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Gene Expression Profiling in *Daphnia magna* Part I: Concentration-Dependent Profiles Provide Support for the No Observed Transcriptional Effect Level

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Ecotoxicogenomic approaches to environmental monitoring provide holistic information, offer insight into modes of action, and help to assess the causal agents and potential toxicity of effluents beyond the traditional end points of death and reproduction. Recent investigations of toxicant exposure indicate dose-dependent changes are a key issue in interpreting genomic studies. Additionally, there is interest in developing methods to integrate gene expression studies in environmental monitoring and regulation, and the No Observed Transcriptional Effect Level (NOTEL) has been proposed as a means for screening effluents and unknown chemicals for toxicity. However, computational methods to determine the NOTEL have yet to be established. Therefore, we examined effects on gene expression in *Daphnia magna* following exposure to Cu, Cd, and Zn over a range of concentrations including a tolerated, a sublethal, and a nearly acutely toxic concentration. Each concentration produced a distinct gene expression profile. We observed differential expression of a very few genes at tolerated concentrations that were distinct from the expression profiles observed at concentrations associated with toxicity. These results suggest that gene expression analysis may offer a strategy for distinguishing toxic and nontoxic concentrations of metals in the environment and provide support for a NOTEL for metal exposure in *D. magna*. Mechanistic insights could be inferred from the concentration-dependent gene expression profiles including metal specific effects on disparate metabolic processes such as digestion, immune response, development and reproduction, and less specific stress responses at higher concentrations.

Introduction

Although metals are naturally occurring, they can accumulate to toxic concentrations because of natural or anthropogenic processes. The ubiquity of metal contamination in the environment creates a pressing need to develop biologically relevant indicators or biomarkers of exposure and effect. Despite their potential in monitoring and risk assessment, biomarkers face challenges because of concern about dose responsiveness and specificity in complex field environments (*1*). An attractive alternative to single biomarker approaches is provided by ecotoxicogenomics in which thousands of end points (mRNA transcript levels, protein expression, or metabolite levels) are monitored for changes after exposure to an environmental toxicant or stressor (*2*). Considering the entire set of genes together as a suite of biomarkers could provide a more robust alternative to single biomarker approaches. Multiple recent studies have demonstrated in environmentally relevant organisms that a characteristic set of genes, an expression profile, are differentially expressed in response to a particular toxicant and studies of protein and metabolite levels show similar promise (*3*). However, like any biomarker, ecotoxicogenomic approaches must be extensively validated through dose−response studies in the laboratory and through field validation.

Of particular interest is the ability to apply ecotoxicogenomic approaches to risk assessment and regulation. However, before this is possible, computational tools must be developed to translate gene expression profiles into regulatory benchmarks. One method developed by Thomas et al. integrates dose−response gene expression data with the benchmark dose (BMD) analysis method to define a level of toxicant predicted to increase risk on the basis of the differential expression of genes belonging to functional categories defined by Gene Ontology (GO) (*4*). However, this method may not be readily applicable to many model ecotoxicology organisms because of incomplete annotation of these organism's genomes and difficulty in assigning GO terms to their genes. An alternative method recently proposed is the No Observed Transcriptional Effect Level (NOTEL), which defines a concentration at which no genes are differentially expressed (*2, 5*). However, to the best of our knowledge, no ecological studies have shown that a NOTEL exists or have developed computational methods to determine the concentration of the NOTEL.

We therefore expanded upon our initial study, which examined gene expression profiles for the metal toxicants, Cu, Cd, and Zn, in the ecotoxicology model organism, *Daphnia magna* (*6*), to include a range of biologically relevant concentrations. This study reported the gene expression patterns for Cu, Cd, and Zn at the 1/10 LC_{50}, representing a sublethal concentration that resulted in chronic effects to reproduction. We found that each metal had a distinct expression profile at these concentrations.

In the present study, we investigated how the expression profiles changed at high and low concentrations and determined whether a NOTEL exists for these metals. We performed chronic toxicity bioassays to determine appropriate exposure concentrations that included a tolerated concentration, resulting in no observable chronic effects and a high concentration close to acutely toxic levels. The high and low concentrations resulted in gene expression profiles distinguishable from the original expression profiles for each metal. There was overlap in the expression pattern of some genes at the higher concentrations, whereas the lowest concentration caused little change in gene expression.

