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Evaluating Acute Toxicity of Cadmium to Chironomid Using Hemoglobin as a Molecular Biomarker

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EVALUATING ACUTE TOXICITY OF CADMIUM TO CHIRONOMID USING HEMOGLOBIN AS A MOLECURAR BIOMARKER

BY

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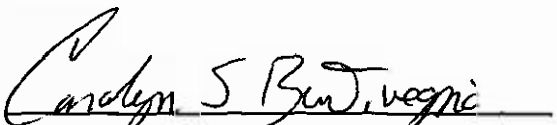

CHAIRPERSON, BIOLOGY DEPARMENT

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Abstract

Effects of cadmium (Cd), a heavy metal contaminant, on hemoglobin were investigated in aquatic midge fly larvae of the Family, *Chironomidae*. In this study, chironomid populations from one polluted site, Kearny Marsh, New Jersey, two reference sites, North East Creek, and Bass Harbor, ME, and a laboratory population were taxonomically identified and tested for their sensitivity to Cd. Heavy metals in tissue, water and sediment were analyzed using Atomic Absorption Spectroscopy. Water quality at each site was assessed by measuring pH, dissolved oxygen (DO), oxidation-reduction potential (eH), salinity and temperature. Hemoglobin proteins in individual larvae were easily detected using polyacrylamide gel electrophoresis (SDS-PAGE). As a model heavy metal toxicant, the effect of Cd on the different hemoglobin proteins was investigated using the following concentrations; 0 μM, 0.3 μM, 3.0 μM, and 30.0 μM. Exposure times included 0 h and 96 h. Hemoglobin band profiles from each individual were distinguished by the presence or absence of bands as well as band intensities and compared to other individuals at different times and concentrations. Results showed that the expression of band profiles varied as concentrations of Cd and duration of exposure time increased. It was noted that the low molecular weight hemoglobin proteins (< 11 KD) became lighter and/or

disappeared in many individuals. This overall trend suggested that the expression of hemoglobin band profiles could give useful information for detecting presence of heavy metals in field studies.

Introduction

Anthropogenic activities, especially those in highly urbanized regions, degrade the quality of water, air, and soil thereby affecting the sustainability of ecosystems. In order to protect ecosystems it is necessary to measure to what extent they can be altered without impacting native organisms. In addition, one must be able to evaluate environmental changes before and after restoration in order to determine the success of remediation. Biomarkers in wild organisms living in a stressed ecosystem provide information on environmental quality. For example, hemoglobin protein in aquatic midge fly larvae (Family: *Chironomidae*) is a promising biomarker for evaluating environmental health in aquatic ecosystem.

Chironomids are abundant benthic macroinvertebrates that are distributed globally, live in a wide range of salinities and are highly tolerant to a range of adverse environmental stressors. Chironomids are a major food source for both vertebrate and invertebrate organisms (Ha, 2008). Environmental contamination can influence chironomids at a molecular and cellular level (Timmermans, 1992; Michailova, 2004; Lee, 2006). Chironomids have abundant levels of hemoglobin in their hemolymph which allows the organism to live in suboxic sediment. The work presented in this research utilized this characteristic to develop a biomarker that has the potential to

identify wild chironomid genus as well as detect toxic concentrations of heavy metal contaminants by measuring changes in levels of hemoglobin proteins. High levels of hemoglobin are easily obtained from individual chironomids (Tichy, 1975).

Hemoglobin in chironomids exists as monomers and dimers. This has been shown for two different genera, *Chironomus ramosus* (CR) and *Chironomus thummi thummi* (CTT) (Das, 1996). The larvae of chironomids contain up to 16 hemoglobin proteins and 12 globin polypeptides (Gruhl, 2000). These many different hemoglobin genes in the larvae of chironomids are expressed in a stage and tissue-specific way (Schmidt, 1988). Using the cyano-methemoglobin method to determine total hemoglobin contents in hemolymph, researchers have found that 60 % ofhemolymph consisted of protein and hemoglobin accounted for 92% of the proteins found (Choi, 2004; Ha, 2004). This high protein content has made it easy to detect to hemoglobin on gels using simple stains such as commaisse blue (Jacobs and Bentivegna, 2005). Hemoglobin proteins show a high degree of polymorphism, high affinity for oxygen and extracellular localization (Osmulski and Leyko, 1986). It is generally accepted that the presence of hemoglobin in invertebrates allows them to adapt to adverse environment conditions under low oxygen level (Lee, 2006).

The proposed biomarker has potential for monitoring environmental health

and also identifying genus. Chironomids are often used for laboratory and sediment toxicity testing. However, they are rarely chosen for field studies because it is difficult to distinguish the larvae of one species from another. A field study done by Jacobs and Bentivegna (2005) found that polymorphic hemoglobin profiles generated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) could be used to confirm taxonomic identifications performed using head capsule morphology (Epler, 2001). While species shared some of the same bands, the pattern of bands was unique to a particular genus. This finding was limited to two species from one location and requires further development by studying more species from a variety of habitats.

For this project, three sampling sites were chosen: Kearny Marsh (KM) in New Jersey and North East Creek (NEC) and Bass Harbor (BH) in Mount Desert Island, Maine (USA). KM is located in a highly urbanized area in the NJ Meadowlands, which has been exposed to chronic environmental abuse including contamination from nearby landfills, urban encroachment, and automobile pollution. During the Industrial Revolution (1760-1830) 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 development was unguided and the area was contaminated with toxic waste disposal from industries that were in close proximity and raw sewage. Recent work evaluated the environmental condition of KM (Bentivegna et al, 2004). A four year study conducted by Bentivegna and coworkers evaluated a novel capping substrate, AquaBlok (clay-based capping material) which was intended to control sediment contamination. This study involved collecting benthic macroinvertebrates for biodiversity studies and analyzing whole chironomid tissues for heavy metal concentrations. Results showed levels of heavy metals above national criteria's for sediment and water at KM (data unpublished). Both NEC and BH are located in a rural area of Mount Desert Island, within or adjacent to Acadia National Park and it was anticipated that they provided a healthy and intact habitat. The Acadia Nation Park has a long term water monitoring program that deals with atmospheric deposition and eutrophication for freshwaters in Mount Desert Island (Acadia National Park, 2004). This monitoring system allows bodies of freshwater to be designated and protected as water supplies or for recreational use across the island. Research has found that sediment mercury levels at NEC and BH in Acadia National Park are 10 times lower than KM (Bank, 2006).

We studied Cd as a heavy metal toxicant. The concentration of Cd in sediment

at KM was above the lowest effects limit (based on Ontario Aquatic Sediment Criterion) before and after capping AquaBlok (Jajeda, 2007). The lowest effects limit was found to be 0.6 mg/kg and all sites had above 2.0 mg/kg of Cd. Interestingly, chironomids continue to survive in that environment as heavy metals tends to bind to sediments and chironomids are most active in close proximity to sediments. Apparently, they survive this type of exposure due to high levels of tolerance to Cd compared to other heavy metals and organic pollutants (Ha, 2008). In chironomid, the 24 h lethal concentrations (LC) of Cd are 93.05 mg/L, 169.5 mg/L, and 308.08 mg/L, for the LC10, LC50 and LC90, respectively (Ha, 2008). Previous studies that used macroinvertebrate as a model organism have focused on physiological effects. Yet, our main interest was neither mortality nor abnormal growth but hemoglobin content as a sublethal molecular biomarker. The relationship between Cd and hemoglobin protein content has not yet been defined. Therefore, the purpose of this study was to develop a biomarker that could detect changes in hemoglobin protein levels of chironomid due to heavy metal exposure and to determine if pre-exposure to sediments contaminated with heavy metals would influence a population's sensitivity to Cd as detected by this biomarker. If populations do show differential sensitivity, it would suggest that there is a genetic component to the mechanism of action which

would support the use of this biomarker for biomonitoring.

In this project, three different populations (KM, NEC and BH) of chironomid were tested for their sensitivity to Cd in 0 and 96 hour concentration-response experiments. Cd was chosen for its high bioavailability in many freshwater systems. Experimental endpoints included mortality and hemoglobin band intensity. Individuals from all three sampling sites and a laboratory population were exposed to Cd. Cd concentrations were low enough to keep the individuals alive and high enough to detect changes. The highest concentration tested was about 20 times lower than the LC10. As an analytical tool, SDS-PAGE was used to compare hemoglobin protein expression among different chironomid individuals. The staining of gels with commaisse blue allows one to detect the presence, absence and intensities of bands using the naked eye. These parameters can then be used to establish a band profile for every individual and evaluate the response of hemoglobin. Hemoglobin responses could then be compared with heavy metal exposures by measuring Cd and other heavy metals in whole body tissue of chironomid, water, and sediments from all field sites. Hemoglobin polymorphic profiles as well as morphological distinctions in head capsules (Epler, 2001) were used to identify the field species. This allowed not only population responses to be evaluated but also the responses of the species found.

Materials and Methods

Sample Collection and Site Selection

Larvae of chironomid populations were collected from three different sampling sites and from our established laboratory culture. The sites were Kearny Marsh (KM) (Figure 1) in the New Jersey Meadowlands, North East Creek (NEC) (Figure 2) and Bass Harbor (BH) (Figure 2) in Mount Desert Island (MDI), Maine. For KM, chironomids were collected from Hester-Dendy samplers placed above sediments for one month. For MDI sites, chironomids were collected by hand from submerged vegetation. Wild chironomids were held in site water for approximately 24 h before use in toxicity experiments. The laboratory population of chironomid was the genus *Chironomus riparius*. They were cultured in 20 gallon fish tanks containing acid-washed play sand (American Stone-Mix, Inc., Towson, Maryland) as substrate. Culture water consisted of tap water that had been particle and carbon filtered using CDPRM1206 and CDFC01204 filters (Millipore Corporation, Billerica, Massachusetts) and had a hardness of approximately 170 mg/L. Chironomids were fed ground fish food (TetraCichlid, Tetra GMBH, Germany) weekly and held at a photoperiod of 12 light/ 12 dark. Temperature was 21 ± 0.5 °C. The same water, sand and culturing conditions were used in toxicity tests with the laboratory population (see

below).

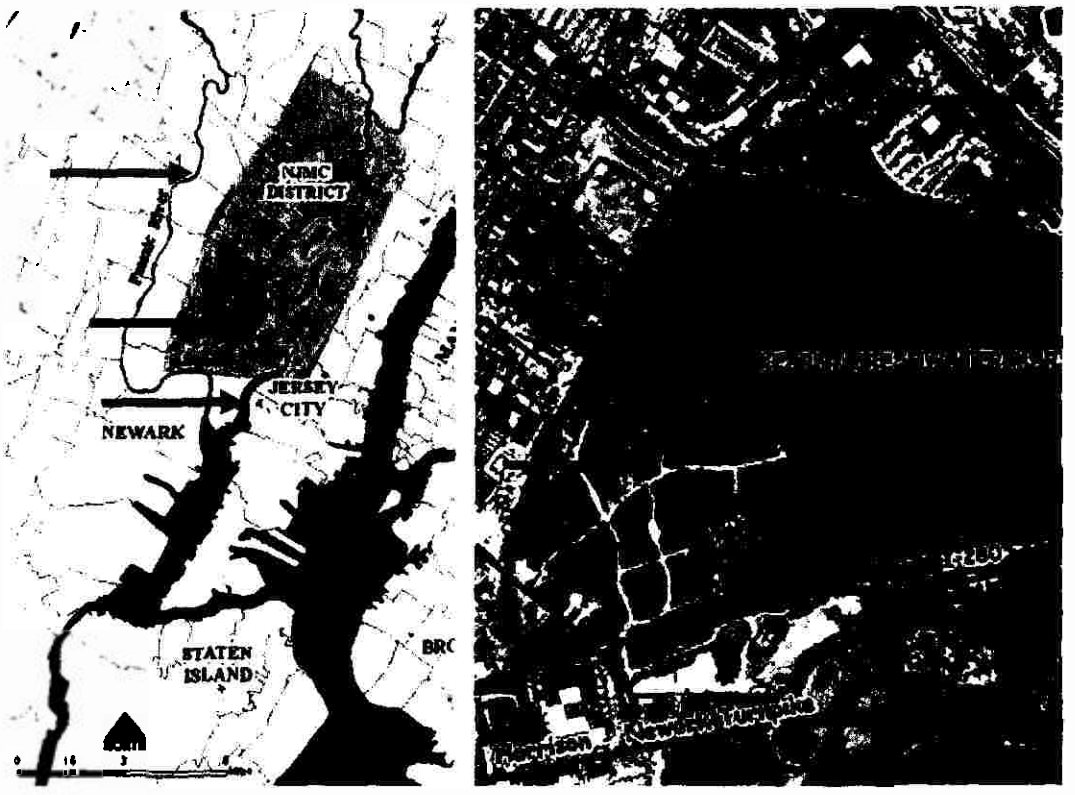


Figure 1: Kearny Marsh. Left: Map showing nearby major cities. Black arrow indicates Kearny Marsh sites. Kearny Marsh is located in the New Jersey Meadowlands between the Passaic River and Hackensack Rivers as indicated by the orange arrows. Right: Map showing nearby major highways. Red arrows indicate landfills. In 2008, methane gas produced by the decomposing trash at the landfills was collected and converted into electricity.

