CORRELATION OF DNA METHYLATION WITH MERCURY CONTAMINATION IN MARINE ORGANISMS: A CASE STUDY OF NOAA MUSSEL WATCH TISSUE SAMPLES

A Senior Scholars Thesis

by

KAYLYN ELIZABETH GERM

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2009

Major: Marine Biology

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Approved by:

Co-Advisors: Robin Brinkmeyer
Robert Taylor

Associate Dean for Undergraduate Research: Robert C. Webb

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ABSTRACT

Correlation of DNA Methylation with Mercury Contamination in Marine Organisms:
A Case Study of NOAA Mussel Watch Tissue Samples. (April 2009)

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American oysters (*Crassostrea virginica*) obtained from the NOAA Mussel Watch program were screened for DNA methylation, a type of epigenetic response to stressors. Oysters were collected from sites in the Gulf of Mexico having high mercury contamination (measured by NOAA) and from sites with little to no measurable mercury. Assessment of anthropogenic stressors such as mercury in the coastal environment has traditionally relied upon species diversity indices or assays to determine lethal doses. However, these indices fail to examine sub-lethal impacts such as gene expression. A 'global' DNA methylation kit, recently introduced by Sigma-Aldrich, was used to spectrophotometrically compare the degree of methylation in DNA extracted from contaminated oysters and non-contaminated oysters. DNA methylation was higher in oysters from pristine sites than in oysters from contaminated sites.

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CHAPTER I

INTRODUCTION

Epigenetic research is a growing field that is thought to be the explanation for gene expression changes over the generations due to environmental conditions, rather than genetic mutations. Bird (2007) defined epigenetics as the study of heritable changes in gene expression and function which are not the results of DNA changes and/or mutations. Epigenetic markers that influence gene expression include i) methlyation of cytosine residues in DNA, ii) acetylation or methylation of histone proteins, and iii) regulatory processes altered by small RNA molecules (Bird, 2007). The epigenetic marker discussed in this paper is direct methylation of cytosine residues in DNA.

The epigenome, literally translated meaning "above the genome," is the genome affected by the outer environment, which cause changes in gene expression. The epigenetic effect of DNA methylation causes differentiation in gene expression, cell differentiation, chromatin inactivation, genomic imprinting, and carcinogenesis (Xu et al., 2000). Studies of the whole genomes of identical twins from birth to adulthood often find that differences in disease between twins are caused by epigenetic differentiation (Bird, 2007). Physiological responses to each individual's environment allows physical, physiological, and developmental differences to occur, which are then incorporated into

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This thesis follows the style of the journal of Environmental Toxicology.

the genomic and ultimately expression. These epigenetic effects also accumulate over time, as older sets of identical twins were found to have significant differences in the total number of methylated sites and therefore expressed different phenotypes; as compared to young sets of identical twins which had minimal differences both in methylated sites and gene expression (Holt and Patterson, 2006).

DNA methylation is heritable. In a series of experiments, rats were exposed to a fungicide (vinclozolin) and CpG sites of several protein encoding genes became hyper-methylated, thus physically preventing transcription (Anway et al., 2005; Anway and Skinner, 2006). (CpG sites in DNA have a cytosine nucleic acid followed by a guanine nucleic acid; these sequences are often clustered in the regulatory regions of genes) The offspring of the F1 generation were never exposed to the original fungicide and yet they as well as the F2 offspring and four generations of rats later exhibited the same suppression or silencing of genes as in the F1 rats after exposure. DNA methylation is important in differential regulation of genes during embryological development (Reik, 2007). Epigenetic studies of cancer have found hypomethylation of genes encoding for growth.

DNA methylation of cytosine residues is the most common and best studied epigenetic effect. Methylation is determined by DNA sequencing, antibody, or restriction enzyme detection of methyl groups bonded to the cytosines in the DNA sequence at the CpG sites (Tweedie et al. 1997). Direct methylation correlates to gene silencing, or “turning

the gene off", because the attached methyl group inhibits DNA transcription by physically blocking transcription factors and RNA polymerase. Alternatively, the loss of methylation allows for DNA transcription of genes.

Natural environmental examples of stressors in the aquatic ecosystems that can cause cell damage include heavy metals, ultra-violet radiation, and salinity. Environmental changes alone can cause problems for developing and physiological cell processes (Pawlak and Deckert, 2007), and it is still unknown what types of lethal and/or sub-lethal effects anthropogenic stressors and changes in the aquatic environment can induce, and in what amounts. Anthropogenic stressors include nutrient enrichment, physical alteration of habitat, altered fresh water inflow, toxic chemicals (heavy metals, organo-chloride compounds, pesticides, fungicides, and petroleum products) released into the air and water, invasive species, pathogens, and resource exploitation. All stressors can potentially alter morphological, physiological, and developmental functions by methylating CpG sites on the DNA and silencing genes in a particular regulatory region (Pawlak and Deckert, 2007). Assessment of stressor impacts has traditionally relied upon species diversity indices or assays to determine lethal doses. However, these indices fail to examine sub-lethal impacts, such as gene expression (Bossdorf et al., 2008).

