Developing an Aquatic Toxicity Biomarker using Hemoglobin Gene Expression

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Abstract Current toxicity protocol involving acute and chronic toxicity assays provide little information about the mechanism of toxicity and are limited in their sensitivity. The use of gene expression as a biomarker for environmental toxicity is a recent concept. By looking at the expression of specific genes, it may be possible to see the effects and mechanisms of toxicity at sub-lethal levels. In this experiment, we studied the expression of the hemoglobin gene in *Daphnia magna* after metal exposure. Hemoglobin RNA was extracted from *Daphnia magna* exposed to different concentration levels of copper, cadmium, and lead. Two exposure levels derived from acute toxicity assays were used for each metal: the NOEC (no observable effect concentration), and the midpoint between the NOEC and LC_{50}. Expression levels of hemoglobin were compared using northern analysis. It was found that the daphnia hemoglobin gene was greatly expressed following lead exposure. There was also a difference in expression between the two lead concentrations. Little to no expression of the hemoglobin gene was expressed following copper and cadmium exposure. The results indicate that the hemoglobin gene may be specific to lead toxicity. More importantly, the difference in expression indicates that hemoglobin gene expression may serve as a biomarker for lead toxicity upon further studies. The analysis suggests that changes in gene expression may be a more biologically relevant predictor of environmental impact because changes were detected at sub-lethal levels, making it a more sensitive test.

Introduction

Economic growth does not come without sacrifice. Over the past two centuries, the increasing need for minerals in industry has created some of the worst hazardous waste areas in the United States (van Geen and Luoma 1999). Although metals are naturally occurring chemicals, human influences have altered their distribution in the environment (van Geen and Luoma 1999; Eaton 1979). By extracting metals from ores, manipulating their chemical speciation in industry, and dumping them into the environment in altered forms, humans have disrupted the natural processes that govern the fate of metals (Waalkes 1995). Despite our extensive knowledge concerning the deleterious effects of metal contamination on aquatic ecosystems, few reliable methods for assessing the level of this contamination exist. Direct measurement of the concentration of metals requires prior knowledge of the contaminants present and can be confounded by the speciation of the metals in the ecosystem, which can drastically alter their toxicity. Acute and chronic toxicity assays, which target a specific organism within the ecosystem, do not reveal the toxicant responsible for the contamination and also provide little information about the mechanism of toxicity (Chapman 2000). A more sensitive test is necessary to accurately assess the ecological impacts of metal contamination.

Changes in gene expression are sensitive indicators of stress to an organism (Far and Dunn 1999). Measuring these changes after toxicant exposure may provide information about the sub-lethal effects of a toxicant to an organism. There is also the possibility of linking changes in gene expression to specific toxicants (Nuwaysir 1999). In a recent study by Custodia *et al.* using *Caenorhabditis elegans*, the xenobiotic response to varying concentrations of a vertebrate steroid hormone was correlated with gene expression changes (Poynton 2003, pers comm; Custodia 2001).

The objective of this research is to develop and validate a new method to assess the effects of toxicants to an ecosystem by measuring the changes in hemoglobin gene expression in a common water toxicity bioassay organism, *Daphnia magna.* Daphnia are often used as indicators of contamination (USEPA 1993); therefore, effects on this organism can be used to predict ecosystem health.

Hemoglobin levels in daphnia are affected by metal exposure. Previous studies have found a complex response pattern of hemoglobin content after exposure to copper,

cadmium, and lead (Dave 1984; Berglind 1985; Berglind *et al.* 1985). This research will further elaborate on these studies by measuring the gene expression of hemoglobin in daphnia after exposure to copper, cadmium, and lead.

This research will address the hypothesis that the hemoglobin gene in *Daphnia magna* is differentially expressed at different levels of metal exposure. These results will serve as a benchmark for further studies on gene expression in daphnia using cDNA microarrays. Further studies may include different genes (such as the metallothioneins) and a wider array of metals. Once gene expression profiles are established, it may become possible to use gene expression as an accurate bio-indicator of the ecological impacts of metal contaminants.

