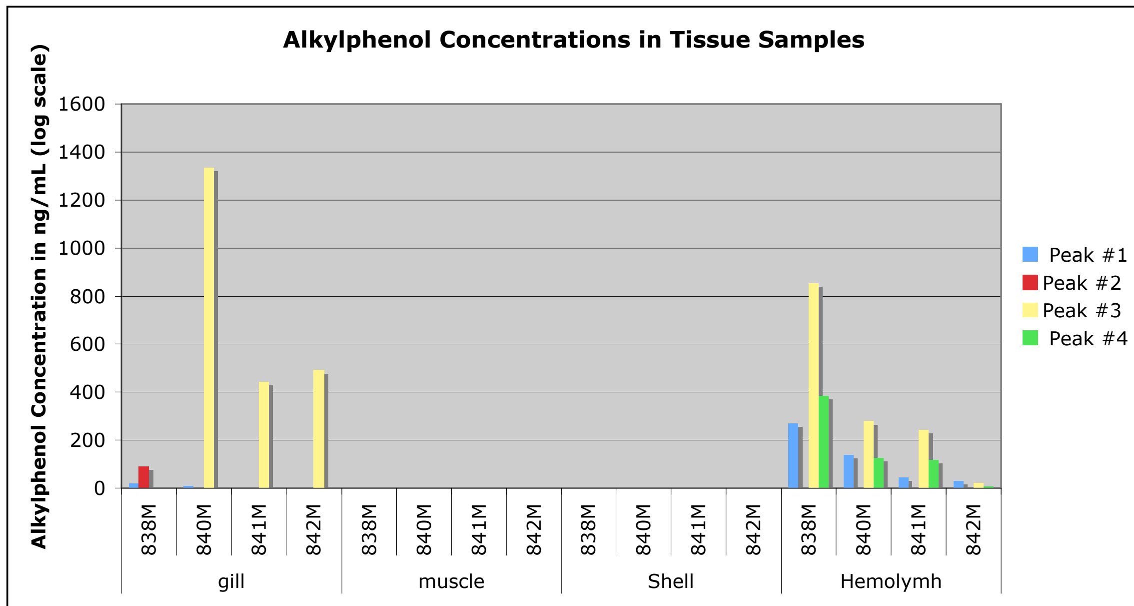

Figure 2—Alkylphenol concentration in tissues of non-injected lobsters: 838M, 840M, 841M, and 842M.

Table 2: Background information and hemolymph contamination data on lobsters injected with compound 3 before date of death. Alkylphenol Contamination is in units of (ng/mL).

Time in lab	Death and Background	Pre-Injection Bleed Date	Injection Dates	Sample	Peak 1	Peak 2	Peak 3	Peak 4
10 days	Died and molted 7/29/08, Cape Cod, M, no SD	7/16/08	7/24/08	901M Hemo	0	0	0	0
2 weeks 3days	Died 6/24/08, Offshore, M, no SD	5/30/08	6/1/08, 6/5/08, 6/12/08, 6/24/08	848M Hemo	**11.0**	0	0	0
3 weeks	Died and molted 7/4/08, Ct East, F, SD	6/11/08	6/11/08, 6/18/08, 6/24/08	1036C Hemo	**128.0**	0	**3.0**	0
3 weeks	Died 7/3/08, Ct East, F, SD	6/11/08	6/11/08, 6/18/08, 6/24/08	1038C Hemo	**18.1**	0	**3.7**	**1.5**
2 weeks	Died 9/3/08 molted 8/27/08, RI, F, SD	8/19/08	8/27/08)	1049R Hemo	0	0	0	0

Table 3: Background on control lobsters, all had low initial hemolymph levels. All hemolymph contamination units are (ng/mL).

Death and background	Bleed times	Sample	Peak 1	Peak 2	Peak 3	Peak 4
Died and molted 7/21/08, Cape Cod, M, no SD	7/21/08	896M Hemo	0	0	0	0
Died and molted 7/18/08, Cape Cod, M, no SD	7/18/08	888M Hemo	0	0	0	0
Died and molted 7/22/08, Cape Cod, M, no SD	7/22/08	883M Hemo	0	**0.6**	0	0

Table 3.1: Alkylphenol contamination levels in muscle samples from injected and non-injected lobsters.