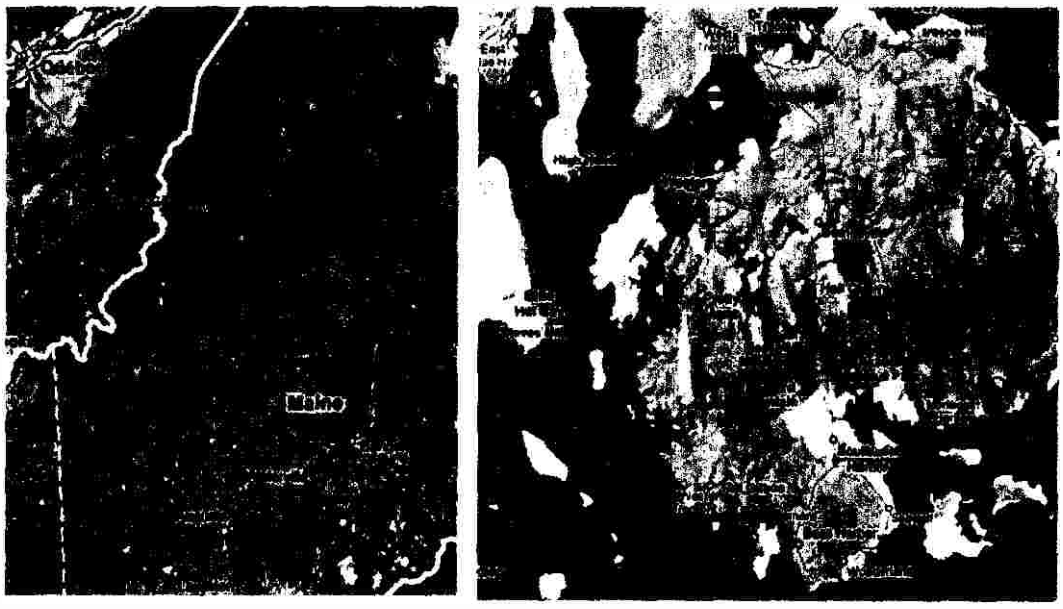


Figure 2: Mount Desert Island, Maine. Left: Map of Maine State. Black arrow indicates location of Mount Desert Island. Right: Map of Mount Desert Island. Red arrow indicates North East Creek and blue arrow indicates Bass Harbor. North East Creek is located in Acadia National Park region and Bass Harbor is located in close proximity to sea water. *Dicrotendipes* was the only species found in North East Creek. Both *Chironomus* and *Dicrotendipes* were present in Bass Harbor.

Water quality

Water quality parameters of pH, redox potential, salinity, dissolved oxygen (DO) and temperature were monitored during chironomid collection at all field sites. The parameters were measured using an YSI meter, model 556 (YSI Environmental, Yellow Springs, OH). The pH was measured for laboratory culture water using a Corning pH meter 240. It was found to be 7.19 in the 30.0 μM Cd treatment at 96 h.

Acute toxicity test

Chironomids were exposed to various concentrations of cadmium chloride (Cd) for 0 or 96 h. For laboratory chironomid population, two replicates of 15 larvae were exposed to 0 μM, 0.3 μM, 3.0 μM, or 30.0 μM Cd. This corresponded to 0, 0.055, 0.55 and 5.5 mg/L. The Cd was spiked into 1500 mL of test water (see above) creating a stock solution. Each stock was held in a separate polypropylene carboy and aerated throughout the experiment. Each treatment contained 15 larvae chironomids, 40 g of sand (see above) and approximately 250 mL of test water. Chironomids were fed 0.5 mL of 0.04 g/ml food (see above) and approximately 80 % of the test water was replaced daily (static renewal) from the stock. The hardness of water was measured one day prior to the start of the experiment (LaMotte test kit, Carolina, Burlington, NC). All field experiments followed the same setup as the laboratory

experiments, except field water was used as the test water and MDI experiments had an ambient photoperiod and temperature range of 19-22 °C. For wild chironomid populations, two replicates of 15 larvae were treated with 0 μM, 0.3 μM, 3.0 μM, 30.0 μM of Cd for 96 h. Test water was made by spiking Cd into approximately 6.0 L of field water and aerating as described above. For all experiments, 10 larvae were used for hemoglobin protein analysis and 5 larvae were used for heavy metal analysis.

Preparation of Head Capsules Samples

Head capsules were collected and mounted for species identification. The process involved decapitating chironomids and fixing head capsules in 70 % ethanol. Mounting of head capsules was done according to Epler (2001). Head capsules were boiled in 10% KOH for 10 minutes and then placed in ddH_2O to wash out KOH (30 seconds finger flicking). They were placed in glacial acetic acid (Pharmco-Aaper, Brookfield, Connecticut) (30 seconds finger flicking) and then in a solution of 50% xylene substitute and 50% Euperal for 24 h. Drops of Euparal were placed on the slides and four heads were mounted per slide. They were used for taxonomic identification according to Epler (2001). Identification was only done to the genus level to date.

Preparation of Hemolymph Samples

Hemolymph was extracted from each larva by decapitation and bleeding out onto a microscope slide. Approximately 2 µL of hemolymph was drawn up and transferred into a 1.5 mL centrifuge tube containing 14 µL of Lithium Dodecyl Sulfide (LDS) sample buffer (Invitrogen, Carlsbad, California), 2 µL of 8M urea (Qiagen, Valencia, California) and 2 µL of 10X 2-mercaptoethanol (Sigma Chemical Co., Saint Louis, Missouri).

Sodium Dodecyl Sulfate (SDS) Polyacrylamide gel electrophoresis

Hemolymph proteins were separated on 16.5% TRIS-GLYCINE SDS polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA). All hemolymph samples were denatured in a heating block at 68 ℃ for 10 minutes. Molecular weight standard solution was prepared with 4 µL of See Blue Molecular Weight Standard (Invitrogen, Carlsbad, California) and 4 µL of LDS sample buffer. Each sample (5 µL) was run on a gel at 100 mA for approximately 2 hours. This was followed by gel washing and staining.

Gel Staining and drying

Gels were rinsed 3 times for 5 min in 200 mL ddH_2O, and then fixed for 15 min in 50 % methanol (Pharmco-Aaper, Brookfield, Connecticut) and 7 % glacial

acetic acid (Pharmco-Aaper, Brookfield, Connecticut). After fixing, the gel was washed with deionized water for 30 min, and then it was shaken in 25 ml Gel Code Blue Strain reagent (Pierce, Rockford, Illinois) for 45 min. The stained gel was rinsed two times using 200 mL of boiled ddH_2O for 30 min each. After rinsing, the gel was placed in 25 mL of drying solution for 5 min (20 % ethanol, 4 % glycerol, 76 % distilled water) and dried on blotting paper using a gel dryer for 50 min at 68 °F.

Hemoglobin protein expression profiling

For hemoglobin, every scorable, polymorphic band was assigned as a variable: hemoglobin (Hb) band 1- 17 (Figure 3). A binary system scored whether or not the individual did or did not have each band and its relative intensity. Profiles were constructed using a 17 band system scored on a 0 to 3 scale where 0 = absent, 1 = present but faint, 2 = prominent band, and 3 = darkest band. Band intensity was estimated qualitatively by visualizing the prepared gel. Further analyses lead to grouping the bands based on size (KD) and sensitivity (band intensity) such that high bands were defined as high molecular weight and numbered from 1 to 7 and low bands were defined as low molecular weight and numbered from 8 to 17.

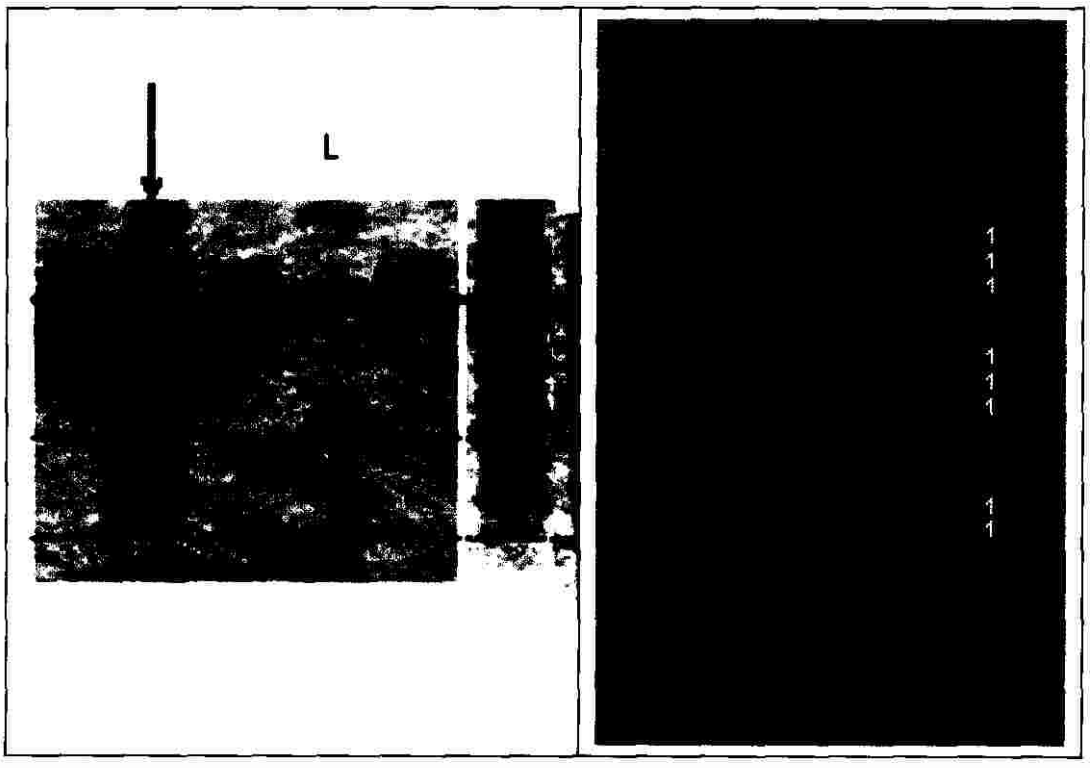


Figure 3: Hemoglobin protein band profiling. **Left**: SDS PAGE showing hemoglobin band patterns for individuals that participated in a toxicity test. Each band pattern represented one individual. The red lines align with the ladder (L): the top, middle and lower lines are 16, 11 and 4 Kd, respectively. The black arrow indicates a specific band profile of an individual generated by the profiling system on the right under band intensity.. All other lanes represent hemoglobin bands of different individuals. **Right:** Each band was numbered from 1 to 17. The profile was determined by presence (1) or absence (0) of a band or by band intensity: dark, moderate, light or absent were 3, 2, 1, or 0, respectively.

Digestion of chironomids for heavy metal analysis

Chironomids collected for heavy metals were stored at -80 ℃ until analyses. Chironomids (5 per sample) were placed together in 1.5 mL centrifuge tubes and oven dried overnight at 80 ℃. After drying, their dry weight was determined and they were digested in 0.2 mL of concentrated 70 % HNO_3 (Pharmco-Aaper, Brookfield, Connecticut). The tube was placed in oven at 80 ℃ overnight for evaporation of nitric acid. After the acid evaporated, 0.2 mL of 30% H_20_2 (VWR International, West Chester, Pennsylvania) was added and it was evaporated at 80 ℃. The trace metals were re-dissolved in 1.5 mL of 0.1 N HNO_3 for atomic absorption spectroscopy (AAS).