Methods

This study was done in Chris Vulpe's lab at the University of California-Berkeley. *Daphnia magna* was cultured according to standard USEPA protocol (1993). The metals involved in this study were copper, cadmium, and lead. These metals were chosen based on their relative concentrations in the environment and their toxicity to aquatic life (Crosby 1998). Concentrations of copper, cadmium, and lead were made using $CuSO_4$, $CdSO_4$, and $PbSO_4$ solutions, respectively. The amount of sulfate added were negligible compared to the amount that existed in the media.

There were four components to the experiment. (1) Test concentrations for copper cadmium and lead were established with an acute toxicity test. LC_{50}'s were determined in order to establish lethal endpoints. Two sub-lethal concentrations below the LC_{50} were used in this study: the NOEC (no observable effect concentrations), and the 1/2 LC_{50} (midpoint between the NOEC and LC_{50}). (2) *Daphnia magna* were exposed for 24 hours to the NOEC and 1/2 LC_{50} of each metal. (3) RNA immediately extracted from the exposed daphnia. (4) Hemoglobin levels were determined by northern analysis. The results were visually analyzed due to the lack of an easily quantifiable technique. A difference in gene expression was shown by a difference in band intensity (brightness and length).

An acute toxicity assay was used to find the NOEC and 1/2 LC_{50}. The test was conducted using a procedure similar to USEPA Whole Effluent Toxicity (WET) protocol (1993). Acute toxicity involved the use of day-old daphnia obtained from the culture. The

daphnia were kept in conditions according to the standard EPA culturing protocol. Following the EPA test acceptability criteria for *Daphnia magna*, four replicates each containing five individuals was made for every concentration. A total of 20 individuals were used for each concentration. Exposure time was 24 hours. Mobile individuals were counted and reported as the number of survivors. Levels of dissolved oxygen, water-hardness, and pH at each concentration were taken and recorded before and after the test was completed. Preliminary concentrations (micrograms/Liter) used for copper were as follows: 0, 0.1, 1, 3, 10, 30, 100, and 300. Preliminary concentrations (micrograms/Liter) for cadmium were: 0, 1, 3, 10, 30, 100, 300, and 1000. Concentrations (micrograms/Liter) for lead were 0, 10, 30, 100, 300, 1000, 3000, 10,000, and 30,000.

The concentration where half of the individuals survived was defined as the LC_{50}. The LC_{50} was statistically determined by applying the probit method. The probit method is a parametric statistical procedure for estimating the LC_{50} and the associated 95% confidence interval (USEPA 1993). This program was developed by EMSL-Cincinnati and can be obtained from the Computer Sciences Corporation on 26 W. Martin Luther King Drive, Cincinnati, Ohio 45268.

To determined hemoglobin expression, RNA was extracted following a 24-hour exposure. The daphnia were exposed to two concentrations of each metal, the NOEC and 1/2 $LC_{50,}$ and an untreated control. Each two liter culture contained 25 adults that were four weeks or older, 25 young adults that were between two and three weeks old, and 50 juveniles that were up to two weeks old. Light and temperature conditions were the same as the conditions used in the acute assay. RNA was isolated with Trizol following the methods described by Invitrogen (2003).

Hemoglobin gene expression was measured by northern analysis and followed the procedures in the handbook, Current Protocols in Molecular Biology (Brown and Mackey 1997). To create the hemoglobin probe used in the northern analysis, RNA was first isolated from 30 untreated adults as described above, using Trizol. cDNA was made following the op Array User Manual (Quiagen 2003). An 890 base-pair hemoglobin probe was synthesized using PCR. The primers described in the appendix (Invitrogen 2003) were designed using Primers 3 (2003) software. The Taq Polymerase manufacturer's protocol (Invitrogen 2003) was followed to make the probe.

Results

NOEC and 1/2 LC_{50} values were found for each metal using the acute toxicity assays. The dose response curves are shown in Fig. 1. From the calculated solubility, lead sulfate is soluble up to 6000 micrograms/Liter. Our results reflect insolubility after 6000 micrograms/Liter and were statistically adjusted. In Table 1, the values from this experiment are compared with published values. The published values were taken from previous studies that used similar conditions to that of this study.