Injection Status	Sample	Peak 1 (ng/g)	Peak 2 (ng/g)	Peak 3 (ng/g)	Peak 4 (ng/g)
Injected	848M	0	0	**70.5**	0
Injected	901M	0	0	**36.3**	0
Injected	1036C	0	0	**350.3**	0
Injected	1038C	0	0	**17.1**	0
Injected	1049R	0	0	**5.0**	0
Not injected	883M	0	0	**8.3**	0
Not injected	888M	0	0	0	0
Not injected	896M	0	0	0	0

Figure 3.1: Alkylphenol contamination levels of peak 3 in muscle samples from injected and non-injected lobsters.

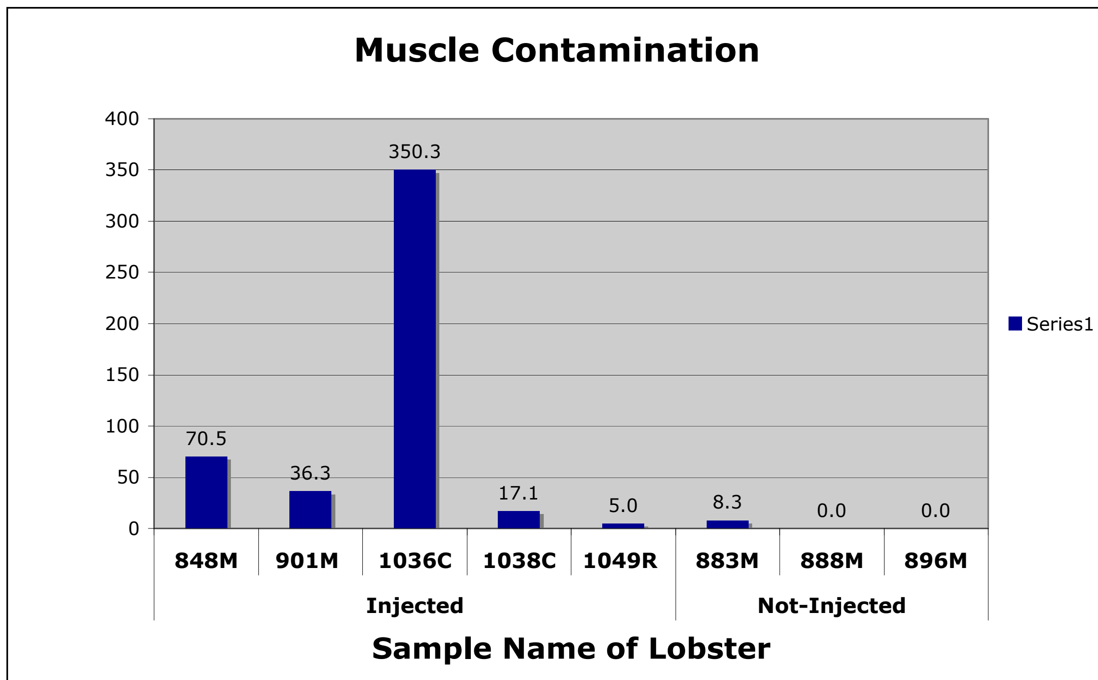

Table 3.2: Alkylphenol contamination levels in hepatopancreas samples from injected and non-injected lobsters.

Injection Status	Sample	Peak 1 (ng/g)	Peak 2 (ng/g)	Peak 3 (ng/g)	Peak 4 (ng/g)
Injected	848M	0	0	**87.6**	0
Injected	901M	0	0	**12.8**	0
Injected	1036C	0	0	**574.4**	0
Injected	1038C	0	0	**177.6**	0
Not injected	883M	0	0	0	0
Not injected	888M	0	0	0	0
Not injected	896M	0	0	0	0

Figure 3.2: Alkylphenol contamination levels of peak 3 in hepatopancreas samples from injected and non-injected lobsters.