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TABLE 1. Exposure Levels for Microarray Hybridizations[a]

Metal	exposure level			
	1/20 EC50 (μg/L)	1/10 EC50 (μg/L)	1/10 LC50 (μg/L)	NOEC (μg/L)
copper	1.0	2.0	6.0	30
cadmium		0.060	18	100
zinc		12	500	1000

[a] *Daphnia magna* were exposed to varying concentrations of copper, cadmium, or zinc for 24 h (acute) or 21 days (chronic). Acute LC_{50} values were determined using the Spearman-Kaber Calculation. Chronic EC_{50} values were determined using the Inhibition Concentration (Icp) Approach. The NOEC for the acute exposures was determined using a Student's *t*-test and defined as the highest concentration with a $p > 0.1$. The chronic 1/20 EC_{50}, chronic 1/10 EC_{50}, acute 1/10 LC_{50}, and acute NOEC were chosen as the exposure levels for the microarray studies.

Together, these results demonstrate the dose-responsiveness of the genomic biomarkers, provide evidence linking gene expression changes and traditional ecotoxicity end points, and suggest the existence of a NOTEL for Cu, Cd, and Zn. In a companion paper, we validated the ability of gene expression profiling to predict the presence of a toxicant in field samples and provide support for the application of the NOTEL approach to field samples (*7*).

Materials and Methods

Maintenance of *D. magna* Cultures. Genetically homogeneous *Daphnia magna* were cultured in COMBO modified for water hardness as described previously (*6*). Following exposure studies, pH, dissolved oxygen (DO), water hardness, and alkalinity were measured and recorded. The pH varied between 7.7 and 8.1; DO ranged from 7.7 to 8.5 mg/L; water hardness was between 125 and 140 mg $CaCO_3$/L; and alkalinity was maintained between 69 and 78 mg $CaCO_3$/L.

Acute and Chronic Toxicity Assays. Acute and chronic toxicity assays were conducted using protocols similar to the U.S. EPA Whole Effluent Toxicity (WET) protocol (*8*) and U.S. EPA chronic toxicity WET protocol (*9*). First instar *D. magna* were placed in 25 mL of media containing varying concentrations of copper sulfate (Fisher Scientific, Hampton, NH), cadmium sulfate (Fisher Scientific, Hampton, NH), or zinc chloride (Sigma-Aldrich, St. Louis, MO). Eight concentrations were tested for each contaminant and a zero concentration control. End points measured in these toxicity tests included lethality for the acute test and reproduction for the chronic test. The LC_{50}, EC_{50}, and NOEC were determined using the statistical procedures outlined by the U.S. EPA (*8*).

Chemical Exposures. Chemical exposures were performed using approximately twenty adult (16−18 day old) *D. magna* placed in 1 L of COMBO media for 24 h. Our primary objective for this study was to develop a tool for environmental monitoring; therefore, we performed 24 h exposures because this is a convenient time point for toxicological studies and it compliments the acute toxicity bioassays described above. Previous work demonstrated that 24 h exposures resulted in reproducible gene expression patterns (*6*). For microarray hybridizations, for each metal, we exposed *D. magna* to two different concentrations as determined in the acute and chronic toxicity bioassay (see Table 1). These concentrations were chosen to represent a tolerated concentration (1/10 EC_{50}) and a no observable effect concentration in acute exposures (NOEC) (see Table S1 in the Supporting Information for a description of each concentration). A zero concentration control was performed alongside each metal exposure, so that each exposure had a complimentary unexposed control for microarray hybridization. For the microarray experiments, three biological replicates were performed for each metal exposure on separate dates. A fourth replicate exposure and an additional exposure at the 1/10 LC_{50} was performed for q-RT-PCR confirmation. Following each 24 h exposure, *D. magna* were collected and RNA was extracted for microarray hybridizations and q-RT-PCR. For the chronic Cu exposures to investigate lectin and β-glucan binding protein (BGBP) expression, we exposed 40 first instar daphnids to $CuSO_4$ at 8, 16, or 0 μg/L concentration control. Daphnids were fed every Monday, Wednesday, and Friday according to standard methods (*9*)and were harvested after 7 or 14 days. Two replicate exposures were performed for each concentration and time point.

RNA Isolation and Microarray Hybridization. *D. magna* were harvested as described previously (*6*), and RNA was isolated using Trizol according to standard methods (Invitrogen, Carlsbad, CA). Before proceeding to reverse transcription, RNA from both the unexposed and exposed *D. magna* was split into two pools, to provide two dye-swapped technical replicates for each metal exposure. Because three exposures were performed for each metal, and RNA from each exposure was hybridized to two different microarrays, there were six hybridizations for each exposure condition as illustrated in Figure S1 of the Supporting Information. Details related to the construction of the *D. magna* microarray and the microarray hybridization procedure have been described previously (*6*). Information about the experimental design, raw signal intensity values, and other MAIME compliant data are available at the Gene Expression Omnibus (GEO) (located at http://www.ncbi.nlm.nih.gov/geo) with the accession number GSE7668.

