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# Chironomid Hemoglobin Genetic Diversity as an Indicator of the New Jersey Hackensack Meadowlands Wetland Health

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Submitted in partial fulfillment of the requirements
for the Degree of Master of Science in Biology
from the Department of Biology of Seton Hall University
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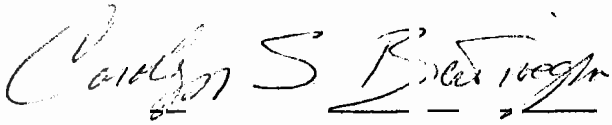
**Committee Member**

**Approved By**



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## Acknowledgements

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## Abstract

Kearny Marsh located within the Hackensack Meadowlands is a freshwater wetland impacted by multiple environmental stressors from human activity. The marsh is located in the northeastern New Jersey metropolitan area. Chironomid larvae, aquatic larvae of midge fly, were analyzed from two Kearny Marsh sample sites, W9 and W22. A previous sediment analysis and toxicity study characterized heavy metal contaminants in the marsh. The data results were applied in this thesis study to determine the correlation between environmental stressors and chironomid genetic diversity. Site W9 sediment was impacted by severe levels of total heavy metal contamination, whereas site W22 had just below to below effect levels of contamination. A primary thesis objective was to determine if chironomid hemoglobin genetic diversity is a suitable bioindicator of multiple environmental stressors.

Prior to the genetic diversity evaluation, the thesis aim was to identify the genus of field chironomid with a molecular biomarker. SDS-PAGE gel electrophoresis was used to analyze hemoglobin protein band patterns. A molecular method was investigated because chironomid identification based purely on taxonomy is difficult, since the various chironomid species have similar morphological features. The hemoglobin protein measured by SDS-PAGE method demonstrated to be an appropriate identification method, because the hemoglobin protein band patterns, characterized by molecular weight and intensity, directly correlated with the respective head capsule morphology. Also, according to phylogenetic analysis with Hierarchical Cluster Analysis the three species evaluated: *Glyptotendipes* and *Endochironomus* clustered separately into two major clades. *Chironomus* and *Glyptotendipes* clustered separately within one major clade however diverged into two separate subclusters. These hemoglobin protein band patterns were applied to the genetic diversity study.

The second objective was to determine if a correlation existed between multiple environmental stressors and genetic diversity. The thesis prediction was that a negative correlation exists between hemoglobin genetic diversity and heavy metal contamination, compounded by multiple environmental stressors. However, analysis of heavy metal concentration levels between the sample sites and Hb genetic diversity based on the SDS-PAGE gel band patterns indicated a positive correlation between *Glyptotendipes* hemoglobin protein diversity and heavy metal contamination levels. Site W9 with higher levels of total heavy metals had a greater Hb diversity level, in comparison to site W22 with lower levels of heavy metals.

This result may indicate several possible endogenous and exogenous explanations for the genetic diversity difference between the sample sites. The thesis study suggested that a physiological adaptation response, population genetic dynamics (i.e., genetic drift and a

bottleneck), and food web cycle alterations played a key role in *Glyptotendipes* genetic diversity levels in response to varying levels of environmental stressors. The thesis study demonstrated that chironomid hemoglobin protein evaluated by the SDS-PAGE biomarker has potential for both identification purposes and to determine genetic diversity of the species which may be applied to bioassessment of wetland environmental health.

## Introduction

Extensive worldwide destruction of our world's wetland ecosystems requires bioassessment in order to restore and protect the remaining wetlands. Wetlands are an integral and valuable part of global environmental health and hence human health. An environmentally healthy wetland is a balanced productive ecosystem that supports a wide array of biodiversity. These ecosystems are transitional environments from aquatic to terrestrial ecosystems with limited standing water and saturated sediments. Cyclic periods of inundation and saturation from surface water and groundwater stabilize wetlands and support a wide array of aquatic flora and fauna (Burkett 2005). Wetlands are beneficial for our society because they support an extensive variety of wildlife habitat, including breeding niches and habitat for fish and migratory birds. Healthy wetlands are import for human society, because they provide natural flood control and water quality improvement through natural filtration of nutrients, suspended sediment and some pollutants. In addition, wetlands support fish and shellfish for human consumption and a natural refuge for outdoor recreation (Burkett 2005). In contrast to a pristine wetland that functions with biological integrity is the urbanized Hackensack Meadowlands located in northeastern New Jersey. This ecosystem is an example a polluted and degraded wetland which once was a balanced healthy ecosystem.

Approximately three hundred years ago, the Meadowlands was an ecologically complex ecosystem composed of 20,500 acres with interconnected waterways, wetlands, Atlantic white-cedar swamps and hardwood forests. Historically, since the European settlement of the northeastern New Jersey in the 1700's, the Hackensack Meadowlands has been exploited for resources and developed for transportation systems with little concern for wildlife protection (Kiviat and MacDonald 2004). During the Industrial Revolution the expanding urbanization of the New York metropolitan area was accompanied by both industrial and residential overdevelopment of the Hackensack Meadowlands (New Jersey Meadowlands Commission

2004). Much of the area was misused for industrial toxic waste and raw sewage disposal. The human community extensively destroyed the ecosystem by deforestation and construction of extensive landfills for industry, transportation and suburbs. In the twentieth century public health officials mandated that 17,000 acres of wetland be drained for mosquito control (New Jersey Meadowlands Commission 2004). Over the years environmental protection has been ignored for economic gain and to support the growing metropolitan population. In contemporary times, the Meadowlands brings to mind images of the massive 130 acre Giant Stadium sports complex surrounded by vast areas of paved parking lots, endless intertwined highways, much industry, and congested residential neighbors. Many call the land a wasteland. Indeed, the pristine land was wasted. Presently, the remaining 8,500 acres of the Hackensack Meadowlands wetland ecosystem (Thiesing 2003) offers a glimpse of the past natural beauty of a pristine wetland and have potential for environmental protection.

Ironically, the urbanized Hackensack Meadowlands continues to support much biodiversity with thousands of species ranging from birds, mammals, fish, reptiles, amphibians, plants, fungi, to microorganisms (Kiviat and MacDonald 2004). The species vary in abundance in comparison to similar species inhabiting uncompromised wetlands. Both native and invasive species survive in the Meadowlands. Urban insects are the least researched species in this area. The surviving biodiversity of flora and fauna requires protection and remediation through scientific research, environmental policy planning, and public awareness.

Kearny Marsh is a much studied site in the Meadowlands by both educational and private research institutes. It lies within the southern region of the New Jersey Hackensack Meadowlands and has been severely stressed by urban sprawl. This marsh is ecologically compromised by urban encroachment in the New York Metropolitan area. Major highways, roads, dikes, pipeline infrastructures and railroads intersect throughout this wetland, fragmenting the once interconnected natural habitats and infringing upon wildlife. Kearny Marsh is a freshwater impoundment wetland detrimentally impacted by multiple pollution sources. Contaminants leach

from surrounding landfills and runoff from bordering industrial and residential developments. Contaminant levels continue to increase in the surface sediment from surrounding non-point and point pollution sources (Meadowlands Research Institute 2004). Keegan Landfill to the southwest corner is known to be a major source of severe contamination. In addition, I-E landfill to the northeast, 15-W landfill, and a metal junkyard which border the marsh may contribute to further pollution of the sediments (Mansoor et al. 2006). Environmental assessment studies indicate Kearny Marsh has high levels of cadmium, mercury, lead, chromium, copper, nickel, zinc, PCBs and pesticides. Heavy metals, particularly lead and cadmium, most likely are entering the environment through groundwater in high concentrations (Meadowlands Environmental Research Institute 2004). Over the years, the productivity of the marsh has declined due to increased water levels within the marsh. This wetland is in need of further environmental scientific research and remediation to restore its biodiversity and habitat.

After investigating several established research sites in the NJ Meadowlands, Kearny Marsh was selected to be the field site in this thesis research project. The marsh has sample sites with a known range of environmental degradation established from previous research. The organism bioindicator was consistently found at two of the sample sites, W9 and W22, investigated in this project. Site W9 was known to have a higher degree of degradation than site W22. Ideally a reference site with minimum degradation is required to establish a baseline for environmental protection research. Without a reference field site, this study utilized a *Chironomus riparius* laboratory population as a reference population that was not exposed to heavy metal contaminants or hypoxic conditions. A possible stress to the laboratory population is several years of inbreeding within the population.

The aquatic larva of midge fly, termed chironomid, was the bioindicator organism selected for this thesis study for several reasons. Chironomids (Diptera: Chironomidae) are an important link in aquatic ecology. They are near the base of the complex wetland food web. Upper trophic level organisms, such as birds, fish, and amphibians feed on these benthos invertebrates. Chironomids

are highly tolerant of pollution and therefore seem to adapt to heavily polluted aquatic ecosystems, such as Kearny Marsh, in relatively great abundance. Since midge larvae inhabit the benthos region of wetlands, the organisms are in close contact with sediment contaminants. In addition, chironomid are known to be good indicators of aquatic environmental health and evaluated in worldwide environmental research which enabled comparisons and conclusions to be drawn based on the support of other research facilities. Chironomids have a short life cycle in comparison to other potential bioindicators, such as fish and amphibians. The larvae are relatively stationary and therefore do not migrate long distances which is an advantage for population genetic diversity comparison studies with pollutants. Lastly, chironomids are easily maintained in a laboratory setting in a tank for research purposes. Although chironomids are a suitable bioindicator organism of environmental health, a common difficulty with the use of chironomids as a bioindicator species is taxonomic identification. Chironomid larvae have similar morphological traits between species and therefore traditional taxonomy requires a high level of expertise for valid identification. Molecular tools, in addition to classic taxonomy, may prove to be a valuable method to identify and classify chironomids at the species level. The first objective of the thesis project was to evaluate and develop a molecular tool to identify chironomid with hemoglobin protein band patterns manifested by the SDS-PAGE molecular method.

A gene marker was required for molecular identification research. Hemoglobin was the molecule of interest in this study for several reasons. Hemolymph contains much hemoglobin and is easily obtained from individual chironomids. Midge larvae hemoglobin is a product of a large gene family (Bergtrom 1997) and therefore the large globin gene family could possibly promote genetic diversity among species. A large gene family with gene duplication and polymorphism evolved in order to produce high levels of hemoglobin protein for adaptation to aquatic habitats. This large Hb gene family may have influenced the divergence of chironomids into more than 5000 species worldwide (Bergtrom 1997). Species-specific hemoglobins and varying number of Hb proteins present in each species may be a result of the gene duplication

from a common ancestral gene (Tichy 1975). "When an intact gene is involved, the act of duplication generates 2 copies of a gene whose activities are indistinguishable. Then copies diverge, as each accumulate different mutations" (Lewin 2004). In general, duplication enables an organism to express proteins at different life stages and tissues. Chironomid hemoglobin displays this adaptation mechanism with larval stage-specific and tissue-specific globin production (Weber and Vinogradov 2001). Also, extensive tandem duplication allows for over expression of proteins, such as hemoglobin, which is advantageous to chironomids inhabiting diverse levels of hypoxic and anoxic aquatic ecosystems (Gruhl, Kao, and Bergtrom 1997). Invertebrate Hbs evolved to adapt to harsh polluted and hypoxic environments (Weber and Vinogradov 2001). Additionally, the adverse effects of mutations in one of the Hb genes may be diminished by having a similar copy of the gene which would continue to be functional. Chironomid polymorphism and gene duplication are characteristic of a large hemoglobin gene family. The hemoglobin protein hypothetically will show the wide diversity within the Chironomidae Family and therefore serve as an identification tool and as a measure of changes in genetic diversity.

Genetic diversity is the fundamental constituent of biodiversity. Species and ecosystem diversity are the other two components of biodiversity (Bagley et al. 2002). Genetic diversity is the variance in heritable traits within a population. Genetic diversity levels fluctuate due to alterations in four strong interacting influences: mutation, migration, natural selection, and genetic drift (Bagley et al. 2002). Environmental changes, both natural and anthropogenic, impact genetic diversity. Populations which are reduced in size are more severely effected by genetic drift, the random alterations in gene frequency each generation, and selection of the fittest, leading to reduced heterozygosity (Bagley et al. 2002). Genetic drift reduces intraspecies population genetic diversity while increasing interspecies population genetic diversity (Bagley et al. 2002). An organism or population with high genetic diversity, gene variation, has a heterozygous advantage over populations with low gene variation. Heterozygosity has the

advantage of containing different alleles of a given gene at a locus, which enables genetic variability in an organism or population. A genetically diverse, heterozygous population has greater fitness and the ability to adapt to a changing environment and evolve in a variety of ecosystems (Reed and Frankham 2003, Bagley et al. 2002).

A currently accepted theory states that "environmental stressors typically reduce genetic diversity, primarily through the forces of selection and genetic drift, so that a recent reduction in genetic diversity is indicative of deteriorating environmental condition. As an indicator of ecological condition, genetic diversity integrates the genetic effects of multiple sources and is cumulative over time" (Bagley et al. 2002). For the thesis project, this prevalent theory was extended to chironomid hemoglobin gene protein diversity, phenotypic variation, in relationship to chironomid Hb genetic diversity changes as an indicator of the Kearny Marsh environmental health. The second objective of the thesis project was to evaluate the usefulness of SDS-PAGE hemoglobin protein band patterns as a biomarker in a comparative genetic diversity study.

The molecular tool applied in this study, the SDS-PAGE method, currently is not a prevalently used tool to determine chironomid hemoglobin identification and genetic diversity, especially for wild type species. Currently alternative molecular tools are available for this type of research. Hypothetically, the following molecular tools have potential for diversity studies: restriction fragment length polymorphism (RFLP), single-stranded conformation polymorphism (SSCP), amplified fragment length polymorphism (AFLP), and randomly amplified polymorphic DNAs (RADP) (Karp, Isaac and Ingram 2001, Bagley et al. 2002). Molecular identification using PCR-RFLP has been applied to identification research of chironomids (Sharley et al., Carew et al.). The degree of detailed genetic information, cost, training and timeframes should be considered in the selection of a tool (Karp, Isaac, and Ingram 2001). Hypothetically, AFLP, a method that evaluates the entire genome of an organism, could be an appropriate tool for research of chironomid identification and diversity in this study and meets the criteria suggested. In comparison, a method, such as SSCP, which examines only one gene, is more laborious because

the establishment of primers sets for one gene, in this case hemoglobin, could be time consuming and may not be in the timeframe of the research project. If time and cost allow for the application of a variety of molecular techniques to determine identification of a species, this may be an advantageous approach.

This thesis study applied the SDS-PAGE protein electrophoresis to identify chironomids to the genus level by examining the hemoglobin protein. This relatively simple technique may be appropriate for both chironomid identification and also genetic diversity studies. The protein data generated by SDS-PAGE method represents Hb genes which are functional and may be responsive to environmental stressors, such as heavy metal contamination. SDS-PAGE method may prove to be a viable molecular tool for bioassessment of environmental health of Kearny Marsh, when applied in conjunction with other environmental research tools, such as traditional chemical analysis of water quality, toxicology studies, DNA genetic diversity studies and biodiversity of both biota and fauna in the Marsh. Ultimately the bioassessment results may support further research in the protection and restoration of the New Jersey Meadowlands wetlands ecosystems.

In summary, the primary objectives of this thesis project were as follows:

1. To determine if the SDS-PAGE gel electrophoresis method used to analyze chironomid hemoglobin protein is a suitable method for molecular identification of chironomids.
2. To determine if a correlation exists between the multiple environmental stressors, in particular, total heavy metal contamination levels and chironomid hemoglobin genetic diversity. If a direct correlation exists between environmental stressors and genetic diversity, chironomid hemoglobin diversity may be a suitable indicator of wetland health.

## Experimental Procedure

### *Collection of Chironomids*

Laboratory stock species, *Chironomus riparius*, were maintained in an aquatic tank containing particle and carbon filtered using CDPRM1206 and CDFC01204 filters (from the Millipore Corporation, Billerica, Massachusetts). The laboratory organisms were fed ground fish food (TetraCichlid, Tetra GMBH, Germany) and received 16 hours of light and 8 hours of darkness. The bottom of the tank was covered with a sand and cerophyll substrate in which larvae inhabit.

Wild type chironomid were collected from the field at the fringe areas of sites W9 and W22 in the highly contaminated Kearny Marsh, New Jersey Hackensack Meadowlands in May, June, and August 2004. Only two species were in the sample populations, genus *Endochironomus* (Chironomidae family) and genus *Glyptotendipes* (Chironomidae family). The larvae were selected by hand from decaying aquatic plant material, clippings of living *Phragmites* submersed plant stalks, submersed rocks, and cement blocks. The majority of the larvae collected inhabited *Phragmites* reed plants. The larvae and plant clippings were transferred to plastic bags which were filled with water from the respective site and the bags were placed in a cooler to transport them back to the laboratory.