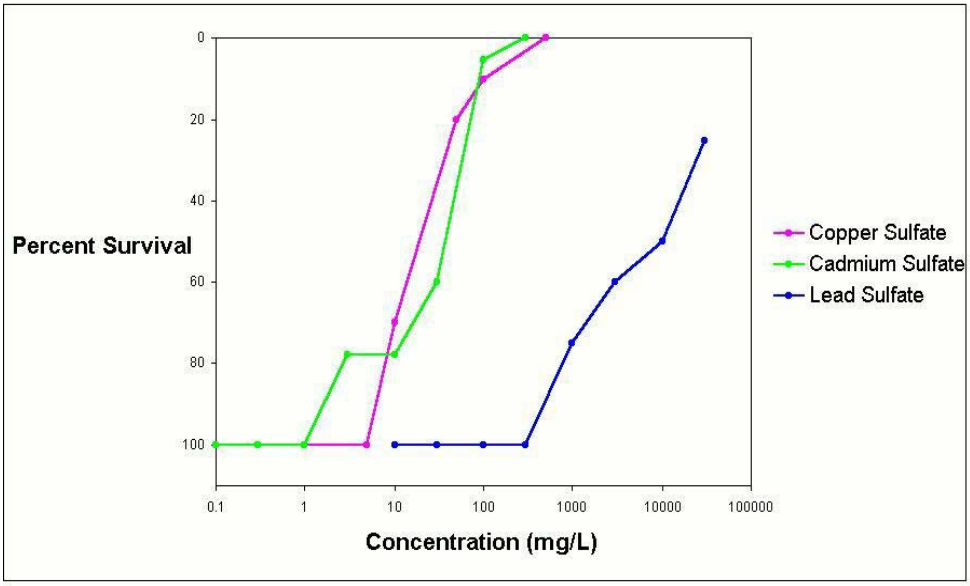

Figure 1. Dose dependent metal toxicity to *Daphnia magna.*

Metal	NOEC (µg/L)	Published LC50 (µg/L)	Reference	Experimental LC_{50} (µg/L)	95% Confidence Interval (µg/L)
Copper Sulfate	3.0	18.9 + 2.3	Mastin and Rodgers 2000	23.4	15.6 - 34.1
Cadmium Sulfate	1.0	156	Braginskiy and Shcherban 1978	22.7	15.4 - 34.4
Lead Sulfate	300	3166 ±1745	Gale *et al*. 1992	3780	2600-6060

Table 1. NOEC and LC_{50} values for copper, cadmium, and lead.

Hemoglobin was successfully extracted following the 24-hour exposure. Fig. 2 is an image of the gel after electrophoresis. The white bands indicate the amount of total RNA. Differences in intensity reflect different amounts of total RNA.