In 1986, the United States National Oceanic and Atmospheric Administration (NOAA) initiated a program to assess the anthropogenic influences on wetland and estuarine

habitats, known as National Status & Trends (NS&T) Mussel Watch Program. Mussel Watch Program focuses on the bivalve species of the seafood industry such as oysters rather than including fish and other invertebrates. *Crassostrea virginica*, commonly known as the American or Eastern oyster, is the only oyster species found in the Gulf of Mexico and is ubiquitous through all associated waters, including bays and estuaries. Mollusks are ideal indicators of aquatic environmental health because of their ability to concentrate chemicals within their tissues; this is known as bioaccumulation. Mollusks are often used as the official measuring devices or sentinels for assessing chemical contamination in aquatic environments. *C. virginica* are a hardy species that can survive in contaminated or polluted areas when other organisms cannot. They have a high tolerance for harsh environments and are readily adaptable to most, including heavily, polluted habitats (O'Connor, 2001). These oysters are also capable of sexual reproduction via broadcast spawning and have relatively efficient development cycles for scientific research.

Mercury, a highly toxic heavy metal, has made its way into bays and estuaries of the U.S. east coast and the Gulf of Mexico via industrial effluent. Fish and shellfish are typically monitored for bioaccumulation of mercury since they are consumed by humans. However, the deleterious impacts of mercury to these organisms are limited to studies of lethal doses or observable, physical deformations and fail to examine the impacts to species diversity or sub-lethal effects such as gene expression.

Inorganic mercury is released into the atmosphere from industrial sources. In the atmosphere it easily returns to the ground or water because of its molecular weight. Much of this inorganic mercury is sequestered in sediments however a significant amount is transformed by bacteria in aquatic environments, including wetland habitats, to highly toxic and bioreactive methyl-mercury. Methyl-mercury is absorbed by phytoplankton and bioaccumulates up the food chain to fish and marine mammals (Kluger, 2006). Human consumption of mercury in fish can cause adverse effects on the central nervous system and development of fetuses. At the molecular level, mercury inhibits protein synthesis by mimicking the 'start' amino acid, methionine, resulting in defective translation (Philbert et al., 2000).

Oysters filter bacteria and phytoplankton from the water column and accumulate methylmercury in their tissues (O'Connor, 2001). The adverse effects of mercury bioaccumulation in oysters is not well studied. Most studies of mercury contamination in oysters or other mollusks are aimed at water quality monitoring and do not assess the health of the study organisms (Fabris et al., 1994; O'Connor, 2001; O'Connor and Lauenstein, 2006). A study of arsenic exposure in the freshwater clam, *Corbicula fluminea*, reported acute toxicity at LC50 (mortality of 50% of test organisms) values of 20.74% mg/L at 96 hours (Liao et al. 2008). Another study examined the influence of elevated temperature on the effects of cadmium toxicity to *C. virginica* (Nikolic and Sokolovic, 2004). Exposure of oyster mitochondria to cadmium while increasing temperature to 35°C markedly decreased respiration rates and enzymatic activity.

Data regarding the epigenetics and methylation of DNA in invertebrates are scarce and data for oysters are non-existent. The percent of methylated cytosines in insects is approximately 0-3%, 5% in birds and mammals, 10% in fish, and possibly more than 30% in plants (Field et al.,2004). One study examining the divergence of invertebrates and vertebrates compared level of DNA methylation as evolutionary adaptations (Tweedie et al. 1997). The genomes of the representative invertebrates, sea urchins and sea squirts, were described as fractionally methylated with a high degree of methylation in functional genes. In contrast, vertebrate genomes were found to be globally methylated (i.e. methylated throughout).

In this study, I examined the effect of mercury contamination on the occurrence of DNA methylation in *C. virginica*. Oyster samples collected by the NOAA Mussel Watch Program from sites in the Gulf of Mexico highly contaminated with mercury and other heavy metals as well as organic pollutants and from 'pristine' sites for comparison of DNA methylation.

Hypothesis: The occurrence of methylation in genomic DNA will be higher in oysters exposed to high levels of mercury than in genomic DNA from oysters collected from pristine areas.

Objective 1: To assess if DNA methylation can be used as a genetic indicator of stress in aquatic organisms.

Objective 2: To test the applicability and effectiveness of a new, commercially available kit to measure global DNA methylation.