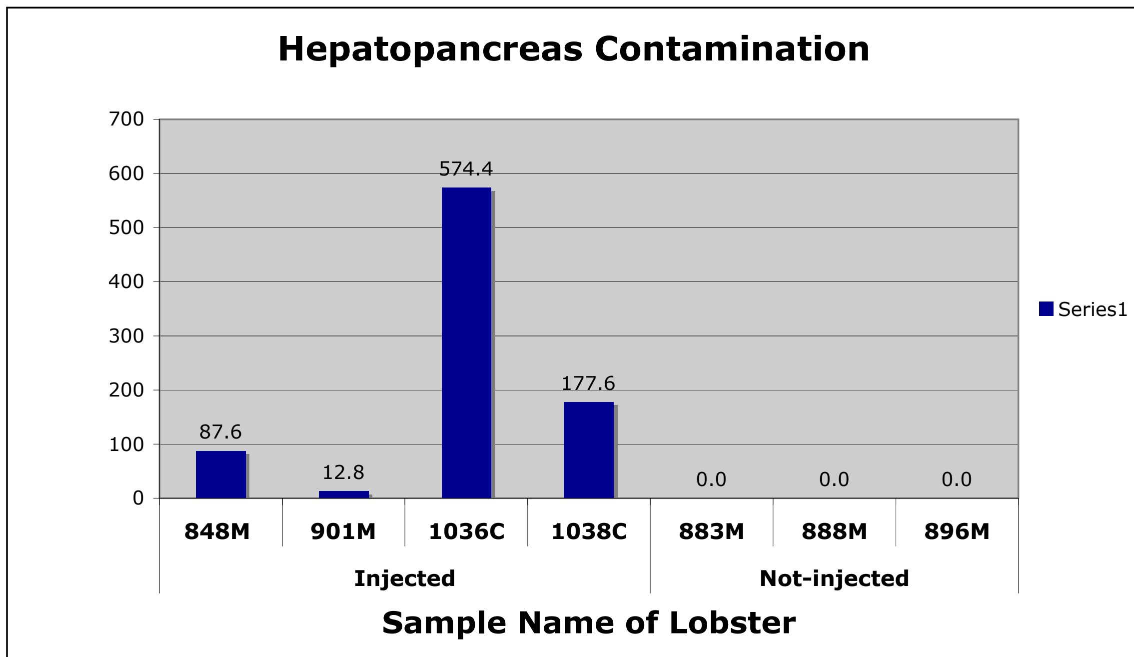

Table 3.3: Alkylphenol contamination levels in gonad samples from injected and non-injected lobsters.

Injection Status	Sample	Peak 1 (ng/g)	Peak 2 (ng/g)	Peak 3 (ng/g)	Peak 4 (ng/g)
Injected	848M	**8.0**	0	**1214.6**	**6.4**
Injected	1036C	0	0	**340.2**	0
Injected	1038C	0	0	**123.0**	0
Not injected	888M	**0.3**	0	0	0
Not injected	896M	0	0	0	0

Figure 3.3: Alkylphenol contamination levels of peak 3 in gonad samples from injected and non-injected lobsters.

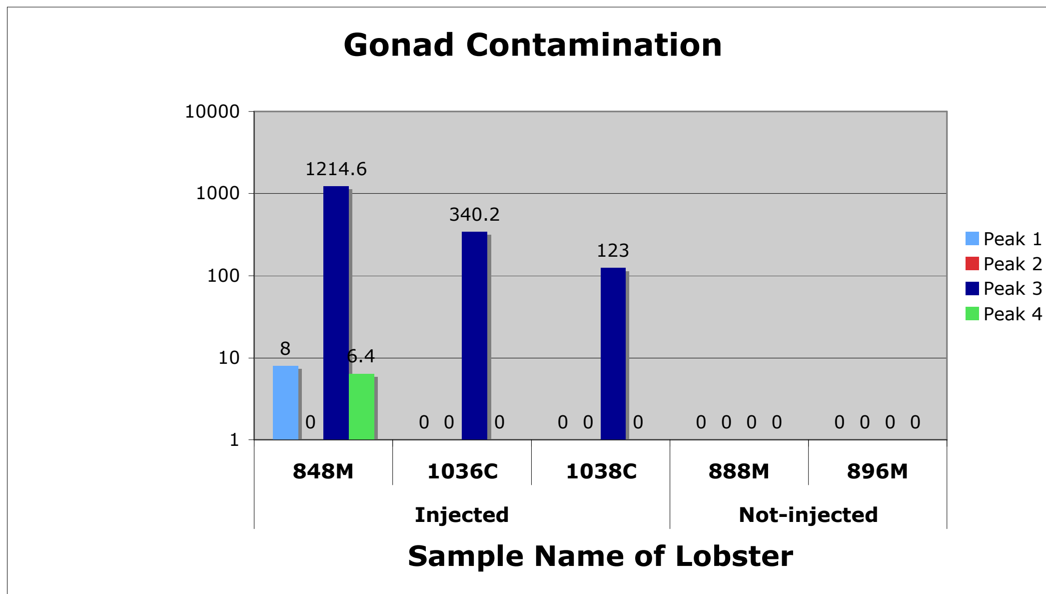

Table 3.4: Alkylphenol contamination levels in gill samples from injected and non-injected lobsters.

