EPIGENETIC VARIATION OF *PHRAGMITES AUSTRALIS* ALONG A SALINITY GRADIENT IN THE HUDSON RIVER ESTUARY

REBEKAH PETROFF
Allegheny College, 520 N Main St, Meadville, PA 16335

MENTOR SCIENTIST: DR. STUART FINDLAY
Cary Institute of Ecosystem Studies, Millbrook, NY 12545

Abstract. Invasive species like *Phragmites australis* are notorious for thriving in drastically different environments. This expansion into new habitats could be assisted by phenotypic plasticity. Phenotypic plasticity may be partially driven by epigenetic changes. Epigenetics, the study how markers on DNA effect transcription, is characterized in part by the patterns of methyl and acetyl groups that attach to either the DNA or histones, thus affecting gene transcription. *Phragmites australis* is a well-known invasive plant in wetland habitats that has been expanding its range in the Hudson River estuary including higher salt concentrations. To investigate the potential relationship of *Phragmites australis* and its epigenetic patterns in varying salinities, I sampled six populations in the Hudson River estuary, from three different salinity ranges. Assays quantifying the amount of methylated DNA were conducted with DNA samples extracted from these populations. Three different tests produced varying results. The only significant results displayed methylation levels that decrease with increased salinity concentrations. Though the other two tests had similar overall trends, neither was statistically significant. These results indicate the need for further examination into environmental epigenetics, to better determine any potential relationship between epigenetics and phenotypic plasticity and understand what may be driving *Phragmites australis* rapid adaptation into saltier waters. Further studies would include investigation into region specific methylation of *Phragmites australis,* as well as studying any changes of methylation while raising clones in varying salinities.

INTRODUCTION

Epigenetics is the study of the genome that exists "above" the genes in DNA (Vandegehuchte and Janssen 2011). Epigenetics incorporates the examination of the gene sequences, amount of gene expression, and external environmental factors through markers. Markers are either identified as acetyl or methyl groups that attach to genes and histones, thus changing gene expression (Head et al. 2012). Though the genes in DNA encode for many essential proteins and phenotypic traits, epigenetics contributes to much more than most people realize. Epigenetic markers are essential to nearly every aspect of genomic transcription. Markers either promote or inhibit transcription, therefore regulating gene expression without altering the genes themselves (Head et al. 2012; Bossdorf et al. 2008). Patterns of the markers may be present or absent in a region of DNA due to a combination of many internal factors, different lifestyles, stresses to the organism, or external environmental factors. Yet even with life history contributing so much to the epigenome, epigenetic differences can be displayed in two genetically identical organisms (Feil and Fraga 2012). Thus, despite environmental factors' impacts on the epigenome, studies continue to demonstrate that these modifications can lead to heritable transgenerational differences in the epigenome expressed several generations beyond the original alteration (Crews and Gore 2012).

Epigenetic modifications can occur in several different ways. One of the ways that epigenetic markers can affect gene expression is by adding or removing methyl or acetyl groups to DNA (Figure 1), thus rendering the DNA unable or able to be transcribed (Feil and Fraga 2012; Hauser et al. 2011). If deviations of the methylation or acetylation patterns of the DNA occur before a new cell develops, then these changes may also lead to a different set of amino acids being expressed at the end of a histone (Smulders and de Klerk 2011; Vandegehuchte and Janssen 2011). If a histones' amino acids are altered, DNA may not bind properly to the histone, and genes may be expressed in irregular ways (Smulders and

de Klerk 2011; Feil and Fraga 2012; Vandegehuchte and Janssen 2011). Histone structure can also be directly affected by methylation or acetylation (Fig. 1), thus disturbing transcription (Hauser et al. 2011). These changes can occur through external stresses from chemicals, social interaction, or environmental stressors, or may be internally inherited from the parents (Smulders and de Klerk 2011; Feil and Fraga 2012; Hauser et al. 2011; Vandegehuchte and Janssen 2011). Both increased and decreased changes in the amount of methylation or acetylation could produce alterations that positively or negatively affect the organism, depending where exactly the epigenomic adjustment occurs. For example, increased methylation levels are associated with a promoter region in ovarian cancer, but decreased methylation is associated with the onset of oncogenesis in various other cancers (Zhang et al. 2013, Akhavan-Niaki and Samadani 2013).

In plants, certain types of epigenetic variation are more likely to be inherited than others. Currently, there is no consensus in the scientific community as to what makes one epigenetic change more likely to be inherited than not, yet we still see the following trends in plants (Grossnilklaus et al. 2013). Acetylation or de-acetylation on either the histones or DNA in plants usually does not have a transgenerational effect (Chen and Tian 2007). With acetylation, the only probable way long-lasting genetic change can occur is if the acetyl groups alter the existing methylation code on DNA (Chen and Tian 2007). Hence it is methylation that proves to have more permanent transgenerational and long-term effects (Chen and Tian 2007). It is methylation or de-methylation directly on the DNA that yield the most profound and long-lasting changes in plants (Hauser et al. 2011; Vanyushin and Ashapkin 2011). DNA methylation occurs after transcription when RNAi directs a methyl group to attach to the fifth carbon on a cytosine (^{m}C) base ring (Vanyushin and Ashapkin 2011). Occasionally, DNA methylation will also occur on adenine bases, but these tend to have little phenotypic effect (Vanyushin and Ashapkin 2011). Methylation of DNA generally silences gene transcription, while de-methylation usually increases transcription (Nishimura and Paszkowski 2007). It should be noted that when detecting methylation in plants, though methylated cytosines can be found next to nearly any base, only the cytosines preceding guanine display true transgenerational effects on the phenotype of the plant (Nishimura and Paszkowski 2007).

Although the knowledge of these heritable epigenetic markers is limited, over the past several years, the clinical and evolutionary importance of epigenetics has become better understood. The presence or lack of markers in specific areas of DNA can contribute to many different aspects of life, ranging from minor changes in protein concentration, to major changes that modify the lifespan of the affected organism (Vandegehuchte and Janssen 2011). In humans alone, epigenetics can contribute to a person's longevity, chances of having disorders such as Fragile X Syndrome, obesity, and even some terminal diseases like cancer (Vandegehuchte and Janssen 2011). Directed epigenetic changes in plants have the potential to be supremely advantageous. Deviations in the epigenome can turn on genes that make a plant flower differently from surrounding plants, grow larger or different shaped leaves, and give the plants an advantage over other competing species, possibly contributing to the rapid success of invasive species (Smulders and de Klerk 2011).