Digestion of water for heavy metal analysis

Water samples included those from each Cd treatment and control (0 μM Cd at 96 h). The control served as the sample for site water analysis. Samples (50 mL) were collected at the same time as the chironomids, fixed with 0.5 ml of concentrated nitric acid (Sigma-Aldrich, St. Louis, MO) and stored at 4 ℃ until analysis. Each water sample (50 mL) was placed in a 100 mL beaker. HNO_3 (3 mL)was added and the sample was heated until the volume of liquid was around 25 mL. The beaker was covered with a watch glass for 30 min. After cooling, the solution was transferred

into a 50 mL volumetric flask. The beaker was rinsed with ddH_2O and the combined contents were diluted to 50 mL with ddH_2O. The contents were transferred into a 50 mL conical for metal analysis.

Digestion of sediments for heavy metal analysis

Sediments were collected from MDI sites only. They were placed in one liter, acid-washed, polypropylene containers and stored at 4 ℃ until analysis. Each sample was processed by placing the sediment into a 300 mL beaker and drying it on a hot plate at 200 ℃ for overnight. Each sample was transferred to a Petri-dish and placed in a fume hood for complete evaporation of any moisture. The dried sample was crushed into powder form and 0.200 g of dry sediment was mineralized with 7 mL of 70% HNO_3 in a 10 mL Teflon bomb in a microwave digester. The solution was micro-waved and sediment/solution was transferred into 25 mL beaker. Teflon bomb was washed out with ddH_2O at least 3 times. The combined sediment/solution was boiled down to a volume of 10 mL using a hot plate. After the sample was cooled, the reduced sediment/solution was transferred into a 10 mL volumetric flask and diluted with ddH_2O to a final volume of 10 mL. This solution was transferred into 15 mL conical for metal analysis.

Heavy metal analysis

Heavy metals in chironomid, sediment, and water were analyzed by Atomic Absorption Spectroscopy (AAS). The equipment used was a Varian AA-220 provided by the Meadowlands Environmental Research Institute (MERI) in Lyndhurst, N.J. Heavy metals were analyzed in chironomids collected from KM, BH and NEC toxicity tests and from untreated chironomids (0 d) representative of field populations. Heavy metals were not analyzed in chironomids from toxicity tests of the lab population. Chironomids collected from toxicity tests were only analyzed for Cd and showed the ability of that population to accumulate Cd. Those representing field populations were collected on day zero of the experiment, usually within 24 h of collection, and showed the pre-existing concentrations of metals. They were analyzed for Cd, Cr, Cu, Fe, Ni, Pb, and Zn. Water samples from Cd treatments were analyzed for Cd only in order to determine actual exposure levels. Water samples from 96 h control (0 μM Cd) were analyzed for Cd, Cr, Cu, Fe, Ni, Pb, and Zn in order to determine heavy metal concentrations in site water. Sediments were analyzed for Cd, Cr, Cu, Fe, Ni, Pb, and Zn in order to determine the environmental levels to which each population had been exposed. Data on sediment from KM was obtained from samples collected in fall 2007 and previously analyzed using the procedures described

here. The AAS method for chironomids and water samples utilized a graphite furnace. The minimum detection limits (MDL) were as follows: Cd= 0.14 µg/L, Cr= 1.03 µg/L, Cu= 0.98 µg/L, Ni= 1.51 µg/L, and Pb= 0.96 µg/L. The method for sediments was flame with MDLs as follows: Cd= 3.73 µg/L, Cr= 29.1 µg/L, Cu= 4.75 µg/L, Fe= 18.2 µg/L, Ni= 21.0 µg/L, Pb= 76.8 µg/L, and Zn= 5.68 µg/L.

Data analysis

Effects of Cd on hemoglobin were determined as follows. The intensity of bands 1-7 or 8-17 was summed for each individual. These values were then averaged for individuals from the same concentrations in order to find treatments effects. Responses to Cd were also determined by averaging band intensities of all individuals within a genus regardless of site. This allowed the sensitivity of one genus to be compared to another. Statistical differences between populations and treatments were determined using one-way ANOVA and Tukey posthoc test, $p \leq 0.05$.

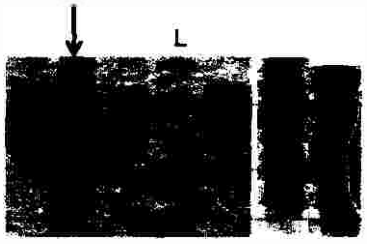
Results

Genus identification

Every individual was taxonomically identified to the level of genus using head capsule morphology and expression of hemoglobin protein bands as detected by SDS-PAGE. Three different genera were found: *Glyptotendipes* (Figure 4), *Dicrotendipes* (Figure 5), and *Chironomus* (Figure 6). KM contained both *Glyptotendipes* and *Chironomus* with *Glyptotendipes* being the dominant genus. NEC contained only *Dicrotendipes*. BH contained both *Chironomus* and *Dicrotendipes* with *Chironomus* being the dominant genus. Each individual's head capsule morphology was compared to its corresponding hemoglobin profile in order to classify the individual. Morphological features of a *Glyptotendipes* included rounded teeth with shallow grooves between them. The head capsule of *Chironomus* possessed sharp and pointy teeth with deep grooves between teeth. *Dicrotendipes'* head capsule was distinguished by one round tooth in the middle with evenly spread out teeth with grooves that are deeper than *Glyptotendipes* but more shallow than *Chironomus*. It was found that a hemoglobin band profile may be used to identify genus by analyzing the first top 3 bands. A common feature of the hemoglobin protein profile for *Glyptotendipes* (Figure 4) was that they showed presence of all 3 bands with strong intensity at band 1 and band 2. *Dicrotendipes* (Figure 5) showed presence of band 1

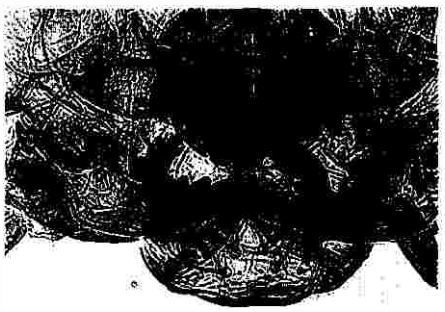
and band 3 but absence of band 2. *Chironomus* (Figure 6) showed opposite profiling to *Dicrotendipes* where it showed absence of band 1 and band 3 (if present, very faint) but showed presence of band 2 with strong intensity.

Head Capsule: Glyptotendipes

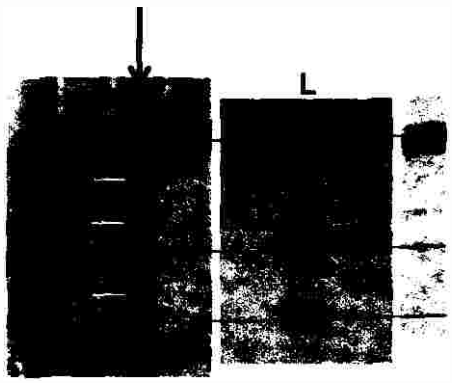


Hemoglobin band	Presence=1 Absence=0	Band Intensity 3 > 1
1	1	3
2	1	2
3	1	1
4	1	1
5	1	1
6	1	2
7	1	1
8	1	1
9	1	1
10	1	1
11	1	2
12	0	0
13	1	2
14	1	1
15	1	1
16	0	0
17	1	0

Figure 4: Hemoglobin profile of *Glyptotendipes* and its corresponding head capsule. *Glyptotendipes* was the dominant genus in KM. A common feature of *Glyptotendipes* is the presence of the first 3 bands with strong band 1. Morphological features of a *Glyptotendipes* are the round teeth with shallow grooves between them. The black arrow indicates the specific band profile on the right under band intensity. All other lanes represent hemoglobin bands of different individuals. The red lines align with the ladder (L): the top, middle and lower lines are 16, 11 and 4 Kd, respectively. Total 17 bands were part of the hemoglobin band system.



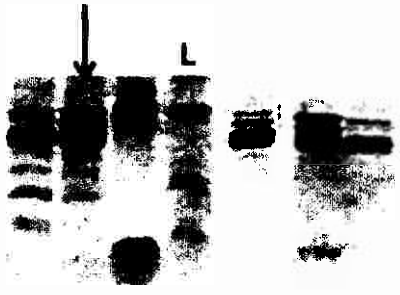
Head capsule: Dicrotendipes



Hemoglobin band	Presence =1 Absence=0	Band Intensity 3 > 1
1	1	3
2	0	0
3	1	3
4	0	0
5	1	2
6	1	2
7	1	1
8	1	1
9	1	2
10	0	0
11	0	0
12	1	2
13	0	0
14	0	0
15	0	0
16	0	0
17	1	2

Figure 5: Hemoglobin profile of *Dicrotendipes* and its corresponding head capsule. *Dicrotendipes* was the only genus found in NEC. A common feature of *Dicrotendipes* is the presence of the strong first and third bands with absence of band 2. Morphological features of a *Dicrotenipes* are the one round tooth in the middle with evenly distributed teeth with grooves that are deeper than *Glyptotendipes* but shallower than *Chironomus*. The black arrow indicates the specific band profile on the right under band intensity. All other lanes represent hemoglobin bands of different individuals. The red lines align with the ladder (L): the top, middle and lower lines are 16, 11 and 4 Kd, respectively. Total 17 bands were part of the hemoglobin band system.

Head Capsule: Chironomus



Hemoglobin band	Presence =1 Absence =0	Band Intensity 3 > 1
1	1	0
2	0	3
3	1	0
4	0	3
5	1	0
6	1	2
7	1	3
8	1	1
9	1	1
10	0	0
11	0	2
12	1	0
13	0	1
14	0	1
15	0	0
16	0	0
17	1	1

Figure 6: Hemoglobin profile of *Chironomus* and its corresponding head capsule. *Chironomus* was the dominant genus in BH. A common feature of *Chironomus* is the absence of the first band with strong band 2. Morphological features of a *Chironomus* are sharp and pointy teeth with deep grooves between teeth. The black arrow indicates the specific band profile on the right under band intensity. All other lanes represent hemoglobin bands of different individuals. The red lines align with the ladder (L): the top, middle and lower lines are 16, 11 and 4 Kd, respectively. Total 17 bands were part of the hemoglobin band system.

Hemoglobin protein expression response to Cd

Exposure of the laboratory chironomid population to Cd caused modifications in the expressions of individual hemoglobin proteins. As concentrations of Cd increased, the low molecular weight hemoglobin proteins (~4 KD to ~11 KD) were affected the most i.e. became lighter and disappeared among many individuals (Figure 7).

Band profiles were constructed for all individuals according to their unique hemoglobin responses to Cd. The profiles were plotted on a graph to visualize the responses. The changes found in profiles among all individuals from KM are shown in Figure 8. Table 1 shows the average band intensities± one standard deviation (SD) for the two genera collected from KM combined. *Chironomus* individuals carried more low bands than *Glyptotendipes*, although *Glyptotendipes* was the dominant genus. The average of low bands at 0day was 13.67 ± 2.25 for *Chironomus* and 2.0 ± 4.40 for *Glyptotendipes*. The averages of low bands for the 0 µM treatment at 96 h for was 11.75 ± 0.50 for *Chironomus* and 4.50 ± 3.15 for *Glyptotendipes*. This indicated that the intensity of low bands increased in *Glyptotendipes* after being removed from the field and held in control conditions. It was observed that *Chironomus* showed a statistically significant reduction in low band intensity at 3.0

μM (3.80 ± 4.27), and no individuals of this genus were found at 30.0 μM. This concentration of Cd may have been too toxic for *Chironomus* to survive. *Glyptotendipes* showed a decrease in band intensity at 3.0 μM (3.70 ± 3.40) and 30.0 μM (1.5 ± 2.62) compared to 0.0 uM (4.50 ± 3.15) Cd at 96 h suggesting an effect of high Cd concentrations over time; however it was not statistically significant.. Overall, the responses at KM appeared to be species specific. *Chironomus* was sensitive to Cd where as *Glyptotendipes* did not appear to be.

