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Toxicogenomics of water chemistry influence on chronic lead exposure to the fathead minnow (*Pimephales promelas*)

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Abstract

Establishment of water quality criteria (WQC), intended to protect aquatic life, continues to rely principally on water hardness (i.e. Ca^{2+}) for lead (Pb) despite growing evidence that other chemical parameters also strongly influence toxicity. To more clearly define the water chemistry parameters mediating Pb toxicity, we evaluated the effects of hardness as $CaSO_4$ and dissolved organic carbon (DOC) as humic acid during chronic (150 days) exposures to the fathead minnow. Measured Pb concentrations ranged from 157 ± 5 nM (33 ± 1 μg/L) Pb in base water to 177 ± 7 (37 ± 1 μg/L) and 187 ± 7 nM (39 ± 1 μg/L) Pb in $CaSO_4$- or HA-supplemented water, respectively. Fish were collected at 2, 4, 10, 30, 63, 90 and 150 days of exposure. Traditional toxicological endpoints were examined alongside gene transcription analyses to help clarify the underlying mechanisms of Pb toxicity and to identify candidate molecular markers that might ultimately serve as robust indicators of exposure and effect. Addition of $CaSO_4$ did not prevent whole body Pb accumulation whereas DOC afforded strong protection (about half the amount accumulated by fish in base water) suggesting that current, hardness-based WQC are likely inaccurate for predicting chronic Pb effects in aquatic systems. Custom-made microarrays were co-hybridized with base water samples ± Pb up to the 30 days time point. Quantitative PCR was employed to verify gene transcription responses and to extend analysis to the $CaSO_4$ and HA treatments and the 150 days time point. Identification of four genes by microarray analysis revealed clear Pb-induced responses over time: glucose-6-phosphate dehydrogenase, glutathione-*S*-transferase, ferritin and β-globin. Results obtained by qPCR were in strong agreement with microarray data by regression analysis ($r^2 = 0.82$, slope = 1.28). The associated pathways implicated herein for these genes provide further evidence supporting roles for anemia and neurological disorders in chronic Pb toxicity. Effects of water chemistry on Pb accumulation and gene transcription responses were in close parallel, though alterations in ionoregulatory and morphological endpoints were not observed. Whereas DOC was protective against Pb accumulation and mRNA expression changes, Ca^{2+} was not. Additionally, several hypothesis-driven genes (ECaC, DMT-1, and ALA-D) were examined by qPCR but revealed either no change or small Pb-induced responses lacking any clear influence attributable to water chemistry. These findings should help pave the way toward development of a new chronic Pb BLM and a Pb-responsive gene transcript profile for fathead minnows, both of which would greatly aid future environmental monitoring and regulatory strategies for Pb.

Keywords: Microarrays; Glucose-6-phosphate dehydrogenase (G6PD); Glutathione-*S*-transferase (GST); Ferritin; Beta-globin (β-globin); Lead (Pb); Hemolysis

1. Introduction

Despite significant reductions in use, most notably in paint production and as a fuel additive, lead (Pb) continues to enter the environment primarily by anthropogenic means, retaining its status as a priority pollutant (USEPA, 2006). While the focus has turned towards remediation with regards to preventing human exposure, much is still needed in the way of determining appropriate measures to monitor and protect the aquatic environment, particularly with regards to point source pollution. In many cases, water quality criteria (WQC) continue to rely principally on water hardness (i.e. Ca^{2+}) despite growing evidence that other chemical parameters (e.g. pH, salinity and dissolved organic carbon (DOC)), which may vary greatly on a local basis, also strongly influence Pb toxicity (Macdonald et al., 2002; Grosell et al., 2006). Efforts to improve WQC for metals have given rise to several toxicity models designed to

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encompass the influences of all major water chemistry parameters. The most widely accepted model, the biotic ligand model (BLM), is currently used by the USEPA to set WQC for copper. In essence, the BLM accounts for site-specific water conditions by considering the competitive effects from other cations and complexation with organic/inorganic agents that prevent the metal from interacting with the site of toxic action (Paquin et al., 2002). The benefits of a site-specific approach to setting regulatory standards would seem agreeable to industry and regulators alike and ideally encourage greater policy compliance.