These epigenetic changes are suspected to play a significant role in phenotypic plasticity in plants. Plants often undergo small, possibly epigenetic, changes to adjust to a slightly different climate, light source, or any other potential abiotic stress (Bossdorf et al. 2008; Prentis et al. 2008). This type of phenotypic plasticity can be easily seen in common plants, especially when comparing more obvious changes in morphology between two individuals or populations of the same species growing in different climates. Occasionally, epigenetic variance can produce differences in the plant's phenotype, such as certain structural changes, like flower malformation, leaf shape, and less visible changes, that can adjust the offspring's epigenotype (Prentis et al. 2008; Smulders and de Klerk 2011). Recently, studies have begun to examine the potential for invasive species to use these small epigenetic changes to become better suited to withstand stresses, and thus prevail as the dominant species in a particular area (Bossdorf et al. 2008;

Prentis et al. 2008). Though little has been published on the relationship between epigenetics and invasiveness, it is suggested that, because epigenetic changes can produce an abnormal phenotype, that is not necessarily permanent, they may be able to result in a phenotypic change that mimics phenotypic plasticity (Bossdorf et al. 2008; Prentis et al. 2008). With added environmental stresses, it is possible that epigenetic change can more rapidly expand the expression of plant DNA functions because a greater number of epigenetic alterations will more likely produce significant phenotypic variation, by turning on novel genes (Prentis et al. 2008). Significant variations can assist a species in becoming more readily adaptable to a new environment (Prentis et al. 2008). Over time, these types of positive evolutionary changes could encourage a species to become more aggressive and suitable across a wide range of habitats (Bossdorf et al. 2008; Prentis et al. 2008).

Phragmites australis is a common invasive wetlands plant, often referred to as common reed. It is a highly invasive and aggressive species, with the ability to overtake a marshy area in a few short years (Meyerson et al. 2000). *Phragmites australis* is typically found in fresh water and brackish water marshes, but is expanding its range into more tidal wetlands with higher salinities (Chambers et al. 2000). Even though it is very aggressive, *Phragmites australis* can prove to be beneficial to the environment through new habitat, food sources, or phytoremediation (Meyerson et al. 2000; Chambers et al. 1999). This aggressive nature is exemplified by its wide range of habitat and salinity tolerance throughout the Hudson River estuary. Salinity concentrations in the Hudson River range from <0.5 ppt to 30 ppt. *Phragmites australis* is found throughout the fresh, brackish, and salt water areas of the river. There have been many studies that have examined both the phenotypic and genotypic differences in populations up and down the river, as well as attempts to understand the genotypic changes overtime (Meyerson et al. 2000; Lipus et al. 2012).

Understanding natural genetic and epigenetic diversity in species is a fairly novel topic in ecology. Recently, microsatellites were used to determine the genetic differences in populations distributed across a salinity gradient and detect any patterns due to salinity. Though the results generally showed microsatellites that were common throughout the populations in water of similar salinities, but absent between populations in different salinities, there were a few unexplained microsatellite sites in the brackish water samples that did not match this trend (Lipus et al. 2012). This unexplained variance may be attributed to epigenetic factors. *Phragmites australis* in the Hudson River present an ideal opportunity to investigate how much epigenetics changes with the salinity gradient, possibly mirroring the patterns found with the microsatellite data, or accounting for the unusual array of data found. I propose that the epigenome of *Phragmites australis*, as seen through DNA methylation, will show evidence of change associated with the salinity of the location on the Hudson River. This change will most likely be seen in decreased methylation with increased salinity, as has been seen in other plants under high salt pressure (Wang et al. 2011). This decreased methylation could be an adaptation that allows for *Phragmites australis*'s rapid expansion into tidal salt marshes. In this study, I compare the global methylation of DNA samples from six different populations of *Phragmites australis* growing across a salinity gradient in the Hudson River estuary.

METHODS

Sample Collection

To collect the samples, protocol mimicked the processes and used the same GPS coordinates as in Lipus et al. (2012). At each of the six sites previously used (Table 1, Fig. 1), approximately 20 leaves from plants of similar heights were collected, through transects and the perimeter of the population. Universal Transverse Mercator (UTM) coordinates were recorded and projected Google Earth to be mapped. Leaves were frozen, freeze dried, and stored at -20°C.

DNA Extractions

0.1 gram of plant material from each leaf was extracted with the *Plant DNAzol* kit (Invitrogen, Grand Island, NY). This commercial kit uses chloroform to extract the DNA from the cells. Each sample was diluted to a concentration of approximately 11 ng/µL, as the initial concentrations ranged from 11 ng/ µL to 40 ng/ µL. All concentrations needed to be consistent to later run PCR analysis (Appendix A). DNA extractions were completed at one time. All extracted DNA in the ELISAs and PCR was from this original extraction.

Enzyme-linked immunosorbent assay (ELISA 1 and 2)

A series of three ELISAs was performed on the original samples. *ABNOVA Global DNA Hydroxymethylation ELISA Kit (5-Hydroxymethylcytosine Quantitation)* was used for ELISA 1 and ELISA 2 (Cell BioLabs, Inc., San Diego, CA). Using the same extracted DNA from the Invitrogen kit, five samples from each of the six sites were diluted to a concentration of 2 ng/ µL in phosphate–buffered saline solution (PBS) (136 mM NaCl, 1.5 mM KH2PO4, 8.2 mM Na2HPO4, 2.7 mM KCl, pH 7.2). Standards included with the kit, blank wells with PBS, and site samples were applied to individual wells, and 100 µL of each solution was distributed to each well. The antigens were then allowed to bind to the plate overnight at 4°C. The plate was washed twice with PBS through aspiration and 150 µL of assay diluent was added to block for one hour at room temperature. After an hour, 100 uL of anti-hydroxymethylcytosine-antibody were added and allowed to incubate for another hour at room temperature. Wells were washed three times with wash buffer, with thorough aspiration and blotting on absorbent pads between each wash. One hundred and fifty microliters of diluted blocking reagent were added and incubated for one hour at room temperature. Wells were washed then with the wash buffer, three times through aspiration, as above. One hundred microliters of the secondary antibody-enzyme horseradish peroxidase conjugate to each to well and allowed to incubate for one hour. Again, the wells were washed three times with the wash buffer as above. Immediately, 100 µL of the substrate solution were added and allowed to incubate for 30 minutes, when color was strongest. To stop the reaction, 100 µL of the stop solution were added to the well. Results were read on a standard microplate reader at a primary wavelength of 450 nm.