Project relevance: The US Environmental Protection Agency as well as other agencies such as the National Oceanographic and Atmospheric Administration are interested in the development and application of novel methods to examine and quantify the impacts of anthropogenic stressors on coastal environments.

CHAPTER II

MATERIALS AND METHODS

Oyster samples

Collection

Oyster samples used in this study were collected by the National Oceanic & Atmospheric Administration (NOAA) as a part of the NOAA Mussel Watch project. Collection protocols are described in the NOAA Technical Memorandum NOS OMA 40 (Shigenaka and Lauenstein, 1988). Protocols included in the document include benthic surveillance, mussel watch, and analysis of compounds. Each protocol describes methods for selection of sampling sites, harvesting the mussels and/or oysters, and analyzing chemicals. NOAA sampling included both blue mussels and oysters, however this study focuses only on oysters and those sample exclusively from the Gulf of Mexico.

Taylor lab protocol

Whole oysters, sampled by NOAA Mussel Watch Program, were sent to Dr. Robert Taylor's lab at Texas A&M University in College Station, Texas. Using trace metal clean facilities, oysters were shucked, the tissues freeze-dried and homogenized, and then digested prior to analysis according to Lauenstein and Cantillo (1998). In Dr. Taylor's lab, the tissues were analyzed for heavy metal concentrations, including mercury. The lyophilized, homogenized oyster tissue samples were stored as dry

powder. For this study, the powdered tissue samples were re-hydrated for DNA extraction.

Selection of samples

Figure 1 illustrates the sampling sites for the NOAA Mussel Watch Program in the Gulf of Mexico. Samples for this study came from sites spanning from Tampa, Florida to Lower Laguna Madre, Texas.

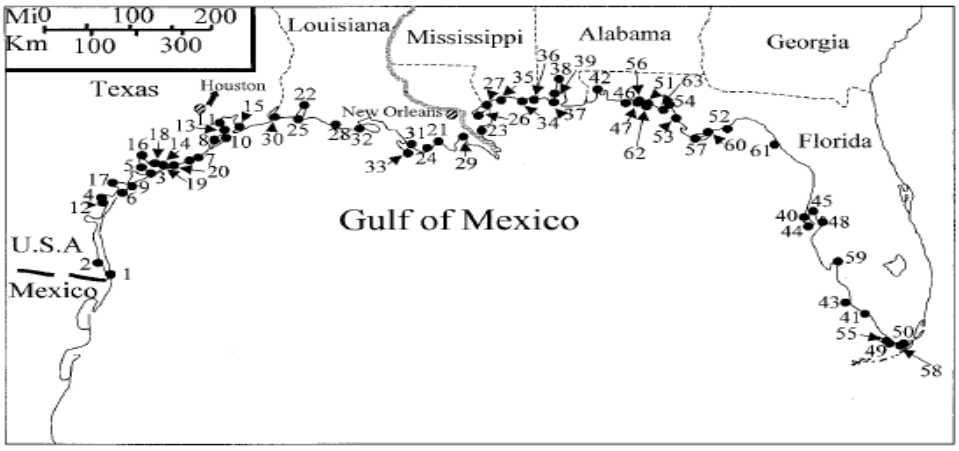

Figure 1. Map of the Gulf of Mexico coastline. Dots indicate NOAA Mussel Watch Program sampling sites. Samples used for this study are (starting in the east): #48 – Hillsborough Bay, #44 – Mullet Key, #60 – Cat Point Bar, #57 – Dry Bar, #56 – Joe's Bayou, #21 – Lake Felicity, #24 – Lake Barre, #11 – Yacht Club, #15 – Hanna Reef (Ship Channel), #16 – Gallinipper Point, #5 – Lavaca River Mouth, #6 – Long Reef, #2 – Port Isabel, and #1 – (Lower Laguna Madre) South Bay (Kannan et al., 2001).

Samples for this experiment were chosen according to contamination levels of mercury and other pollutants determined previously (O'Connor, 2001). Samples were collected

between 2006 and 2008, and reflected most of the inventory available at Dr. Taylor's lab.

Data from O'Connor (2001) were used to differentiate contaminated from non-contaminated sites. A specific site or area was designated as contaminated if it oyster tissues had a significant amount of any of the following elements/toxins/substances: 2 and 3 ring polycyclic aromatic hydrocarbons (tPAH) consisting of low molecular weights (tLMW) and high molecular weights (tHMW), tributyltin (tBT), tDDT, Lead (Pb), Mercury (Hg), Copper (Cu), Cadmium (Cd), Cr, Nickel (Ni), dieldrin (tDield), chlordane (tCdane), and/or polycholorinated biphenols (tPCB). Limits for these compounds were set by the US Food and Drug Administration on a dry-weight basis. All contaminated sites contained tPAH and mercury (Hg). According to USFDA regulations, high levels of contamination are defined as concentrations in the 85th percentile. Concentration percentiles can be found at http://ccmaserver.nos.noaa.gov (O'Connor, 2001). Non-contaminated sites were considered to be absent of approximately all organic contaminants, heavy metals, and other toxins. These samples, designated as pristine, and were used as controls for this study (Table 1).