### *Preparation of Hemolymph Samples*

Hemolymph was extracted from each larva by decapitation and then bleeding onto a microscope slide. The hemolymph was drawn with a 10 microliter pipet. Before clotting occurred the hemolymph was pipeted into a 0.5 ml centrifuge tube which contained a solution of 14 microliters Lithium Dodecyl Sulfate (LDS) sample buffer, 2 microliters 8 M urea and 2 microliters 2-mercaptoethanol (10 X). Approximately 2 microliters of hemolymph was extracted per larva. The body and head were placed in 700 microliters Trireagent (Sigma, St. Louis, MO) and stored in a -20 ° C freezer. The heads were used for morphological identification of species and the bodies were used to isolate DNA for PCR-DNA genetic diversity studies including: design of

hemoglobin gene-specific primer and SSCP. The DNA studies were not within the scope of this thesis project.

***Overview of SDS-PAGE Gel Electrophoresis Technology***

The thesis project applied the classic SDS-PAGE gel electrophoresis as a molecular marker to identify chironomids to the genus level by analyzing the hemoglobin polypeptide gel band patterns. The SDS-PAGE gel electrophoresis technique separated solute macromolecules, such as protein, with an electrical field. In this research study each individual chironomid collected had hemolymph extracted in order to separate the hemoglobin protein polypeptides from the various macromolecules in the chironomid hemolymph solution. The electric field applied to the solution, prompted the negatively charged molecules to migrated from the anode to the cathode, where as the positively charged molecules migrated from cathode to anode (Mathews, Van Holde, and Ahern 2002). The SDS-PAGE technique separated proteins in a solution as they migrate through the electric field in a discontinuous polyacrylamide gel medium and sodium dodecyl sulfate, hence the term SDS-PAGE (Caprette 2005). This method enabled proteins to become denatured, unfold into their primary structures, and fractionate into polypeptide chains of varying molecular weight (KDa) and lengths.

The SDS anionic molecules bound to the polypeptide chains proportionally to the length and respective molecular weight. The polypeptides surrounded by the negatively charged SDS molecules moved towards the anode, which was positively charged, in the electric field at varying rates. Polyacrylamide gel was a cross linked polymer and agarose medium that acted as a sieve separating proteins by molecular weight and length (Mathews, Van Holde, and Ahern 2002). The heavier molecules (KDa) had a slower mobility than the lighter molecules through the polyacrylamide gel towards the anode. The gel was stained with various stains for visualization of the bands.

***SDS-PAGE Gel Electrophoresis***

***Materials:***

NuPAGE MOPS SDS, 3-(n-Morpholine) Propanesulfonic Acid Sodium Dodecyl Sulfate running buffer (20 X), (Invitrogen Corporation, Carlsbad, CA)

4-15% Ready Gel Tris-HCl gel, (Bio-Rad Laboratories, Hercules, CA)

SeeBlue Pre-Stained Molecular Weight Standard (Invitrogen Corporation, Carlsbad, CA)

Lithium Dodecyl Sulfate (LDS) sample buffer (Invitrogen Corporation, CA)

10x Tris/Tricine/SDS running buffer (Bio-Rad Laboratories, Hercules, CA)

15% Ready Gel Tris-HCL gels (Bio-Rad laboratories, Hercules, CA)

GelCode Blue Stain (Pierce, Rockford, IL)

The 15% Ready Gel Tris -HCL gels and 10x Tris/Tricine SDS running buffer were substituted in place of 4-15% Ready Gel Tris-HCL in an attempt for better quality resolution in the later experiments. The resolution improved with the 15% Ready Gel Tris-HCL gels.

***Electrophoresis Method***

Prepared 1 liter of 5% MOPS SDS running buffer solution with 950 ml deionized water and 50 ml running buffer. Denatured the hemolymph solution for 10 minutes in a heating block at 68° F. Electrophoresis was run from cathode to anode horizontally for 1 to 1.5 hours at 30 mA per gel. The molecular weight standard solution was prepared with 10 microliters LDS sample buffer and 5 microliter SeeBlue molecular weight standard and then pipetted into the first gel well. Three microliters of each larva hemolymph sample were pipetted into the Tris-HCL gel with wells labeled 2-10, respectively or as many wells required.

***Stain and Dry Gel***

Rinsed gel three times for 5 minutes in 200 ml deionized water in a plastic tray. Prefixed gel for 15 minutes in 50% methanol and 7% glacial acetic acid. Prefix solution was prepared with 25 ml

methanol, 3 ml acetic acid, and deionized water to total 50 ml. Washed gel with deionized water for 30 minutes. Added 20 ml GelCode Blue Stain reagent to gel and gently shook for 30 minutes. Washed gel with deionized water until the gel background was clear (30 to 60 minutes). The gel was photographed with a digital camera and then dried on blotter paper for ½ hour at 80° C on a slab gel dryer.

***Statisitical Analysis Procedure for the Identification and Hemoglobin Genetic Diversity Studies***

***Kruskal Wallis Statistical Method***

A statistical analysis was conducted on the total distinct hemoglobin band patterns from all three species with Kruskal-Wallis test and Dunn's nonparametric post hoc test. Kruskal-Wallis test is a nonparametric, qualitative data, test for multiple groups not necessarily with normal distribution. Eighteen chironomid hemoglobin profiles total, including one of each distinct chironomid Hb band pattern, were statistically analyzed for significant differences. The qualitative data, band weight and intensity were transformed into quantitative data using the diagnostic identification system described below. Statistical significance was determined with the Kruskal-Wallis test, followed by the Dunns nonparametric post hoc test, $p \leq 0.05$.

***Shannon Wiener Diversity Method***

The Shannon Wiener Diversity Index (H) was used to determine if a significant difference in diversity existed between chironomid inhabiting the two sample sites reflected by the SDS-PAGE hemoglobin band patterns. Shannon Wiener Index, as described by Maryland Sea Grant, University System of Maryland (2006), quantifies biological diversity by accounting for both species richness and evenness in a specific sample population from an environmental location. Species richness is the total number of different species in an environmental site. Species evenness, equitability, is a measure of the distribution among different species in an environmental site. A community with equal distribution, evenness of species, is considered to

have greater diversity than a community dominated by one species. The Shannon Wiener Index provides raw data, nonlinear data, which requires conversion to biologically significant values to interpret the results. A conversion formula to a linear value is exp H. The diversity data may be analyzed as relative values with a minimum value of zero to a maximum value of log (1/#catergories). The greater the diversity, the closer the value reaches the maximum value. The Shannon Wiener index was calculated for the sample populations *Glyptotendipes, Endochironomus,* and *Chironomus riparius.* Although this index does not give threshold values between diversity and pollutant levels, the Shannon Wiener Index is useful in providing relative values of diversity between the two sample sites. These index values enable the investigator to determine which site has higher or lower diversity in comparison to overall environmental degradation.

***Phylogenetic Method***

A phylogenetic analysis was conducted on the three chironomid sample populations in order to elucidate interspecies and intraspecies population hemoglobin genetic relationships. Also to support that the hemoglobin band patterns were suitable for identification. A dendrogram was constructed using Hierarchical Cluster analysis from the SDS-PAGE Hb protein band patterns, i.e. phenotypes, composed from the total sample populations. The Hb band patterns were analyzed for Between Groups Linkages using and Euclidean Squared Distance. Statistical analyses were carried out with SPSS software, version 14.

***Statistical Method for Hemoglobin Genetic Diversity Study***

First, a statistical analysis was applied to the Simpson Diversity results to determine if a significant difference in genetic diversity existed between the field chironomid species from the sample sites W9 and W22. Secondly, the statistical analysis calculated whether or not a significant difference exists between the three analyzed species (*Glyptotendipes, Endochironomus,*

and *Chironomus*). The statistical method applied in the thesis was adapted from a method described in the *Journal of Clinical Microbiology* (Grundmann et al. 2001). The method distinguished the sampled species genetic diversity from the two sample sites by comparison of the Confidence Intervals (CIs) for the respective Simpson's Diversity index (D) values of the organisms' phenotypic or genotypic markers. If the CIs for the varying species Simpson Diversity values (1-D) did not overlap, the genetic diversity values are distinct.

A limitation to this statistical method was that for highly diverse populations, as the sample size increases, the number of genotypes (phenotypes) diversity will increase. In order to reduce this effect, approximately the same size sample was analyzed. The chironomid samples collected for the thesis were not similar with the exception of the *Glyptotendipes* sample populations collected from sites W9 and W22. Therefore, for the statistical purposes the samples were randomly reduced to a similar size using Microsoft EXCEL software program.

The following equations (Grundmann et al.2001) were applied for statistical analysis of the thesis genetic diversity study:

$\sigma^2 = 4/n\ [\sum \pi_j^3 - (\sum \pi_j^2)^2]$

$CI = [D - 2(\sigma^2)^{1/2},\ D + 2(\sigma^2)^{1/2}]$

Where, n is the sample size, D is the diversity index value, $\sigma^2$ is variance, and $\pi_j = n_j/n$ is the# of categories/total sample size

**Results for Identification and Genetic Diversity Studies**

**I. *Chironomid Identification Results***

***Collection of Sample Population***

A total of 179 chironomids were collected and analyzed for the thesis research project. Seventy-seven *Chironomus riparius* larva were selected from the laboratory stock population with a known identity to the species level. The chironomid larvae collected from two field sites in Kearny Marsh (figure 1) were identified by head capsule morphology with a taxonomic guide (Bode 1980) (figure 2 and 3). The sample population larvae were identified to the species level. Two species were collected in Kearny Marsh, *Glyptotendipes lobiferus* and *Endochironomus subtendens* (possibly 1 *Endochironomus nigricans*). A total of 66 *Glyptotendipes* and 36 *Endochironomus* were identified with the taxonomy guide. Thirty-four *Glyptotendipes* larva were collected from site W9 and 36 *Glyptotendipes* were collected from site W22. Twenty-three *Endochironomus* were identified from site W9 and 13 larvae from site W22 (figure 4).

Site W9 was known to have a higher degree of environmental degradation than site W22 (Bentivegna et al. 2004). Most species were collected from clippings of *Phragmites* aquatic reed plants which inhabited the fringe of the marsh and also from fallen *Phragmites* stalks floating on the fringe water of Kearny Marsh. The majority of larva dwelled in protective tubes attached to the interior of plant reeds. The tubes were constructed from sediment and chironomid mucous. In the laboratory the largest larva, most likely the 4$^{th}$ instar larval stages, were selected for SDS-PAGE gel electrophoresis analysis, because sufficient hemolymph was required for this technique. Mouthpart deformities were noted in the laboratory records for future research.

***Data Analysis of SDS-PAGE Hemoglobin Protein Gels for the Identification Method***

The first objective of the thesis study was to isolate the hemoglobin protein from the hemolymph of each individual in the sample populations applying the SDS-PAGE method. Then devise a

diagnostic identification system based on the gel polypeptide band patterns which was verified by taxonomy.

The SDS-PAGE bands visualized on the protein gels of 179 chironomid larva were analyzed and characterized. *Chironomus tentans* hemoglobin molecular weight was recorded to be approximately 15.9 KDa (Schaller and English 1976) and 40% of the total larval hemolymph protein was hemoglobin (Thompson et al. 1968). A mass spectrometric study of the extracellular hemoglobins from *Chironomus thummi thummi*, synonomous to *Chironomus riparius*, indicated 20 components ranging from approximately 14.4 KDa to 17.3 KDa in the 4$^{th}$ instar larvae (Green et al. 1998). Also, *C. thummi thummi* has 27 globin genes with only several globin genes expressed in the 4$^{th}$ instar larvae (Green et al. 1998). These literature references provided insight and were comparable to the data results for the thesis project.

The major polypeptide bands on the SDS-PAGE gels were observed between 14 KDa and 3 KDa. The bands represented fractionated proteins as polypeptides with varying degrees of abundance, intensity, degradation, and resolution. These polypeptides were manifested as one or more bands in the gel. If two polypeptides of similar molecular weight did not resolve, they may have appeared as one distinct band pattern. This was possibly observed in one of the hemoglobin protein profiles for *Endochironomus.* Doublets, defined as "two bands of similar density and thickness that migrated close together, which usually indicated that the polypeptides are closely related in structure" (Caprette 2005) were common for all band patterns except *Endochironomus* (figure 3). The band quality varied from distinct to not distinct bands and smears which were all reproducible and consistent for each species (figure 3). The dark intense bands indicated a dominant form of hemoglobin polypeptide (Caprette 2005) where as less intense bands were less predominant.

Upon continual examination of the protein gel collection, distinct reproducible band patterns became easily recognized. The number of Hb bands in a profile pattern ranged from 1 to 6 bands. Chironomid larval hemoglobin structure existed as monomers, homodimers, and dimers (Green et

al. 1998). Heterozygote hemoglobin monomers were depicted as doublets, hemoglobin dimers were visualized as 3 bands with one intermediate hybrid band, while hemoglobin tetramers appeared as 5 bands with 3 hybrid bands, and polymers were observed as multi-banded or a smear (Caprette 2005). These various hemoglobin structures were represented in one or more chironomid individual band patterns in the thesis project using SDS-PAGE gels. For *Glyptotendipes* the most predominant gel band pattern characteristic was an intense doublet (13 KDa and 9 KDa). Where as, most *Endochironomus* Hb profiles featured an intense single band at 11.5 KDa. *Chironomus riparius* manifested a common band pattern feature which was an intense doublet (9 KDa and 7.5 KDa).

Next, the head capsules, mounted on slides were compared to each respective chironomid individual's hemoglobin gel band pattern. According to the protein gel analysis supported by the taxonomy evaluation, *Glyptotendipes lobiferus* had 5 distinct reproducible Hb profiles, *Endochironomus subtendens* had 4 profiles, and *Chironomus riparius* had 9 profiles (figure 5). With combined information from literature and thesis data, a diagnostic identification system was devised (Table 1). The identification band system was based on a six band pattern was observed by Schaller and English for *Chironomus utahensis* in their cellulose acetate electrophoretic separation which was applied to the construction of a system for identifying hemoglobin band patterns. The bands were assigned a number 1 through 6 according to molecular weight (KDa) with reference to a molecular weight standard (Table 1, figure 3). The bands were distinguished by intensity on a scale of 0 to 3: absent band (0), present band (1), increased intensity band (2), and more possible bands, smear (3). The identification system was first established for *Chironomus riparius* which had the maximum number of bands and profile patterns. *Chironomus* was grown in a controlled laboratory tank and therefore easily accessible for numerous studies before field work with wild type species. Then this system was applied to both *Endochironomus* and *Glyptotendipes* wild type species which were collected from Kearny Marsh. Each hemoglobin profile was given an identification name, such as, Gp1, Ep2, or Cp4, where "G"

symbolized *Glyptotendipes*, "E" symbolized *Endochironomus*, and "C" indicated *Chironomus*; "p" indicated polypeptide; the numbers equal specific Hb band patterns within a species.

***Statistical Analysis of Hemoglobin Band Patterns for Identification***

According to statistical analysis (Kruskal-Wallis Test, Dunns Post Hoc test) the null hypothesis that "No difference exists between hemoglobin protein band patterns" was rejected. When the total characteristic bands from the three species were examined for this study, a statistical difference existed between the 18 distinct Hb band patterns (Table 2). The calculations indicated $p=0.005$, Kruskal-Wallis test, $H=13.296$ with 17 degrees of freedom, and $p< 0.05$ for Dunns Post Hoc test.

***Phylogenetic Analysis for Identification***

The dendrogram showed two major clades with two subclusters for each clade (figure 5). The second major clade was composed of only *Endochironomus*. The first clade contained *Glyptotendipes* in one subcluster and *Chironomus* in the other subcluster. This dendrogram relationship could have indicated a closer relationship between *Chironomus* and *Glyptotendipes* Hb profiles than *Endochironomus*. One rare *Endochironomus* Hb profile was in the first clade subcluster 2. The phylogenetic analysis supported the SDS-PAGE method with hemoglobin protein band patterns for identification of chironomid to the genus level as a suitable technique. The dendrogram indicates that *Glyptotendipes, Chironomus*, and *Endochironomus* Hb profiles clustered separately with the exception of the rare Ep1 band pattern. Therefore, the species could be identified by unique hemoglobin patterns.