Figure 9 presents hemoglobin responses of all individuals from NEC. Table 1 shows the average band intensity of all individuals from NEC. A concentrated-related response was observed for the NEC population. The average of low band intensity for 0day was 3.13 ± 2.17. The averages of low band intensities at 96 h were 3.71 ± 2.21, 5.54 ± 1.92, 1.62 ± 1.50 and 0.84 ± 0.07 for 0 μM, 0.3 μM, 3.0 μM, and 30.0 μM, respectively. The above averages for band intensity were calculated by combining both replicates. The only genus found in NEC, *Dicrotendipes*, was observed to have a naturally low number of lower bands regardless of Cd exposure. A slight induction of low bands apparently occurred in 0.3 μM Cd treatment: however, it was not significantly different from 0 μM. The intensities of low bands at 3.0 and 30 μM were statistically reduced compared to 0 μM.

Figure 10 presents hemoglobin responses of all individuals from BH. Table 1 shows the average band intensity of the two populations from BH, *Chironomus* and *Dicrotendipes*. Both populations from BH showed a concentration-related response for low bands as well. *Chironomus* was the dominant genus and showed similar responses similar to those of *Dicrotendipes* from NEC. At 0 day, the averages of *Chironomus* and *Dicrotendipes* were 4.75 ± 2.71 and 2.0 ± 0 respectively. Another slight induction of protein expression was observed in *Chironomus*. The average of the low bands of *Chironomus* at 0.3 μM of Cd exposure was 6.17 ± 1.94 which was higher than 0 day. When compared to 0 μM Cd exposure, it was observed that the averages of both *Chironomus* and *Dicrotendipe* at 3.0 μM and 30.0 μM Cd exposure had decreased to 0.75 ± 1.16 and 1.43 ± 0.98 respectively, suggesting concentrated-related responses.

Figure 11 presents hemoglobin responses of all individuals from the laboratory population. Average band intensities of *Chironomus* are shown in Table 2. The averages of low bands at 0 day and 0 μM at 96 h were 2.0 ± 2.247 and 7.714 ± 6.798, respectively. This indicated that the intensity of low bands increased after being removed from the culture and held in control conditions. The averages of low bands decreased as concentration of Cd increased. Compared 0 μM at 96 h (7.714 ± 6.798),

the responses for 0.3 μM, 3.0 μM, and 30.0 μM Cd at 96 were 4.8 ± 3.334, 2.526 ± 2.874 and 1.2 ± 1.908 respectively. The data showed that low bands were significantly reduced suggesting an effect of Cd on hemoglobin levels in this laboratory population of *Chironomus*.

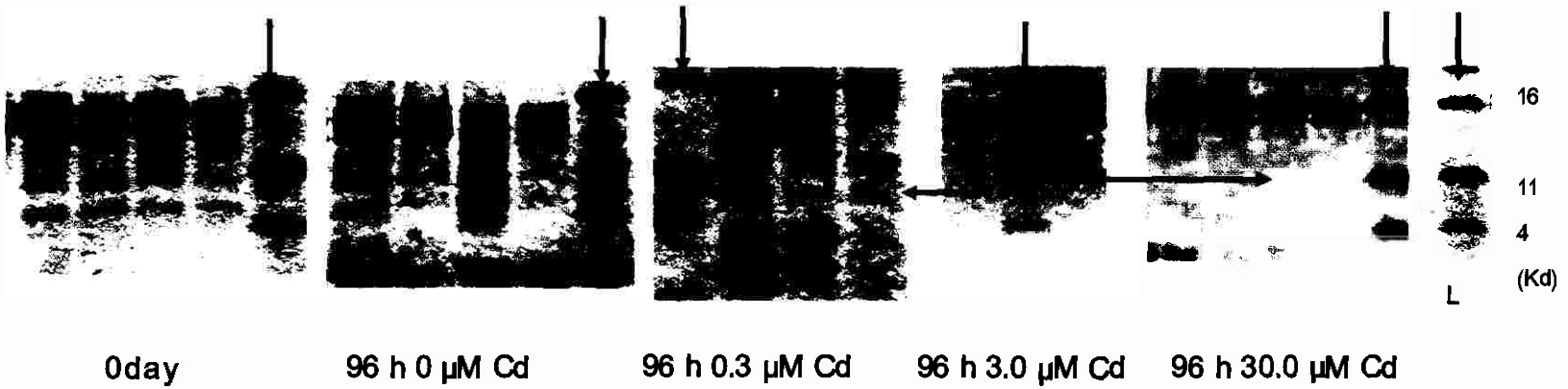


Figure 7: Hemoglobin band patterns of individuals that participated in 96 h Cd toxicity tests. Red arrows indicate alteration in hemoglobin band expression. Black arrow indicates molecular standard weight. The affected regions are mainly from ~4 Kd to ~14Kd. Increased time and concentration (µM) of Cd resulted in decreased expression of low MW proteins.

Table 1: Average band intensity value of individuals from Kearny Marsh, North East Creek, and Bass Harbor. Ave High represents bands from 1-7 and Ave Low represents bands from 8-17. All individuals within in a treatment were separated by species. Band intensities were summed and averaged along with others within in same treatment/species. C = *Chironomus* and G = *Glyptotenipes,* D = *Dicrotenipes*.. ND = No Data.

Site	Treatment	Species	High bands	Low bands
Kearny Marsh	0-d	C	5.67 ± 1.51	13.67 ± 2.25
		G	10.63 ± 1.22	2.0±4.40
	0µM	C	8.50 ± 1.0	11.75 ± 0.50
		G	10.83 ± 1.47	4.50±3.15
	0.3 µM	C	ND	ND
		G	ND	ND
	3.0µM	C	8.80 ± 2.05	3.80 ± 4.27
		G	10.50 ± 2.22	3.70 ± 3.40
	30.0 µM	C	ND	ND
		G	10.0 ± 1.51	1.5 ± 2.62

Site	Treatment	Species	High bands	Low bands
North East Creek	0-d	D	7.38± 1.77	2.88 ± 1.73
	0µM	D	9.14 ± 1.86	4.14 ± 1.95
	0.3 µM	D	9.58 ± 1.44	5.83 ± 1.40
	3.0µM	D	8.08 ± 1.62	1.67 ± 1.67
	30.0 µM	D	6.62 ± 1.61	0.92 ± 0.76

Site	Treatment	Species	High bands	Low bands
Bass Harbor	0-d	C	11.38 ± 0.52	4.75 ± 2.71
		D	9.0 ± 1.41	2.0 ±0
	0µM	C	10.71 ± 0.76	4.71 ± 2.14
		D	10.0 ± 0	6.25 ± 2.63
	0.3 µM	C	11.17±2.04	6.17 ± 1.94
	3.0µM	C	10.90 ± 1.73	2.70 ± 2.87
	30.0 µM	C	10.0±1.31	0.75 ± 1.16
		D	6.43 ± 0.98	1.43 ± 0.98

Table 2: Average band intensity value of individuals from Laboratory population. Average High represents bands from 1-7 and Average Low represents bands from 8-17. All individuals were identified by genus. Band intensities were summed for each individual and averaged by treatment for each genus. C = *Chironomus*

Site	Treatment	Species	High bands	Low bands
Laboratory	0-d	C	12.6 ± 2.233	2 ± 2.247
	0 µM	C	10.35±2.3	7.714 ± 6.798
	0.3 µM	C	9.9 ± 2.511	4.8 ± 3.334
	3.0µM	C	7.315 ± 2.944	2.526 ± 2.874
	30.0 µM	C	7.4 ± 2.702	1.2 ± 1.908

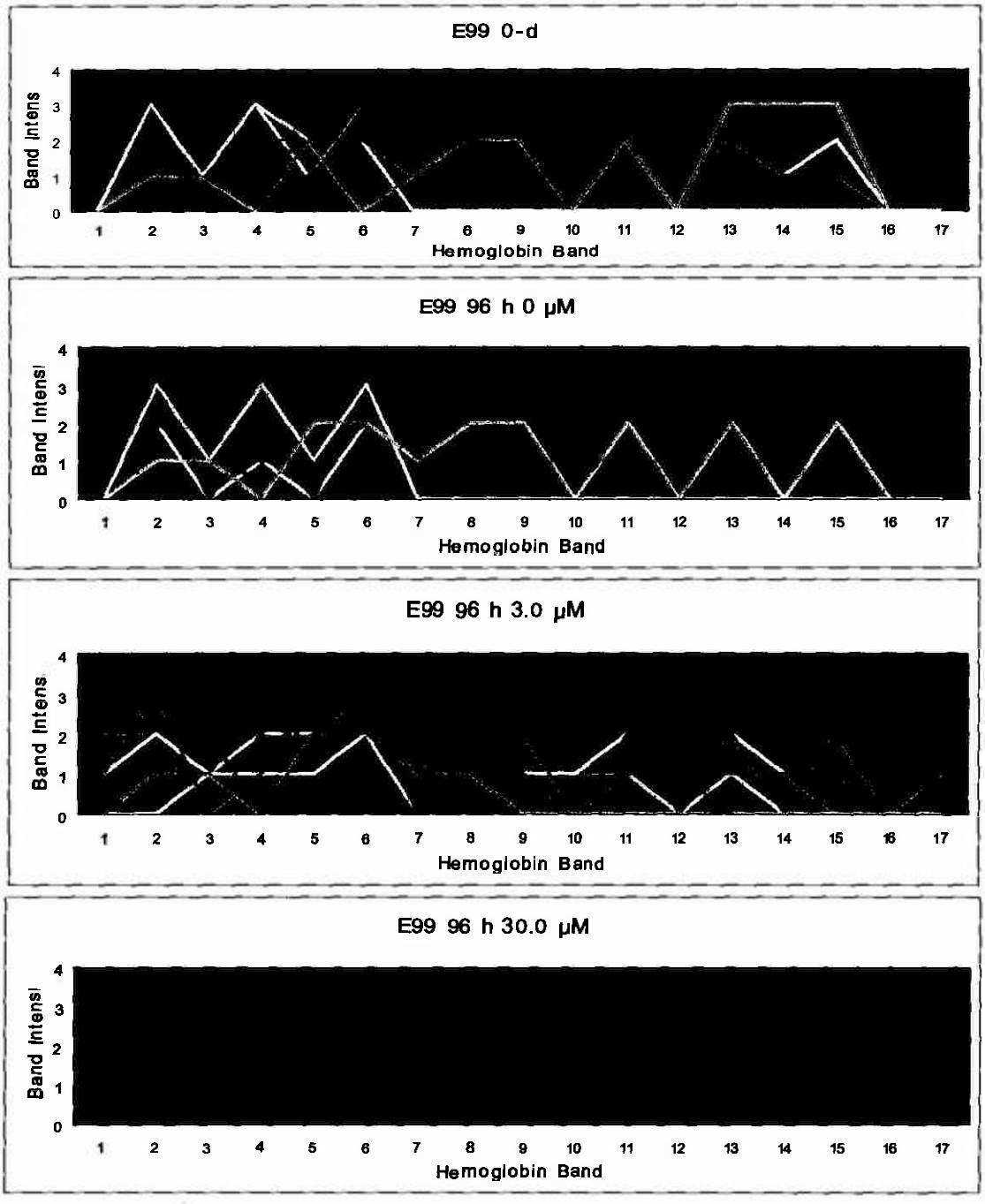


Figure 8: Graph of hemoglobin responses of Kearny Marsh (KM) chironomids to Cd. Data are for 0-d (no Cd) and concentrations of 0, 3 and 30.0 µM at 96 h (no 0.3 µM). The x-axis represents hemoglobin bands defined as 1-17 based PAGE gel profiles. The y-axis represents band intensity based on a qualitative scale of 0-3 with 3 being the darkest. The profiles of all individuals within a given treatment were plotted on a same graph. Each line color represents a profile of an individual or individuals with same profile.