TABLE 1. Samples Used in This Study. Samples 1-7 were designated as 'contaminated' and samples 8-14 were used as controls and considered 'pristine' (O'Connor, 2001). Contaminated samples had mercury (Hg) and other pollutants at levels within the 85th percentile as defined by the USFDA.

Sample	Location	Taylor Lab ID #	Year Collected	Contaminant[1]												
				Hg	Cu	Pb	Cd	Cr	Ni	tDield	tCdane	PCB	tLMW	tHMW	tPAH	tBT
1	Tampa Bay - Hillsborough Bay	T6020-050	2006										X	X	X	X
2	Choctowhatchee Bay - Joe's Bayou	T6020-036	2006	X		X							X	X	X	X
3	St. Andrew's Bay - Watson Bayou	T7027-002	2007		X								X	X	X	
4	Galveston Bay - Ship Channel	T6128-001	2006				X			X	X	X		X	X	X
5	Galveston Bay - Yacht Club	T6128-002	2006							X	X					X
6	Matagorda Bay - Gallinipper Point	T7013-003	2007	X				X	X							
7	Matagorda Bay - Lavaca River Mouth	T6135-006	2006	X				X								
8	Tampa Bay - Mullet Key Bayou	T6020-044	2006													
9	Apalachicola Bay - Dry Bar	T7027-005	2007													
10	Apalachicola Bay - Cat Point Bar	T7027-004	2007													
11	Terrebonne Bay - Lake Barre	T6020-021	2006													
12	Aransas Bay - Long Reef	T6135-003	2006													
13	Lower Laguna Madre - Port Isabel	T6020-009	2006													
14	Lower Laguna Madre - South Bay	T6020-008	2006													

[1]Contaminant concentrations fell within the 85th percentile with the following values: Hg ≥0.23 µg/g; Cu ≥360 µg/g; Pb≥3.0 µg/g; Cd≥5.9 µg/g; Cr≥4.7 µg/g; Ni≥3.1 µg/g; tDield≥8.5 ng/g; tCdane≥34 ng/g; PCB≥420 ng/g; tLMW≥320 ng/g; tHMW≥770 ng/g; tPAH≥1100 ng/g; tBT≥300 ng of Sn/g.

DNA extraction

Fifty mg of powdered oyster tissue from each sample was weighed and placed into a sterile 2 ml tubes. Forceps and other instruments used for sample handling were sterilized with 100% ethanol before use and between samples. DNA was extracted from oyster tissues using the DNeasy kit by Qiagen (Valencia, CA) according to the protocol for rat tails. Approximately 200 µL of PCR water was added to the tissues for rehydration prior to extraction. Once homogenously rehydrated by vortexing, 180 µL of Buffer ATL and 20 µL of Proteinase K were added to the tubes, followed by vortexing. Tubes were incubated in a heat block for 3 hours at 55° C. Next, samples were vortexed and 200 µL of Buffer AL was added before further vortexing. The tubes were then inserted back into the heat block at 70° C for 10 minutes. After this second incubation, 200 µL of 100% ethanol was added to the tissue, and the sample was transferred to a spin column. Columns were centrifuged at >6000 × g for 1 minute. Flow-through was discarded. Next, 500 µL of Buffer AW1 was added to the column before centrifuging a second time at >6000 × g for 1 minute. The flow-through was discarded. 500 µL of Buffer AW2 was then added to the spin column and then samples were centrifuged at >20,000 × g for 3 minutes, flow-through was discarded. The spin column was placed in a new 2ml tube and 200 µL of Buffer AE (elution) was added. The samples were incubated at room temperature for 3 minutes and were then centrifuged at 14,000 ×g for 1 minute. This step was repeated, for a total flow-through volume of 400 µL. Nucleic

acids concentration and purity was determined by $A_{260/280}$ measurements using an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE) spectrometer.

DNA methylation detection

The Imprint Methylated DNA Quantification kit (Table 2), introduced by Sigma Aldrich in October 2008, was used to determine at global DNA methylation, or total methylation of the genomic DNA in each sample. In short capture and detection antibodies that bind to CpG sites in the genomic DNA are quantified colorimetrically.