## II. *Results for Chironomid Hemoglobin Genetic Diversity Study*

### *Abundance of chironomid sample populations*

A total of a 106 chironomid were collected from the field sample sites and 50 individuals were analyzed from the laboratory population. Thirty-four *Glyptotendipes* larvae were collected from site W9 and 36 larvae from site W22, whereas 23 *Endochironomus* larvae were sampled from W9 and 13 from W22. Fifty *Chironomus riparius* from the laboratory population were selected for analysis as a reference population. *Glyptotendipes* were twice as abundant as *Endochironomus* in the pooled sample populations. *Glyptotendipes* abundance was distributed evenly between the sites in contrast to *Endochironomus* which had higher abundance in the more degraded site W9. This significant *Endochironomus* abundance difference between sample sites may indicate that *Endochironomus* was sensitive to an environmental factor at site W22 other than the one evaluated for the thesis project.

### *Analysis of SDS-PAGE gels hemoglobin band patterns*

The hemoglobin polymorphism and gene multiplicity apparent in the SDS-PAGE hemoglobin band patterns were applied to this genetic diversity study and used to calculate genetic diversity with the diversity indices. Initial observation of the SDS-PAGE gels indicated varying degrees of hemoglobin protein polymorphism between the three chironomid species analyzed: *Chironomus*, *Glyptotendipes*, and *Endochironomus*. The hemoglobin polymorphism, allelic variation expressed as different hemoglobin protein phenotypes, was visualized on the gels as varying distinct band patterns. The different Hb profiles were composed of polypeptide bands of different molecular weight, abundance and intensity. All of the chironomid hemoglobin patterns were species-specific, with the possible exception of the *Endochironomus* Hb band pattern labeled Epl. *Endochironomus* Epl band pattern, hemoglobin phenotype, may be a hybrid species. The unique Hb profile appeared to have a combination of both *Glyptotendipes* and *Endochironomus* Hb

polypeptide band patterns. This convergence of two different species Hb band patterns could be an indication of interspecies inheritance (figure 3). The presence of a possible hybrid Epl in the sample populations may indicate a heterosis advantage which is a genetic mechanism to maintain variability.

The sample populations from Kearny Marsh had apparently less polymorphism than the laboratory reference population. *Glyptotendipes* showed only 5 distinct Hb band patterns in the gels examined, whereas *Endochironomus* had 4 different Hb band patterns. The reference population, *Chironomus*, had the greatest degree of polymorphism with 9 different hemoglobin band patterns. The number of hemoglobin band patterns could have demonstrated genetic diversity such that both wild type species from the polluted Kearny Marsh had less diversity than laboratory sample population which was not exposed to toxic contaminants or oxygen stress.

***Comparison of Chironomid Populations from the Two Kearny Marsh Sample Sites***

*Heavy metal contamination in detritus and sediment in sites W9 and W22*

The second objective of this thesis was to determine if a biologically significant correlation existed between chironomid hemoglobin protein diversity and heavy metal contamination compounded by multiple stressors in Kearny marsh sites W9 versus W22. Although many interrelated biological parameters play a role in hemoglobin protein diversity, the most substantial data pertaining to hemoglobin protein diversity that was provided for this thesis study was heavy metal contamination analyzed in Kearny Marsh (2002) and therefore will be the focus of the study.

The sediment and detritus in the Hackensack Meadowlands, including Kearny Marsh sites W9 and W22, were characterized by a previous heavy metal contamination analysis and toxicity study conducted in 2002-2003 (Bentivegna 2004). The sediment analysis was applied to a three year project on the environmental health of the Hackensack Meadowlands (Bentivegna 2003). The thesis project evaluated chironomid samples collected in May to August 2004 was within the

timeframe of the sediment contamination study. Valuable and pertinent heavy metal contamination published data was applied in the thesis project to examine the correlation between heavy metal contamination and Hb gene protein diversity (Tables 3, 4, and 5). Heavy metal analysis included cadmium (Cd), chromium (Cr), copper (Cu), iron (Fe), mercury (Hg), manganese (Mn), nickel (Ni), lead (Pb), and zinc (Zn).

In addition to heavy metal contamination, this thesis study acknowledged that possible multiple stressors at both sample sites which were not assessed at the time of the chironomid sample population collection in 2004 existed and had a confounding effect on chironomid genetic diversity. The multiple stressors included unnatural water parameters and sediment composition, habitat loss, possible elevated nutrient and organic air pollutants.

With this in mind, heavy metal contamination data analysis was evaluated applying the heavy metal tables and data (Tables 3 and 4) which were highlighted in gold and yellow for clarity. The orange highlighted data values in the tables have Severe Effect Levels (SEL) which indicates that the heavy metal concentrations are more likely to affect benthos organisms' health. Whereas, the yellow highlighted data values in the tables have Low Effect Levels (LEL) which indicates the metal concentrations have little effect on the majority of sediment dwelling organisms. The total SEL heavy metal contaminant levels in both sediment and detritus were highest at W9 and lowest at W22. The total heavy metal contamination concentrations in detritus were 1090 mg/kg at site W9 and approximately half this total concentration at site W22, 499 mg/kg. The total heavy metal concentrations in sediment were 2468 mg/kg and 222 mg/kg at sites W9 and W22, respectively.

Several specific metals evaluated in the sediment and toxicity study (2002 to 2003) had a possible higher detrimental impact on chironomid species than the other metal contaminants. First, cadmium concentrations correlated the best with reduced growth in toxicity tests performed by Bentivegna and coworkers and had SELs, 22.2 mg/kg in June at site W9 while site W22 had LELs of 8.8 mg/kg. Also, lead had LEL levels in June at both sites in the detritus. Site W9 had 235 mg/kg concentrations of Pb, where as site W22 had 49 mg/kg concentrations of Pb. Lead has

been shown to have detrimental effects on chironomid health by causing anemia in the toxic organisms (Bentivegna 2001). Lastly, mercury has been shown to increase, biomagnify, as the metal cycles through the food web (Boening 1999, Hope 2003). The mercury levels tested in June sediment were above SELs at site W9 and above LELs at site W22. Overall these particular heavy metals which are known to effect benthic invertebrates were higher at site W9 than at W22.

*Shannon Wiener Index diversity results between sites W9 and W22*

Next, in order to determine a correlation between heavy metal pollutants and genetic diversity the Shannon Wiener diversity index was applied to calculate hemoglobin genetic diversity levels between sites and between species using the hemoglobin phenotypes represented by SDS-PAGE band patterns. As previously stated, the thesis prediction was that since site W9 had higher levels of total heavy metal toxicity than site W22, the chironomid hemoglobin genetic diversity would be lower at site W9 than W22. This thesis prediction was in agreement with the prevalent theory which suggests a negative correlation may exist between environmental stressors and genetic diversity (Bagley et al.). So, as environmental stressors, such as pollutants increase, genetic diversity will decrease. Comparison of heavy metal concentrations data with diversity indices calculation values may reveal the correlation between the two parameters.

The Shannon Wiener index of diversity was used to measure the genetic diversity of the 3 chironomid species hemoglobin phenotypes. This diversity index was effective and advantageous because it calculated both richness and evenness of the hemoglobin phenotypes. Richness was the total number of distinct hemoglobin band patterns, phenotypes, and evenness was the similarity in their relative abundance distribution. The Shannon index indicated greater diversity when either evenness or richness increased. The formula for Shannon Wiener index is $H = [\text{sum } (p_i \log (p_i)]$ where $p_i$= relative abundance (#of profiles/total# of profiles in the sample).

Each species' hemoglobin phenotypes at each sample site had varying abundance, richness, and evenness (Table 6, figure 6). The laboratory reference population, *Chironomus riparius* had

the greatest richness with 9 characteristic hemoglobin phenotypes and the most evenness of band patterns with the least dominance. In comparison to *Chironomus,* the field samples from the degraded marsh had significantly lower richness. *Glyptotendipes* showed 5 total distinct hemoglobin band patterns in the gels examined, whereas E*ndochironomus* had merely 4 total different Hb band patterns. Both field species had low evenness with one or two most abundant, dominant hemoglobin phenotypes, which indicated reduced heterozygosity and possibly reduced fitness due to inbreeding. Site W9 showed dominance of Hb profiles: Ep4, Gp3 and Gp4, whereas site W22 was dominated by Hb profiles: Ep3, Gp3, and Gp4 (figure 6).

The chironomid hemoglobin was found to have varying levels of genetic diversity in of both wild type species from the polluted Kearny Marsh and the laboratory sample population as a reference chironomid larvae population which was not exposed to toxic contaminants or hypoxic conditions. *Endochironomus* had a decrease in hemoglobin phenotypes richness from 4 Hb patterns to 3 Hb patterns, from site W9, the more degraded site to W22, the least degraded site. *Glyptotendipes* also had a decrease in richness from 4 Hb protein patterns at site W9 to 3 at site W22.

The hemoglobin genetic diversity was confirmed by the Shannon Wiener index of diversity. The diversity index values were applied to a diversity study comparing 2 sample field sites, W9 and W22, to determine if a change in diversity correlated with a change in environmental stressors, in particular heavy metal contamination. This correlation may show that hemoglobin genetic diversity was a suitable indicator of pollutants in Kearny Marsh.

The results of these calculations were *Glyptotendipes* at site W9 had an (H) value of 0.44 and at W22 the (H) value was 0.38, whereas *Endochironomus* at site W9 had an (H) value of 0.37 and at W22 had an H value of 0.34 (Tables 6 and 7). In order to compare the results, the ratio was calculated between each field species (H) value and the laboratory reference species, *Chironomus riparius* which had the highest (H) value of 0.77. These ratios indicated a relative diversity difference between the marsh and the reference laboratory populations which was not impacted

by stressors. Wild type reference species from an equivalent ecosystem to Kearny Marsh with minimal human impact for both *Endochironomus* and *Glyptotendipes* would be ideal for Shannon diversity index analysis. However, in substitution the thesis project used the laboratory reference species for diversity analysis. The Shannon diversity index ratio values calculated were *Glyptotendipes* at W9 equaled 57% and decreased significantly at W22 to 49%. In comparison, *Endochironomus* at W9 had a ratio value of 48% with a less significant decrease at W22 of 44%. These data indicated that the marsh populations were about 50% less diverse than the laboratory population.

The Shannon Index (H) value is a nonlinear value therefore the (H) value may be converted to (exp H), true diversity, which is a linear value. This linear value was used to compare the diversity of the same species at different sites. *Glyptotendipes* at site W9 had an exp H of 1.55 and site W22 had an exp H value of 1.46. The ratio of these linear exp H, 1.46 (W9) to 1.55 (W22), was 94% which hypothetically may imply a significant biological difference between genetic diversity at the two sample sites (figure 8). Differences between sites were confirmed by statistical analyses below.

The Shannon Wiener Index may be influenced by sample size. *Glyptotendipes* had approximately equal and larger sample sizes from sites W9 and W22, 34 and 36 individuals, respectively, where as *Endochironomus* had unequal sample sizes of 23 (W9) and 13 (W22) individuals. Therefore *Glyptotendipes* was the principle chironomid species analyzed in this study. In addition, the Shannon Index indicated *Glyptotendipes* Hb profiles had the greater difference in H values between sites W9 and W22, than *Endochironomus*. Shannon Index values for *Endochironomus* Hb profiles did not appear to be significantly different between the sample sites. Because *Glyptotendipes* had the greater Shannon Index diversity difference between sample sites, it therefore seemed to be more responsive, sensitive to pollutants. *Glyptotendipes* was the more useful bioindicator species in this genetic diversity study based on the Shannon Wiener index

analysis. The Shannon Wiener diversity index values for *Glyptotendipes* correlated positively between total heavy metal levels at sites W9 and W22.

To support the Shannon Index results, the Evenness index (E) for the hemoglobin phenotypes was calculated. Evenness is the similar distribution of abundance of categories, in this case hemoglobin phenotypes. A high degree of evenness, the lack of a population composed of both dominant and rare species, is an indication of a diverse population. The formula for evenness is E=H/log S, (His the Shannon index value and S is richness of categories). When (E) equals 1 the distribution of categories is similar, whereas (E) less than 1 indicates dissimilar abundance distribution (Table 7).

The data results indicated the laboratory reference species, which was not exposed to heavy metal contaminants or hypoxic conditions, had the highest (E) value of 0.8, and therefore had the highest diversity. The Evenness values for *Glyptotendipes* were lower than *C. riparius* and decreased from site W9 to site W22, 0.63 to 0.54. This difference in evenness values was significant at 85%, which supports the values calculated by the Shannon index. The graph (figure 6) visually portrays Evenness differences between species. Site W9 *Glyptotendipes* sample population had higher evenness, hence higher diversity, than site W22. *Endochironomus* had similar Evenness index values between site W9 and W22, 0.62 and 0.57, respectively. The percentage difference is 92% for *Endochironmus* evenness values. The Evenness index results support the Shannon index values for *Endochironomus.*

Next, the Simpson's Diversity index (D) was also calculated to support the other measures of diversity. The results were consistent with the Shannon and Evenness indices. The Simpson's Diversity index takes into account the number of different species, richness, (in this case hemoglobin phenotypes) present in a sample population in addition to the percentage of each species in the population. The Simpson index formula is D=sum [n (n-1)]/N (N-1), where n is the number of categories (Hb phenotypes) and N is the total sample size. The calculated (D) values were converted to (1- D) with a standard range of low to high diversity with the respective range

of values from 0 to 1, where 0 is the minimum diversity and 1 is the maximum diversity. *Glyptotendipes* site W9 had D=0.61 and W22 had D=0.57, so the diversity was higher at site W9 than W22 (Table 7). *Endochironomus* site W9 had D=0.49 and W22 had D=0.50 therefore the values were similar. The Simpson Diversity index results were fairly consistent with Shannon and Evenness indices values.

Lastly, a significant difference between *Endochironomus* intraspecies abundance was apparent, 23 individuals were collected in the pooled sample population at site W9 and 13 individuals at site W22. While *Glyptotendipes* intraspecies abundance was relatively similar between the sample sites, with 34 individuals collected at site W9 and 36 individuals at W22. These abundance differences were informative to the thesis evaluation of genetic diversity as an indicator of environmental stressors. Site W9 had twice the abundance of *Endochironomus* in contrast to site W22. Possibly *Endochironomus* was sensitive to another pollutant or factor at site W22 indicated by the lower abundance number. Two of the three measures for *Endochironomus* diversity, while all three diversity indices for *Glyptotendipes* indicated a positive correlation between genetic diversity and heavy metal contamination levels between the sites. While the differences between sites were less apparent, the response was in keeping with that for *Glyptotendipes*. This finding is contradictory to the thesis hypothesis prediction that as environmental stressors, in particular heavy metal, increase, genetic diversity decreases (Bagley 2002).

In summary the diversity indices values for *Glyptotendipes* seemed to be biologically significant and so further statistical analysis was conducted to confirm a statistical difference existed between *Glyptotendipes* hemoglobin protein diversity between sites W9 and W22. If a statistical difference exists for *Glyptotendipes* and/or *Endochironomus* Hb diversity(s) may prove to be responsive to multiple pollutants and therefore may be a suitable bioindicator(s) of environmental stressors.

***Statistical analysis results for genetic diversity study***

The statistical analysis method (Grundmann et al. 2001) indicated a significant difference existed between *Glyptotendipes* genetic diversity at site W9 and W22. The Confidence Interval (CIs) for *Glyptotendipes* diversity at site W9 ranged from 60.6% to 61.4% for a Simpson's Diversity index (1-D) valueof 61%. The *Glyptotendipes* collected from site W22 had a genetic diversity (1-D) value of 57% and the CIs were from 56.9% to 57.1%. The Confidence Intervals for the two sample sites did not overlap and therefore the *Glyptotendipes* genetic diversity values between the sample sites were statistically distinct.

In contrast, *Endochironomus* genetic diversity values were calculated to have overlapping CIs between sites W9 and W22 and therefore were not statistically different values. The *Endochironomus* CIs ranged from 48.3% to 49.7% at site W9 whereas the CIs ranged from 49.3% to 50.8% at site W22. The genetic diversity values measured by Simpson's Diversity index (1-D) were 50% and 49% for sites W9 and W22, respectively.

Based on the support from this statistical analysis, the thesis project analyzed *Glyptotendipes* rather than *Endochironomus* hemoglobin genetic diversity for the diversity study which compared heavy metal contamination levels, compounded by multiple environmental stressors, and hemoglobin genetic diversity in Kearny Marsh sites W9 and W22.