Enzyme-linked immunosorbent assay (ELISA 3)

Global DNA Methylation ELISA Kit (5'-methyl-2'-deoxycytidine Quantitation) was used for ELISA 3 (Cell BioLabs, Inc., San Diego, CA). Using the same extracted DNA from the Invitrogen kit, three samples from each of the six sites were selected and diluted to 10 ng/ µL in water. DNA samples were converted to single stranded samples by heating them at 95°C for five minutes, and then chilling them on ice. DNA was then incubated with 5 units of nuclease P1 (Sigma-Aldrich, St. Louis, MO) in 20 mM Sodium Acetate for 2 hours at 37°C. After incubation, DNA samples were treated with 5 units of alkaline phosphatase (Sigma Aldrich, St. Louis, MO) in 100mM Tris buffer for 1 hour at 37°C. Digested samples were centrifuged for 5 minutes at 6000 g.

The plate was prepared for addition of the DNA by coating it with a DNA conjugate. This was completed by adding to each well 100 µL of 1X 5MedCyd DNA Conjugate that has been heat-denatured by heating it at 95°C for 10 minutes and then chilling the plate on ice. The 1X 5MedCyd DNA Conjugate was incubated on the plate for 2 hours at 37°C. The conjugate solution was aspirated and the plate was washed once with 1X PBS (136 mM NaCl, 1.5 mM KH2PO4, 8.2 mM Na2HPO4, 2.7 mM KCl, pH 7.2) and blotted on a paper towel. Two hundred microliters of assay diluent were added to each well and blocked for 1 hour at room temperature. The assay diluent was removed immediately before the plate's use.

From the digested DNA, the samples were added to wells in 250 µL increments. Fifty microliters of the standards were added to blank wells. Any remaining wells were given 50 µL of assay diluent. All samples and standards were duplicated. The samples were incubated for 10 minutes at room temperature. After ten minutes, 50 µL of anti-5MedCyd antibody, diluted in assay diluent 1:200, were added to each well. This mixture was incubated at room temperature for 2 hours. After the two hours, the solution was discarded through aspiration, and the wells were washed three times with 250 µL of 1X wash buffer. The plate was blotted on paper towels to remove excess liquid. Then 150 µL of 1X blocking reagent was added to each well and allowed to incubate for 1 hour. Once again, the plate was washed three times with wash buffer as above. One hundred microliters of secondary antibody-enzyme horseradish-peroxidase conjugate diluted 1:1000 in assay diluent was added to each well. This was incubated for one hour at room temperature. After the hour, the plate was again washed with wash buffer three times. Immediately after the wash, substrate solution was added to each well in increments of 100 µL. The reaction was stopped with 100 µL of stop solution after 15 minutes, when color was seen in all wells. Results were immediately read on a plate reader using a 450 nm wavelength.

Statistical Analysis

In all three of the ELISAs, none of the standards displayed a regular standard curve, with data points showing an irregular pattern. Many to all of the absorbances in each ELISA were less than the lowest standard sample, indicating a below zero amount of methylation, a biologically implausible phenomena. This means that the absorbances of the samples could not be properly converted into quantitative methylated DNA. Because the ELISAs used were direct ELISAs, instead of converting the absorbances to quantified methylation, raw absorbances can be compared. I compared the average absorbances of each site from the individual ELISAs. From this, I was not able to quantitate any definitive differences in methylation for the sites. However, I was able to see any trends, or significant overall differences through comparison of the raw absorptions. For each ELISA, a one-way ANOVA and Tukey post-hoc test was completed to detect any significant differences in the groups. A p-value of less than 0.05 was considered significant. The average absorbances for each site within each ELISA were graphed with standard error as the error bars.

RESULTS

In all three of the repeated ELISAs, the following results were achieved. Overall, every ELISA showed decreasing absorbances with increasing salinity. Because all three of the ELISAs used were direct ELISAs, this decrease in absorbance can be directly related to a decrease in global methylation. However, only one ELISA was found to have any statistically significant results demonstrating this trend.

In ELISA 1, all of the water types were found to have significantly different levels of absorbance (Fig. 3, $p = <0.0001$). Fresh water samples were found to have the greatest absorbance, while samples from higher salinity sites had the least amount of absorption. Within each of the environment types, several individual sites were found to have statistically significant variations (Fig. 4, $p = 0.00014$). Specifically, significant differences were found between absorbance levels in Hudson and Rye ($p=0.01$), Hudson and Staten Island ($p=0.005$), Albany and Berry's Creek ($p=0.013$), Albany and Rye ($p=0.0009$), and Albany and Staten Island ($p=0.0005$).

In ELISAs 2 and 3, overall trends showed similar results to ELISA 1. In both ELISA 2 and 3, the fresh water sites showed the highest levels of absorbance, while the salt water sites showed the lowest levels. However, no significant differences in these absorbances were detected between any of the environments (Fig. 5, $p=0.288$ and Fig. 6, $p=0.338$). No significant differences were seen in any of the geographic site specific analyses of both ELISA 2 and ELISA 3($p= 0.4928$ and $p= 0.7358$).

DISCUSSION

With the diverse results from each of the ELISAs, little can be concluded about any true differences in methylation levels from each of the sites. Though results showed overall trends of decreased absorbance and methylation with increased salinity levels, only the results from ELISA 1 were statistically significant (Fig. 3 compared to Fig. 5 and 6). Some of the differences between the significant levels in each ELISA could be caused by several conditions. Halfway through the analysis phase of this study, the ELISA product used for ELISA 1 and 2 was discontinued. Though the new ELISA used for ELISA 3 was approved for use of plant DNA, it was designed for use in mammalian DNA or urine. Because of this, several steps had to be adapted for use for the plant DNA samples. These adjustments, combined with lower than desired concentrations of DNA could have skewed the results of ELISA 3. In fact, the duplicated samples produced several absorbances that were drastically different from each other, despite initiating from the same DNA sample. This indicates that somewhere along the process, the third ELISA most likely did not produce reliable results. Despite all of this, even if the measurements observed were the true values, the results of ELISA 3 still should not be compared to ELISA 1 and ELISA 2, as they are two different ELISA kits, with different processes to measure DNA methylation. With all of the above information considered, I decided to focus on the results from the first two ELISAs, and only lightly consider those from ELISA 3.

When accounting for the differences between ELISA 1 and ELISA 2, there could have been several factors to which the differences could be attributed. The statistical values from the ANOVAs indicate that the differences seen in ELISA 1 between sites are much more likely to be real differences (Figure 1, $p = <0.0001$, $f = 19.54$) than the lack of differences seen in ELISA 2 (Figure 3, $p=0.288$, $f = 1.303$). However, even though this leads us to believe that ELISA 1 demonstrated epigenetic variation in *Phragmites australis*, none of the ELISAs were DNA site-specific. Instead, they measured overall total methylation on the DNA. This means that there lies the possibility that, even though ELISA 2 does not support global variation between the site populations, there could be specific regions of the DNA that are more methylated in one type of the environment over the others.