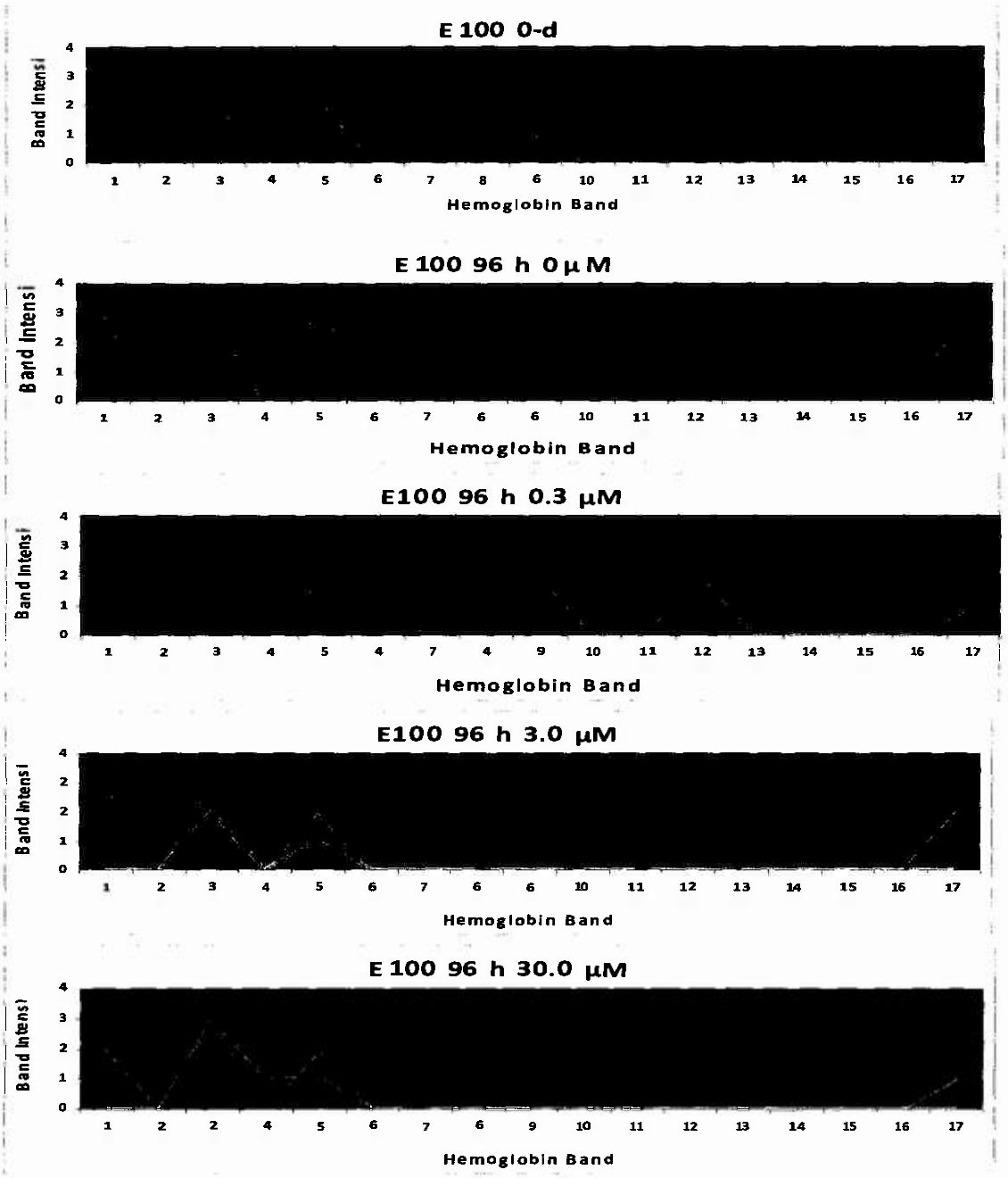


Figure 9: Graph of hemoglobin responses of North East Creek (NEC) chironomids to Cd. Data are for 0 d (no Cd) and concentrations of 0, 0.3, 3 and 30.0 µM at 96 h. The x-axis represents hemoglobin bands defined as 1-17 based PAGE gel profiles. The y-axis represents band intensity based on a qualitative scale of 0-3 with 3 being the darkest. The profiles of all individuals within a given treatment were plotted on a same graph. Each line color represents a profile of an individual or individuals with same profile.

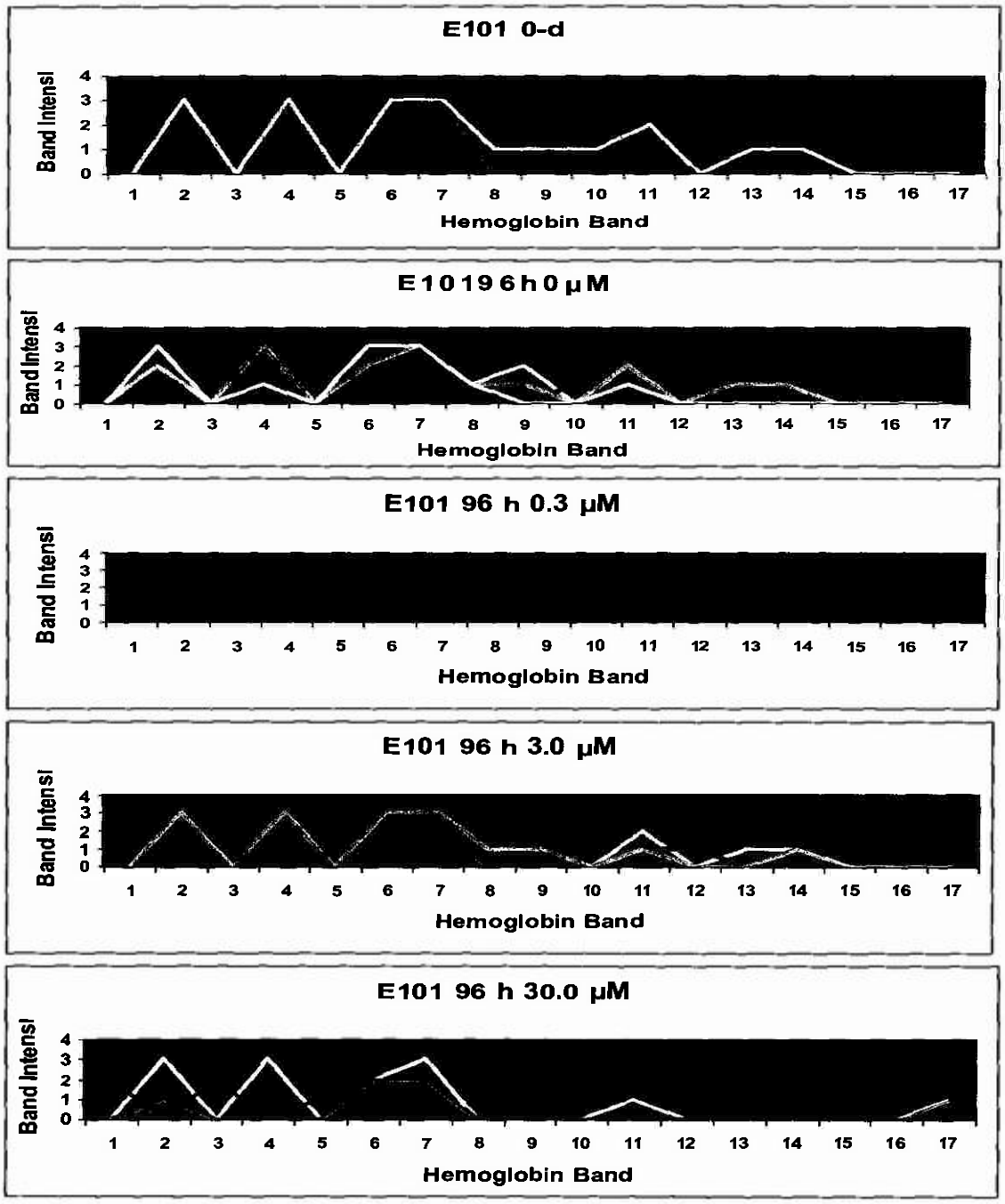


Figure 10: Graph of hemoglobin responses of Bass Harbor (BH) chironomids to Cd. Data are for 0 d (no Cd) and concentrations of 0, 0.3, 3 and 30.0 µM at 96 h. The x-axis represents hemoglobin bands defined as 1-17 based PAGE gel profiles. The y-axis represents band intensity based on a qualitative scale of 0-3 with 3 being the darkest. The profiles of all individuals within a given treatment were plotted on a same graph. Each line color represents a profile of an individual or individuals with same profile.

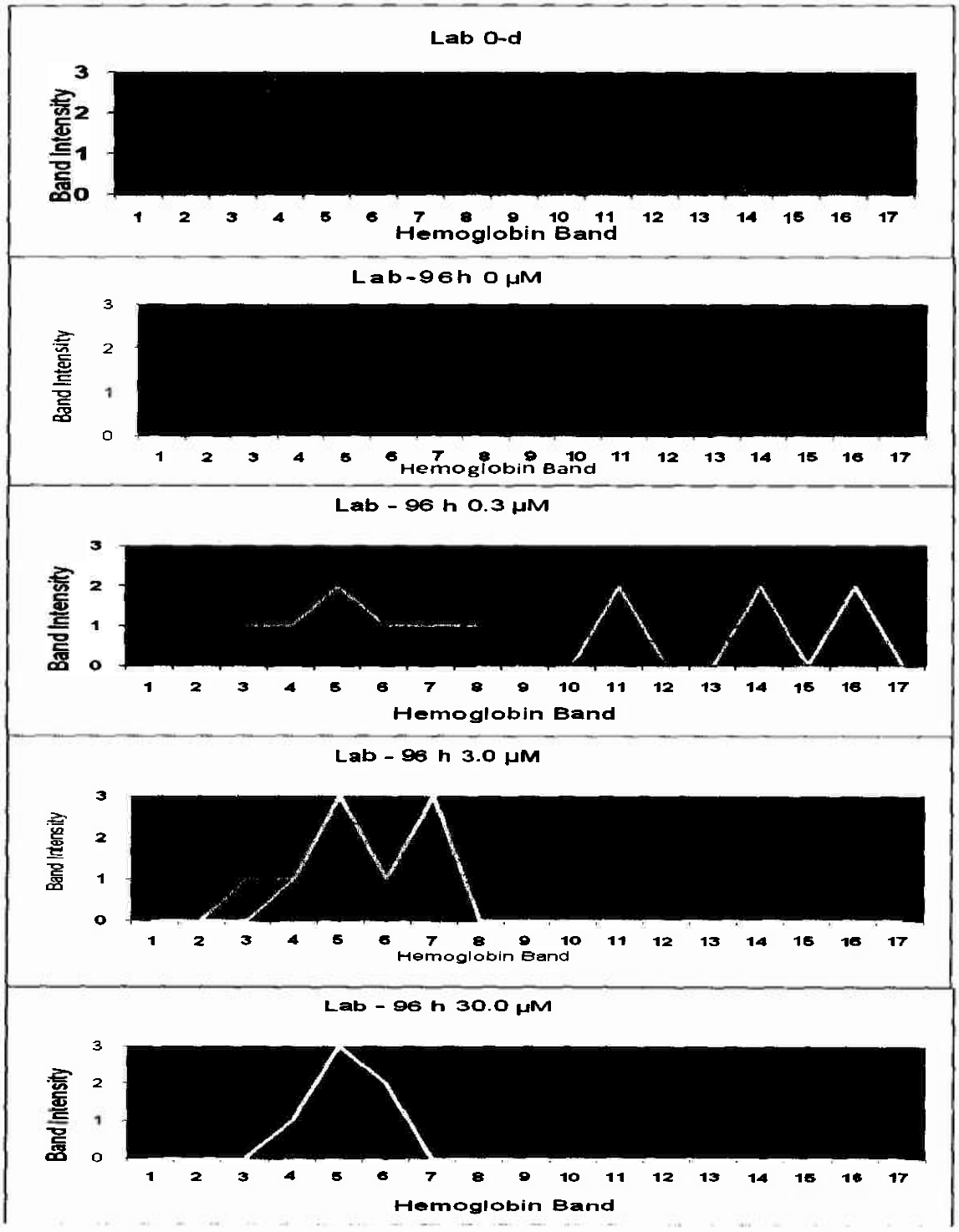


Figure 11: Graph of hemoglobin responses of Laboratory chironomids to Cd. Data are for 0 d (no Cd) and concentrations of 0, 0.3, 3 and 30.0 µM at 96 h. The x-axis represents hemoglobin bands defined as 1-17 based PAGE gel profiles. The y-axis represents band intensity based on a qualitative scale of 0-3 with 3 being the darkest. The profiles of all individuals within a given treatment were plotted on a same graph. Each line color represents a profile of an individual or individuals with same profile.