Lastly, the statistical difference between the species: *Chironomus*, *Glyptotendipes*, and *Endochironomus* genetic diversity values (1-D) were evaluated. The original sample populations from sites W9 and W22 were randomly reduced to 36 individuals to enable more accurate statistical analysis. The statistical calculations indicated that the CIs for the three species did not overlap. Hence these species genetic diversity (1-D) values between the species were statistically distinct. The Simpson Diversity index value (1-D) for *Chironomus* was 89%, *Glyptotendipes* was 55% and *Endochironomus* was 58%. The CIs for *Chironomus* ranged from 86% to 92%, *Glyptotendipes* from 54.9% to 55.1%, and *Endochironomus* from 57% to 59%.

*The heavy metal analysis in comparison to hemoglobin genetic diversity values*

Heavy metal contamination concentrations were higher at site W9 than site W22. In comparison, *Glyptotendipes* hemoglobin protein diversity also was significantly higher at site W9 than site W22 (figure 8). In reiteration the data results indicated that sediment total heavy metal contamination at site W9 was considered to be severe with 2468 mg/kg concentration, in comparison to site W22 which had moderate to low total sediment heavy metal concentrations of 222 mg/kg (Tables 3 and 4). Whereas, detritus total heavy metal concentration had less severe contamination concentrations than in the sediment with approximately half the heavy metal contamination concentrations of 1090 mg/kg at W9 and 499 mg/kg at site W22 (Tables 3 and 4).

A similar pattern between sites W9 and W22 resulted with the Shannon Wiener index values (H), the Evenness index values, and Simpson's Diversity index values. *Glyptotendipes* had higher diversity values for site W9 populations and lower values for site W22 populations. The *Glyptotendipes* hemoglobin genetic diversity seemed to increase at site W9, due to higher heavy metal contamination concentrations (figure 8). A positive correlation existed between heavy metal levels hemoglobin diversity. Both total heavy metal contamination concentrations and hemoglobin protein diversity were higher at site W9 and lower at W22.

The heavy metal contamination in relationship to the diversity index results were contradictory to the thesis prediction which stated that as total heavy metal contamination concentrations, compounded by other environmental stressors, increased, and the chironomid hemoglobin genetic diversity values would decrease. A negative correlation existed between pollutants and genetic diversity. However, based on the heavy metal data from the previous study, in relation to the calculated diversity results, the predicted hypothesis appeared to be refuted. A direct positive correlation appeared to exist between heavy metal contamination and hemoglobin genetic diversity.

A confounding observation to the heavy metal results in relation to genetic diversity calculations was that *Endochironomus* seemed to be a more sensitive species based on

interspecies abundance levels. *Glyptotendipes* were twice as abundant in the pooled sample populations as *Endochironomus*, 70 individuals collected in comparison to 36, respectively. The intraspecies abundance of the *Endochironomus* pooled sample population seemed significantly more abundant with 23 individuals from the degraded site W9 versus 13 individuals from the less heavy metal contaminated W22. Possibly *Endochironomus* was also a sensitive species to an unmeasured stressor(s) not present at site W9. This *Endochironomus* intraspecies abundance difference between sites may have indicated further substantial testing of potential environmental stressors at site W22 is required to make validate or refute the thesis prediction that as environmental stressors increase at a field site, genetic diversity decreases.

Further information was provided by the reference laboratory population, *Chironomus riparius*, which had the highest Shannon diversity index value of 81%. The laboratory population was not exposed to hypoxia, heavy metal contamination, or other toxics. The only stress may be inbreeding over a 9 year period. Published data has reported 12 Hb phenotypes (Green et al. 1998), however, only 9 Hb band patterns were found in our laboratory population. The laboratory reference population was not stressed by pollutants and therefore did not require physiological adaptation response to stress to maintain heavy metals. The high (H) value did not appear to correlate with a physiological adaptation feedback response to heavy metal contamination or hypoxic conditions. However, chironomid inhabiting a confined tank may have been stressed by unnatural ecological habitat conditions and therefore may have a metabolic stress response to their unnatural habitat.

The thesis project evaluated chironomid hemoglobin genetic diversity data measured by several diversity indices and compared diversity values to the total heavy metal concentration tested in a previous sediment analysis and toxicity study. Based on these analyzed data results, the thesis hypothesis prediction that a negative correlation exists between the diversity and heavy metal contamination concentrations seemed to be refuted. According to the results a positive correlation occurred between chironomid hemoglobin protein diversity and heavy metal

contamination levels, compounded by multiple stressors, between sites W9 and W22. Of significance to these results was that the molecule under investigation was protein which represented the morphology, physiology, and biochemistry of an organism. The chironomid hemoglobin phenotypes were encoded by functional genes manifested in the gel band patterns. Further investigation of hemoglobin genes should reveal information on the Hb genes genotype structure and possibly functional genes encoding Hb polypeptide.

## Discussion of Identification Method and Genetic Diversity Study

### I. *Analysis of SDS-PAGE as a Molecular Tool for Chironomid Identification*

SDS-PAGE electrophoresis as a molecular tool for identification of chironomid with the hemoglobin protein genetic marker had strengths and weaknesses. The hemoglobin band patterns were limited to combinations of hemoglobin polypeptide chains, which lacked the detailed information manifested by analysis of the higher degree of possible combinations created by the 20 amino acid sequences composed of 61 codons. Although amino acid sequencing is more laborious and costly, this method would provide more detailed genetic information on chironomid identification and possibly allow for more accurate identification to the species and subspecies level. Amino acid sequencing currently is more widely accepted and found in worldwide databases than the SDS-PAGE method for identification of organisms. However, the hemoglobin band patterns in the thesis study were easily recognized, consistent, reproducible and suitable for identification of chironomids inhabiting Kearny Marsh.

Molecular tools which investigate one gene protein, such as hemoglobin gene(s) protein, are advantageous for molecular evolution and environmental human impact studies because they are subject to environmental stressors, both natural and due to human activity (Bentivegna 2003). This may be a disadvantage for identification of chironomid due to a degree of physiological, ontogenetic and environmental variation. For instance, hemoglobin is a hypoxia-induced protein in chironomid which enables the larva to adapt to range of habitats with a high to low level of dissolved oxygen (Green et al. 1998). Hemoglobin genes are expressed at a diversity of levels as required for metabolism and homeostasis of the organism in different levels of oxygenated habitats. Therefore, for identification purposes inconsistency in Hb protein band patterns may arise due to hemoglobin gene protein expression as a function of environmental levels of dissolved oxygen.

Ontogeny is another variable which may lead to inconsistent Hb protein band patterns. Published data on *Chironomus thummi thummi* demonstrated that hemoglobin protein expression is developmental stage-specific with 12 globins existing in the 4th instar and 7 globins existing in the 3rd instar (Green et al. 1998). The following is a published theory on chironomid larvae ontogeny: "one can reasonably assume that stage-specific Hbs perform stage-specific functions. Though these functions are still undefined, one could envision special oxygen-binding needs in embryos which develop in well-oxygenated surface waters. Oxygen carrying requirements would increase as 1st instar larvae move from the surface to the benthos. The 2nd and 3rd instar larvae spend all of their time in the benthos, 'nesting' in tubes of their own creation. During the 4th instar particularly near the end, the larvae consume much energy preparing for metamorphosis, which could explain the existence of 4th instar globins expressed to meet this new energy need. Hbs are produced at any one time, the overlapping but different set of multiple Hbs at each developmental stage may simply ensure the production of ample levels of Hbs whose average oxygen binding characteristics are best suited to a given stage" (Green et al. 1998). The knowledge from this published concept on ontogeny specific Hb expression, brought to light another weakness of hemoglobin protein as a genetic marker for chironomid identification. The larval stage requires identification in order to have consistent reliable identification results. Ontogeny adds complexity and more intense labor than considered in the thesis study. Lastly, another variable became apparent when evaluating the data results.

Environmental parameters may confound identification results. Chironomid biodiversity including, genetic diversity, increases or decreases with environmental health. This is currently a widely accepted theory and indicated in the thesis study. So in an ecologically compromised environment the most tolerant species will survive and may adapt to the increased degradation. In other cases, the genetic diversity may decrease due to intolerance to poor environmental health causing a negative chain of events. A bottleneck may cause a crash in population leading to a small population which inbreeds with lower fitness. Ultimately the gene pool is decreased for this

population with lower diversity of Hb genes and lower ability to adapt to hypoxia. Whereas in a pristine environment, the same chironomid species may have a higher level of genetic diversity and therefore have the capacity to maintain a larger Hb gene family. When examining chironomid from various levels of environmental health, a varying degree of hemoglobin may be expressed. This concept tends to confuse identification by hemoglobin band pattern, unless the investigator is only evaluating one site. Future study is required to reduce the physiological, ontogenetic, and environmental parameters when using hemoglobin gene protein as identification marker for chironomid. Possibly growing and developing an identification method with stock *Endochironomus* and *Glyptotendipes* species in the controlled laboratory setting could reduce complicating variables. Another possibility is to evaluate and apply the SDS-PAGE biomarker to a different protein which does not fluctuate as readily to exogenous and endogenous factors.

An alternative method to SDS-PAGE electrophoresis as a method for identification may be AFLP, RAPD or RFLP which evaluate DNA genome, rather than protein, and do not examine one specific gene. These DNA molecular tools currently are prevalently applied in identification research of various organisms. This is advantageous for comparison with worldwide research and to obtain information on chironomids from genetic databases and literature. The results may be more consistent because nucleotides compose the polymorph band patterns which do not fluctuate in band patterns due to environmental, physiological, and ontogenetic parameters. Each alternative tool has strengths and weaknesses that require conceptual and pilot studies to arrive at the most suitable to tool for identification of an organism. AFLP and RAPD are known to be less laborious and more cost effective than RFLP. RAPD has issue with reproducibility of bands, so AFLP would be a more likely candidate for identification based on the chironomid genome. The possible weaknesses, of the SDS-PAGE technique for identification of chironomid, variability due to the environment and endogenous factors, that were discussed above may be an advantage to genetic diversity, environmental health and molecular evolution studies. Each hemoglobin

variable can be overcome but require consideration and must be controlled during sample collection and research analysis, in order to arrive at significant conclusions.

## II. *Comparison of Chironomid Hemoglobin Genetic Diversity between the Sample Sites*

### *Additional possible uninvestigated environmental stressors which influenced chironomid hemoglobin genetic diversity in Kearny Marsh*

The tested heavy metal contamination data and genetic diversity results were advantageous in arriving at a theory pertaining to the correlation between these two parameters however heavy metal contamination is only one of many stressors influencing hemoglobin protein diversity. Conceptually, in order to view the wider environmental scope, the chironomid sample populations' environmentally stressed habitats were further described in reference to the genetic diversity study. Further bioassessment of the multiple stressors in Kearny Marsh will provide substantial data to determine whether or not chironomid hemoglobin protein diversity is a suitable indicator of varying levels of pollution between the two sample sites. The following discussion will review a several potential stressors impacting the chironomid sample population habitat.

*The Endochironomus and Glyptotendipes ecotype*

Firstly, of significance to the thesis evaluation was that the sample populations from sites W9 and W22 were selected from similar habitat ecotypes in May, June, and August 2004. The larvae inhabited the fringe area of the Kearny Marsh water edge. The chironomid larvae were found dwelling in protective tubes formed by the larvae with fine sediment and mucous. The larvae in tubes were collected from the submerged aquatic reed plants, *Phragmites australis*, within the interior of both living plant stalks and fallen decaying stalks floating on the water edge. The larval tubes were composed from sediments contaminated with toxic heavy metals, of particular relevance are lead, cadmium, mercury, copper and zinc (Table 3 and 4) which leached into the

marsh habitat from the surrounding landfills and abandoned industrial factories. Also, the chironomid larvae sample population fed on plant matter and detritus which had taken up contaminants from the marsh sediment and possibly from air pollution. Although the ecotype was similar at both sites W9 and W22, *Phragmites* reed plant, the level of heavy metal contamination concentrations varied in each site. Habitat variability was reduced by collecting from one ecotype.

*Oxygen as an influence on chironomid hemoglobin diversity*

Although chironomid are known to adapt well to suboxic conditions, periods of severely low dissolved oxygen was considered to be a possible environmental stressor to chironomid organisms in Kearny Marsh. Oxygen is a major requirement for most wetland organisms and therefore a commonly used indicator of wetland health. Dissolved oxygen (DO) levels change daily with daylight photosynthesis of plants providing oxygen as a byproduct of photosynthesis. Dissolved oxygen levels also differ seasonally with changes in water depth, temperature, stratification, and organic matter from flora and fauna life cycles. Human activity impacts dissolved oxygen levels with runoff of chemicals and waste including nitrogen and phosphates from residential and industrial sources. Increased organic matter may cause eutrophication which decreases oxygen available to biota.

Water parameters could be directly correlated to hemoglobin genetic diversity because an apparently direct relationship exists between hemoglobin protein physiological function, such as respiration and metabolism, because hemoglobin protein binds oxygen. However data was not tested at the time of chironomid sample collection and since oxygen levels are known to fluctuate further research is required to draw valid conclusions on the relationship between Hb genetic diversity and biologically available oxygen to chironomid.

In lieu of water parameter data, historical dissolved oxygen levels data were reviewed and considered as an additional stressor to heavy metal contamination because DO appeared to correlate with genetic diversity levels between sites. Both Kearny Marsh sample sites, W9 and

W22, were tested to be suboxic in 2002 and August 2003. In the summer 2003 the dissolved oxygen levels were tested to be suboxic at both sites, and was measured to be lower at site W9, (0.1 ppm) and higher at site W9 (2.34 ppm). In past evaluations site W9 had lower dissolved oxygen levels than site W22, which seems to have a positive correlation with hemoglobin protein diversity. Timely and substantial data and analysis in conjunction with chironomid sample population collection is required to arrive at valid conclusions on the relationship between DO and Hb genetic diversity.

Hypothetical analysis shows that the chironomid collected in 2004, included *Glyptotendipes* and *Endochironomus* were collected from the same total pooled sample population from the ecotype at site W9 and then collection was taken at W22. Therefore, the dissolved oxygen parameters were similar for the sample populations containing a mixture of the two species. Their shallow fringe water habitat most likely had higher oxygenated water than the benthos region of the deeper central region of Kearny Marsh. Many aquatic plants grew in the fringe water which added oxygen to the water as a byproduct of photosynthesis. Also the larvae collected inhabited the interior of fallen decaying reed plant stalks. The fallen aquatic plant matter inhabited by chironomid samples lay on the oxygenated surface water. In comparison to heavy metal contamination the dissolved oxygen parameter at the thesis sample collection habitat may be considered as a less detrimental environmental stressor because the chironomid inhabited one of the more highly oxygenated regions of the marsh. Chironomid are known to adapt to suboxic conditions, however this hypothesis requires bioassessment and evaluation to arrive at valid theories.

*Invasive vegetation*

The Hackensack Meadowlands, including Kearny Marsh, historically had a diverse plant community which supported diverse fauna populations. *Phragmites australis*, an invasive foreign aquatic reed plant has flourished and dominated the natural variety of wetland plants in the

Hackensack Meadowlands (Boening 2000). The native *Spartina alterniflora* reed plant population decreased due to the invasive *Phragmites*. The detrimental colonization of *Phragmites australis* reduced a variety of niches for organisms dependent on wetland plants. The marsh habitat became more homogeneous which may lead to a decline in biodiversity of animals, birds, fish, invertebrates, fungi, and microbes due to a decrease in habitat variety to support biodiversity.

Decayed plant material found in detritus is a food source for chironomid larvae. Published studies indicate that heavy metals are taken up by *Phragmites* reed plants during the cycling of metals (Weis 2003) which are beneficial to water quality, but may not be beneficial to organisms feeding on *Phragmites*. For instance, chironomids feed on detritus composed of decayed contaminated *Phragmites* matter in Kearny Marsh. The larvae were also in direct contact with heavy metals when dwelling and feeding within the *Phragmites* reed stalks. Additionally, the older leaves were shown to have higher concentration of heavy metals than younger leaves (Weis 2003). During the decay process heavy metal metals in the plants increased in concentration, especially Cu and Zn, compared to the heavy metals in the surrounding sediment (Weis 2003). Hence, the Cu and Zn levels consumed by wetland organisms may be higher than standard heavy metal analysis of sediment would indicate. Lastly, chironomid are near the base of the food web therefore organisms in higher trophic levels are exposed to contaminants accumulated in chironomids which feed on detritus.