There is no demonstrated biological need for Pb, thus uptake and toxicity is likely mediated through the mimicry of other cations (Ballatori, 2002), the most probable candidate being Ca^{2+} given the strong evidence that Pb acts as a Ca^{2+} antagonist (Busselberg et al., 1991; Rogers and Wood, 2004). However, the identification of a specific ligand for Pb remains elusive. As in mammals, the principal effects of chronic Pb exposure to fish are presumably hematological (Hodson et al., 1978), neurological (Davies et al., 1976) and renal (Patel et al., 2006) impairment. Studies have also examined reproduction and behavior (Holcombe et al., 1976; Weber, 1993), though the nature of the observed effects is unclear with respect to the influence of water chemistry.

Another factor lending uncertainty to the regulatory decision-making process is that metals and other toxicants are commonly present as mixtures in the environment. Genomic approaches are well suited to address such problems, filling in where more conventional methods prove insufficient to pinpoint key environmental stressors or elucidate the contributions and additive effects from multiple toxicants. Furthermore, microarrays provide opportunities not only for establishing the molecular basis of toxicity, but potential for gaining insights into modes of action and higher order effects. Thus, defining toxicant-specific mechanisms that link signature gene transcript profiles to chronic effects would greatly aid in monitoring and diagnosing water quality and also prioritizing higher-tier tests in ecological risk assessment. The significance of genomics in this regard was recently addressed by the USEPA as outlined in the Interim Genomics Policy (Dix et al., 2006).

We undertook this study to garner a more comprehensive understanding of chronic Pb toxicity by integrating traditional toxicological endpoints with mRNA expression analyses using one of the long-standing USEPA vertebrate test organisms, the fathead minnow. Specifically, we sought to achieve three goals: (1) further examine the influence of Ca^{2+} and DOC on the chronic toxicity of waterborne Pb, (2) identify Pb-responsive genes and determine whether transcriptional changes reflect the influence of ambient water chemistry in modulating toxicity and (3) gain additional insights into the specific mechanisms underlying chronic Pb toxicity. A time course of multiple endpoints including molecular, ionoregulatory, and morphological effects were analyzed. Microarray analysis revealed four genes in particular that may provide additional clues to the molecular mechanisms involved in response to sublethal Pb exposures. Although no ionoregulatory or morphological effects were observed at the Pb concentrations employed, whole body burdens differed in a manner that was closely paralleled by mRNA expression responses with respect to the influence of the different water chemistries. Whereas DOC was protective against Pb accumulation and changes in mRNA levels, Ca^{2+} was not. The observed lack of protection by increased Ca^{2+} is an important finding given that Pb WQC are hardness-based, suggesting the current approach is likely inaccurate for predicting chronic effects of Pb exposure. These findings should help pave the way toward development of a new chronic Pb BLM and a Pb-responsive mRNA expression profile for fathead minnows, both of which would greatly aid future environmental monitoring and regulatory strategies for Pb.

2. Materials and methods

2.1. Experimental animals

Fathead minnows (*Pimephales promelas*) were obtained from Aquatic BioSystems Inc. (Fort Collins, CO) at <24 h post-hatch on arrival, distributed evenly among 18 one-liter plastic beakers (~70 fish per beaker, three replicates per treatment) and gradually acclimated to test media without Pb for 1 week. Fish were reared in these chambers under flow-through conditions for the first 30 days of exposure, beyond which the remaining were pooled from each replicate and maintained in single 6 L plastic containers. Fish were fed freshly hatched *Artemia* nauplii once daily for 1 month followed by 1 week of an *Artemia* and Tetramin flake food mixture and then flake food only thereafter. Prior to feeding, any fish that had perished were removed, the mortality recorded and leftover food and feces siphoned out.