Identification of Candidate Differentially Expressed Genes. The statistical methods used to preprocess the data and identify differentially expressed genes are described in detail in Loguinov et al. (*10*). Briefly, raw intensities in each channel were corrected, local background was subtracted, and log (base 2)-transformed intensity values were normalized by print-tip groups to remove possible nonlinearity, if any. We applied an approach based on sequential single-slide data analysis utilizing the α-outlier-generating model and outlier regions approach to identify differentially expressed cDNAs on each slide correcting raw *p*-values for multiplicity of comparisons with *q*-values (*10*). As an alternative to between-slide normalization, we utilized a method of multiple-slide data analysis for replicates that does not require a scale estimator: binary outcomes for each gene in replicates are treated as Bernoulli trials (each replicate in a group of 6 as a trial) then applying exact binomial test to associate a p-value with each gene: 6 "successes" of 6 trials. Otherwise we utilized a more liberal empiric decision rule: 4 "successes" of 6 trials. Our algorithms are implemented as software written in S-plus language (R version of the software for single-slide data analysis is available in Loguinov et al. (*10*)). We applied an average false positive cutoff of 1 to identify candidates for differential gene expression. cDNAs differentially expressed in both technical replicates, and in 2 of the 3 biological replicates were chosen as candidate differentially expressed cDNAs and should offset the allowance of 1 false positive in each experiment. Candidate differentially expressed cDNAs were sequenced as described previously and closest protein homologues were determined by translated BLAST searches (http://greengene.uml.edu/Batch.html) and PredictProtein (http://www.embl-heidelberg.de/predictprotein/predictprotein.html) (*6*). The final list of candidate differentially expressed genes is shown in Table S3 of the Supporting Information.

Quantitative Reverse Transcription PCR (q-RT-PCR). To confirm differential expression, we chose several genes

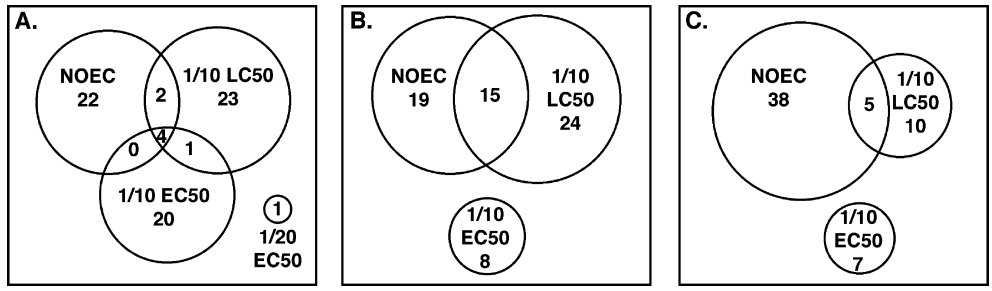

FIGURE 1. Number of differential expressed genes at each exposure concentration. The number of differentially expressed genes specific to each exposure concentration are shown. The number of genes differentially expressed in more than one concentration are shown in the overlapping portion of the Venn diagrams. (A) Cu exposures, (B) Cd exposures, (C) Zn exposures.

for q-RT-PCR analysis. Total RNA isolated following metal exposures was treated with DNase I and repurified using the Ambion DNA*free* kit (Ambion, Austin, TX). The purified RNA was reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). PCR amplification was performed using a SYBR GREEN PCR Master Mix (Applied Biosystems, Foster City, CA) and the following program: 95 °C for 2 min, 40 cycles of 95 °C for 15 s, 60 °C for 1 min. PCR products were quantified in real time using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). A dilution series of cDNA from unexposed *D. magna* was used to create standard curves for each primer set according to the standard curve method employed by the System Software. PCR reactions, performed in triplicate, were compared to the standard curve to determine fold induction of each gene relative to the unexposed samples. 18S rRNA was also assayed to compare total cDNA in each sample. The expression ratio of each gene was divided by the quantity of 18S rRNA relative to the unexposed sample to normalize for cDNA content. Primer sequences used in qPCR are available in Table S2 of the Supporting Information, and q-RT-PCR results are shown in Table S4 of the Supporting Information.