*Organic air pollutants from surrounding highways*

An additional potential pollutant stressor impacting the chironomid habitat, especially attracted by the marsh vegetation, is organic air pollutants emitted from heavy vehicular traffic and runoff pollutants from the adjacent three major highways (the NJ Turnpike, Route 280, and Route 7) all intersecting together by site W22. The highways are directly adjacent to site W22 and relatively distant from site W9 which may have a greater significant environmental impact on chironomid hemoglobin genetic diversity at site W22 than chironomid dwelling in site W9. Automotive air

emissions, including hydrocarbon and nitrogen oxide, may be eliminated from the atmosphere by rainwater, photochemical reactions, and uptake by plants (Manahan 1994). Lipophilic plant cuticles have an attraction for organic substances, such as organic air pollutants (Manahan 1994). So, chironomid may digest organic air pollutants absorbed by *Phragmites* and other aquatic plants as the plants decay to detritus matter. In addition the *Glyptotendipes* and *Endochironomus* inhabiting these reed plants are in close physical contact with the contaminants which may be absorbed by their gills and cuticles.

*Unnatural sediment composition as an influence on hemoglobin genetic diversity*

An additional environmental stressor impacting hemoglobin protein diversity could be unnatural fine sediment (sand, silt, and clay) composition. Most chironomid species' protective tubes are composed of fine sediment, regardless of their various habitats (sediment, plants, and rocks). Although the *Endochironomus* and *Glyptotendipes* collected for this study were found dwelling within submerged marsh vegetation, as previously discussed, their protective tubes were constructed of contaminated fine sediment and mucous. Therefore sediment composition was a relevant parameter in determining changes in hemoglobin genetic diversity.

In general variation in the levels of gravel, sand, silt, and clay are an influence on the habitat diversity for benthos organisms. Natural levels of embedded silt are a desirable habitat for benthos dwelling chironomid; however sediment composition change due to human activity may have a negative effect on chironomid habitat. Chironomid larvae aggregate towards fine sediment habitats in environmentally healthy aquatic ecosystems. Macroinvertebrates have adapted to natural levels of fine sediment (sand, silt, clay) embedded in the substrate (Gale et al. 1995). Yet, if the fine sediments become unnaturally high, detrimental biological effects occur in the aquatic ecosystem (Gale et al. 1995). In general, substrates embedded with greater than 67% fine sediment in aquatic substrates may cause alterations in macroinvertebrates structure (Gale et al. 1995).

Sediment composition was not analyzed at the time of the thesis collection of chironomid in 2004. However, the previous heavy metal contamination analysis study included characterization of the sediment (Table 5) which provided some insight on Kearny Marsh benthos habitat. Both sites W9 and W22 had higher than 67% sand: site W9 had 84.2% and W22 had 69.5% sand of the total sediment concentration. Site W22 had a higher percentage of silt and clay than W9, 30.1% and 15.8% respectively. In fact the percentage of clay and silt at site W22 in 2002 was the highest of all the 6 sites tested in the previous study in June 2002 (Bentivegna 2004). High clay content may be a negative parameter decreasing the Hb genetic diversity in *Glyptotendipes* in site W22. Clay is negatively charged which attracts positively charged pollutants (Gale et al. 1995). Site W22 had double the percentage of clay and silt in comparison to site W9 in June 2002.

Without a baseline for a normal healthy substrate, one may suggest a significant difference of embeddedness existed between the two sample sites. The sediment composition was not characterized at the time of chironomid sample collection, however these results (2002) suggested that Kearny Marsh has possible fluctuations of sediment composition that may be detrimentally unnatural to the chironomid health and therefore decrease normal hemoglobin genetic diversity values. Further monitoring of sediment in conjunction with chironomid sampling is required to assess conclusions.

*Turbidity as an influence on genetic diversity*

An additional possible stress in both sample sites W22 is suspended fine sediment, causing increased turbidity. Data from a previous study indicated a change in silt and clay at site W22 from June to October in 2002, from the highest silt and clay concentrations of all six sites to the lowest concentrations of silt and clay of all sites analyzed in the previous study (Bentivegna 2004). The levels dropped from 30.1% to 4.5% (the lowest clay and silt level of all sites analyzed in the previous study). Although this data was not collected at the time of the thesis sample

collection, this change may imply a flow of sediment causing turbidity during June 2004 and possibly during various other periods in Kearny Marsh from human activity or natural sources.

Turbidity will decrease available light for photosynthetic activity and therefore plants will not thrive in Kearny Marsh (Gale et al. 1995). Hence, a resulting chain reaction may occur in the wetland ecosystem food web. The food source for chironomid is detritus which may initially increased with plant death, and benefit the chironomid. However, with time and increased organic matter from decayed plants, eutrophication may result with lower dissolved oxygen available to the marsh biota. Eventually, the decreased plant population communities will cause a decrease in both food source and habitat for chironomid and other aquatic biota. This could partially explain a decrease in the *Glyptotendipes* hemoglobin genetic diversity due to a decrease in the *Glyptotendipes* population size and fitness.

Also, suspended fine sediment, turbidity, may have interfered with physiological functions, in particular digestion and respiration (Osmond et al. 1995). Chironomid gills may be clogged by fine suspended sediment which interferes with respiration and therefore decreases the uptake of oxygen. Increased turbidity could have influenced hemoglobin genetic diversity reduction in *Glyptotendipes* dwelling in Kearny Marsh.

***Phylogenetic Analysis of Hemoglobin Phenotypes***

The following hypothetical evaluation of the dendrogram was applied to gain insight on chironomid population dynamics in Kearny Marsh. In addition to indicating relationships between interspecies and intraspecies chironomid hemoglobin phenotypes, a possible explanation of the sample populations' hemoglobin phenotype divergence patterns may be manifested in the dendrogram (figure 4). A subjective time scale was applied to the dendrogram scale (Rescaled Distance Cluster Combine, CASE) in which 0 was representative of relative current time and 25 was the most distant past (figure 4). The points of divergence indicate a time of environmental selection; an environmental pressure caused the species with a particular hemoglobin protein

phenotype to change genetically. The species with the varying hemoglobin phenotypes diverged further creating even more phenotypes, which was shown in the dendrogram (figure 4).

Possible explanations for Hb phenotypes to diverge were adaptation, extinction, hybridization, and speciation due to environmental selection of the fittest Hb phenotypes. Both natural selection and stress from human activity lead to species divergence due to hemoglobin gene selection. Hemoglobin genes are functionally significant to chironomid and are well conserved. In general, more conserved gene sequences are appropriate for older events, divergence (Karp, Isaac, and Ingram 2001). However, the dendrogram illustrated Hb genetic divergence at varying distances on the scale from 0 to 25, which is subjective time in this discussion. The majority of the diverging Hb profiles occurred between approximately 2.5 to 0, considered to be more recent in this discussion. Possibly, a recent severe environmental pressure, such as industrialization accompanied by development of the area surrounding Kearny Marsh, and eventually degradation due to human activity, caused the once healthy chironomid population which had evolved over a long term to possess a well conserved hemoglobin gene pool to show an increased divergence pattern. The divergence pattern may indicate hemoglobin population dynamics, such as a bottleneck, physiological adaptation, or possibly a change in the large conserved hemoglobin gene family.

Further analysis of the dendrogram, indicated that abundant Hb profiles may reflect chironomid with certain Hb phenotypes which are dominant, an indication of the tolerant species able to adapt to current environmental stressors. The few tolerant species may be inbreeding; however, through inbreeding they may have the potential to speciate over time. The rare species may be in the process of extinction because they are unable to adapt to environmental change. The Hb phenotypes: Gp3, Gp4, and Ep4 all found at both sample sites were the most abundant Hb profiles and logically seem to adapt to the severely degraded Marsh. These three Hb profiles types diverged more recently, approximately 0 to 2 on the dendrogram distance scale, in relation to the other *Endochironomus* and *Glyptotendipes* Hb phenotypes. The rare Hb phenotypes from

the field species: Gp2 and Ep1 were found at site W9 which may indicate these species could become extinct, possibly because of sensitivity to the high total heavy metal concentrations at W9 compounded by other stressors at this site. *Endochironomus* individuals with the Ep4 Hb phenotype were collected from both sites W9 and W22. Ep4 phenotype was abundant, dominant and indicated an older lineage than most Hb phenotypes. The *Endochironomus* species with Ep4 Hb phenotype may be the best adapted species to environmental change occurring in Kearny Marsh over the relatively longest period of time including both the past and present eras.

Ep1 was abundant and the only *Endochironomus* Hb phenotypes in the 1st clade, which is dominated by *Glyptotendipes* and *Chironomus* (figure 4). The Ep1 Hb phenotype was rare and was closely related to Gp4 phenotypes. The Ep1 Hb phenotype was found only at site W9 and may be a hybrid with a *Glyptotendipes* and an *Endochironomus* from site 9. According to SDS-PAGE gel analysis, Ep1 phenotype appeared to be a combination ofEp4 of site 9 and Gp5 or Gp3 phenotypes (figure 2). Ep2, Gp1, and Gp5 were rare with older lineages. These Hb phenotypes appeared to be declining towards extinction possibly these species with these Hb phenotypes were unable to adapt to environmental change in Kearny Marsh over time.

Lastly, *Endochironomus* and *Glyptotendipes* may have different physiological adaptation responses to stressors, such as heavy metal contamination and hypoxia which is discussed in the following section. Therefore *Endochironomus* and *Glyptotendipes* diverged into two major clades based on physiological adaptation mechanisms and population dynamics due to a changing environment. This divergence pattern constructed by hemoglobin protein band patterns, representing phenotypes, may reflect their different hemoglobin physiological responses to pollutants and suboxic conditions.

### *Molecular Ecology: Integration of Genetics and Environmental Factors*

According to the results *Glyptotendipes* hemoglobin protein diversity appeared to be indicative of environmental stressor levels in Kearny Marsh which were manifested by a change in SDS-

PAGE band patterns variation between the sample sites. As the previous sediment analysis study confirmed the total heavy metal contamination of detritus and sediment was greater at site W9 than site W22 in June 2002. Similarly, Hb protein diversity was higher at site W9 than W22. A direct correlation appeared to exist between heavy metal contamination and *Glyptotendipes* Hb protein diversity. The following discussion elaborates upon three explanations for this correlation:

**1.** This species at both sites most likely had an energy demanding response to heavy metal toxicity which may be an indication of Hb phenotypic variation between sites at the time of sample collection (Table 8). As heavy metal contamination concentrations increased, *Glyptotendipes* and *Endochironomus* hemoglobin diversity values increased. A direct positive correlation seemed to exist. This hemoglobin diversity correlation may be a result of an increased demand for hemoglobin protein synthesis to meet increased metabolic rates due to toxicity stress, compounded by additional stressors. The organisms may have required increased energy to detoxify heavy metal toxins from their body and to maintain homeostasis under physiological stress (Forbes and Calow 1996). The chironomid population may have attempted to adapt to the degraded environment by expending energy to detoxify the heavy metals from their bodies to maintain homeostasis and survival. "A general prediction arises from models involving graded physiological responses that are metabolically costly is that metabolic rate should increase with increasing levels of toxicant (exposure time and/or concentration) until irreversible pathological effects impair metabolism itself" (Forbes and Calow 1996).

A physiological adaptation feedback mechanism played a role in the regulation of increased energy requirements to survive in highly polluted habitat. As energy demands increased, oxygen for metabolism requirements increased, which triggered an increase in hemoglobin protein production to bind increased amounts of oxygen. Increased hemoglobin protein production may be encoded by an increase in hemoglobin genes in the *Glyptotendipes* Hb gene family, as metabolic demands increase (figure 9). When the heavy metal contamination concentration in an

ecosystem is lower, metabolic demands may decrease, and therefore fewer Hb genes encode less amounts of hemoglobin protein. Possibly a variety of number and types offunctional hemoglobin genes encode Hb protein which is reflected as hemoglobin protein variation, diversity, and is visualized on the SDS-PAGE gels as varying band patterns with varying numbers of distinct bands. Hemoglobin genetic diversity potentially may be explained by the varying quantities of hemoglobin protein required for metabolism to adapt to varying levels of environmental stressors. The *Glyptotendipes* hemoglobin protein diversity analyzed with the SDS-PAGE gels, the Shannon Wiener, Evenness and Simpson's Diversity indices and the dendrogram, could have indicated a physiological adaptation response to the environment stress (figure 9).

Of interest is an observation that *Glyptotendipes* was shown by the SDS-PAGE gels to have a larger cluster of Hb genes expressed as protein, than *Endochironomus*. The *Glyptotendipes* Hb protein was visualized as 5 hemoglobin phenotypes equivalent to the 5 distinct Hb band patterns versus the 4 Hb protein phenotypes for *Endochironomus*. At site W9 *Glyptotendipes* and *Endochironomus* had similar Evenness diversity index values (Table 7) of E= 0.63 and E= 0.62, respectively. The two species were collected from the same sample populations from the same habitat and environmental parameters including heavy metal and dissolved oxygen levels. Conceptually, *Glyptotendipes* with more functional Hb genes produced increased levels of hemoglobin protein to meet higher metabolism requirements for detoxification of heavy metals. Possibly *Endochironomus* regulated oxygen needs with fewer Hb phenotypes; however these Hb phenotypes may have a higher binding affinity for oxygen, and so both species adapted to the stressed ecosystem by binding oxygen with different physiological metabolic mechanisms. If one gene encodes one polypeptide, the SDS-PAGE gels with each band representing one polypeptide (figure 2) appeared to support this explanation. The *Endochironomus* with the Ep4 hemoglobin appeared to over express Hb protein in comparison to Ep, Ep2, or Ep3 which was manifested by one intense band. However this band may be two bands which have a very similar weight. Regardless of the physiological adaptation mechanism, *Glyptotendipes* with a greater Hb protein

diversity difference and a statistically significant difference between sample sites provided to be the more appropriate bioindicator species to determine overall degradation in Kearny Marsh.

2. Population genetic dynamics may have played a major role in the change in genetic diversity values between sample sites (T. Eeva et al. 2006). The prevalent hypothesis states that as multiple stressor levels increased, the genetic diversity should have decreased (Bagley et al. 2002) (Table 8). This hypothesis indicates a negative correlation between multiple environmental stressors and genetic diversity. This theory states that genetic diversity is not an indicator of specific contaminants, but may predict the effect of multiple stressors, such as, habitat loss/fragmentation, habitat degradation, exotic/admixtures, and environmental mutagens. Changes in genetic diversity are an indicator of cumulative effects over decades or years of breeding generations exposed to multiple stressors and the change in diversity is not apparent until after a lag period (Bagley et al. 2002).

The thesis prediction states that a negative correlation between environmental stressors and genetic diversity which seemed to be in agreement with the prevalent theory. Possibly over the past years, unevaluated compounded stressors impacted both sample sites and caused a reduction in both *Endochironomus* and *Glyptotendipes* population sizes. The measured decrease in genetic diversity at both sites W9 and W22 in comparison to the laboratory reference population could have been evidence of a reduction in both *Endochironomus* and *Glyptotendipes* population. This reduction in population size may have been caused by either natural or anthropogenic stressors. Site W22 chironomids appeared to be exposed to more severe multiple stressors which were demonstrated by the relatively lower genetic diversity at site W22.

Either genetic drift, random changes in allelic frequency, over a period of time or a bottleneck, a severe sudden reduction in population size, may have occurred in Kearny Marsh which resulted from severe environmental pressures. Genetic drift in a small population has a more intense effect than in a large population (Bagley et al. 2002). Both genetic drift in a small population or a

bottleneck may have caused decreased heterozygosity in the hemoglobin gene family. In addition to reduced heterozygosity, selection of the fittest was a factor in reduced genetic diversity (Bagley et al.). The resulting reduction in chironomid population induced inbreeding of the tolerant species with certain hemoglobin phenotypes which allowed the species to adapt to the habitat impacted by multiple stressors. When only the tolerant chironomid survived through selection the population genetic dynamics altered and a dominant species population pattern evolved. Theoretically, when heterozygosity decreased, fitness decreased. The tolerant survivors possessed only a portion of a total naturally evolved hemoglobin gene pool. The population may have become more homogenous with less evenness or genetic diversity.

Possible evidence of a change in the total number of hemoglobin phenotypes is shown in figure 6. *Glyptotendipes* had a reduction in hemoglobin phenotype abundance from 5 total analyzed Hb phenotypes to 4 Hb phenotypes at site W9 and to 3 at site W22. Hence, the most tolerant survived and the reduced populations inbreed which decreased genetic fitness to adapt to a changing environment. Dominance of species with the hemoglobin phenotypes, Gp3 and Gp4, is evident in this graph. The tolerant, dominant species most likely had the hemoglobin phenotype which was able to over express hemoglobin to adapt to a multistressed environment, by having the ability to bind the required amounts of oxygen for maintenance of homeostatsis and possibly detoxification of xenobiotics.