Injection Status	Sample	Peak 1 (ng/g)	Peak 2 (ng/g)	Peak 3 (ng/g)	Peak 4 (ng/g)
Injected	848M old gill	0	0	**2766.5**	0
Injected	901M old gill	0	0	**2375.0**	0
Injected	1036C new gill	0	**3.8**	**5323.6**	**40.2**
Injected	1036C old gill	0	0	**357.7**	0
Injected	1038C old gill	0	0	**916.6**	**2.2**
Not injected	883M old gill	0	0	0	0
Not injected	888M old gill	0	0	0	0
Not injected	896M old gill	0	0	0	0

Figure 3.4: Alkylphenol contamination levels of peak 3 in gill samples from injected and non-injected lobsters.

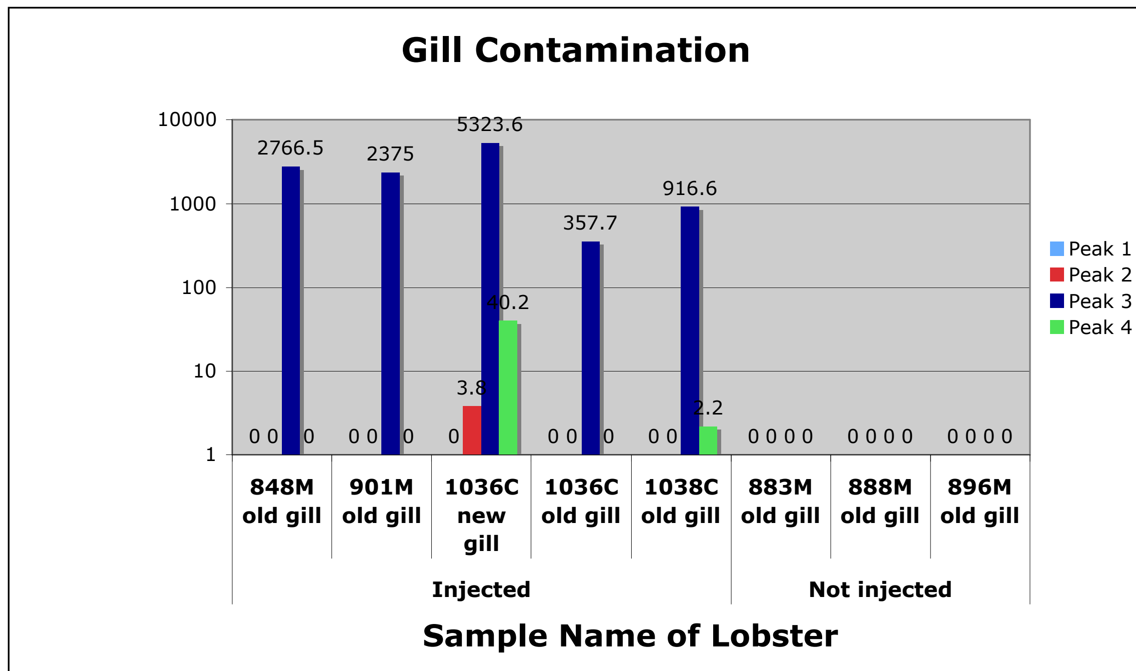

Table 3.5: Alkylphenol contamination levels in epidermis samples from non-injected lobsters. There were no injected lobster epidermis samples to analyze.

Injection Status	Sample	Peak 1 (ng/g)	Peak 2 (ng/g)	Peak 3 (ng/g)	Peak 4 (ng/g)
Not injected	883Mepidermis	0	0	0	0
Not injected	888Mepidermis	**0.6**	0	0	0
Not injected	896Mepidermis	**1.6**	0	0	0

Figure 3.5: Alkylphenol contamination levels in epidermis samples from non-injected lobsters. There were no injected lobster epidermis samples to analyze.