Calculation of No Observed Transcriptional Effect Level (NOTEL). We fit a dose−response curve with 95% confidence intervals based on empirical data for the binary response: "portion of differentially expressed cDNAs versus concentration," using a generalized linear model with binomial family for logit analysis (*11*). The portion of differentially expressed cDNAs was based on the number of cDNAs that were differentially expressed in six out of six microarray hybridizations (p-value = 0.0156 using exact binomial test) applying the number of spots on each slide (4992) as a total (see Table S5 in the Supporting Information). To estimate noise (no signal) we counted the number of systematic outliers in "same versus same" unexposed control pairs. We then applied bootstrapping software from S-plus (library *boot*) to calculate location estimator and 95% confidence intervals for the control pair empirical data. We applied the following definition for NOTEL: NOTEL is a maximum concentration of a chemical for which the 95% confidence interval for outliers based on control pairs is still overlapping with the 95% confidence interval for dose−response curve for the chemical.

Results and Discussion

Exposure Concentrations. To determine appropriate exposure concentrations for the gene expression studies, we performed toxicity bioassays with *Daphnia magna*. Figure S2 in the Supporting Information shows the effect of metal exposure on survival after acute exposure and reproduction after chronic exposure. To compare gene expression profiles across different metals, we choose concentrations that would result in a similar toxic response. The exposure concentrations were based on the following end points as described in Table S1 in the Supporting Information: chronic 1/20 EC_{50} (for Cu only), chronic 1/10 EC_{50}, acute 1/10 LC_{50}, and the acute NOEC. The concentrations of each metal at these end points are given in Table 1.

Patterns of Gene Expression at Different Metal Concentrations. Three replicate exposures for each metal concentration and two dye swap hybridizations were performed for a total of six microarray hybridizations at each concentration (see Figure S1 in the Supporting Information). We identified candidate differentially expressed genes on each array using the EDGE method of Loguinov et al. (*10*). Figure 1 shows the number of differentially expressed genes at each exposure condition including overlapping genes differentially expressed in multiple concentrations. The complete list of differentially expressed genes organized into their functional categories is shown in Table S3 in the Supporting Information. We also included data from our previous study at the 1/10 LC_{50} for comparison (*6*). The $\log_2$ ratio averaged over all six microarray hybridizations is also shown so expression levels can be compared across the different treatments.

Confirmation of Concentration-Dependent Gene Expression. To confirm microarray results and determine the suitability of sets of genes as biomarkers of exposure, we selected eight genes for q-RT-PCR analysis. Genes including monooxygenase (DV437798), cellulase (DV437797), and slit homologue (DV437805) responded generally to all three metals. Candidate biomarkers that responded specifically to one metal include inositol monophosphatase (IMPase) (DV437806), which responded to Cu; glutathione-S-transferase (GST) (DV437830), which responded to Cd; and chitinase (DV437858), which responded to Zn. The results of the q-RT-PCR analysis compared to microarray ratios are shown in Table S4 in the Supporting Information. We previously carried out confirmatory q-RT-PCR for several genes at one concentration including monooxygenase, IMPase and GST (*6*); in the present study, we assayed the differential expression of these genes across different concentrations. For the majority of these genes, the q-RT-PCR results confirm the differential expression determined by microarray analysis thereby providing support for their use as biomarkers of exposure. Two metallothionein (MT) genes, MT (a) (DV437799) and MT (b) (DV437826), were chosen for further investigation by q-RT-PCR because of their historical use as biomarkers of metal exposure. As shown in Figure S3 in the Supporting Information, MT (a) is specifically induced by Cd at low concentrations, but responds to all three metals at higher concentrations, whereas MT (b) responds generally to metal exposure at both concentrations. These results are consistent with the microarray analysis (see Table S3 in the Supporting Information).

Loss of Specificity at High Metal Concentrations. We examined how the specificity of the gene expression profiles varied by metal concentration and found that the expression profiles became less distinct with increasing concentration.