The chironomid hemoglobin large gene family, possessing both gene duplications and polymorphism, which evolved over millions of years in order to adapt well to aquatic environments and the benthos region, could have been altered with inbreeding. Possibly the gene family became more homogenous. Therefore, the survivors of the bottleneck may have lost genetic resilience, fitness to adapt and survive. The resulting small Kearny Marsh chironomid population of tolerant species will inbred and continue to reduce fitness. "Because loss of genetic diversity is related to inbreeding and inbreeding reduces reproductive fitness, a correlation is

expected between heterozygosity and population fitness" (Environmental Protection Agency 1998)

Of interest was the small *Chironomus riparius* lab population which inbred for nine years. Initially the thesis prediction was that the laboratory population had lower Hb genetic diversity than the wild type chironomid in a natural habitat. Decreased diversity most likely occurred in the laboratory population however as the thesis data results demonstrated the Hb genetic diversity was less severely decreased than the chironomid sample populations from Kearny Marsh. Without a baseline for the total hemoglobin gene family size only predictions may be suggested for *Endochironomus* and *Glyptotendipes* inhabiting Kearny Marsh, site W9 and W22.

*Chironomus thummi thummi*, synonymous with *Chironomus riparius*, is known to have 12 different hemoglobin protein phenotypes (Green et al. 1998). The laboratory population showed 9 different band patterns which may represent 9 different hemoglobin phenotypes. This result indicated a 25% reduction in the total 12 hemoglobin phenotypes found in a healthy gene pool. According to the dendrogram analysis *C .riparius* was more closely related to *Glyptotendipes* than *Endochironomus*. So possibly *Glyptotendipes* has a similar size hemoglobin phenotype size of 12 different phenotypes which was reduced to 5 Hb phenotypes in Kearny Marsh. The *Glyptotendipes* from the Kearny Marsh sample sites may have been reduced greatly by the many environmental stressors, including heavy metal toxicity, which was not present in the lab population. Noteworthy was that not all hemoglobin genes are expressed at the same developmental stage and expression varies as the need for oxygen varies, so the reference species, *Chironomus riparius* collected and analyzed may not have expressed all phenotypes.

In conclusion, an alteration in chironomid population genetic dynamics may have occurred, in particular inbreeding of the most tolerant species reducing genetic diversity, hence heterozygosity, and increasing dominance of species caused by severe multiple environmental stressors, including one of many unevaluated stressors at site W22 such as organic air pollutants, at the time of the thesis chironomid sample population collection. In conclusion, this conceptual interpretation

partially explains that a possible negative correlation exists between hemoglobin genetic diversity and the possible severe unevaluated multiple stressors in site W22.

3. The third explanation addressed was Kearny Marsh food web as relevant to alterations in the hemoglobin genetic diversity. A change in chironomid genetic diversity due to a habitat and food sources which were contaminated by pollution may suggest a change in the food web. This discussion will focus on mercury contamination in detritus and sediment, but acknowledges the overall scope with a compound effect of stressors as possibly playing a role in food web changes. Anaerobic microbes inhabiting the benthos of a wetland are at the base of the food web trophic structure and are known to increase mercury toxicity through methylation of mercury (Boening 2000). When MeHg enters the aquatic food web at the base, the methylated mercury becomes biomagnified, from the lower to higher trophic levels. The concentration of the metal continues to increase as it is acquired by organisms in higher trophic levels of the food web (Kendall 2004; Hope 2003). Although standard water quality testing or heavy metal toxicity testing may indicate acceptable levels of mercury in an aquatic ecosystem this may be misleading. Low levels of mercury which biomagnifies in the food web actually are putting biota in the upper trophic levels at risk (Hope 2003). In Kearny Marsh the mercury contamination may be methylated by anaerobic microbes in the sediment. Then the MeHg next is taken up by Phragmites plants. When the plant decays and becomes detritus mercury concentrations increase (US Fish & Wildlife 2005). Chironomids feed on the MeHg contaminated detritus and the mercury concentration become bioconcentrated in their tissues. Fish then consume the chironomid and birds eat the fish. The contaminated fish die and are biodegraded by microbes. The food web cycle continues to repeat and the methylated mercury concentrations continue to increase as the metal accumulates in upper trophic levels.

In general plants are not sensitive to mercury (Boening 2000); however, larval stage aquatic invertebrates are more susceptible to Hg toxicity, than many aquatic organisms (Boening 2000). Therefore chironomid larvae may not tolerate MeHg, and the population will decrease which in

turn decreases a food source for many aquatic animals. Not only is the food web altered by Hg, but a decreased chironomid population may decrease genetic diversity. Evidence of mercury in the Meadowlands ecosystem food web is the substantial numbers of White Perch fish species found in the Hackensack Meadowlands in a study (2001-2003) which determined that Hg levels were above FDA/EPA standards (Meadowlands Environmental Research Institute 2006). The White Perch are in the upper levels of the food web and most likely fed on contaminated lower levels organisms, such as invertebrates. Mercury biomagification in the food web is an example of the advantage of using a bioindicator in conjunction with standard chemical testing to determine environmental health. Although the standard testing may indicate the water and sediment parameters are acceptable for environmental health, bioassement of indicator species in the foodweb will demonstrate whether the wetland organisms are at risk to exposure by certain heavy metals.

***SDS-PAGE as a Molecular Tool to Evaluate Chironomid Genetic Diversity***

Lastly, the chironomid hemoglobin protein diversity as measured by the SDS-PAGE method demonstrated to be a useful molecular tool in studying the effect of heavy metal contamination on chironomid hemoglobin gene protein diversity. The SDS-PAGE method was simple and appropriate for environmental research. The Hb gel band patterns were easily recognized, reproducible, and distinct for both interspecies and intraspecies evaluations of the chironomid inhabiting Kearny Marsh. The use of *Glyptotendipes* hemoglobin protein as a gene biomarker proved to be suitable for diversity studies because Hb protein was polymorphic and a product of a large gene family with gene duplication. Theoretically the chironomid Hb gene bioindicator was responsive to multiple stressors and therefore a potential candidate as an indicator of multiple environmental stressors. This thesis study investigated heavy metal stress, however dissolved oxygen levels and related parameters (nutrients, water levels, salinity, and so on) were apparently directly linked to the chironomid Hb gene indicator and Hb genetic diversity. Further research of

chironomid species genetic diversity, both phenotypic and genotypic, and water parameters, including organic pollutants, warrants in depth investigation.

## Conclusion

### I. *Identification Method*

Preliminary research conducted in this thesis study showed that molecular identification of chironomid with the SDS-PAGE analysis of the hemoglobin protein has potential to be a useful method of identification. The hemoglobin protein band patterns were species-specific easily recognized, consistent, and reproducible. The possible variables, ontogeny and environmental parameters, may be overcome during collection of species samples and in the laboratory experimental procedure.

### II. *Glyptotendipes Hemoglobin Genetic Diversity as an Indicator of Multiple Environmental Stressors*

Preliminary research conducted in this thesis indicated that *Glyptotendipes* hemoglobin protein genetic diversity has the potential to be a viable indicator of multiple environmental stressors, in particular heavy metal contamination in sediment, which affects the biota in Kearny Marsh. A direct correlation seemed to exist between the level of multiple stressors and the level of *Glyptotendipes* hemoglobin protein diversity. The change in diversity has three possible explanations, physiological adaptation response to toxic pollutants, a decrease in the phenotype size expressed due to genetic drift or a bottleneck, and biomagnification of mercury, copper and zinc, in the Kearny Marsh food web. The hemoglobin protein diversity change between the two sample sites, W9 and W22, was most likely a result of three factors.

Firstly, at some undetermined time a bottleneck may have occurred with only the most tolerant species surviving the severe environmental stress in Kearny Marsh. Possibly a bottleneck, a crash in the chironomid population size, resulted from human activity substantially increasing during industrialization of the NJ Meadowlands which detrimentally impacted the marsh with severe pollutants and degradation. The possible reduced population had lower fitness due to inbreeding

and a diminished heterozygous hemoglobin collective gene pool. Chironomid genetic diversity decreased as a result of the possible bottleneck.

Evidence of a bottleneck was the relatively low number of hemoglobin phenotypes in the field species sampled for the thesis project. *Endochironomus* had merely 4 hemoglobin phenotypes and *Glyptotendipes* had only 5 hemoglobin phenotypes. In comparison, *Chironomus riparius*, the laboratory reference species, had 9 hemoglobin phenotypes and according to literature is known to have 12 hemoglobin phenotypes (Green et al. 1998). Most likely only the tolerant species possessing the advantageous hemoglobin phenotypes became the most dominant chironomid species throughout Kearny Marsh and continued to survive chronic severe environmental stress and inbreed in their degraded habitat. Although chironomid are widely accepted to be categorized as tolerant aquatic benthos invertebrates of pollutants, the Evenness diversity calculations indicated dominance of both the *Endochironomus* and *Glyptotendipes* species which shows low diversity. A tolerance for pollutants threshold may have been exceeded in Kearny Marsh by certain intra- and interspecies chironomid.

Secondly, the surviving chironomid population from the bottleneck may have adapted to environmental stressors by a physiological adaptation response mechanism that caused increasing hemoglobin protein production in order to meet metabolic requirements for detoxification and maintenance of homeostasis. Detoxification of contaminants and maintenance of homeostasis under physiological stress required increased energy accompanied by a greater demand for oxygen; and therefore, a rise in metabolic rate. Conceptually the increased demand for oxygen induced an increased production of hemoglobin protein to bind oxygen. Increased hemoglobin production is possibly regulated by encoding increased numbers of hemoglobin genes which was manifested as increased hemoglobin protein diversity. As stressors increased, chironomids regulated homeostasis by increasing production of hemoglobin protein. As stressors decrease, hemoglobin production decreased.

The SDS-PAGE gel electrophoresis method used to analyze chironomid hemoglobin separated the protein, in this case hemoglobin, in polypeptide bands and therefore that which is shown in the electrophoresis gels is protein band patterns. The genetic diversity was calculated from phenotypic diversity, not genotypic diversity. Because protein represents physiological and biochemical characteristics of an organism, the hemoglobin protein genetic diversity visualized on SDS-PAGE gels as band patterns, most likely manifests hemoglobin physiological function. The physiological function demonstrated in this study may be hemoglobin physiological adaptation response to environmental stressors which was partially supported by the correlation between heavy metal contamination and hemoglobin protein diversity.

The correlation between the sample sites heavy metal contamination levels, compounded by other environmental stressors, was a positive correlation (figure 8). Hence, as heavy metal contaminants were increased at site W9, hemoglobin protein diversity was increased as a physiological metabolic response to maintain adequate levels of hemoglobin to bind oxygen for increased metabolic rates due to increased energy for maintenance of homeostasis under the physiological stress of toxicity from heavy metal contaminants, compounded by other stressors. Increased numbers of hemoglobin genes may have encoded for increased hemoglobin production which was manifested as increased hemoglobin protein diversity as environmental stressors increased.

In conclusion, the *Glyptotendipes* hemoglobin genetic diversity difference between sites W9 and W22 may indicate a positive correlation between environmental stressors and hemoglobin protein production diversity which fluctuates to meet metabolic requirements to varying degrees of environmental stressors levels at the time of collection. The thesis research indicated that *Glyptotendipes* hemoglobin protein diversity evaluated with SDS-PAGE method has the potential to measure present environmental stressors, in particular heavy metal contamination because chironomids seemed to have the ability to physiologically adapt to environmental degradation with varying amounts of hemoglobin production to meet metabolic requirements due to stress.

Possibly varying numbers of genes were encoded to produce varying types of Hb protein phenotypes. *Glyptotendipes* hemoglobin protein diversity may be a suitable bioindicator of multiple environmental stressors in a wetland ecosystem. Varying species, such as *Endochironomus* and *Glyptotendipes*, may have different detoxification capacities (T. Eeva et al. 2006). This variation in a species ability to detoxify heavy metals may explain the calculated genetic diversity difference between *Glyptotendipes* and *Endochironomus*. Further research is necessary to validate the findings determined in the preliminary research conducted in this thesis project.

Lastly the thesis prediction that a negative correlation existed between genetic diversity and multiple environmental stressors was not supported by the heavy metal contaminant data. However, the field sample populations, *Glyptotendipes* and *Endochironomus*, from Kearny Marsh had a decrease in genetic diversity in comparison to the reference laboratory species. Also, the *Glyptotendipes* sample populations demonstrated a statistically significant reduction in genetic diversity from sites W9 to W22. This reduction in genetic diversity most likely indicated increased exposure to cumulative unmeasured multiple stressor from the urbanization of Kearny Marsh over the years. The thesis findings demonstrated that hemoglobin genetic diversity measured by SDS-PAGE analysis of hemoglobin protein has the potential to be a suitable indicator of cumulative multiple stressors over the past decades or years.

**Future Research**

Further investigation of chironomid hemoglobin diversity on the nucleotide level would be interesting and provide more detailed information on hemoglobin protein genetic diversity. AFLP molecular method coupled with SDS-PAGE analysis of functional Hb genes which were expressed as polypeptide band patterns in the gels could be enlightening to Kearny Marsh ecological health. The information acquired would be supportive of future remediation efforts. Ultimately the information acquired from genetic diversity studies of Kearny Marsh may be shared by researchers who aim to protect our remaining wetlands.

The following are suggested study based on insight from the thesis research:

1. Locate a reference site that has minimal urban influence as a baseline for both chironomid identification and genetic diversity studies.
2. Further investigation is necessary to determine which stressor(s) actually caused a change in the genetic diversity between the sample sites. Dissolved oxygen and organic air pollutants are potentially significant additional stressors to heavy metal in Kearny Marsh. Possibly both stressors contributed to the change in diversity observed in the thesis project.
3. DNA analysis is required to determine if the change in diversity is due to a bottleneck and/or physiological adaptation.
4. Evaluate several molecular tools, benthos invertebrates and genes to determine which tool(s), species and gene are best suited for which environmental stressor(s).
5. Conduct extensive monitoring of several sites with a wide range of pollution and degradation over a long period of time to arrive at conclusive results.
6. Apply the knowledge acquired from these studies to support wetland protection and restoration efforts.

**Table 1.** Chironomid hemoglobin protein band patterns were produced by SDS-PAGE gel electrophoresis. Hemoglobin protein profiles were characterized by a six band pattern based on the molecular weight (kDa) and intensity of the bands. The band intensity was based on a numerical range: absent (0), present (1), increased intensity (2) and more possible bands (3).
Key: Endochironomus: (E); Glyptotendipes: (G); Chironomus: (C); Phenotype (band pattern: p and numbered (1-9) for each distinct phenotype within a species.

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Gp1 | 2 | 2 | 2 | 0 | 1 | 1 | 0 | 2 |
| Gp2 | 0 | 0 | 2 | 0 | 1 | 1 | 1 | 0 |
| Gp3 | 2 | 1 | 0 | 2 | 0 | 3 | 13 | 18 |
| Gp4 | 2 | 0 | 0 | 2 | 0 | 0 | 17 | 16 |
| Gp5 | 2 | 2 | 0 | 2 | 0 | 3 | 3 | 0 |
| Ep1 | 2 | 0 | 2 | 0 | 3 | 0 | 1 | 0 |
| Ep2 | 0 | 2 | 0 | 0 | 3 | 0 | 1 | 1 |
| Ep3 | 0 | 2 | 0 | 0 | 0 | 0 | 5 | 9 |
| Ep4 | 0 | 2 | 0 | 1 | 3 | 0 | 16 | 3 |
| Cp1 | 1 | 0 | 2 | 2 | 0 | 0 | 6 lab | |
| Cp2 | 1 | 1 | 2 | 2 | 2 | 2 | 6 lab | |
| Cp3 | 1 | 0 | 2 | 2 | 2 | 2 | 6 lab | |
| Cp4 | 1 | 0 | 2 | 2 | 1 | 1 | 12 lab | |
| Cp5 | 1 | 0 | 2 | 0 | 0 | 3 | 3 lab | |
| Cp6 | 1 | 0 | 2 | 1 | 2 | 2 | 3 lab | |
| Cp7 | 1 | 1 | 2 | 2 | 1 | 1 | 10 lab | |
| Cp8 | 1 | 1 | 2 | 2 | 1 | 0 | 1 lab | |
| Cp9 | 1 | 1 | 2 | 1 | 2 | 2 | 3 lab | |

**Figure 1.** An aerial map of Kearny Marsh. Chironomid samples were collected from sites W9 and W22. Site W9 was previously tested to be more degraded than site W22.