Comparison of low bands by genus and site

The low bands of all individuals within a particular genus were compared to other genera from the same or different collection sites. As mentioned above, a certain genus might dominate a site and be found at other sites as well. *Dicrotendipes* for example was the only genus found in NEC but was observed in BH as well. Each genus was separated out from others and the average band intensity was calculated. Figures 12-15 present the average band intensity of each genus from each site. In Figure 12, *Chironomus* from the laboratory population showed concentration-related responses with average band intensity decreasing as Cd concentration increased. The average band intensities of 0 μM and 0.3 μM Cd were not statistically different. However, the average band intensities of 0 μM compared to 3.0 μM and 30.0 μM were statistically different, which indicated a concentrated-related response by the individuals.

Responses of individuals in KM are shown in Figure 13. Average band intensities of *Chironomus* at 0 day and 0 μM showed no statistical differences. Average band intensities at 3.0 μM was in fact significantly different from 0 day and 0 μM. *Chironomus* were not present in higher concentrations of Cd (30.0 μM). *Glyptotendipes* had reduced band intensity at 30.0 μM Cd compared to 0 μM and 3.0 μM but it was not statistically significant from the 0 day.

Figure 14 presents responses of *Dicrotendipes* from NEC. Although 0.3 μM of Cd did not show statistical differences to 0 day and the control, 3.0 μM and 30.0 μM did show statistical differences compared to 0 day and the control. In Figure 15, *Chironomus* was observed to have similar response to *Dicrotendipes* population in NEC. The average band intensities of 0 day, control, and 0.3 μM were not significantly different from each other. However, 3.0 μM and 30.0 μM did show statistical differences to 0 day, control, and 0.3 μM of Cd. The average band intensity of 30 μM of Cd did show statistical difference to the controls and 0.3 μM of Cd. *Dicrotendipes* were not present in both 0.3 μMand 3.0 μM treatments. No significant differences were found in 0day and 0 μM of Cd treatment. However, at 30.0 μM of Cd, the average band intensity did show statistical difference to the control.

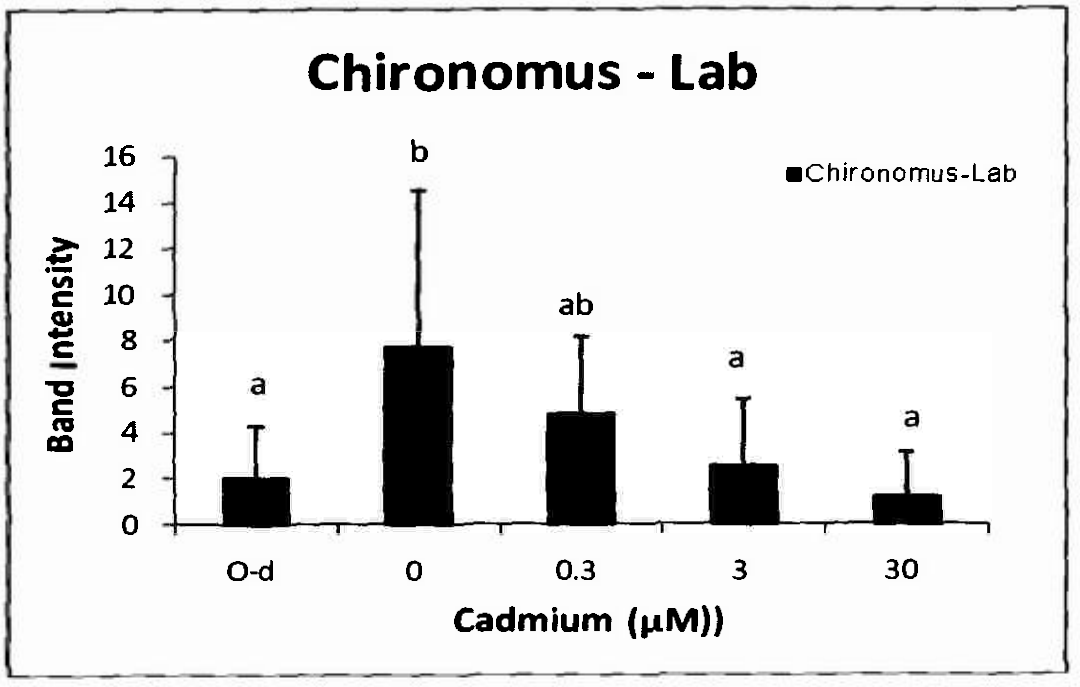


Figure 12: A graph of average low band intensity of *Chironomus* genus in laboratory population. The x-axis represents concentrations of Cd that were exposed to *Chironomus* genus. The y-axis represents band intensity. Treatments that share letters were not statistically different, $p > 0.05$.

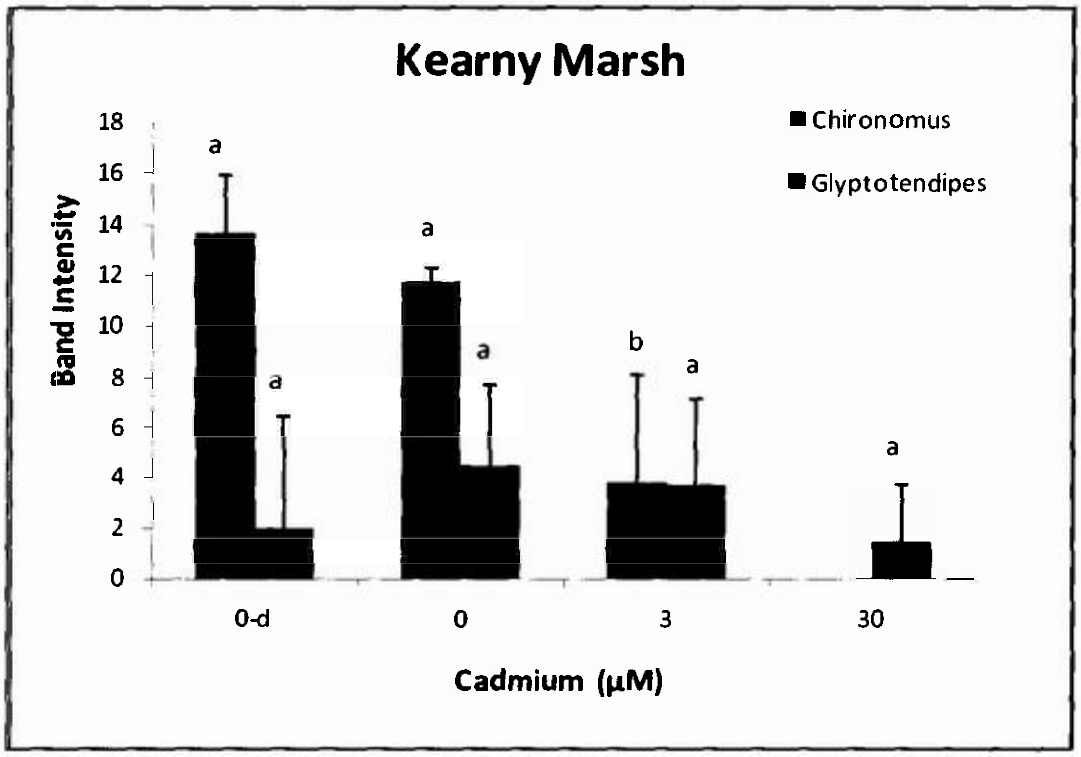


Figure 13: A graph of average low band intensity of *Chironomus* and *Glyptotendipes* genus in KM. The x-axis represents concentrations of Cd that were exposed to both *Chironomus* and *Glyptotendipes* genera. The y-axis represents band intensity. Each genus was analyzed separately. Treatments that share letters were not statistically different, $p > 0.05$.

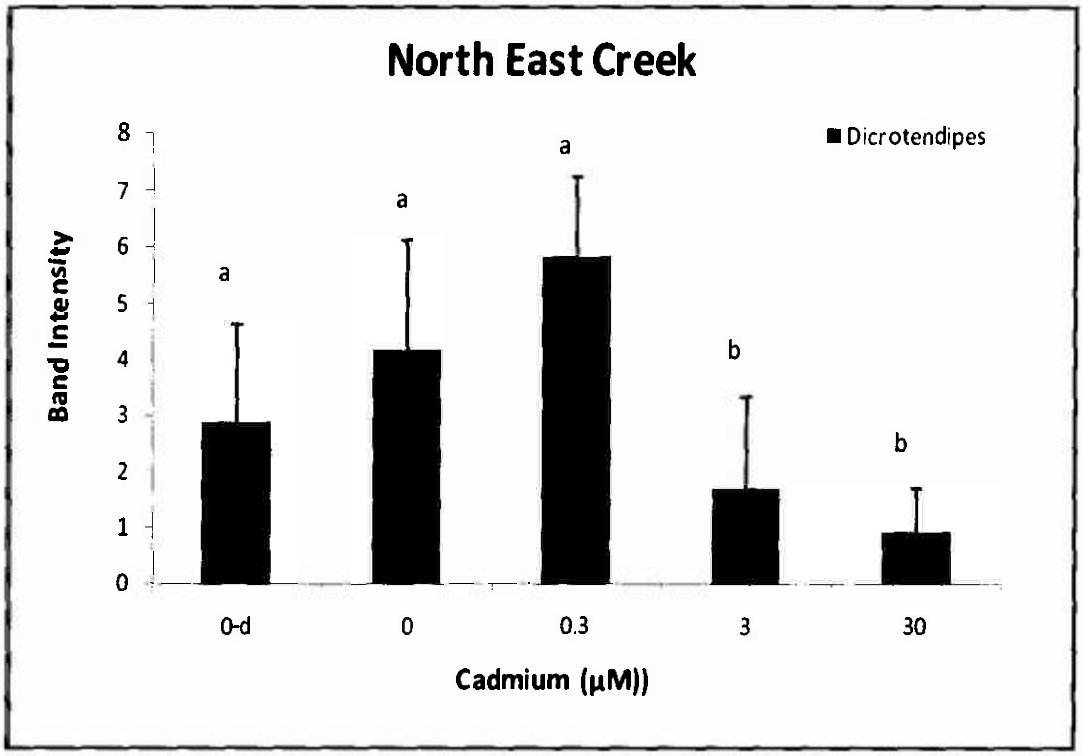


Figure 14: A graph of average low band intensity of *Dicrotendipes* genus in NEC. The x-axis represents concentrations of Cd that were exposed to *Dicrotendipes* genus. The y-axis represents band intensity. Treatments that share letters were not statistically different, $p > 0.05$

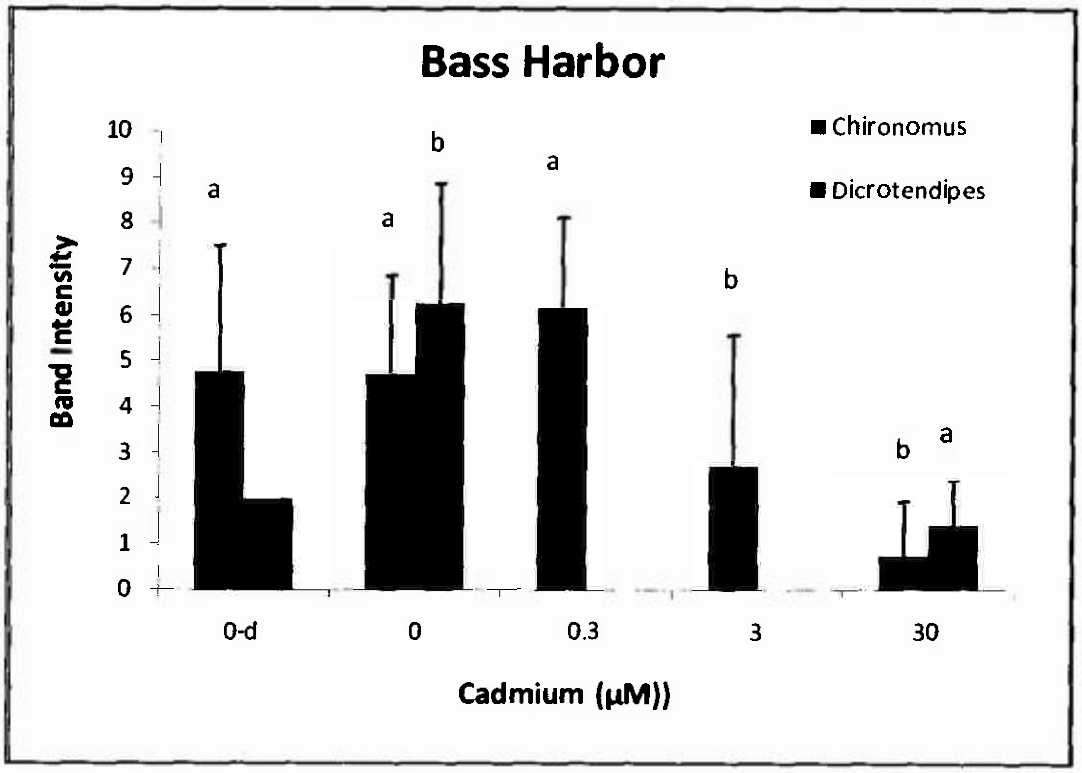


Figure 15: A graph of average low band intensity of *Chironomus* and *Dicrotendipes* genera in BH. The x-axis represents concentrations of Cd that were exposed to *Chironomus* and *Dicrotendipes* genera. The y-axis represents band intensity. Each genus was analyzed separately. Treatments that share letters were not statistically different, $p > 0.05$.