It should also be noted that neither of these ELISAs were replicated in duplicate, due to limited resources. Also, the standard curves for each of the ELISAs indicated that many of the absorbances in ELISA 1 and all of the absorbances in ELISA 2 correspond to negative DNA methylation levels. This was most likely due to contamination in the PBS used for the blank wells, and for DNA dilutions, possibly skewing the overall results of the absorbance levels. Some samples were diluted with more PBS than others, so different wells could have displayed different amounts of contamination. This contamination and skewing does not allow for translation of the absorbance levels into DNA methylation levels. It should also be noted that because of limited resources, only 3-5 samples from each site were able to be tested in each ELISA. For sampling a population of any species, Hale et al. (2012) suggest that a minimum of twenty-five samples from each population is required. This allows the researcher to account for any within population variation and compare any variation between populations. By using so few samples tested in each ELISA, any conclusions could only be considered as support for further investigation into epigenetic variation.

With all of this in mind, there is evidence supporting the need for further, DNA site-specific investigation into the exact epigenetic patterns of *Phragmites australis*. With the statistically significant results from ELISA 1, the differences between groups should be addressed. These results are supported by studies by Chwedorzewska and Bednarek (2012) investigating the methylation patterns of invasive species with MS-AFLP techniques. They found epigenetic variation in accordance with the extremely different habitats in which an invasive grass was growing. While studying the patterns of expansion, they brought forth several theories that could explain possible variation in populations. Non-native *Phragmites australis* is a

traditionally fresh or brackish water plant that has adapted to grow in high salt water concentrations (Chambers et al. 2000). It is possible that a weak bottleneck could have occurred during the *Phragmites australis* expansion into the saltier waters of the Hudson River estuary, thus accounting for the differences in methylation patterns seen in ELISA 1. As discussed above, previous research has found genetic variation of microsatellites in *Phragmites australis* on the Hudson River estuary (Lipus et al. 2012). These investigations showed that the microsatellites in DNA that were consistent among individuals in one population varied significantly between individuals in many populations growing in differing salt concentrations, especially when comparing salt water individuals to fresh or brackish water samples. When investigating genetic variation in another invasive plant, Chwedorzewska and Bednarek (2012) found genetic variation to be associated with similar epigenetic variation. Because *Phragmites australis* was found to be so genetically varied based on the salt concentrations, it follows that the epigenome may vary in tandem with the genome. Other studies have found that because genetic and epigenetic variation are so closely related, they are often inherited together in a traditional Mendelian fashion (Riddle and Richards 2005). Results from this report showing differences in epigenetic patterns, combined with the results supporting differences in the genome, may indicate that *Phragmites australis* could have adapted both genetically and epigenetically to be able to thrive in the saltier environments (Lipus et al. 2012, Chwedorzewaska and Bednarek 2012, Wang et al. 2011). Even though *Phragmites australis* is usually a fresh or brackish water plant, it is also possible that the reverse is true – the original Hudson River invasion started in saltier waters and adapted genetically and epigenetically to fresh water environments.

These results also support the idea that part of *Phragmites australis*'s aggressive nature may be associated with its genetic and epigenetic variability. The phenotypic plasticity needed to thrive in different environments enables *Phragmites australis* to remain a cohesive species but possess varying physical characteristics to better suit its environment. Even though the results of this study found epigenetic variability associated with changing environments, these data do not truly relate phenotypic plasticity to the molecular patterns. Nevertheless, they present a vital step in beginning to understand the relationship between these two qualities. Once we more fully understand the relationship between phenotypic plasticity and genetic and epigenetic variation in plants, we have the potential for developing mechanisms to best control the spread of invasive species like *Phragmites australis*.

However, more support with conclusive data must first be collected to support my original hypothesis. First, DNA site-specific investigation of at least 25 samples from each site must be completed. Though I attempted to do so, limited time and resources did not allow for successful completion of this step (Appendix A). Using AFLP and MS-AFLP would allow an investigator to see the variation of the genome and epigenome, and correlate any specific differences to differences in environment. To see if these differences were due to changes in the plant's environments, experiments manipulating the environments of offspring from each of the different salinities to something other than its parents would be necessary. In addition to carrying out these experiments with those samples on the Hudson River, it could prove beneficial to sample individuals from native populations in Australia and compare them to the populations in similar environments in the United States. This would allow us to clearly see the genes and epigenes associated with the plant's expansion in its new territory. With more evidence from experiments like these, we would be able to better determine any decisive patterns in epigenetic variation in *Phragmites australis*, as well as see if these patterns were associated with a plant's ability to thrive in a particular salinity.

CONCLUSION

My results provide preliminary evidence supporting the hypothesis that *Phragmites australis* displays decreased DNA methylation with increased salinity concentration in its habitat. Because the results did not show any quantitative differences and displayed uncertain overall differences, more research is needed to conclude any true variations. With more conclusive results, better management practices in

managing the spread of invasive species like *Phragmites australis* could be developed. Management practices using demethylating agents, like azacitidine, on plants in fresh water areas are a potential controlling tool if we better understand the true patterns of methylation in this species growing in various environments on the Hudson River.

ACKNOWLEDGEMENTS

I would like to thank Dr. Stuart Findlay and Dr. Jim Palmer for guiding me so well on this project.

I would also like to extend much appreciation to Bradley Hersh, Susan Rankin, Jill Devine, Kelly Oggenfuss, Joseph Stabile, Jason Lopiccolo, Patti Smith, Cary Institute of Ecosystem Studies, Marist College, and Allegheny College for the support throughout this experience.

A special acknowledgement goes to Cary and Allegheny for funding the research in this thesis.

Lastly, an enormous thank you is extended to all of my friends and family for encouraging the development of this project and supporting me throughout the entire process (and beyond).

LITERATURE CITED

Akhaven-Niaki, H. and A. Samadani. 2013. DNA methylation and cancer development: molecular mechanism. Cell Biochemistry and Biophysics **67:**501-513.

Blouin, M.S., V. Thuillier, B. Cooper, V. Amarasinghe, L. Cluzel, H. Araki, and C. Grunau. 2010. No evidence for large differences in genomic methylation between wild and hatchery steelhead (*Oncorhynchus mykiss*). Aquatic Science 67:217-224.

Bossdorf, O., C.L. Richards, and M. Pigliucci. 2008. Epigenetics for ecologists. Ecology Letters 11:106-115.

Chambers, R., L.A. Meyerson, and K. Saltonstall. 1999. Expansion of *Phragmites australis* into tidal wetlands of North America. Aquatic Botany **64:**261-273.

Chen, J.Z., and L. Tian. 2007. Roles of dynamic and reversible histone acetylation in plant development and polyploidy. Biochimica et Biophysica Acta **1769:**295-307.