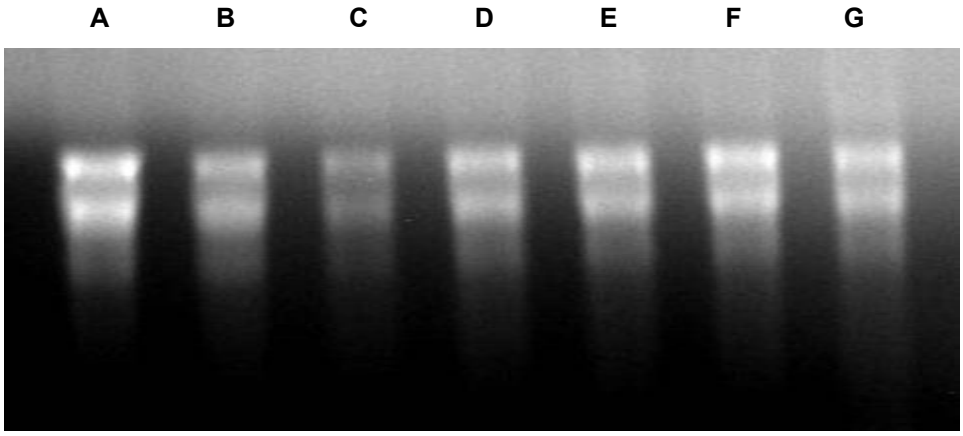

Figure 2. Total RNA isolated from metal exposed *Daphnia magna.* (A) Daphnia cultured under normal conditions, (B) 3.0 µg/L Cu, (C) 13.0 µg/L Cu, (D) 1.0 µg/L Cd, (E) 12.0 µg/L Cd, (F) 300 µg/L Pb, (G) 2000 µg/L Pb.

Results from northern analysis show that there was significant hemoglobin gene expression following lead exposure (Fig. 3). Little to no expression of the gene was found at the control and after copper and cadmium exposure. There was also a visible decrease in expression at the 1/2 LC_{50} of lead.

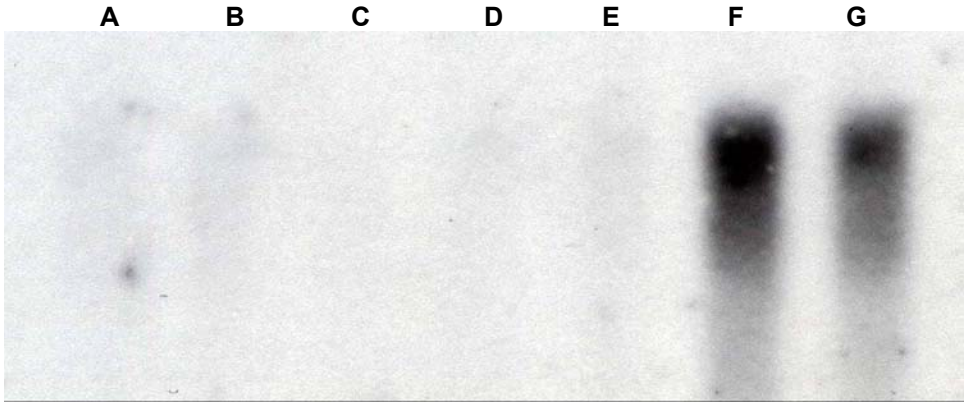

Figure 3. Results from northern analysis. (A) Daphnia cultured under normal conditions, (B) 3.0 µg/L Cu, (C) 13.0 µg/L Cu, (D) 1.0 µg/L Cd, (E) 12.0 µg/L Cd, (F) 300 µg/L Pb, (G) 2000 µg/L Pb.

Discussion

The objective of this study was to establish hemoglobin gene expression as a effective biomarker for aquatic toxicity. There were two indicators for the achievement of this objective. First, there was a visible difference in hemoglobin gene expression between the three metals exposed. The hemoglobin gene was more expressed after lead exposure compared to the negligible expression after copper and cadmium exposure. This indicates that induction of hemoglobin gene expression was more specific to lead toxicity than to copper and cadmium toxicity. Because only three metals were tested, there is still a possibility that hemoglobin gene expression could be induced following exposure to other metals. Further studies using more metals would strengthen the findings of this study.

Second, there was a visible difference in hemoglobin gene expression between the NOEC and 1/2 LC_{50} of lead. These results correlate with the study by Berglind et al. (1985); it was found that ALA-D activity was inhibited after lead exposure. Increased hemoglobin gene expression at the NOEC was a reflection of increased heme synthesis, a result of a positive response to the inhibited ALA-D activity. Near death and extreme stress was a possible explanation for the decreased expression at the 1/2 LC_{50}. In any case, the difference in expression between the NOEC and 1/2 LC_{50} exemplifies the sensitivity of the use of gene expression for sub-lethal lead toxicity assessment.