2.2. Chronic Pb exposures

To obtain the three test media (Table 1), gravity flow-through conditions were employed as previously described (Grosell et al., 2006). Briefly, a base water of 2:1 deionized water:dechlorinated Virginia Key tap water was adjusted for hardness or DOC by the addition of $CaSO_4$ or Aldrich humic acid (HA) stock solutions, respectively. Following 1 week of acclimation, Pb exposures were carried out for 150 days by

Table 1
Chemistry of test media (in μM except for hardness which is expressed as mg/L as calculated by APHA Standard Methods; $n = 6$ except for pH $n = 22$ and for DOC as noted in parentheses)

	$[Na^+]$	$[K^+]$	$[Ca^{2+}]$	$[Mg^{2+}]$	$[Cl^-]$	$[SO_4{}^2-]$	$[CO_2]$	[DOC]	Hardness	pH
Base water	235 ± 8	25 ± 1	241 ± 12	53 ± 1	355 ± 27	20 ± 6	332 ± 11	82 ± 1 (3)	30 ± 1	7.5 ± 0.1
500 μM Ca^{2+}	251 ± 12	23 ± 1	618 ± 41	45 ± 2	284 ± 12	414 ± 41	305 ± 7	74 ± 2 (3)	68 ± 4	7.4 ± 0.1
4 mg/L humic	242 ± 7	26 ± 1	247 ± 21	54 ± 2	341 ± 13	16 ± 7	345 ± 25	149 ± 6 (14)	31 ± 2	7.6 ± 0.1

dispensing concentrated $PbNO_3$ solutions via Mariotte bottles to test media targeting final concentrations of approximately 170 nM (~35 μg Pb/L). All chemicals used for modifying our target parameters were obtained from Sigma–Aldrich (St. Louis, MO). Average water temperature throughout the exposures was 22 °C ± 1.

2.3. Water chemistry and whole animal Pb and ion accumulation

Fish were collected and euthanized at 2, 4, 10, 30, 63, 90 and 150 days of exposure for analysis of whole body ion and Pb composition as previously described (Grosell et al., 2006). Sample sizes were 18, 18, 12, 12, 10, 7 and 10, respectively (except for 150 days $CaSO_4$ ± Pb $n = 6$). To ensure sufficient material for analytical purposes, six larval fish from each beaker were pooled into a single tube at 2 and 4 days due to the small sizes at these time points. For the remaining time points, sizes were large enough to allow for collection of each fish into a single tube. Significant mortality, likely due to a pathogen infection, was observed in the $CaSO_4$ (no Pb) group around 45 days of exposure that was abated by several days of malachite green treatment. To ensure adequate numbers for the full term exposure we elected not to collect fish from this group at 90 days.

Water chemistry was also analyzed once or twice per week. Samples were measured for Pb concentration after filtering through a 0.45 μm cellulose syringe filter (Acrodisc, Pall Life Sciences, MI) and acidification to 1% HNO_3 (Fisher Scientific, trace metal grade). All Pb measurements were performed by graphite furnace atomic absorption spectroscopy (Varian 200Z, Varian, Australia). Concentrations of Na^+, K^+, Ca^{2+}, and Mg^{2+} were determined by flame atomic absorption spectroscopy (Varian, 220FS, Varian, Australia), Cl^- and $SO_4{}^{2-}$ by anion chromatography (DIONEX DX120, CA), and total CO_2 using a Corning 962 carbon dioxide analyzer (UK). To measure DOC, high temperature catalytic oxidations were performed using a Shimadzu total organic carbon-VCSH analyzer (Kyoto, Japan) (Hansell and Carlson, 2001).