Concentration-response of Chironomus genus

Differences in sensitivity (i.e. environmental quality) to Cd exposure by *Chironomus* were compared among sites. Figure 16 shows all the average band intensities and SD of *Chironomus* genus only. Although populations showed concentration-related responses, their sensitivities did not appear to be due to environmental quality. The responses from each site compared to one another were not statistically different.

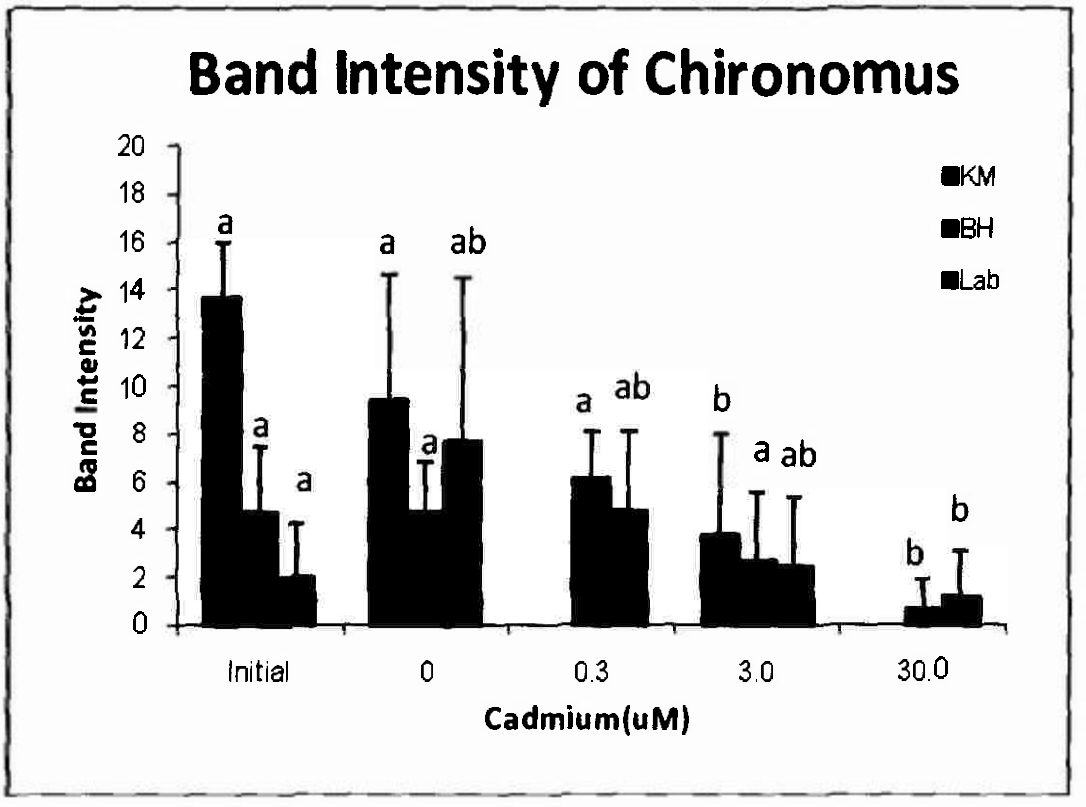


Figure 16: A graph of average low band intensity of only *Chironomus* genus in each site. The x-axis represents concentrations of Cd that were exposed to *Chironomus* genus. The y-axis represents band intensity. KM = Kearny Marsh, BH = Bass Harbor, and Lab = laboratory. Each site was compared to the other for a particular treatment. Treatments that share letters were not statistically different. No statistical differences were found between sites: one-way ANOVA, Tukey posthoc test, $p > 0.05$.

Water quality of the test sites

Water quality of the three sites were measured at the time of chironomid collection and compared to each other (Table 3). Temperature of KM was warmer than MDI sites by approximately 8 °C. The average water temperature of KM was 28.5 °C, NEC was 20.2 °C, and BH was 20.05 °C. Salinity was similar in all three sites. The average salinity of KM was 1.69 ppt, NEC was 0.645 ppt, and BH was 2.9 ppt. MDI sites showed normal levels of dissolved oxygen (DO) level which ranged from 5.57 to 6.28 mg/L. The DO levels in AquaBlok (ranged from 7.32 to 11.90 mg/L) sites was supersaturated and had approximately 5 mg/L higher level than uncapped sites (ranged from 0.65 to 7.47 mg/L). KM had higher pH than MDI sites by 1-2 units. The pH averages in KM, NEC, and BH were 8.04, 6.39, and 6.325 respectively. The redox potential in MDI sites was similar to the uncapped site in KM. The average redox potentials of NEC, BH, and uncapped were -110, -93.5, and -107.5 respectively. The redox potential in AquaBlok KM at uncapped sites ranged from -62 to -153 and was generally lower than MDI sites. The supersaturated DO, high pH and low redox all indicated eutrophication of KM compared to MDI sites.

Table 3: Water quality parameters. Temperature, salinity, benthic dissolved oxygen, pH, and redox potential were measured at Kearny marsh and North East Creek, and Bass Harbor. AB stands for AquaBlok and CN for Control.

Site		Temp. (°C)	Salinity (ppt)	Benthic DO (mg/L)	pH	Redox (eH)
Kearny Marsh	plot 1 & 4(AB)	28.1 to 30.3	1.73 to 1.74	7.32 to 11.90	9.09 to 8.59	-65 to 21.9
	plot 9 & 10 (CN)	26.7 to 28.1	1.68 to 1.73	0.65 to 7.47	7.49 to 8.48	-62 to -153
North East Creek		19.4 to 21.0	0.45 to 0.84	5.57 to 6.60	5.92 to 6.86	-106 to -114
Bass Harbor		18.4 to 21.7	2.10 to 3.7	6.02 to 6.28	6.16to 6.49	-45 to -142

Cd uptake and accumulation tissue, water

Table 4 presents nominal and actual Cd concentrations in water and chironomid tissues on 0 day. Water samples were taken from 96 h toxicity tests with 0 μM Cd added. Since there was no Cd added, Cd levels represent what was in site water. The actual concentrations of Cd in KM replicates A and B water were 0.0147 μM and 0.0120 μM respectively. Chironomids exposed to this water had tissue concentrations of 0.0336 μmol/g for A and 0.0100 μmol/g for B. High deviations were observed on actual Cd concentrations for NEC. The actual concentration of Cd was found to be 0.1667 and 0.1703 μM for replicates A and B, respectively, which was approximately 10 times higher than found in KM. This suggested that the control was contaminated during the experiment. Tissue samples from NEC were not collected due to insufficient size of the animal. Cd in BH water and chironomid tissue was similar to those in KM. Water concentrations were 0.0940 and 0.0115 μM Cd for replicates A and B, respectively. Tissue concentrations were 0.0063 and 0.0133 μmol/g for A and B, respectively. Overall, results showed that chironomids collected at KM and BH had similar Cd levels as did the site water.

Table 5 presents nominal and actual Cd concentrations in water and chironomid tissues at 96 h. All three sites showed similar responses- as nominal

concentrations increased, the actual concentrations increased as well. BH replicate B had 0.0115 μM of actual Cd concentration for 0 μM of nominal Cd. The actual Cd concentration of BH replicate B increased to 0.149 μmol/g at 0.3 μM, 1.314 μmol/g at 3.0 μM and 14.829 μmol/g at 30.0 μM of nominal Cd. Overall, the actual concentrations were approximately 50 % of nominal. The lower nominal concentrations were likely due to Cd binding to sand in the containers.

Cd concentrations in tissue from all three sites showed similar concentration-related responses. Cd concentration in tissue at 0 day for KM was slightly higher than BH. Tissue concentration at 0 day for KM replicate B was 0.0717 μmol/g and for BH replicate B was 0.0063 μmol/g. Increase in tissue concentration was observed as nominal concentration increased. At 3.0 μM of nominal Cd, tissue concentrations of KM replicate B and BH replicate B were increased to 0.6409 μmol/g and 0.4468 μmol/g respectively. Tissues in both KM and BH replicate B showed a 10 fold increase from 0 μM to 3.0 μM. As mentioned above, tissues were not collected for NEC site due to insufficient size of chironomids.

Table 4: Heavy metal analysis of chironomid tissues and water samples from Kearny marsh (KM), North East Creek (NEC), Bass Harbor (BH) at 0 day. Nominal and actual concentration of Cd in water was measured in μM/L and Cd uptake by tissues was measured in μM/g by using Varian AA-220 as an Atomic Absorption Spectroscopy (AAS). Tissues were not collected at NEC due to insufficient size of the chironomids at the time of collection. Replicates contain all individuals regardless of their genus

Treatment	**Replicate**	**Water nominal**	**Water actual**	**Tissue weight**	**Tissue Cd**
units		(μM/L)	(μM/L)	(g)	(μM/g)
KM0d	A	0	0.0147	0.0026	0.0336
	B	0	0.0120	0.0014	0.0100
NEC0d	A	0	0.1667	ND	ND
	B	0	0.1703	ND	ND
BH0d	A	0	0.0940	0.0019	0.0447
	B	0	0.0115	0.0017	0.0720

Table 5: Heavy metal analysis of chironomid tissues and water samples from Kearny marsh (KM), North East Creek (NEC), Bass Harbor (BH) at 96 h. Nominal and actual concentration of Cd in water and Cd uptake by tissues were analyzed by Atomic Absorption Spectroscopy (AAS). The water was measured in µM/L and the tissue was measured in µM/g using Varian AA-220 as an Atomic Absorption Spectroscopy (AAS). Tissues were not collected at NEC due to insufficient size of the chironomids at the time of collection. KM 96 h 0.3 µM did not performed. Treatments with grey shade were not considered as data due to insufficient weight of the tissue.

Site	Replicate	Water nominal	Water actual	Tissue weight	Tissue Cd
		(µmol/L)	(µmol/L)	(g)	(µmol/g)
KM96h	A	0	0.0147	0.0012	0.0312
	B	0	0.0120	0.0025	0.0717
NEC96h	A	0	0.1667	ND	ND
	B	0	0.1703	ND	ND
BH96h	A	0	0.0940	0.0011	0.0133
	B	0	0.0115	0.0009	0.0063
KM96h	A	0.3	ND	ND	ND
	B	0.3	ND	ND	ND
NEC96h	A	0.3	0.07	ND	ND
	B	0.3	0.22	ND	ND
BH96h	A	0.3	0.1062	0.0007	0.0840
	B	0.3	0.1498	0.0007	0.0392
KM96h	A	3.0	0.3310	0.0021	0.3879
	B	3.0	0.3676	0.0019	0.6409
NEC96h	A	3.0	0.8681	ND	ND
	B	3.0	0.9385	ND	ND
BH96h	A	3.0	1.3859	0.0001	5.8101
	B	3.0	1.3141	0.0011	0.4468
KM96h	A	30.0	1.8574	0.0003	3.3974
	B	30.0	2.9263	0.0008	1.2469
NEC96h	A	30.0	ND	ND	ND
	B	30.0	ND	ND	ND
BH96h	A	30.0	15.3356	0.0014	0.2122
	B	30.0	14.8291	0.0002	4.7409

Heavy metal analysis of chironomid tissue

Table 6 presents heavy metal accumulation in chironomid tissues that were collected in KM and BH. Tissues from both sites were found to have similar concentrations of Cd, Cu, Fe, Ni and Zn as values were within one order of magnitude of one another. Tissues from MDI showed less Cr and Pb than KM. Cr was approximately 3x higher, Pb was approximately 4x higher at KM. The Pb in KM may have come from automobile exhaust.