Chwedorzewska, K.J. and P.T. Bednarek. 2012. Genetic and epigenetic variation in a cosmopolitan grass *Poa annua* from Antarctic and Polish populations. Polish Polar Research **33:**63-80.

Crews, D. and A.C. Gore. 2012. Epigenetic synthesis: a need for a new paradigm for evolution in a contaminated world. F1000 Biology Reports **4:**18. doi:10.3410/B4-18

Feil, R. and M.F. Fraga. 2012. Epigenetics and the environment: emerging patterns and implications. Nature Reviews: Genetics **13:**97-109.

Hale, M.L., T.M. Burg, T.E. Steeves. 2012. Sampling for microsatellite-based population genetics: 25 to 30 individuals per population is enough to accurately estimate allele frequencies. PLoS ONE **7:**e45170. doi:10.1371/journal.pone.0045170

Hauser, M.-T., W. Aufsatz, C. Jonak, and C. Luschnig. 2011. Transgenerational epigenetic inheritance in plants. Biochimica et Biophysica Acta **1809:**459-468.

Head, J.A., D.C. Dolinoy, and N. Basu. 2012. Epigentics for ecotoxicologists. Environmental Toxicology and Chemistry **31:**221-227.

HRECOS: Historical Data. HRECOS. Web. 1 Dec. 2013. http://www.hrecos.org/joomla/index.php?option=com_content&view=article&id=58&Itemid=54>.

Lipus, D., J. Stabile, and I. Wirgin. 2012. Assessment of temporal and geographic population structuring of *Phragmites australis* along the Hudson River using microsatellite DNA markers. Section III:

1-27 pp. *In* D.J. Yozzo, S.H. Fernald, and H. Andreyko (eds.). Final Reports of the Tibor T. Polgar Fellowship Program, 2011. Hudson River Foundation.
Meudt, H.M. and A.C. Clarke. 2007. Almost forgotten or latest practices? AFLP applications, analyses and advances. Trends in Plant Science **12:**106-117.
Meyerson, L.A., K. Saltonstall, L. Windham, E. Kiviat, and S. Findlay. 2000. A comparison of *Phragmites australis* in freshwater and brackish marsh environments in North America. Wetlands Ecology and Management **8:**89-103.
Nishimura, T. and J. Paszkowski. 2007. Epigenetic transitions in plants not associated with changes in DNA or histone modification. Biochimica et Biophysia Acta **1769:**393-398.
Prentis, P.J., J.R.U. Wilson, E.E. Dormontt, D.M. Richardson, and A.J. Lowe. 2008. Adaptive evolution in invasive species. Trends in Plant Science **13:**288-294.
Riddle, N.C. and E.J. Richards. 2005. Genetic variation in epigenetic inheritance of ribosomal RNA gene methylation in *Arabidopsis*. Plant Journal **41:**524-532.
Sainani, K. 2010. The epigenome: a new view into the book of life. Bio-medical Computation Review. Web. 18 June 2013. <http://biomedicalcomputationreview.org/content/epigenome-new-view-book-life>.
Santos, S.R. 2000. Amplified fragment length polymorphism (AFLP) protocol. http://www.auburn.edu/~santosr/protocols/AFLPProtocol.pdf
Schrey, A.W., M. Alvarez, C.M. Foust, H.J. Kilvitis, J.D. Lee, A.L. Liebl, L.B. Martin, C.L. Richards, and M. Robertson. 2013. Ecological epigenetics: beyond MS-AFLP. Integrative and Comparative Biology **53:**340-350.
Singh, M., K. Chabane, J. Valkoun, and T. Blake. 2006. Optimum sample size for estimating gene diversity in wild wheat using AFLP markers. Genetic Resources and Crop Evolution. **53:**23-33.
Smulders, M.J. and G.J. de Klerk. 2011. Epigenetics in plant tissue culture. Plant Growth Regulation **63:** 137-146.
"USGS Current Water Data for New York." USGS National Water Information System. USGS. Web. 1 Dec. 2013. <http://waterdata.usgs.gov/ny/nwis/rt>.
Vandegehuchte, M.B. and C.R. Janssen. 2011. Epigenetics and its implications for ecotoxicology. Ecotoxicolgy **20:**607-624.
Vanyushin, B.F. and V.V. Ashapkin. 2011. DNA methylation in higher plants: past, present and future. Biochimica et Biophysica Acta **1809:**360-368.
Wang, W., X. Zhan, Y. Pan, L. Zhu, B. Fu, and Z. Li. 2011. DNA methylation changes detected by methylation-sensitive amplified polymorphism in two contrasting rice genotypes under salt stress. Journal of Genetics and Genomics **38:**419-424.
Zhang L., R. Bacares, M. Bisogna and D.A. Levine. 2013. Evaluation of pyrosequencing for the detection of BRCA1 promoter hypermethylation in ovarian cancers. Journal of Molecular Diagnostics **15:**943-943.

APPENDIX

MS-AFLP Techniques

In the lab, approximately 20 samples from each population, for a total of approximately 140 individuals, were chosen to perform epigenetic analysis. Twenty samples is a great enough number where differences in individuals within a population will be able to be determined and not affect the comparisons among populations (Lipus et al. 2012; Singh et al. 2006; Hale et al. 2012). MS-AFLP protocol will follow Blouin et al.'s (2008) version based on the protocol by Hazen et al. (2002). This protocol was attempted because MS-AFLP is the most accurate technique to assess the epigenome of a very genetically diverse species with polyploids and multiple hybridizations (Meudt and Clarke 2007). MS-AFLP consists of three individual steps (Fig. 7). First, enzymes called HpaII methyltransferase, MspI methyltransferase, AciII, and EcoR I, were used to restrict and cut the DNA. All enzymes in this experiment were ordered from Invitrogen. The cut pieces were amplified through PCR with the following primer sequences:

GATGAGTCTAGAACGGTCC – AciII/HpaI, and GACTGCGTACCAATTCCTG - EcoRI. The PCR products were analyzed through gel electrophoresis and software compatible with the Kodak imaging machine. The software removes any noise from the image and gives the user a numeric score for each sample.

TABLE 1. Shows the sampling sites, water types, and associated salinity. Salinity ranges from real-time data available at hrecos.org and usgs.gov.

Site	**Habitat**	**Conductivity (millasiemens /cm at 25°C)**
Albany	Fresh Water	0.07-0.17
Hudson	Fresh Water	0.01-0.20
Piermont	Brackish Water	0.09-15.38
Berry's Creek	Brackish Water	0.1-21.97
Rye Marsh	Salt Water	17.1-27.9
Staten Island	Salt Water	Estimated 17.0-32.0

TABLE 2. Elisa 1 Raw Data.