TABLE 2. **Imprint™ Methylated DNA Quantification Kit Components.** (Sigma-Aldrich, 2008)

Kit Components

Reagent	Catalog Number	48 Rxn	96 Rxn
10× Wash Buffer	W4267	11 mL	22 mL
DNA Binding Solution	D8568	1.5 mL	3 mL
Methylated Control DNA (50 ng/µL)	M8570	16 µL	32 µL
Block Solution	B6561	10 mL	20 mL
Capture Antibody	C3493	10 µL	20 µL
Detection Antibody	D8818	10 µL	25 µL
Developing Solution	D8693	6 mL	12 mL
Stop Solution	S3447	3 mL	6 mL
8 well Assay Strips	A4231	6 strips	12 strips

Prior to analysis, the 10× Wash Buffer was diluted to 1×. Eleven mL of 10× Wash Buffer was added to 99 mL of sterile PCR water to make a 1× Wash Buffer solution. DNA extracted from the oyster samples was diluted as necessary to obtain a

concentration of 50 ng/µl for methylation detection in subsequent steps. Finally, total DNA in the binding solution (30 µL) was normalized to 50 ng. Tests trials using binding solution as 'blanks' and replicates of the control methylated DNA were used to evaluate the kit's protocol. I tested low (10 ng) to high (200 ng) total DNA to determine optimal concentrations for sample analysis.

Once the optimal concentration of DNA for methylation detection was determined (50 ng) 30 µL of DNA- Binding Solution, was added to a well of the Assay Strip supported by a 96 well plate. The well-plate was then tilted side to side to expose the entire bottom of the well to the DNA solution. The wells were covered with transparent, laboratory-sealing tape to prevent contamination and evaporation. The entire well-plate was incubated at 37° C for 60 minutes. After initial incubation, 150 µL of the Block Solution was added directly to each well. Adhesive tape was reapplied and the samples continued to incubate at 37° for another 30 minutes. Following the incubation, the DNA solution and Block Solutions were removed from the wells with a pipet and each well was washed three times with 150 µL of the 1× Wash Buffer by pipeting Wash Buffer into each well and then immediately removing it.

During the previous incubations, both the Capture and Detection Antibodies were prepared by dilution in 1× Wash Buffer at 1:1000. Following the three washes (above), 50 µL of the diluted Capture Antibody was added to each well. The wells were covered and incubated at room temperature for 60 minutes. Next, the diluted Capture Antibody

was removed each well was washed four times with 150 µL of 1×Wash Buffer. After washing, 50 µL of the diluted Detection Antibody was added to each well. The well-plate was covered and incubated at room temperature for 30 minutes. The diluted Detection Antibody was then removed from each well after 30 minutes, and then each well was washed five times with 150 µL of 1× Wash Buffer.

After the final wash, 100 µL of the Developing Solution was added to each well. The well-plate was then covered and incubated at room-temperature in the dark for approximately 9 minutes. The solutions were observed for an immediate to gradual color change to blue. Once blue, and with less than 10 minutes of development, 50 µL of the Stop Solution was added to the wells to induce another color change to yellow. The time at which the Stop Solution was added was recorded since the overdevelopment was found to affect the color change.

Analysis

96-well plates were read for absorbance at 450 nm on a PowerWave X spectrophotometer. Data was displayed on the software that was a grid of the well plate, and each absorbance was determined and reported from the spectrophotometer. Absorbance values were then converted using the 'global methylation calculation' below:

$$\frac{\text{Sample Average} - \text{Blank Average}}{\text{Control Average} - \text{Blank Average}} \times 100 = \text{Percentage of global methylation compared to the control DNA sample}$$

Resulting percentages were then compared directly between replicates and then analyzed for statistical differences using the Student's T-test. Student's t-test calculated and compared means and variances of each of the replicates. Within each replicate, the first seven samples (1-7) were compared to the second seven (8-14). A table was created by Excel to obtain a P-value. The probability value that was used was 0.05, where our confidence interval was 95%. The null hypothesis for the t-test analysis was that there was no difference between the amount of global methylation at the contaminated sites and the pristine sites.

CHAPTER III

RESULTS AND DISCUSSION

A series of tests were performed to determine optimal DNA concentration for detection of methylation. Since the kit was first introduced by Sigma-Aldrich in October 2008, no published evaluations of kit use and performance were available. These test trials also examined varied time of color development for the effect upon final colorimetric measurements. Several practice runs were required to work out the protocol since the kit instructions were vague and misleading. The kit instructions did not specify how to wash the assay strip wells and this may have affected the final results if non-bound antibody was not completely removed.

Trial run #1

The pilot run for this experiment was a test to carry-out the methods, gauge time-spans, and find out if the kit was working properly to produce results. Three samples were tested: 1 blank of Binding Solution, 1 control DNA sample, and 1 test DNA sample which was randomly chosen from the set of NOAA oysters samples. This run produced predicted absorbance levels as compared to the examples in the protocol, despite minor inconsistencies in carrying out the protocol. The absorbance reading for the blank was 0.283. The Sigma-Aldrich protocol identifies a troubleshooting guide for the blank absorbance readings being >0.2, as shown in Table 3. The development time is noted to be no longer than 10 minutes, whereas the development time for this pilot run was 10

minutes and 23 seconds. This seemed to be the most logical explanation for the outcome of this trial. After 'methylation calculation', the test sample was found to have 102.60% global methylation as compared to the control DNA sample.