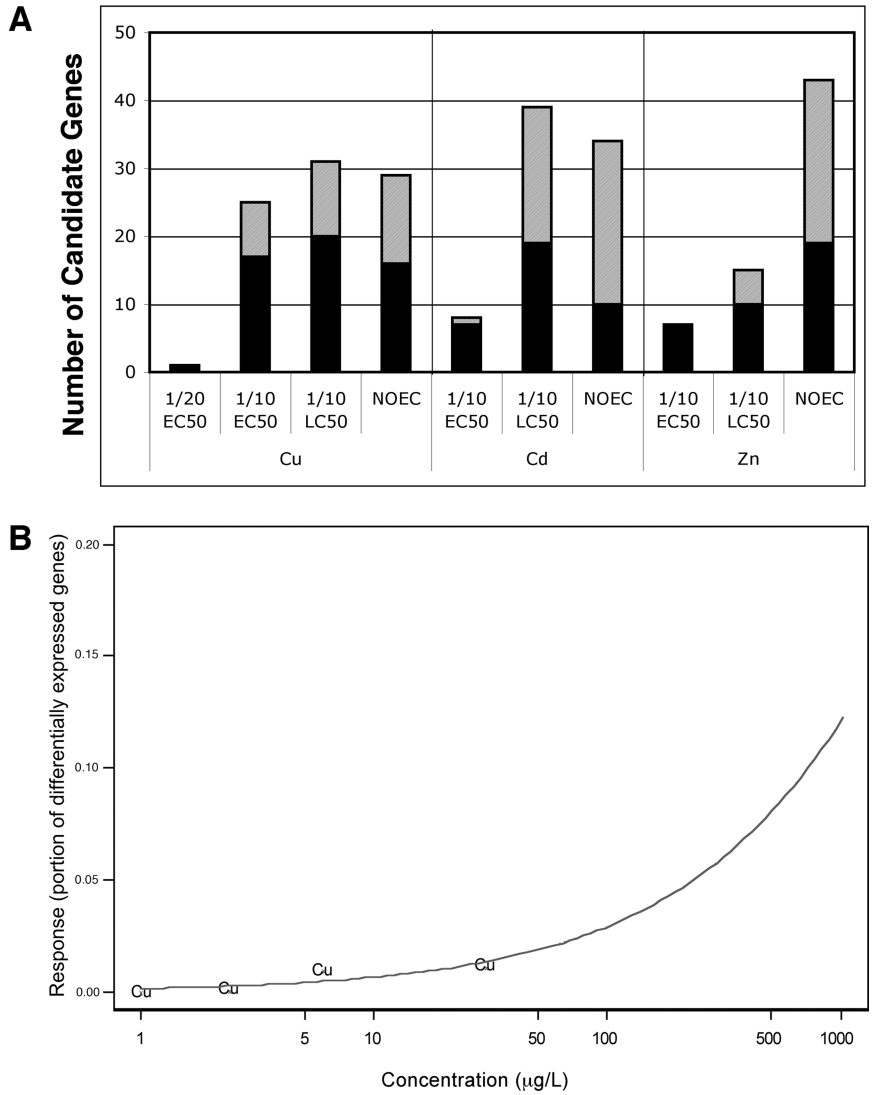

FIGURE 2. Concentration-dependent responses in global gene expression to metal exposure. (A) Specificity of the gene expression patterns at different metal concentrations. The number of candidate differentially expressed genes is shown for each exposure condition. The black bars represent the number of genes specific to the individual metals. The gray portion of the bars represents the number of genes that overlap in differential expression with other metals. (B) Copper dose−response curve for global gene expression. The dose−response curve is the probability for a spot (cDNA) to be differentially expressed versus Cu concentration and was created using a generalized linear model with binomial family for logit analysis (*11*). Empirical points (observed frequencies) used for the fitting are labeled as "Cu". The portion of differentially expressed cDNAs was based on the number of cDNAs differentially expressed in six out of six microarray hybridizations (*p*-value = 0.0156 using exact binomial test). The number of spots on each slide (4992) was applied as the total when calculating the observed frequencies.

For example, at the 1/10 EC_{50}, 88% of the differentially expressed genes are unique to Cd. However, at the higher concentration, the acute NOEC, only 29% of the differentially expressed genes are Cd specific. Figure 2A illustrates how the number of differentially expressed genes appears to increase at the higher metal concentrations, but the number of genes specific to the metal exposure (shown in black) represents a smaller proportion of the total differentially expressed genes.

In addition to loss of specificity in the gene expression profiles, we also found that the expression profiles of the sublethal concentration, 1/10 LC_{50}, did not overlap extensively with the highest concentration, the NOEC (see Figure 1). At lower concentrations, we expect to find an active compensatory response to the toxicant and toxicant specific toxicity. Resulting gene expression changes would likely be specific to that compound's mode of toxicity. However, at higher concentrations, a less- specific response could occur as tissues become significantly compromised. General stress-related genes become more prevalent and the specific mode of toxicity would be overshadowed by a common stress response. A similar observation was reported following arsenic exposure in a human lung cell line. At low concentrations, the arsenic response was distinct but a higher dose caused a very different gene expression profile composed of mostly general stress response genes (*12*). Another study in rainbow trout fry reported similar gene expression profiles in response to high doses of four different toxicants (β-naphthoflavone (βNF), Cd, CCl_4, and pyrene). They suggested this could represent nonspecific toxic effects at high doses (*13*). Because of poor annotation of *D. magna* genes, it is difficult to discern the role of the differentially expressed genes at the NOEC. The genes affected at the highest metal concentrations include peptidases and cell signaling kinases, but their specific function is unknown (see Table S3 in the Supporting Information). These genes may represent general stress-related proteins such as caspases and heat shock proteins, but without functional studies, it is difficult to speculate. However, the general pattern presented by this study, that high metal concentrations cause a less-specific

expression profile, suggests that general stress-related processes become more predominant as the concentration nears acutely toxic levels.