Improvements of the test design could have been made. Repeated acute and chronic toxicity tests would validate the test concentrations that were used. In general, the experimental values matched up with the published values for the copper and lead exposures (Table 1). The lead concentrations may have been slightly skewed due to the insolubility after 6000 micrograms/Liter. Lead chloride should have been use instead, as it is more soluble than lead sulfate. The experimental results from cadmium exposure were more sensitive than the published results. This may have been due to the extremely high variations within the published LC_{50} values of cadmium (EPA 2003; Suedel *et al.* 1997). Variations in LC_{50} values in this study were also found in the acute toxicity tests for cadmium exposure. Results from three acute tests showed statistically insignificant values. After combining the results, a statistically significant value was found. Fluctuations may have occurred as a result of experimental error or as a result of the unknown responses that daphnia have to cadmium.

Overall, the slight variations between the experimental and published values may have been a result of the use of unfed daphnia as opposed to fed daphnia used in the published experiments. Other variations occurred because exposure times in the published results varied from 24-hr tests to 96-hr tests. To normalize the differences in total RNA, a separate test with a known expression level independent of toxicity could have been done.

Results from this study and future studies will lay the groundwork to establish the use of cDNA microarrays as biomarkers of aquatic toxicity (Aardem and MacGregor 2002). Microarrays can be used as tool for visualizing global changes in gene expression in response to a toxic chemical and will provide a gene expression profile for each metal tested. Further studies with microarrays will aid in the early detection of aquatic toxicants and help curb further detriment to polluted aquatic ecosystems. Communities suffering from environmental injustice will be able to use microarrays to identify sources of pollution in order to hold offenders accountable. (Poynton 2003, pers comm)

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Appendix

Hemoglobin sequence with primer sequences highlighted:

1 aaaacacatt gcaattctac ggaagagcat cagttagctg tctgctattc ccagagctaa
61 accaacccgc aacaatggct tcttttaaga ttgcccttct cctcggcgtc atcgccttcg
121 tcaacgcttg ctcccaagcg cccggaacta ccaccaccac tgtgactacc actgtcacca
181 ccgtgtcagc tgatgatgga agcgaagccg gccttctgag cgctcacgag cgcagcctca

241 tccgcaagac atgggaccag gccaagaagg acggagatgt cgctccccag gtcctcttcc
301 gcttcgtcaa ggcccacccc gagtaccaga aaatgttcag caagttcgcc aacgttcctc
361 aatctgagct tttgagcaac ggaaacttct tggctcaggc ctacaccatc ttggccggtt
421 tgaacgtcgt catccagtcc ctgttcagcc aggagctgat ggccaaccaa ctgaacgctc
481 tcggtggtgc ccatcaaccc cgtggagcta cccccgtcat gttcgagcaa ttcggtggca
541 tcctcgagga agtcctggct gaagagcttg gaagcggatt cactgctgag gccagacaag
601 cctggaagaa cggacttgcc gctttggttg ctggcatcgc caaaaacctc aagaaagctg
661 aagatttggc tgatccccag actaaactga ctccccacca gatccgcgat gtccagagga
721 gctgggaaaa catcagaaac gaccgtaacg ccctcgtctc ctccatcttc gtcaagctct
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841 ctttggccgg caatgccgaa tacgagaaac aaattgctct ggttgccgac cgtctggaca
901 ccatgatctc ggctatggat gataaactgc aacttttggg taacatcaac tacatgagat
961 acacccacac cgaacgtgga attccccgtg ctccttggga ggacttctct cgtcttcttc
1021 ttgacgttct tggctctaag ggagtttcaa ctgatgactt ggactcatgg aagggcgttt
1081 tggccgtctt cgtcaacgga gtttcaccca tcaagaaata aattcaaacg tggcactcaa
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1201 aattatgatt tgctcaaata caaacaattg attggaatct aaaaaaaaaa aaaaaaaaaa
1261 a

Primers:

LIGO	start	len	tm	gc%	any	3'	seq
LEFT PRIMER	193	20	59.95	50.00	8.00	2.00	tgtcagctgatgatggaagc
RIGHT PRIMER	1171	20	59.97	50.00	2.00	1.00	cttgatgggtgaaactccgt

SEQUENCE SIZE: 1329
INCLUDED REGION SIZE: 1329