TABLE 3. Troubleshooting Guide. (Sigma-Aldrich 2008)

Observation	Cause	Recommended Solution
High Background Present in Blank (>0.2 OD)	Inappropriate/inconsistent washing	Perform all wash steps as indicated in the protocol. If necessary, one wash can be added at each of the wash steps.
	Contaminating DNA	Ensure a clean tip is used when applying DNA Binding Solution. When handling plate, ensure that no splashing occurs.
	Over-development	Monitor development of blue color once the Developing Solution has been added. Do not allow the reaction to incubate more than ten minutes.
No Signal for DNA-Containing Samples (<0.2 OD)	Addition of Reagents	Ensure reagents are added in the appropriate order and none of the procedure steps have been omitted.
	Washing	Ensure the wells of the assay strip are not washed prior to the application of the DNA sample.
	Incubation time and temperatures	Ensure incubation time and temperatures correspond to the written protocol.
	Addition of DNA to well	Ensure DNA is mixed thoroughly prior to application. The wells of the assay strip bind up to 200 ng of total DNA. The methylated control DNA (Catalog Number M8570) included in the kit can be detected at the 10 ng level.
	Degradation of DNA	Ensure DNA is stored at the appropriate temperature.

Trial run #2

Another trial run allowed us to experiment with the developing times more precisely, using only the blank solutions and 10ng of control DNA. A set consisted of one blank and one control sample, and three sets were run. The first set developed for 5 minutes, the second for 6 minutes and 15 seconds, and the third for 8 minutes. The goal was to find the optimum time of colormetric development. In trial #1, variability of color change was observed with increased time of development. For 5 min development time, absorbance value for the blank was 0.486 and therefore elevated > 2 × the suggested reading of 0.2 as recommended by the kit protocol. As such, the blank could not be used for calculation of DNA methylation in samples. Development time of 6 min produced an

'appropriate' blank reading at 0.169. However, the highly methylated control DNA reading was only slightly greater than that of the blank, indicating a problem with color development. For development time of 10 min, the absorbance readings were found to be the most "correct" as compared to the kit's definitions. The blank read out at 0.156, less than the blank in the second set, and the DNA sample read at 0.288, showing a greater difference between the blank and the highly methylated control DNA. No calculations were performed for this experimental run because no sample DNA was examined; developing time was the primary concern of this run.

The resulting absorbance levels from this run show that the numbers become more "favorable" the longer the solutions develop. However, the pilot run for this kit showed that over-development, longer than 10 minutes, also gives skewed results by making the blank readings higher, > 0.2. It was concluded that the longer the solution develops, but not developing longer than 10 minutes, the more accurate the readings.

Trial run #3

A replicate trial was run just as in the previous trial, again experimenting with developing time. Concentration of control DNA was varied at 50 and 100 ng. According to the kit protocol, up to 200 ng total DNA per sample can be tested. Results showing optimal DNA concentrations are shown in Fig. 2. Again, three replicates of the blank and control DNA sample were tested. Developing times also varied and also confirmed the data and results from the previous run: the longer the solution develops, the more

accurate the readings will be. The first set developed for 5 minutes, with a blank reading of 0.209 and the control sample was 0.167. The second set developed for 6.5 minutes. The blank read an absorbance of 0.134, which was within the appropriate range, and the control sample read out at 0.167. The third set gave the best numeric results after developing for approximately 9 minutes. The blank reading was 0.129 and the control DNA was 0.209. Due to the lack of a test DNA, the global methylation calculation was not applied to the data from this run.

Results from the test trials lended confidence for performing the protocol and we concluded that 10 ng of DNA, of which was the lowest end of the range at which the kit could detect methylation, was not a sufficient amount for the control and sample DNA. The development time was found to be optimal between nine and ten minutes. The kit cautioned that overdevelopment, developing for more than ten minutes, would lead to skewed absorbance data from the spectrophotometer (readings of >0.2). This warning was confirmed in our trial runs and was applied throughout the experiment.