Few Genes Are Differentially Expressed at Low Concentrations. One goal of our study was to determine if low metal concentrations, which do not result in chronic toxicity, would induce a similar pattern of differentially expressed genes as higher metal concentrations. As shown in diagrams B and C in Figure 1, the 1/10 EC_{50} for Cd and Zn causes the differential expression of only a few genes, which are not found in the expression profiles of the higher metal concentrations. In contrast, the gene expression profile for the 1/10 EC_{50} Cu contained 25 genes and had some similarity to the 1/10 LC_{50} (see Table S3 in the Supporting Information and Figure 1A). This was probably because there was only about a 2-fold difference in the Cu concentration at the 1/10 EC_{50} (2.4 μg/L) and the 1/10 LC_{50} (6.0 μg/L). Therefore, we decided to investigate the gene expression changes at a lower concentration of Cu, 1/20 of the EC_{50} (1.0 μg/L). At the 1/20 EC_{50} we found that only one gene was differentially expressed (see Figure 1A).

Environmental monitoring of toxicants such as metals, which are ubiquitous in the environment, requires tools that can differentiate between toxic and nontoxic concentrations of contaminants. The No Observed Transcriptional Effect Level (NOTEL) has been suggested to describe the concentration of a chemical, which results in no significant changes in gene expression (*5*). In the first study introducing this concept, Lobenhofer et al. performed gene expression analysis of MCF-7 cells, an estrogen responsive cancer cell line, exposed to four concentrations of estrogens including two low doses, a physiological dose, and a cytotoxic dose. The higher doses generated gene expression responses, but the two lowest doses failed to elicit differential expression in any of the 2000 genes represented on the ToxChip. This study established a threshold concentration for transcriptional responses to estrogen and demonstrated that low doses of chemicals may result in no changes to gene expression (*5*). Because of the potential utility of the NOTEL in environmental monitoring, we were encouraged that our study shows that low metal exposures result in only subtle changes to the gene expression profile. These results suggest a threshold toxicant level for transcriptional changes. As shown in Figures 1 and 2A, very few genes are differentially expressed at the lowest concentrations tested. These concentrations represent tolerated exposure levels, causing no chronic effects to survival or reproduction. The few genes differentially expressed at the low concentrations suggest that a NOTEL may exist where no genes are differentially expressed. Another important observation is that there was no overlap in the expression profiles of the lowest concentrations and the higher, toxic metal concentrations. Therefore, the toxic profile may be used for as a reference profile for determining if the concentration of an environmental sample is toxic. The results of this study support the existence of a NOTEL for metal exposure and suggest a means for differentiating between toxic and compensatory responses to contaminant exposure. In a companion paper, we applied this concept to field samples to show that upstream samples with undetectable levels of metals also had few differentially expressed genes. This field example also provides an illustration of using a subset of genes, shown in this study to be affected only by the toxic levels of Cu, to distinguish between samples with toxic or nontoxic levels of metals (*7*).

Computational Analysis for NOTEL Determination. In addition to field monitoring, there is an interest in applying the NOTEL to environmental risk assessment in chemical screening and whole effluent testing. In these examples, a method is needed to extrapolate the gene expression data to estimate a concentration or percent effluent that results in no gene expression changes. In this study, we found that the number of differentially expressed genes increased as the metal concentration increased (see Figures 1 and 2A). Because we had gene expression profiles for four concentrations of Cu, we were able to create a dose−response curve that related the proportion of differentially expressed genes to the Cu concentration (see Figure 2B). As described in the methods, the NOTEL is a maximum concentration of a chemical for which the 95% confidence interval for outliers based on unexposed control pairs is still overlapping with the 95% confidence interval for dose−response curve for that chemical. As explained in Table S5 in the Supporting Information, this value was determined to be 0.58 μg/L for Cu with a 95% confidence interval [0.26, 1.30], within which falls our experimental value of 1 μg/L that elicited the differential expression of only one gene. In chemical screening, this method could be employed to define a NOTEL. If the predicted environmental concentration of a new chemical is below the predicted NOTEL, then the chemical would not require further testing (*3*). In whole effluent toxicity (WET) testing, the NOTEL would represent a percent of the effluent and permits could be written to require that the NOTEL be above a certain percent of the effluent. Although further validation with other chemicals is required, this method provides a potential application for ecotoxicogenomics in environmental risk assessment.