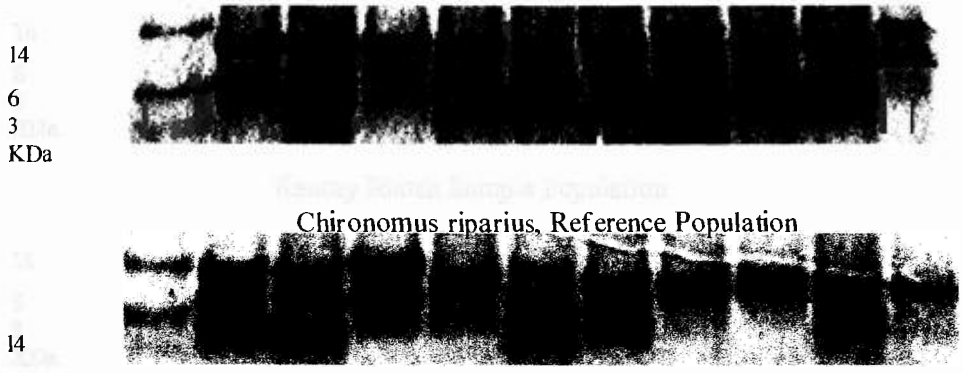



**Figure 2.** Examples of the SDS-PAGE gels analyzed for chironomid identification and the genetic diversity study

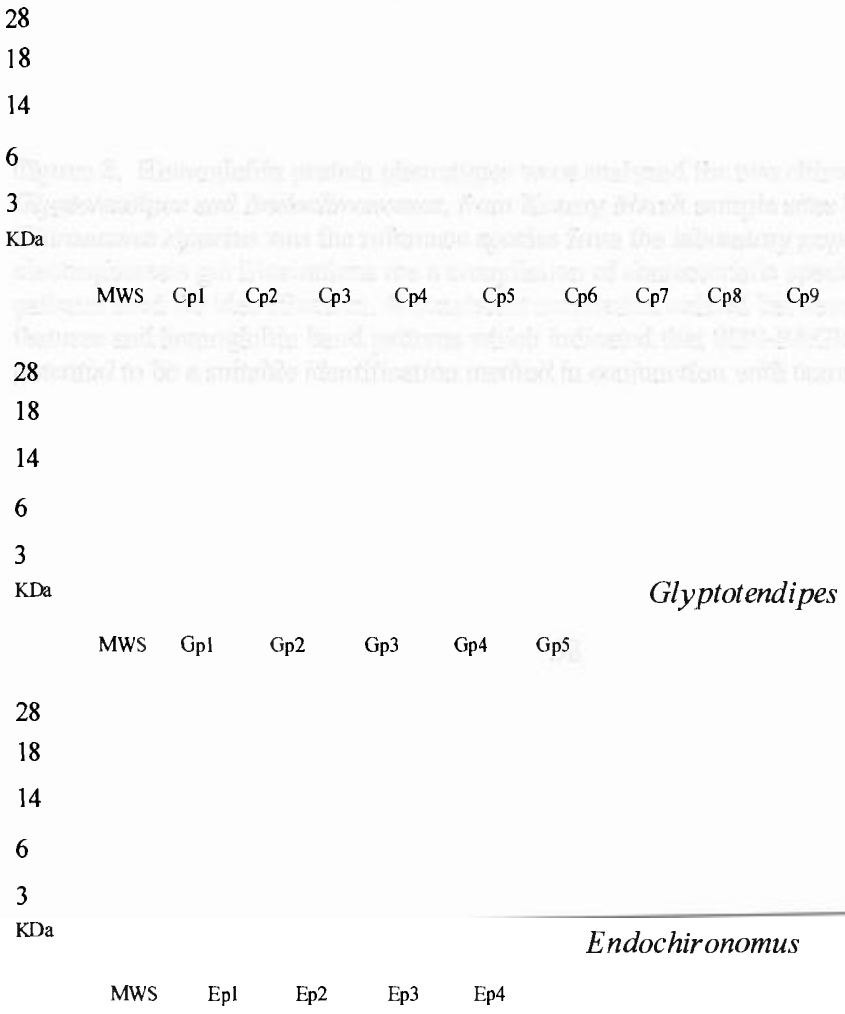



**Figure 3.** Hemoglobin protein phenotypes were analyzed for two chironomid species, *Glyptotendipes* and *Endochironomus*, from Kearny Marsh sample sites W9 and W22. *Chironomus riparius* was the reference species from the laboratory population. These electrophoresis gel illustrations are a compilation of characteristic species-specific hemoglobin patterns used for identification. A consistent correlation existed between chironomid taxonomic features and hemoglobin band patterns which indicated that SDS-PAGE analysis of Hb has potential to be a suitable identification method in conjunction with taxonomy evaluation.

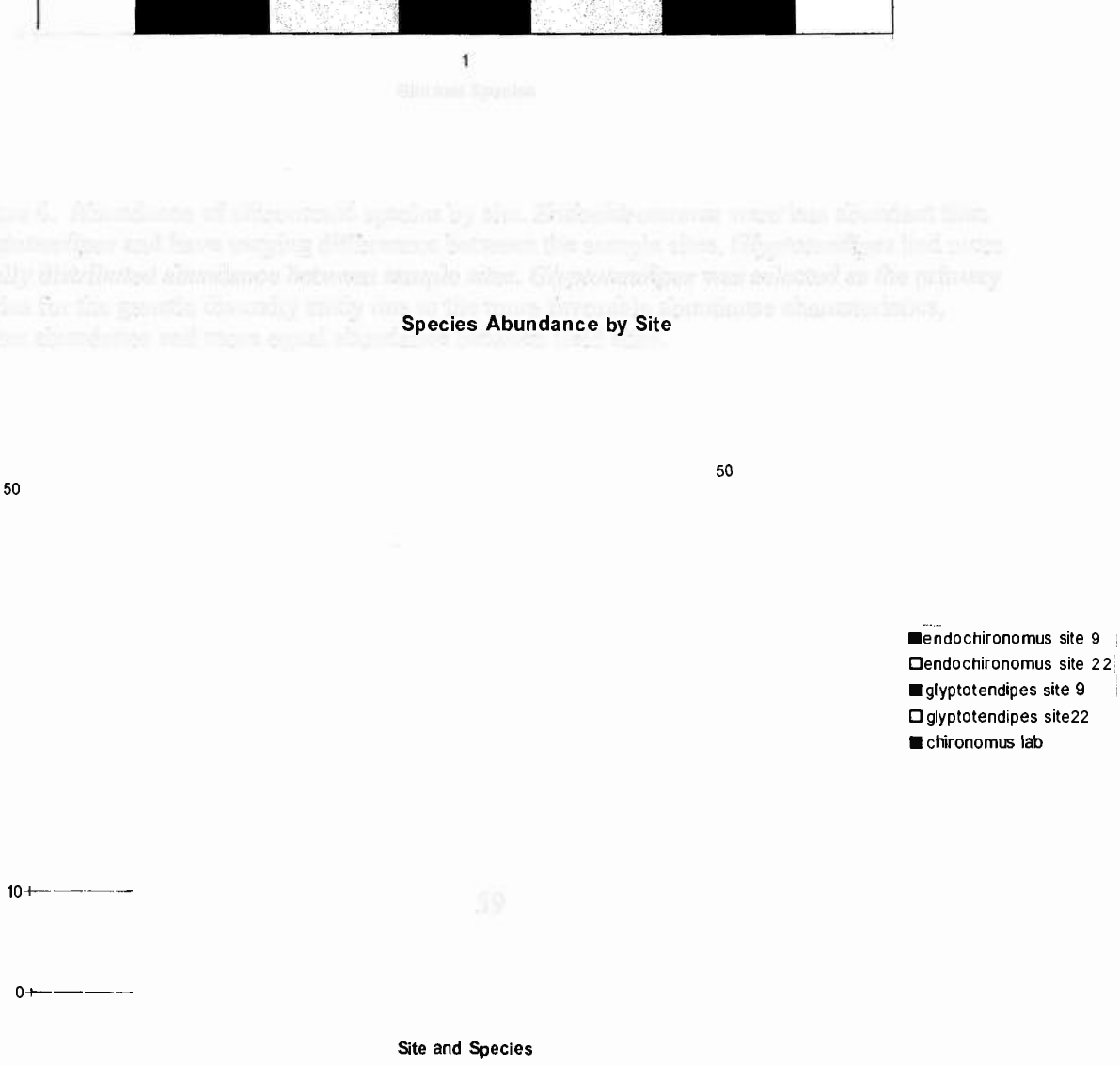



**Figure 4.** Abundance of chironomid species by site. *Endochironomus* were less abundant than *Glyptotendipes* and have varying differences between the sample sites. *Glyptotendipes* had more equally distributed abundance between sample sites. *Glyptotendipes* was selected as the primary species for the genetic diversity study due to the more favorable abundance characteristics, greater abundance and more equal abundance between field sites.

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 0 | 0 | 0 | 3 | 3 | 0 | 3 | 0 | 2 | 2 | 1 | 0 | 2 | 1 | 1 | 2 |
| 1 | 1 | 3 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 1 | 3 | 2 | 1 | 0 | 2 |

**Table 2.** Statistical analysis with Kruskal Wallis test followed by Dunns Post Hoc test. The statistical analysis was applied to determine if a significant difference existed between the total hemoglobin band patterns for all three species. The table shows the data set applied to the analysis. Each column is a distinct band pattern determined for each species with the SDS-PAGE method and described in Table 1. The results indicated a statistical difference existed between the bands because $p<0.05$, Kruskal Wallis test and $p<0.05$ for Dunns Post Hoc test.

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | | 0 | 2 | 0 | 2 | 2 | 2 | 0 | | 0 | 0 | 0 | 0 | | 1 | 1 | |
| 2 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | |
| 0 | 2 | 2 | 2 | 0 | 0 | 0 | | 2 | 2 | 2 | 2 | 0 | 1 | 2 | 2 | 1 | |
| | 0 | 0 | 0 | 3 | 3 | 0 | 3 | 0 | 2 | 2 | 1 | 0 | 2 | 1 | 1 | 2 | |
| | 3 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 1 | 3 | 2 | 1 | 0 | 2 | |

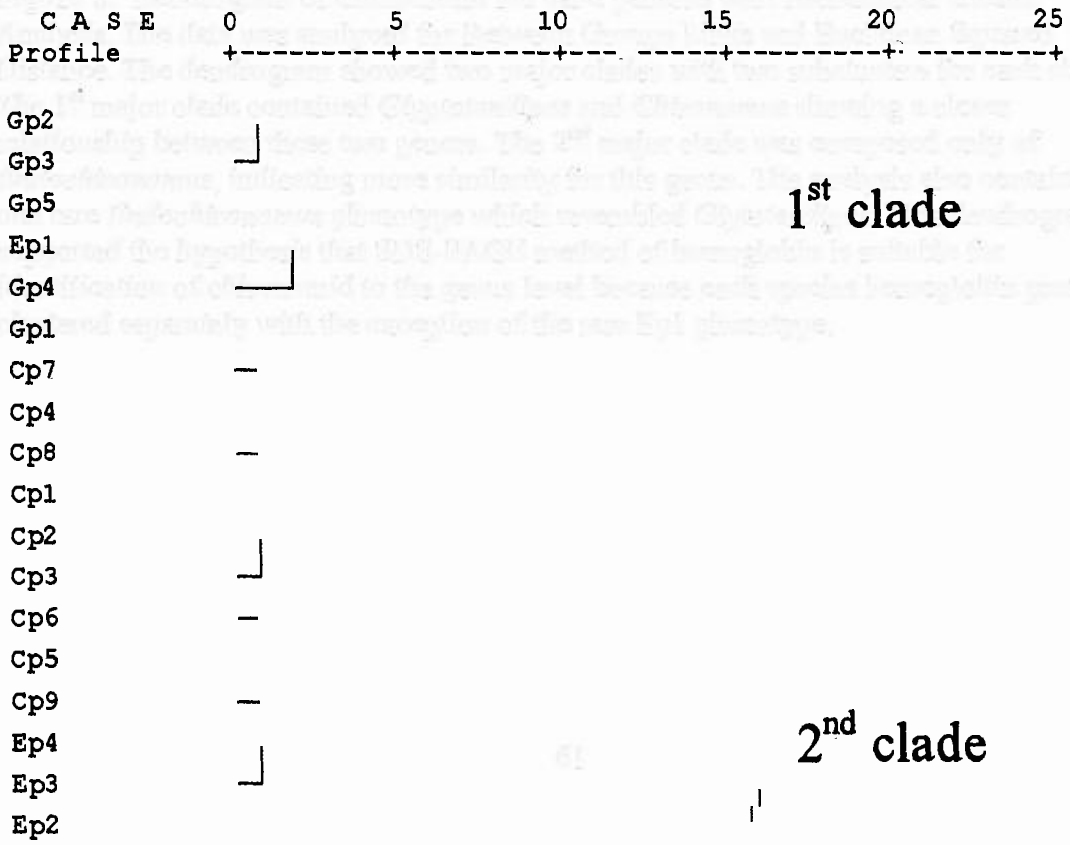



**Figure 5.** Dendrogram of Chironomid Hb band patterns with Hierarchical Cluster Analysis. The data was analyzed for Between Groups Links and Euclidean Squared Distance. The dendrogram showed two major clades with two subclusters for each clade. The 1st major clade contained *Glyptotendipes* and *Chironomus* showing a closer relationship between these two genera. The 2nd major clade was composed only of *Endochironomus*, indicating more similarity for this genus. The analysis also contained one rare *Endochironomus* phenotype which resembled *Glytotendipes*. This dendrogram supported the hypothesis that SDS-PAGE method of hemoglobin is suitable for identification of chironomid to the genus level because each species hemoglobin profile clustered separately with the exception of the rare Ep1 phenotype.

| Site | Mon | Gravel | Sand | Silt +Clay | TOC | SEM | AVS | SEM-AVS |
|---|---|---|---|---|---|---|---|---|
| 9 | Jun | 0.01 | 84.2 | 15.8 | 52.9 | 24.6 | 79.1 | -54.5 |
| 22 | Jun | 0.48 | 69.5 | 30.1 | 11.8 | 1.7 | 5.7 | -4.0 |

**Table 3.** Heavy metal concentrations (mg/kg) in Kearny Marsh Sediments, 2002

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Jun | 1.94 | 15.7 | 40.6 | 12032 | 0.21 | 183 | 23.3 | 61.2 | 79.1 | 222 |
| | | 0.60 | 26 | 16 | NS | 0.20 | NS | 16.0 | 31 | 120 | |
| | | 10.00 | 110 | 110 | NS | 2.00 | NS | 75.0 | 250 | 820 | |

**Table 4.** Heavy Metal Concentrations (mg/kg) in Kearny Marsh Detritus, 2002

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| LEL | 0.6 | 26 | | 0.20 | NS | 16 | 31 | 120 |
| SEL | 10.0 | 110 | | 2.00 | NS | 75 | 250 | 820 |

**Table 5.** Characterization of Sediments from June 2002 Collections.

Total= Includes concentrations for Cd, Cr, Cu, Hg, Ni, Pb, and Zn (mg/kg)
LEL = Lowest Effects Limit based on Ontario Ministry of the Environment guidelines
SEL = Severe Effects Limit based on Ontario Ministry of the Environment guidelines.