Experiment run #1

Following the test runs to experiment with the variable options in the kit, *C. virginica* samples were tested at concentrations of 50 ng total DNA. Seven contaminated and seven non-contaminated (pristine) samples were tested, two replicates each. All fourteen samples and their name and number labels are found in Table 2, and are sequential to the order they were placed in the 96-well plates. In addition to oyster samples, additional

samples for variability testing of the kit were included: two wells with blank, two wells with 10 ng of control DNA, two wells with 50 ng of control DNA, two wells with 100 ng of control DNA, and one well with 200 ng of control DNA. Developing time was precisely at 9 minutes per each well. A total of five wells did not show any color change during development, perhaps indicating incomplete washing or removal of antibody. Wells containing 100 ng control DNA, did not have any color change, indicating that 100 ng of DNA is not processed effectively by the kit reagents. Also, sample number 11, TBLB, did not show any color change, possibly due to insufficient vortexing of DNA or dilution error.

The absorbance results for all but the five undeveloped samples fell within in the acceptable ranges as defined by the kit, however, the 50 ng control DNA samples, replicates 1 and 2 were elevated to 1.070 and 1.209 respectively. For samples tested at 50 ng total DNA (Experiment Run #1, methylation was higher in pristine or uncontaminated sites for both replicate tests 1 and 2 (Table 4).

Experimental run #2

A second experimental run was repeated, differing only in the amount of sample DNA, which was increased to 100 ng. Variation of the control DNA replicates were not repeated, as the run focused on the oyster DNA samples. The first replicate of all of the samples either did not exhibit any color change, or a very faint color change, while the second replicate of all fourteen samples had very distinct color changes after

development. These results concur with experimental run #1 where 100 ng of DNA did not yield any colorimetric development or absorbance reading. Data is not presented due to inconsistencies as compared to the kit and previous the previous trial and experimental runs.

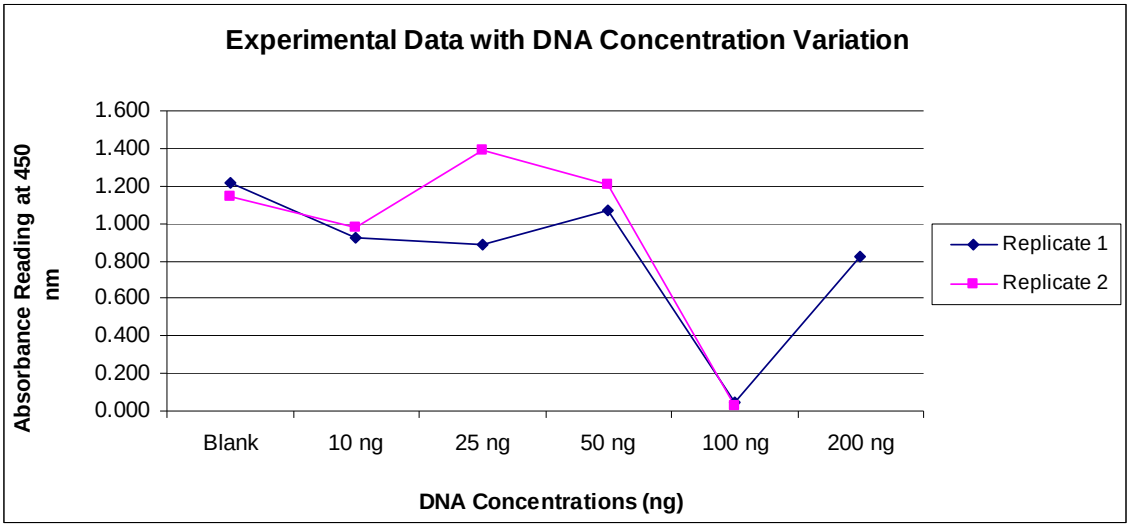

Figure 2. Graph shows the data from the trial run involving experimentation with DNA concentrations using the control DNA. A total concentration of 50 ng was determined as the optimal concentration for consistent kit performance.

TABLE 4. Raw Data from Experimental Run #1 with Total Sample DNA Concentrations of 50 ng. Contaminated samples in blue (1-7) and non-contaminated samples in red (8-14). Columns 3 and 5 show each sample as it results from the global methylation calculation from the protocol.

Sample	Absorbance Reading at 450 nm	% from Control DNA	Absorbance Reading at 450 nm	% from Control DNA
	Replicate 1		Replicate 2	
1	0.501	1658.54	0.786	963.41
2	0.776	987.80	0.724	1114.63
3	0.907	668.29	0.689	1200.00
4	0.894	700.00	0.717	1131.71
5	0.047	2765.85	0.789	956.10
6	0.668	1251.22	0.498	1665.85
7	0.728	1104.88	0.932	607.32
8	0.464	1748.78	0.741	1073.17
9	0.347	2034.15	0.800	929.27
10	0.470	1734.15	0.844	821.95
11	0.046	2768.29	0.039	2785.37
12	0.467	1741.46	0.588	1446.34
13	0.527	1595.12	0.383	1946.34
14	0.674	1236.59	0.801	926.83

Student's t-test

The Student t-test yielded a p-value for each replicate. For Experiment #1, the p-value was 0.028, less than 0.05, therefore the null hypothesis that no change in DNA methylation will be observed is rejected. In other words, there is a significant difference between global methylation levels at the contaminated sites and the pristine sites Experimental Run #1- replicate 2 had similar results, with the calculated p-value of 0.038. Again, this p value is less than 0.05, so the null hypothesis can be rejected. The variance around the means for Experiment 1, replicate 1 [Fig. 3-1] and replicate 2 [Fig.3-2] are plotted.