	FRESH	BRACKISH	SALT
	0.673	0.521	0.25
	0.414	0.528	0.335
	0.691	0.364	0.322
	0.522	0.466	0.4
	0.37	0.417	0.316
	0.697	0.402	0.25
	0.511	0.372	0.335
	0.805	0.395	0.322
	0.583	0.462	0.4
	0.511	0.429	0.316
AVERAGE	0.5777	0.4356	0.3246
ST. DEV.	0.137349	0.057533	0.05041869
POPULATION	10	10	10
ST. ERROR	0.043433	0.018194	0.01594379

TABLE 3. Elisa 1 one-way Anova table.

	Sum of sqrs	df	Mean square	F	p(same)
Between groups:	0.32191	2	0.160955	19.54	5.67E-06
Within groups:	0.222451	27	0.00823892		
Total:	0.544361	29			
omega^2:	0.5527			F-crit	3.35

Tukey's pairwise comparisons: Q below diagonal, p(same) above diagonal			
	Fresh	Brackish	Salt
Fresh		0.004592	0.0001288
Brackish			0.02856
Salt			

(**TABLE 3,** continued)

TABLE 4. Site specific Elisa 1 raw data.

	Albany	Hudson	Piermont	Berry's Creek	Rye	Staten Island
	0.697	0.673	0.521	0.402	0.25	0.25
	0.511	0.414	0.528	0.372	0.335	0.335
	0.805	0.691	0.364	0.395	0.322	0.322
	0.583	0.522	0.466	0.462	0.4	0.4
	0.511	0.37	0.417	0.429	0.316	0.316
AVERAGE	0.6214	0.534	0.4592	0.412	0.3246	0.3246
ST. DEV.	0.127745059	0.146125	0.069719	0.03456154	0.0535	0.053477098
POPULATION	5	5	5	5	5	5
ST. ERROR	0.057129327	0.065349	0.031179	0.01545639	0.0239	0.023915685

TABLE 5. Site specific Elisa 1 one-way Anova table.

	Sum of sqrs	df	Mean square	F	p(same)
Between groups:	15041.5	5	3008.3	9.48	0.0001459
Within groups:	5711.95	18	317.331		
Total:	20753.4	23			
omega^2:	0.6386				

Tukey's pairwise comparisons: Q below diagonal, p(same) above diagonal						
	Hudson	Albany	Piermont	Berry's Creek	Rye	Staten Island
Hudson		0.8429	0.5971	0.1342	0.01009	0.005042
Albany			0.1043	0.01326	0.000948	0.0005379
Piermont				0.8995	0.2284	0.1295
Berry's Creek					0.7782	0.5852
Rye						0.9994
Staten Island						

TABLE 6. Elisa 2 raw data.

	FRESH	BRACKISH	SALT
	0.53	0.392	0.358
	0.416	0.503	0.392
	0.426	0.411	0.409
	0.379	0.513	0.475
	0.404	0.386	0.441
	0.6	0.368	0.307
	0.48	0.391	0.281
	0.42	0.385	0.417
	0.453	0.477	0.42
	0.471	0.425	0.585
AVERAGE	0.4579	0.4251	0.4085
ST. DEV.	0.066177	0.053064845	0.085775
POPULATION	10	10	10
ST. ERROR	0.020927	0.016780577	0.027125

TABLE 7. Elisa 2 one-way Anova table.

	Sum of sqrs	df	Mean square	F	p(same)
Between groups:	0.0126392	2	0.0063196	1.303	0.2883
Within groups:	0.130974	27	0.0048509		
Total:	0.143614	29			
omega^2:	0.01979				

TABLE 8. Elisa 3 raw data.

	FRESH	BRACKISH	SALT
	0.526	0.462	0.494
	0.518	0.555	0.647
	0.695	0.593	0.573
	0.719	0.651	0.623
	0.613	0.647	0.476
	0.553	0.577	0.601
	0.387	0.486	0.469
	0.534	0.564	0.452
	0.655	0.532	0.498
	0.71	0.592	0.497
	0.665	0.567	0.655
	0.673	0.548	0.697
AVERAGE	0.604	0.5645	0.556833
ST. DEV.	0.100894	0.055683358	0.085404
POPULATION	12	12	12
ST. ERROR	0.029126	0.016074401	0.024654

TABLE 9. Elisa 3 one-way Anova table.

	Sum of sqrs	df	Mean square	F	p(same)
Between groups:	0.0153749	2	0.00768744	1.121	0.3381
Within groups:	0.226315	33	0.00685802		
Total:	0.24169	35			
omega^2:	0.006674				

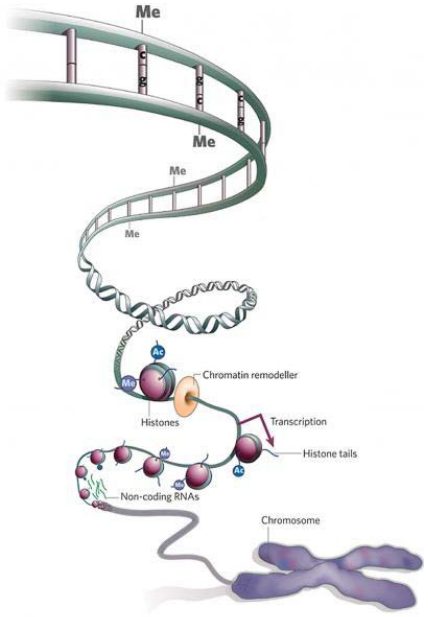

FIGURE 1. Shows examples of DNA and histones modified with epigenetic markers (Sainani 2010).

FIGURE 2. Map showing the sample sites on the Hudson River.