Table 6: Heavy metal analysis on tissues of chironomids collected in Kearny Marsh and Bass Harbor. All metals were measured in mg/kg using Varian AA-220 as an Atomic Absorption Spectroscopy (AAS) then recalculated to µmol/g. No tissues were collected for North East Creek. Only Pb was consistently higher in KM than BH

Site	Replicate	Tissue g	Cd µmol/g	Cr µmol/g	Cu µmol/g	Fe µmol/g	Hg µmol/g	Ni µmol/g	Pb µmol/g	Zn µmol/g
KM 1+4	A	0.0026	0.033	0.162	0.552	65.47	ND	8.28	0.459	0.636
KM 9+10	B	0.0014	0.01	0.126	0.305	29.6	ND	2.38	0.473	0.026
	A	0.0019	0.045	0.043	0.061	39.39	ND	6.02	0.009	0.216
BH	B	0.0017	0.072	0.052	0.608	52.59	ND	11.64	0.108	0.04

Heavy metal analysis of water

Result of heavy metal analysis of water is shown in Table 7. Trace metals of Cd, Cr, Cu, Fe, Hg (not measured in NEC and BH), Ni, Pb, and Zn were analyzed by using the graphite furnace method of AAS. Cd concentrations for NEC were higher than for BH and KM; as mentioned above; samples may have been contaminated during the experiment. Copper concentrations were higher in KM than MDI sites. All other metals were within one order of magnitude of each other.

Table 7: Heavy metal analysis of water samples from Kearny Marsh (KM), Bass Harbor (BH), and North East Creek (NEC). Water was taken from 96 h test samples with 0 µM Cd. All metals were measured in µM using Varian AA-220 as an Atomic Absorption Spectroscopy (AAS). Note the higher levels of Cu in KM water. Other values are similar. *NEC samples may have been contaminated with Cd during daily water renewals.

Site	Replicate	Cd µM	Cr µM	Cu µM	Fe µM	Hg µM	Ni µM	Pb µM	Zn µM
KM	A	0.015	0.022	0.222	4.310	ND	0.074	0.014	0.014
	B	0.012	0.018	0.193	1.277	ND	0.054	0.016	0.016
BH	A	0.094	0.011	0.080	5.885	ND	0.122	0.008	0.008
	B	0.012	0.013	0.053	5.237	ND	0.036	0.013	0.013
NEC	A	0.167*	0.092	0.041	6.825	ND	0.011	0.009	0.009
	B	0.170*	0.014	0.032	6.718	ND	0.001	0.007	0.007

ND =no data

Heavy metal analysis of sediments

Table 8 presents heavy metal analysis of sediments collected from all three sites. KM replicates 1 and 4 showed the heavy metal concentrations of the AquaBlok covering sediments, and replicates 9 and 10 showed the concentrations in sediment at the uncapped site. The metals Cd, Cr, Cu, Fe, Hg and Ni were 10 times higher in uncapped versus capped sites. Pb was approximately 100 times higher in uncapped sites: Zn was similar in capped and uncapped. Cd concentrations in KM Aquablok was actually less than MDI sites. In comparing KM and MDI sediments, uncapped KM sediments had 10 times more Cd and Pb than those for MDI sites. Concentrations of Cr, Fe, Ni and Zn were similar. Hg was not measured for MDI sediments.

Table 8: Heavy metal analysis of sediment samples from Kearny Marsh (KM), Bass Harbor (BH), and North East Creek (NEC). Replicates 1 and 4 are AquaBlok sites and 9 and 10 are control (uncapped) sites of KM. All metals were measured in mg/kg using Varian AA-220 as an Atomic Absorption Spectroscopy (AAS). Note high levels of Cd, Hg and Pb in KM uncapped sediments.

Site	Replicate	Cd mg/kg	Cr mg/kg	Cu mg/kg	Fe mg/kg	Hg mg/kg	Ni mg/kg	Pb mg/kg	Zn mg/kg
KM capped	1 A	0.15	7.1	5.9	4088	0.11	7.2	22	37
	1 B	0.19	7.8	15.6	10043	0.04	6.9	51	66
	1 C	0.03	6.2	1.9	1065	0.06	3.9	6.7	14
	4 A	0.13	9.7	6.7	3080	0.12	13	19	34
	4 B	0.12	7.7	2.4	2664	2.01	8	26	29
	4 C	0.34	15	33	21575	0.17	12	80	106
KM uncapped	9 A	3.80	91	150	33848	1.39	72	597	69
	9 B	3.04	56	83	28139	0.88	56	509	39
	9 C	4.07	83	195	23075	1.39	69	648	55
	10 A	2.72	36	76	16895	0.64	41	352	37
	10 B	3.50	79	164	27738	1.41	75	620	51
	10 C	2.35	23	47	15710	5.69	32	254	17
BH	A	0.26	19	29	14726	ND	19	35	95
	B	0.82	17	122	12412	ND	23	61	85
NEC	A	0.68	24	200	15502	ND	25	90	117
	B	1.50	27	287	14217	ND	26	74	131

ND =no data

Discussion

In this study, changes in hemoglobin protein expression of different chironomid populations along with genus identification and heavy metal analysis on tissue, water and sediments were performed. The primary objectives were to develop hemoglobin proteins as a means of identifying wild chironomid genus and detecting toxic levels of heavy metals. To do this, chironomid populations were collected from an impacted site, Kearny Marsh, NJ (KM) and two potential reference sites, North East Creek (NEC) and Bass Harbor (BH) from Mount Desert Island (MDI), ME. They were evaluated for their hemoglobin protein profiles using SDS-PAGE and their relative sensitivity to Cd in concentration-response experiments.

This study contributed to the development of a new means of taxonomic identification of chironomids using hemoglobin protein profiles on SDS PAGE. Previous identification of genus relied solely on larval morphology, in particular, head capsule morphology (Epler 2001). From study sites, three distinct genera of chironomids were found at these sites: *Glyptotendipes* and *Chironomus* from KM, *Dicrotendipes* from NEC, and *Dicrotendipes* and *Chironomus* from BH. Results showed that most individuals of a particular genus had one or two common profiles, but sometime several profiles for a single genus were found. In these cases, head capsule morphology could confirm the genus identification. As shown above in Figures 4-6, the first three bands constituted a common feature for each genus. *Glyptotendipes* always has a very dark band 1 and lighter band 2. *Dicrotendipes* has strong band 3 and band 1. *Chironomus* does not have band 1 but has strong band 2 with absence or very faint band 3. It was observed that many individuals had variety

of profiles even within a genus. Some individuals had profiles that were not anticipated in such treatment (i.e. no low bands in 0 μM of Cd exposure). Majority of individuals had very little effects on the high bands regardless of the level of Cd exposure.

While head capsule morphology is the standard for genus identification, it is not always dependable. In some cases, the head capsule can be damaged when mounting or be deformed due to environmental contaminants (Madden, 1992). Studies have observed that deformities found in head capsules of chironomids living in fresh water reflect sublethal effects and can be used as indicator for environmental contamination (Nazarova, 2004).

Concentration-response experiments showed that Cd caused changes in hemoglobin protein levels (Figures 12-15). Low molecular weight bands were particular sensitive. The trend observed was a slight induction in band intensity at 0.3 μM Cd (NEC and BH) followed by a concentration-related declined at 3.0 and 30.0 μM at for *Dicrotendipes* and *Chironomus* at all sites (Figures 13, 14, and 15). *Glyptotendipes*, which was found only at KM, did not show a concentrated-related response (Figure 13). It was anticipated that the chironomid populations from less urban sites, i.e. MDI sites, would be more sensitive to Cd than those populations from KM. They should have responded to Cd at lower concentrations. Although 30.0 μM is well below the LC10 for Cd at 24 h (507.5 μM), only *Glyptotendipes* from KM did not show a significant response: this difference may be genus or site related. Tissue analyses from concentration-response experiments showed that increasing concentration of Cd were associated with higher body burdens (Table 4). However, there did not appear to be site associated differences between KM and BH. Data at

30.0 μM Cd were limited due to low tissue weights and numbers of replicates making it difficult to draw conclusions. Overall, results indicated that hemoglobin protein as detected by SDS-PAGE can be modulated by heavy metal exposure. It suggested that at least one genus from KM, the impacted site, was less sensitive to Cd even though tissue levels were similar to the reference site.

For field populations, AAS results showed that chironomid tissue (0 d) and water levels from KM were similar to MDI sites, except for higher levels of Cr and Pb in tissue and Cu in water at KM (Table 5 and Table 6). KM sediments (uncapped) had higher levels of heavy metals than MDI sites. Since chironomids have been known to bioaccumulate metals from diet and substrates (Choi, 2004), tissue levels should have been different between sites but they were not. The lack of bioaccumulation might have been an adaption to high heavy metals in sediment at KM. This pre-exposure may also have accounted for the lack of hemoglobin response in *Glyptotendipes*. *Glyptotendipes* from KM showed no statistically significant effects in concentration-response experiments even though tissue concentrations were similar to those from BH. In addition, hemoglobin bands were much more intense in *Chironomus* from KM than *Chironomus* from BH indicating that hemoglobin expression was different between the impacted and reference site for this genus. *Chironomus* tissue from KM had higher levels of Cr and Pb than tissue from BH. Addition of Cd during experiments might have exceed their defense mechanisms and possibly accounted for increased sensitivity to Cd seen in acute toxicity tests for *Chironomus* at KM. For example, they had a statistically significant response at 3.0 μM Cd compared to 30.0 μM for BH and no *Chironomus* were found at 30.0 μM for KM where as they were found for BH. All together, there appeared to be differences between impacted and

reference sties.

Chironomids have high tolerance to Cd compared to other environmental contaminants (Ha, 2008). According to our heavy metal analyses, Cd uptake by tissues of chironomid populations in both KM and BH, at 0 day, was similar, which suggests that they can control Cd levels in their tissues. In concentration-response experiments, actual water concentrations increased 10X between 3.0 and 30.0 μM treatments. However, this was associated with only a doubling of tissue concentrations, again indicating an ability to modulate heavy metal concentrations. One possible mechanism has been proposed by Yamamura and co-workers (1983). They found metal-binding proteins in gut tissue of chironomid and proposed that shedding gut cells allowed them to eliminate Cd.

Although most of the individuals showed concentrate-related decreases in low Kd bands of hemoglobin proteins, *Dicrotendipes* found in NEC and *Chironomus* found in BH showed a slight increase at 0.3 μM of Cd. Additionally the low bands disappeared at higher concentrations. This suggested a new type of defense mechanisms: these small hemoglobin proteins might have possessed the ability (i.e. defense mechanism) to form metal-hemoglobin complexes leading ultimately to metal elimination. This would have transiently increased the production of hemoglobin proteins and then reduced them. As more metals bind, the hemoglobin production may have discontinued due to the fact that the metals may enter their system and altered the gene expression.

The overall results suggest that different genera of chironomids do respond to heavy metal exposure in an acute toxicity test. Nonetheless, more molecular investigations are needed on these organisms. These should include repeating the

experiments conducted here, analyzing more chironomids *in situ* at impacted and reference sites as well as correlating hemoglobin protein levels with RNA expression. This pilot project indicates that the expression of hemoglobin proteins has potential as a biomarker of heavy metal exposure. Biomonitoring field populations overtime could show changes in environmental exposure and therefore indicate the success of different remediation strategies or other types of changes in ecosystem quality. Since chironomids are a dominant genus in the NJ Meadowlands, the hemoglobin biomarker could help make risk management decisions in this wetland system.

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