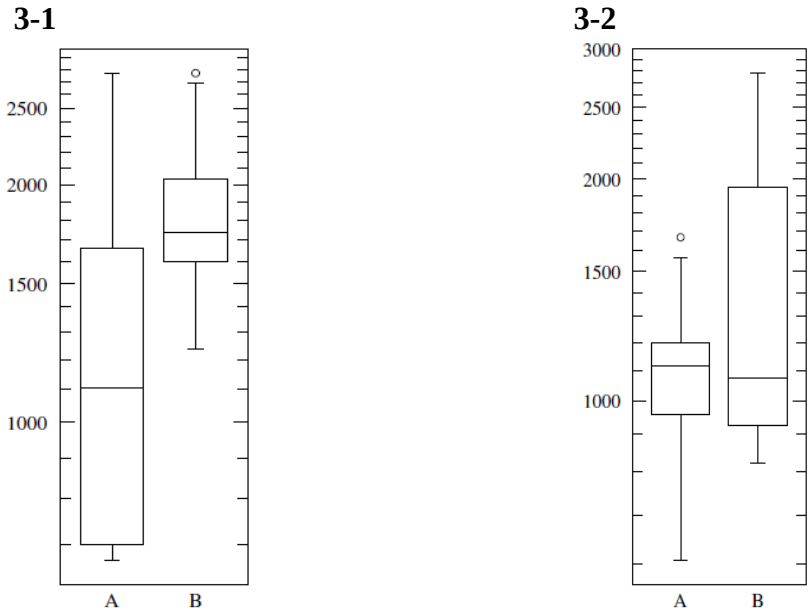

Figure 3. Logarithmic plots of student's t-test for experiment run #1. (3-1) is replicate 1, and (3-2) is replicate 2. The boxes are the data presented around the mean, and the error bars represent the standard error. In both figures, (A) signifies the contaminated samples, and (B) signifies the pristine samples.

Figure 3 illustrates the difference in global methylation levels between contaminated and pristine sites. Surprisingly, however, the pristine sites were found to have higher levels of global methylation than the contaminated sites. This result is contrary to my hypothesis that predicted higher methylation of DNA in the samples from contaminated sites.

Discussion

The literature regarding epigenetic markers for evaluation of health of aquatic organisms is scarce, thus emphasizing the need for these types of studies. Despite some problems with the efficiency of the Imprint™ DNA Methylation Quantification Kit, differences in the DNA methylation of oyster tissues from contaminated versus pristine sites were observed. DNA methylation was higher in oyster tissues from pristine sites. This result was unexpected. However, further reading of the body of literature for vertebrate DNA methylation provided some possible explanation. Nikolic and Sokolovic (2004) found that mercury has an inhibitory effect on some enzymes, such as aminotransferases. This may impose a similar inhibition of DNA methyltransferases, which catalyze DNA methylation. This could perhaps explain why oysters from contaminated sites had lower DNA methylation concentrations if mercury somehow inhibits DNA methyltransferase. DNA methylation is also variable from species to species and may also play different roles such as repression of intragenomic parasites (Regev et al., 1998). Therefore, higher concentrations of DNA methylation may be normal in oysters and function as a

protection mechanism. Another explanation for this disparity of DNA methylation in Gulf of Mexico oysters could be regional differences. Geographic variation in nuclear genes of *C. virginica* were determined among a host of functional genes. Allelic frequencies of several nuclear genes in Atlantic coast oysters (from Florida to N. Carolina) are statistically different from Gulf of Mexico oysters (Hoover and Gaffney, 2005). All of the oyster samples analyzed in this study came from the Gulf of Mexico so variability of DNA methylation due to differences in allelic frequency should not be a factor. Moreover, samples from contaminated (and pristine) sites located throughout the Gulf of Mexico coast were pooled, thus minimizing variability.

To my knowledge, this study is the first to examine DNA methylation in *C. virginica*. Moreover, DNA methylation appears to be a useful indicator of oyster health and a viable method to examine the sub-lethal effects of mercury contamination. Additional studies are needed to confirm these preliminary results. The Imprint™ DNA Methylation Quantification Kit was not user friendly and the results were often inconsistent. Other methods such as DNA bisulfite sequencing detection of CpG sites are proposed for future studies.

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