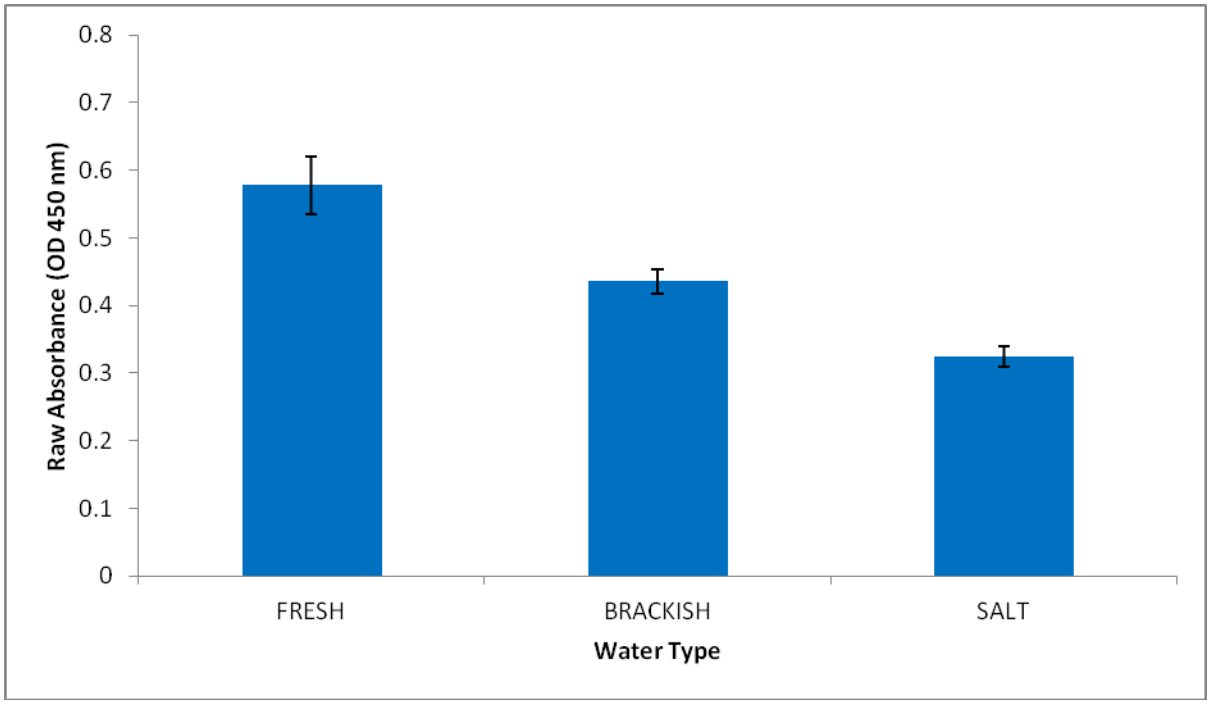

FIGURE 3. Shows the absorbance levels from the ELISA 1 in each site. ELISA 1 used the ABNOVA Global DNA Hydroxymethylation ELISA Kit (5-Hydroxymethylcytosine Quantitation). Error bars are represented by standard error ($p = <0.0001$, $f=19.54$, f-critical $_{(2, 27)} = 3.354$).

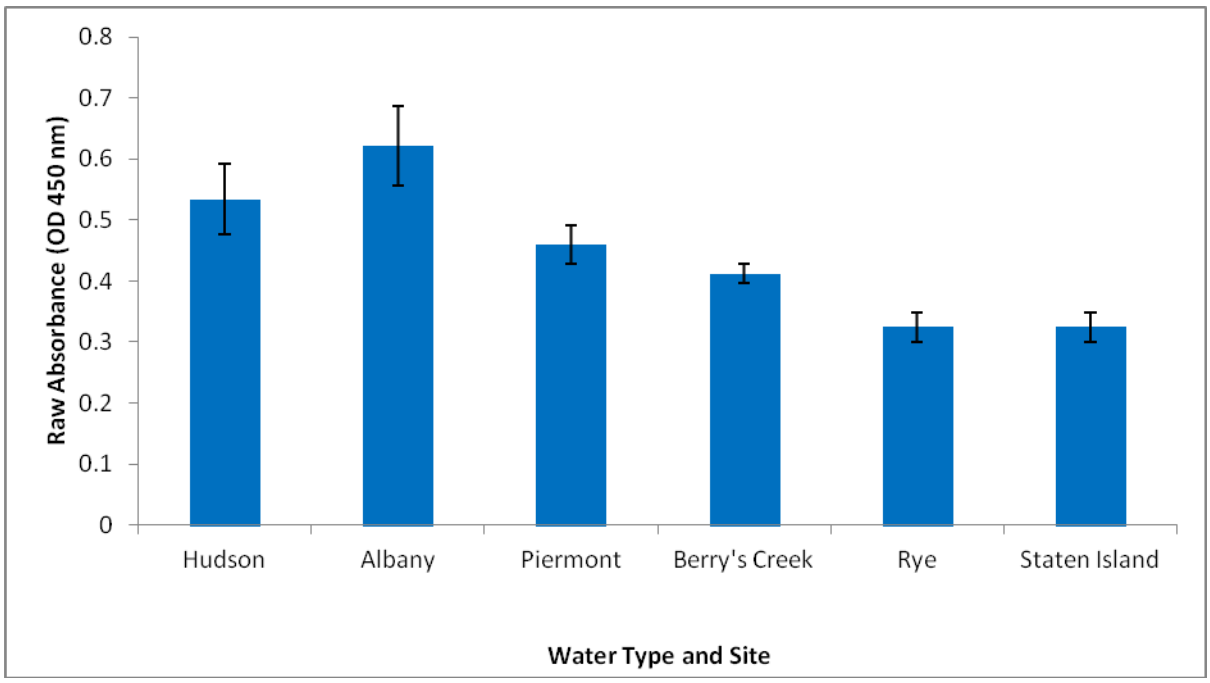

FIGURE 4. Shows the absorbance levels from the ELISA 1 in each site, ordered by water type. ELISA 1 used the ABNOVA Global DNA Hydroxymethylation ELISA Kit (5-Hydroxymethylcytosine Quantitation). Error bars are represented by standard error ($p = 0.00015$, $f=9.48$, f-critical $_{(5, 18)} = 2.773$).