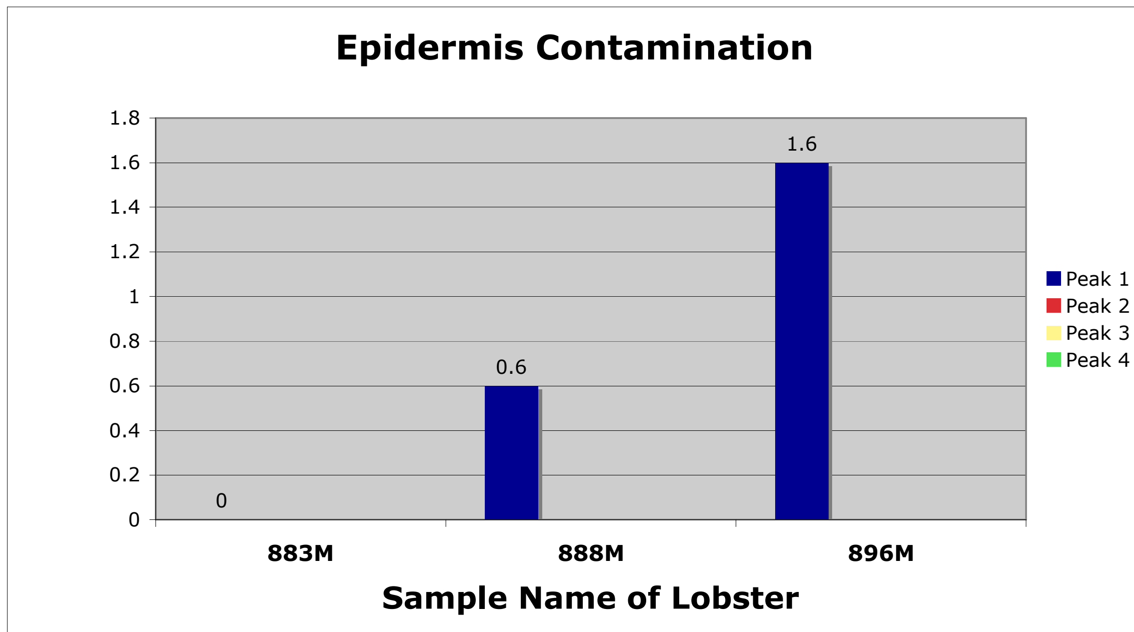

Table 3.6: Alkylphenol contamination levels in new shell samples from non-injected lobsters. There were no injected lobster new shell samples to test.

Injection Status	Sample	Peak 1 (ng/g)	Peak 2 (ng/g)	Peak 3 (ng/g)	Peak 4 (ng/g)
Not injected	883M new shell	**0.2**	0	0	0
Not injected	888M new shell	**0.1**	0	**2.9**	0
Not injected	896M new shell	**0.5**	0	0	0

Figure 3.6: Alkylphenol contamination levels in new shell samples from non-injected lobsters. There were no injected lobster new shell samples to analyze.