>LEL


>SEL

This data was published in *Urban Habitats.* Volume 2. Number 1. ISSN 1541-7115, "Influence of Sediment Characteristics on Heavy Metal Toxicity in an Urban Marsh" Carolyn S. Bentivegna, Joy-Elaine Alfano, Sean M. Bugel, and Katarzyna Czechowicz

| | | | | | | |
|---|---|---|---|---|---|---|
| | 16 | | - | | | - |
| | 23 | | - | - | - | - | - |
| | - | - | | | - | - | |
| | - | - | | | - | - | |
| | - | - | | | - | - | |

undance of Hemoglobin Protein Profiles by Species and Sample Site.

f species hemoglobin profiles, phenotypes, varied between sites and species. ndance was used as a potential indicator of environmental degradation in lso, abundance of hemoglobin phenotypes were used to calculate the diversity ices applied to analyze differences in genetic diversity between species and sample ites. A difference in diversity may have indicated levels of environmental

Key: *Glyptotendipes*: G; *Endochironomus*: E; *Chironomus*: C; p: profile

| | | | | |
|---|---|---|---|---|
| 1 | | 1 | 0/34 | 1/32 |
| 0 | | 1 | 1/34 | 0/32 |
| 16 | | 29 | 13/34 | 16/32 |
| 15 | | 32 | 17/34 | 15/32 |
| 0 | | 3 | 3/34 | 0/32 |
| 36 | | | | |
| 0 | | 1 | 1/23 | 0/13 |
| 1 | | 2 | 1/23 | 1/13 |
| 9 | | 14 | 5/23 | 9/13 |
| 3 | | 19 | 16/23 | 3/13 |
| 13 | | | | |
| | 6 | 6 | | 6/50 |
| | 6 | 6 | | 6/50 |
| | 6 | 6 | | 6/50 |
| | 12 | 12 | | 12/50 |
| | 3 | 3 | | 3/50 |
| | 3 | 3 | | 3/50 |
| | 10 | 10 | | 10/50 |
| | 1 | 1 | | 1/50 |
| | 3 | 3 | | 3/50 |
| | 50 | 50 | | |

**Table 7.** Genetic Diversity Indices. The chironomid hemoglobin genetic diversity levels for the chironomid sample populations inhabiting sites W9, W22, and the laboratory were measured with the Shannon Wiener and Simpson's diversity indices. Both indices account for richness, the number of different categories, and evenness, the degree of equal distribution of abundance. The calculations indicated that the field species had reduced genetic diversity relative to the reference species, *Chironomus*. *Glyptotendipes* had a significantly lower genetic diversity level at site W22 than W9. *Endochironmus* did not have a statistically different genetic diversity level between sites. Yet, *Endochironomus* had twice the abundance of individuals collected in the sample populations at site W9 versus W22. The change in diversity indices may be an indicator of the cumulative environmental degradation degree in Kearny Marsh over the past decades or years.

| Hb Protein Phenotype | Richness (no.of different profiles) | | Evenness (degree of equal abundance) | | | Shannon Index (H) | | | Simpson's Diversity Index (1-D) | | | Intraspecies Abundance | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | W9 | W22 | W9 | W9 | W22 | W9 | W9 | W22 | W9 | | | W9 | | |
| 5 | 0.63 | 0.54 | | 0.44 | 0.38 | | 0.61 | 0.57 | | 36 | 34 | |
| 4 | 0.62 | 0.57 | | 0.37 | 0.34 | | 0.49 | 0.50 | | 23 | 13 | |
| 9 | | | 0.80 | | | 0.77 | | | 0.87 | | | 50 |

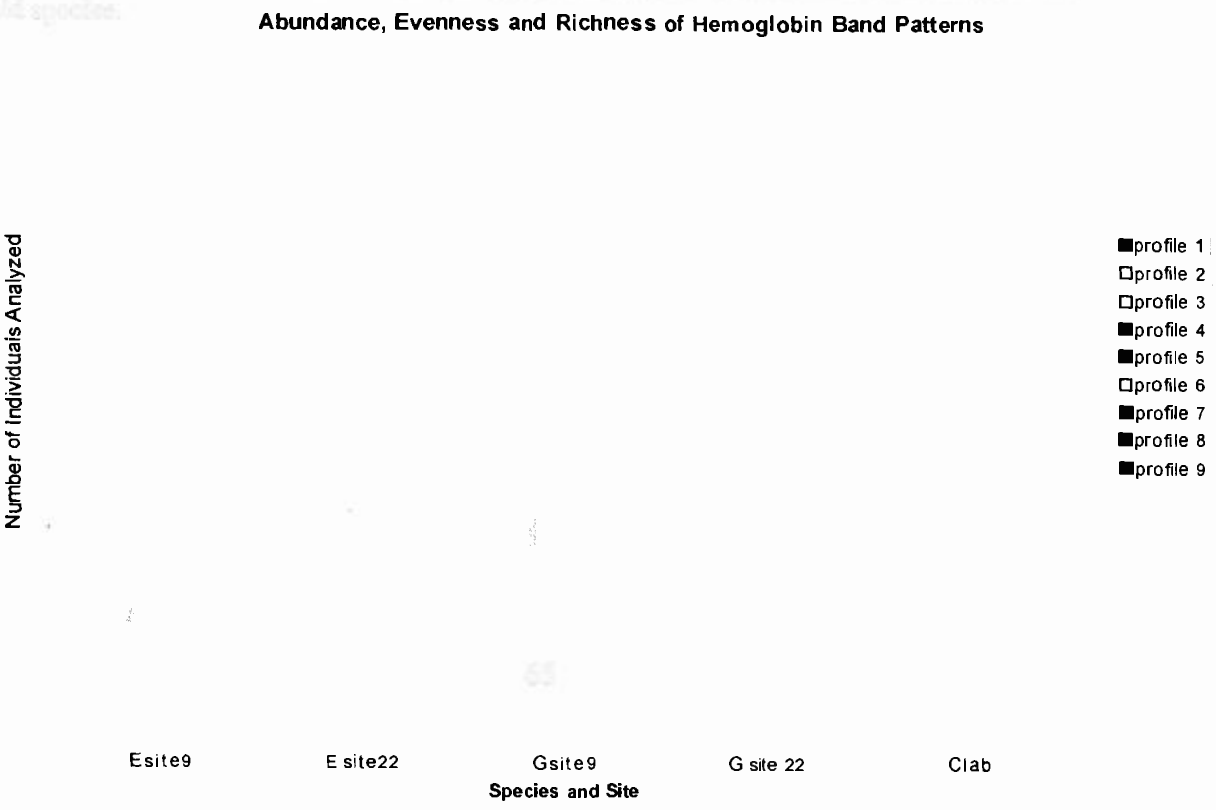



**Figure 6.** Abundance of different hemoglobin gene protein profiles by site and species. The graph indicates richness, evenness, and dominance of species by site. The field species have both lower richness and evenness than the reference species which may indicate low diversity in the field species.

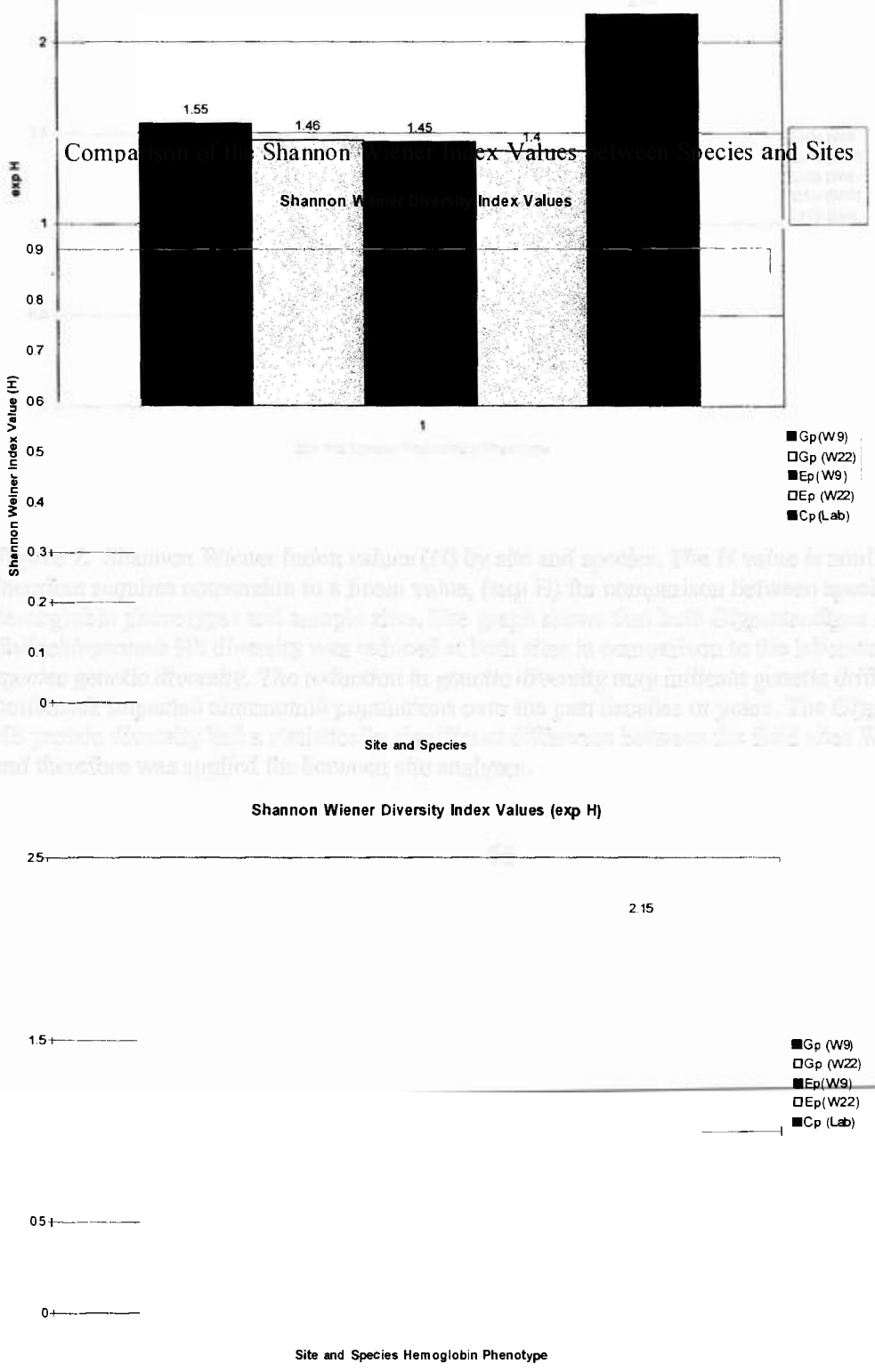
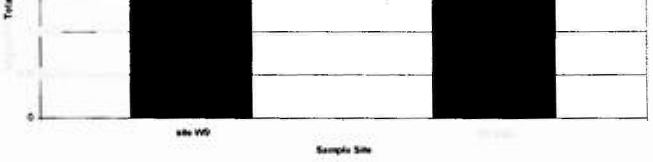
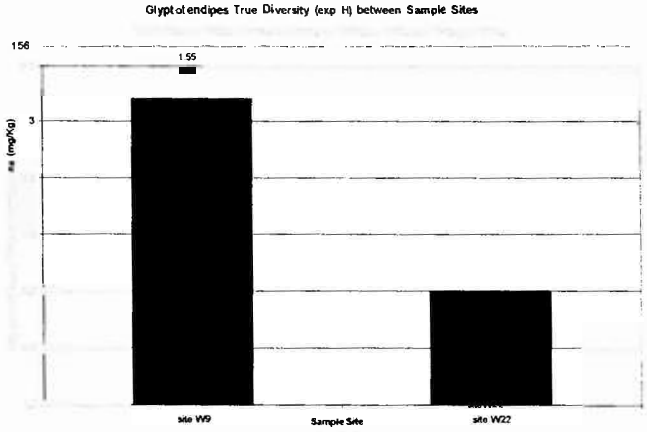



**Figure 7.** Shannon Wiener Index values (H) by site and species. The H value is nonlinear and therefore requires conversion to a linear value, (exp H) for comparison between species hemoglobin phenotypes and sample sites. The graph shows that both *Glyptotendipes* and *Endochironomus* Hb diversity was reduced at both sites in comparison to the laboratory reference species genetic diversity. The reduction in genetic diversity may indicate genetic drift or a bottleneck impacted chironomid populations over the past decades or years. The *Glyptotendipes* Hb protein diversity had a statistically significant difference between the field sites W9 and W22 and therefore was applied for between site analyses.





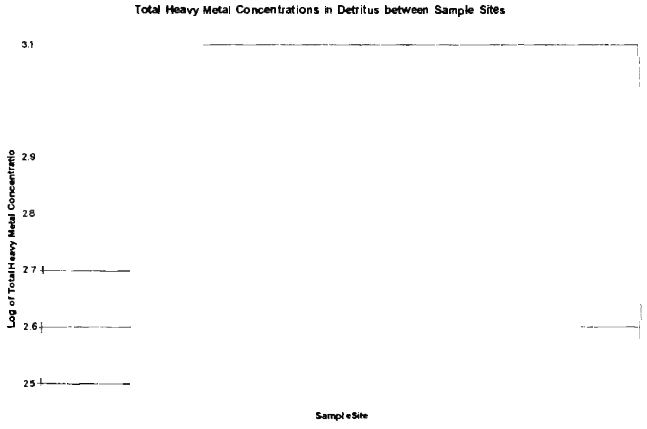



**Figure 8.** Comparison of these three graphs showed a positive correlation between *Glyptotendipes* hemoglobin phenotype true diversity, linear diversity values (exp H) and heavy metal concentrations between the two sample sites. As the heavy metal levels increased, the hemoglobin protein diversity increased. The logarithm of heavy metal concentrations was calculated to narrow the range of scales for easier comparison between the graphs.

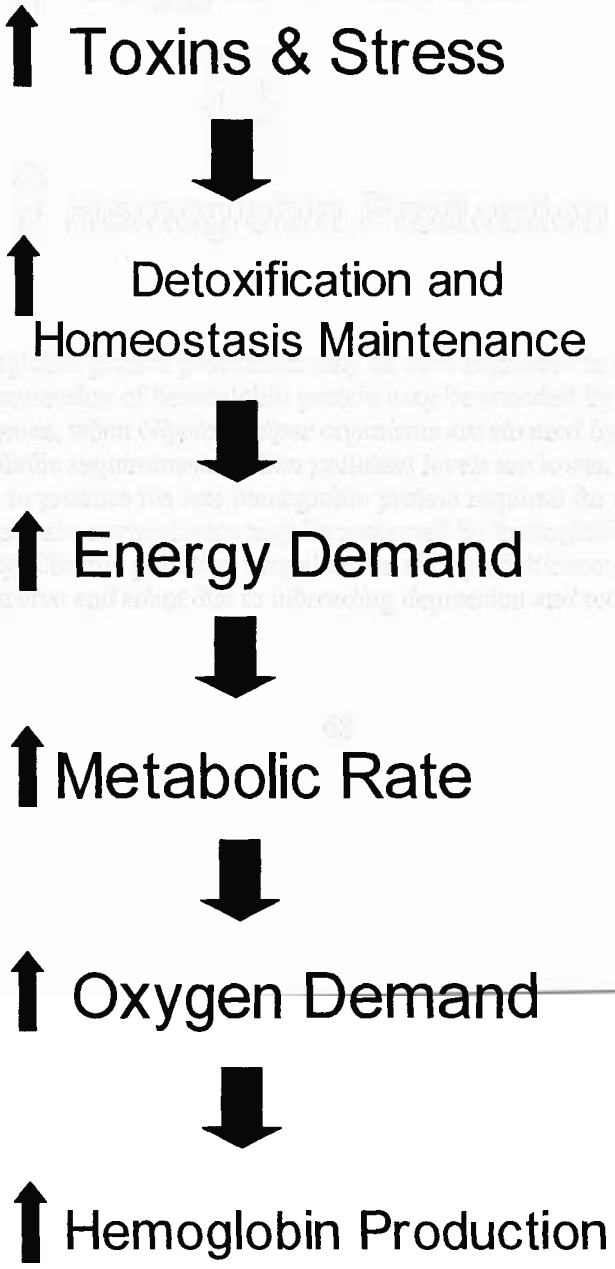



**Figure 9.** Hemoglobin protein production may be over expressed to adapt to environmental stressors. Over expression of hemoglobin protein may be encoded by an increased number of hemoglobin genes, when *Glyptotendipes* organisms are stressed by pollutants as a function of metabolic requirements. When pollutant levels are lower, less hemoglobin genes may be encoded to produce the less hemoglobin protein required for metabolism. Hypothetically, certain contaminants may be measured by hemoglobin protein physiological function diversity. Chronic pollution may alter the ability of chironomid to use this mechanism to survive and adapt due to inbreeding depression and reduced heterozygosity.

| | |
|---|---|
| Not applicable | |
| Increased as more bands are expressed | |
| Organism responds immediately to stressor based on phenotype. | stressors over time. |
| | Negative |
| Increased numbers and types of Hb genes are expressed to increase Hb protein production for metabolism requirements | Reduction in polymorphism of hemoglobin genes |
| | Decreased evenness & increased dominance pattern evolved due to exposure to stressors |
| | Decreased in stressed habitat due to loss of polymorphism at genetic level |
| Temporary based on presence of stressor | An actual change in Hb genotypes in population results in fewer band patterns |

Table 8. Two possible explanations for the change in hemoglobin protein diversity between sample sites and the reference laboratory population. Hypothetically, physiological adaptation mechanisms or population genetic dynamics could have played a role in the change in the hemoglobin diversity measured by SDS-PAGE method and several diversity indices. Further investigation is required to arrive at valid theories on genetic diversity changes due to stressors.

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