Downregulation of Several Gene Families Suggests Functional Impairment. By grouping differentially expressed genes into functional categories based on their similarity to known genes, we were able to make predictions into how the metals exert toxicity in *D. magna*. Although many of these gene families were first discovered in our previous work at a single concentration (*6*), in the present study, we investigated the dose responsiveness of these genes and expanded upon our discussion of these findings. The additional information provided in this dose response study revealed that these genes are not differentially expressed at low concentrations, but are affected at higher concentrations thereby linking their responses to a toxic outcome. In addition, a major criticism of biomarkers in ecotoxicology is that many do not respond in a dose-responsive manner (*1*); therefore, it was imperative to show that genes that constitute candidate biomarkers are responsive at toxic concentrations but do not respond at low, nontoxic levels.

Digestion. The downregulation of several classes of genes including glucanases, peptidases, and fatty acid binding proteins by the three metals suggests that the digestive system and nutrient uptake are commonly affected by metal exposure. Several genes involved in carbohydrate digestion are downregulated at different metal concentrations. Although these genes were not always identified as differentially expressed, their expression ratios across different concentrations of Cu, Cd, and Zn are consistently low, with the exception of the lowest concentration tested (see Table S3 in the Supporting Information). The downregulation of two of these genes, cellulase and preamylase, was confirmed by q-RT-PCR (see Table S4 in the Supporting Information). Genes involved in other areas of digestion were downregulated by metal exposure including trypsin, whose primary function is in protein digestion, and fatty-acid binding proteins, whose expression is linked to lipid intake in other animals (*14*). There are several possible mechanisms that could explain the decreased expression of these three classes of related genes. First, general toxicity and ill health caused by the metal exposure may disrupt *D. magna* feeding patterns. This idea is supported by experimental evidence illustrating a decrease in feeding and food intake during metal exposure (*15*). A second possibility is that the digestive organ, comprised of the intestine and hepatopancreas, is a direct target of metal toxicity. This is supported by morphological

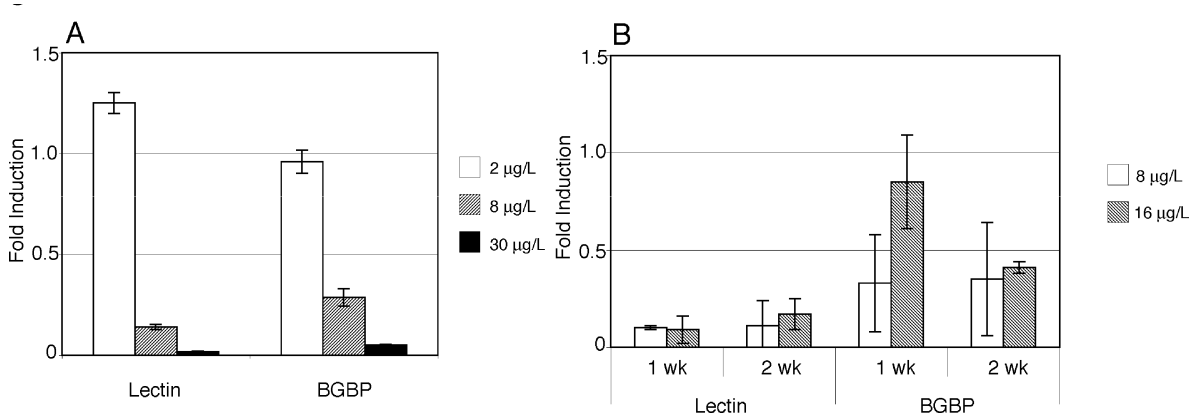

FIGURE 3. Quantitative reverse transcription PCR investigation of immune function genes following metal exposure. In each experiment, the expression level of each gene was normalized to 18S rRNA and is shown as the fold induction compared to an unexposed control. (A) To confirm the dose-dependent downregulation of lectin (DV437813) and β-glucan binding protein (BGBP) (DV437823) observed by microarray analysis, gene expression was investigated at different Cu concentrations using q-RT-PCR. Error bars represent the standard deviation across three technical replicates. (B) The differential expression of lectin and BGBP was determined during chronic Cu exposure. First instar *D. magna* were exposed to $CuSO_4$ or an unexposed control for 1 or 2 weeks. Following the exposure, q-RT-PCR was conducted to determine the expression level compared to the unexposed control. Error bars represent the standard deviation across two replicate exposures and three technical replicates for each exposure.