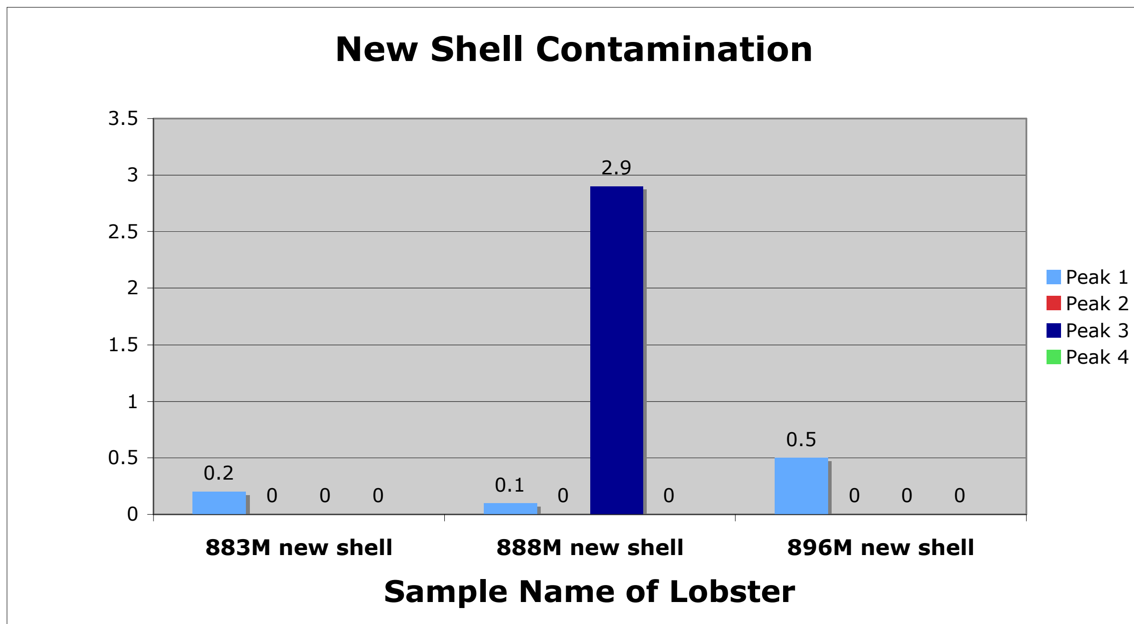

Table 4: Hemolymph Contamination values shown are closest to date of death for non-injected lobsters. *Shell Disease (SD)

# weeks in lab	Death and Background	Bleed and Injection Times	Sample	Peak 1 ng/mL	Peak 2 ng/mL	Peak 3 ng/mL	Peak 4 ng/mL
1 week	Died and molted on 7/28/08, M, Cape cod, no SD	Bleed 7/16/08	878M	0.0	5.5	27.9	6.0
2 weeks	Died 6/20/08, F, Offshore, no SD	Bleed 6/1/08	852M	8.7	4.7	0	0

1 week	Died 6/18/08, M, Mass South, no SD	Bleed 6/14/08	858M	10.6	1.4	11.3	0.8
2 weeks	Died 6/25/08, F, CT East, SD	Bleed 6/11/08	1041C	37.8	0.4	2.7	1.8
1 week	Died 6/27/08, M, RI, no SD	Bleed 6/16/08	1027R	0	0	0	0
2 weeks	Died 7/5/08 Molted 7/5/08, F, RI, SD	Bleed 6/16/08	1033R	0	0	7.4	0
1 week	Died 6/26/08, F, Mass South, no SD	Bleed 6/14/08	864M	4.6	0	4.1	1.5
3 weeks	Died 6/19/08, M, Offshore, no SD	Bleed 5/30/08	843M	0	0	0	0
2 weeks	Died 7/7/08, F, RI, SD	Bleed 6/16/08	1029R	0	0	0	0
2 weeks	Died 9/3/08, F, RI, SD	Bleed 8/19/08	1039R	0	0	6.3	0
Same Day	Died 3/14/08, M, CT East, SD	Bleed 3/14/08	1030C	0	0	0	0
1 week	Died 7/21/08, F, Cape Cod Bay, no SD	Bleed 7/16/08	889M	0	0	0	0
1 day	Died 6/12/08, F, CT East, SD	Bleed 6/11/08	1033C	26.1	1.1	1.7	1.4
1 day	Died 7/17/08, F, Cape Cod, no SD	Bleed 7/16/08	879M	0	0	2160.3	0
1 week	Died 7/31/06 Molted 7/31/06, M, Offshore, SD	Bleed 7/17/06	817M	0	0	0	0
1 week	Died 3/17/08, F, CT East, SD	Bleed 3/14/08	1023C	0	0	0.1	0
2 weeks	Died 6/19/08, F, Offshore, no SD	Bleed 5/30/08	850M	15.4	10.2	30.4	3.3
2 weeks	Died 7/2/08, M, RI, no SD	Bleed 6/16/08	1025R	Still in freezer			

Table 5: Muscle samples from non-injected lobsters.

Sample	Peak 1 (ng/g)	Peak 2 (ng/g)	Peak 3 (ng/g)	Peak 4 (ng/g)
878M muscle	1.5	0	5.9	3.5
852M muscle	0.7	0	0	0
858M muscle	0.3	0	0	0
1041C muscle	0.9	0	15.5	0
1027R muscle	0	0	28.2	0
1033R muscle	0	0	6.1	0
864M muscle	0	0	2.8	0
843M muscle	1.7	0	0	11.0
1029R muscle	0.5	0	25.3	1.4
1039R muscle	0.1	0	0	7.8
1030C muscle	0	0	0	0
889M muscle	0	0	0	0
1033C muscle	0	0	0	0
879M muscle	0	0	0	0
817M muscle	0	0	0	0
1023C muscle	0	0	0	0
850M muscle	0.6	0	35.8	1.6
1025R muscle	0.4	0	7.7	5.1

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