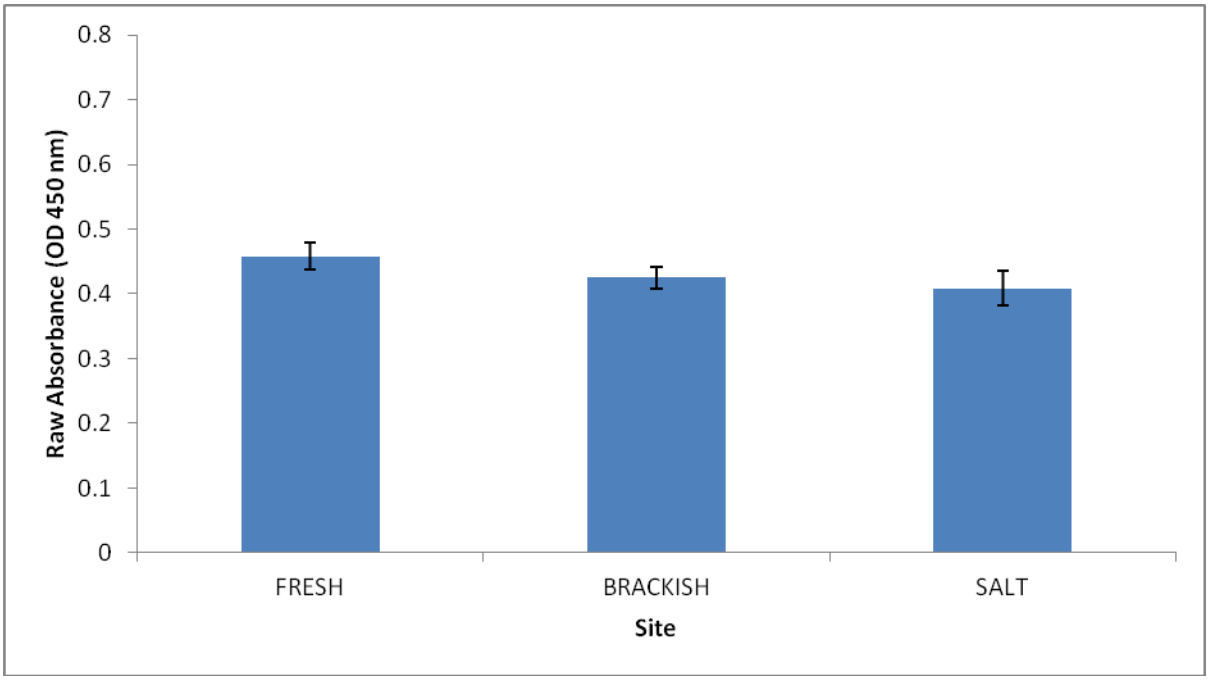

FIGURE 5. Shows the absorbance levels from the ELISA 2 in each environment type. ELISA 2 used the ABNOVA Global DNA Hydroxymethylation ELISA Kit (5-Hydroxymethylcytosine Quantitation). No significant differences were found. Error bars are represented by standard error ($p = 0.288$, $f=1.303$, f-critical $_{(2, 27)} = 3.354$).

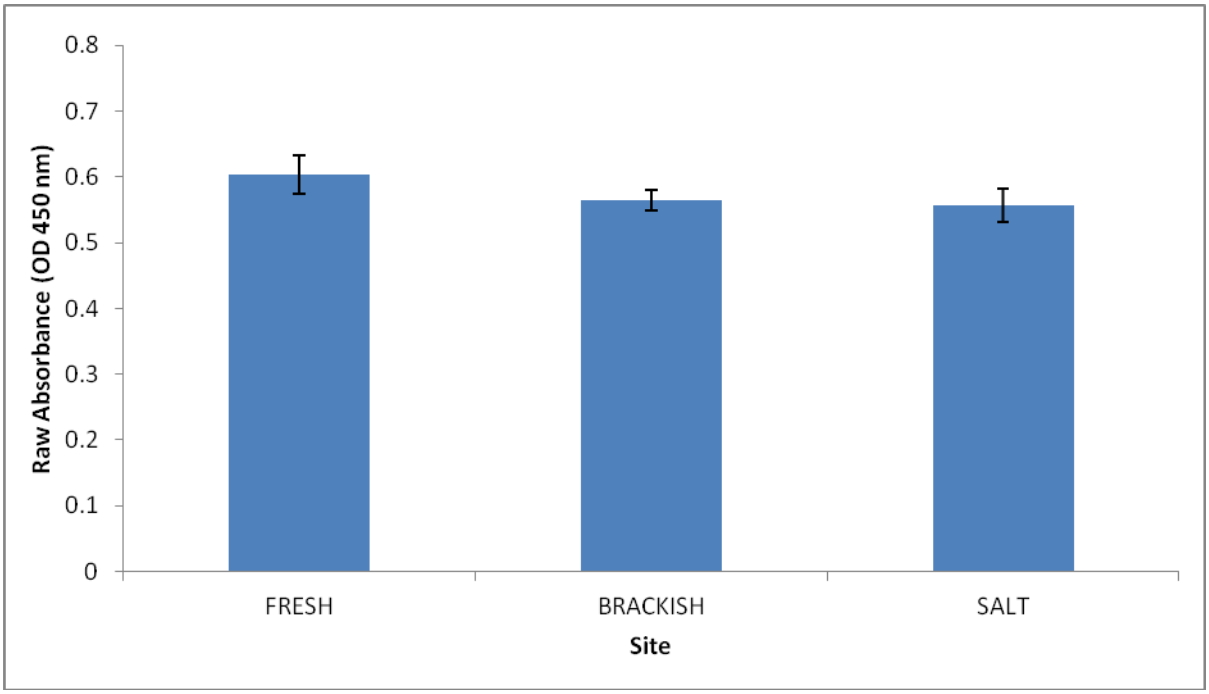

FIGURE 6. Shows the absorbance levels from the ELISA 3 in each type of environment. ELISA 3 used the Global DNA Methylation ELISA Kit (5'-methyl-2'-deoxycytidine Quantitation). No significant differences were found. Error bars are represented by standard error ($p = 0.338$, $f=1.121$, f-critical $_{(2, 33)} = 3.285$).

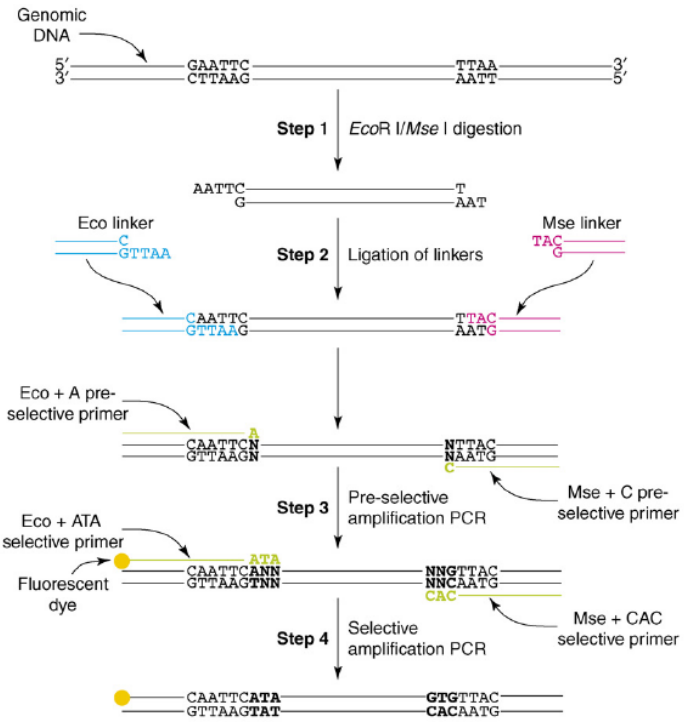

FIGURE 7. Process of AFLP with example enzymes. MS-AFLP uses the same techniques but different enzymes to detect the fragments containing ^{m}C (Meudt and Clarke 2007).