evidence, depicting a shrunken and deformed intestine following Cd exposure (*16*). Necrosis caused by metal exposure would result in fewer cells in the digestive organ able to contribute to the overall pool of RNA. With fewer represented cells, there would appear to be a downregulation of transcripts that are specific to that tissue. From studies in other crustaceans (*18*), it appears the hepatopancreas is a major target site of metal toxicity. Thus, the downregulation of these digestive genes may indicate cellular toxicity to the hepatopancreas and digestive organ of *D. magna*.

Immune Function. The effect of sublethal metal exposure on the immune system of *D. magna* poses important questions regarding the ecological health of daphnids. An investigation of two immune related genes, β-glucan binding protein (BGBP) (DV437823) and lectin (DV437821), in *D. magna* following metal exposure revealed that Cu affects the expression of these genes even at very low concentrations, whereas Cd and Zn cause some downregulation at high concentrations (see Table S3 in the Supporting Information). We confirmed the downregulation of these genes from Cu exposure by q-RT-PCR, showing a dose-dependent downregulation of the immune related genes (Figure 3A). We also investigated the expression of these genes during long-term Cu exposure. As shown in Figure 3B, lectin and BGBP are consistently downregulated during 1 and 2 week exposures, suggesting that the downregulation is not episodic or fluctuating, but that these genes are continuously suppressed by Cu.

As discussed previously, these genes encode proteins with similarities to BGBP and lectin, important proteins involved in the arthropod innate immune response (*6*) and other studies have shown a connection between susceptibility to disease and Cu exposure in crustaceans and mollusks (*19, 20*). The additional evidence presented here that these genes are down-regulated in a dose-responsive manner, and their continuous repression during chronic Cu exposure further implicates Cu in immune suppression and raises concerns about exposure to sublethal levels of Cu, which are not predicted to cause mortality.

Development and Exoskeleton Proteins. The final class of genes downregulated by metal exposure includes genes involved in reproduction or molting. The ability of daphnids to reproduce is directly related to their molting activity. Molting is a highly coordinated process regulated by arthropod hormones and requires Chitinase activity and the synthesis of new cuticle proteins (*21*). Several chitinases and cuticle proteins were downregulated by Cu and Zn at high concentrations (see Table S3 in the Supporting Information). Previously, we showed that Zn also decreases Chitinase activity in a dose-dependent manner (*6*). The downregulation of chitinases and cuticle proteins and subsequent decrease in Chitinase activity suggests molting activity will be decreased, adversely affecting reproduction. Vitellogenin (VTG) and several other genes involved in reproduction and development are downregulated by the high metal exposures (see Table S3 in the Supporting Information). VTGs are important proteins in development providing nourishment for the growing embryos. They are synthesized by the mother and carried through hemolymph to the developing eggs. In other crustaceans, it has been shown that VTG synthesis occurs in the hepatopancreas (*22, 23*). If the hepatopancreas is a target organ of metal toxicity as suggested by downregulation of digestive enzymes, the downregulation of VTG may be a result of cellular damage in the hepatopancreas. The decreased expression of exoskeletal and reproductively important genes suggests that the metals cause sublethal effects on reproductive capacity. Indeed, suppression of these genes is first seen at a sublethal level (1/10 LC_{50}), resulting in chronic effects to reproduction (see Figure S2 in the Supporting Information).

Together, our results suggest possible modes of action for copper, cadmium, and zinc toxicity in *Daphnia magna,* demonstrate the dose-responsiveness of genomic biomarkers, as well as link gene expression changes with traditional ecotoxicity end points. In addition, we provide evidence for a NOTEL in an environmentally relevant organism and suggest that the determination of the NOTEL could have applications in environmental monitoring and risk assessment.

Acknowledgments

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Supporting Information Available

Description of the exposure concentrations used in this study (Table S1), q-RT-PCR primer sequences (Table S2), a complete list of differentially expressed genes (Table S3), q-RT-PCR

confirmation of the microarray results (Table S4), and a further explanation of the NOTEL estimation (Table S5). In addition, an illustration of our exposure design (Figure S1), graphs illustrating toxicity of each metal (Figure S2), and q-RT-PCR results of MT (a) and (b) expression (Figure S3) (PDF).This material is available free of charge via the Internet at http://pubs.acs.org.

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