2.4. RNA extractions

Except for 150 days, from which six fish were collected for both $CaSO_4$ treatments and nine for all others, pooling and sample numbers were the same as above. Fish were euthanized, placed into cryotubes and immediately snap-frozen in liquid nitrogen. Total RNA was isolated from whole fish using RNA STAT-60 solution (Tel-Test, Friendsworth, TX) and a Polytron homogenizer. From each isolate 10 μg was treated with DNase I to remove any traces of genomic DNA (Turbo DNA-free kit; Ambion, Austin, TX). Integrity of RNA was confirmed by gel electrophoresis prior to cDNA synthesis.

2.5. Microarray hybridization and analysis

Arrays were constructed using 5000 randomly picked clones from a cDNA library representing the full fathead minnow life history (Wintz et al., 2006). For each time point equal amounts of RNA from all no Pb controls were pooled and hybridized with each of three biological replicates corresponding to the respective Pb exposures described above. Synthesis of *Cy*5/*Cy*3-labeled cDNA was accomplished using the 3DNA Array 900 kit (Genisphere, Hatfield, PA). All hybridizations were performed per the manufacturer's instructions and repeated using dye-swapped cDNAs. An ArrayWoRx Biochip Reader (Applied Precision, Issaquah, WA) and GenePix software Version 3.01 (Molecular Devices, Sunnyvale, CA) were used to scan and quantify hybridization signals, respectively.

For preprocessing, microarray data sets were $\log_2$-transformed and normalized by print-tip-groups to remove any possible non-linearity (Yang et al., 2002). Candidate genes are outliers from the line of equivalence ($Cy5 = Cy3$) and the majority of other data points lie in the vicinity of the line forming a concentration ellipse possibly distorted with heteroscedasticity and/or non-linearity (Loguinov et al., 2004). Specifically, for exploratory differential mRNA expression analysis we used "M vs. A" scatter plots, where $M = \log_2(Cy5/Cy3)$ and $A = \log_2(Cy5Cy3)/2$. To quantify heteroscedasticity (if any) on the "M vs. A" scatter plots we applied a robust non-parametric regression (*lowess*) and the outliers are treated in terms of α-outlier-generating model and outlier regions (Loguinov et al., 2004). To correct for multiplicity of comparisons we applied a q-value approach that is a modification of the FDR method (Storey and Tibshirani, 2003). As a result, each gene was characterized with two numbers: (1) $\log_2$-normalized intensity value, and (2) q-value. As a threshold for q-values we use one assuming one false-positive outcome at average (Storey and Tibshirani, 2003) and it always was less than 0.05 in terms of q-values.

2.6. 5′ RACE

To obtain poly(A) RNA as template for RACE reactions, aliquots from the 30 days total RNA samples were pooled and processed using the MicroPoly(A) Purist kit (Ambion, Austin, TX). Poly(A) RNA (1 μg) was reverse transcribed and amplified using the BD SMART RACE cDNA Amplification Kit (BD Biosciences, Franklin Lakes, NJ; Table 2). Touchdown PCR cycling conditions were as follows: five cycles of 94 °C for 30 s, 69 °C for 30 s and 72 °C for 3.5 min followed by 5 and 20–25 cycles as previously except at 67 and 65 °C annealing temperatures, respectively. Products were gel purified, cloned into the pCR 2.1 vector (Invitrogen, Carlsbad, CA) and sequenced.

2.7. cDNA synthesis and primer design

For qPCR, cDNA was synthesized from 1 μg DNase I-treated total RNA using the SuperScript II First-Strand System (Invitrogen, Carlsbad, CA). Following RNase H treatment, all reactions were diluted tenfold in TE buffer. To obtain fathead minnow-specific sequences ~1.5 kb of the epithelial calcium channel (ECaC) and 326 bp of δ-aminolevulinic acid dehydratase (ALA-D) were cloned by degenerate PCR; all qPCR primers were designed from the coding regions of each target gene (Table 2).

Table 2
Primers used for qPCR and cloning. Accession numbers for fathead minnow sequences from which primers were designed are provided

Primer	Accession no.	Sequence (5′ → 3′)	Product size (bp)
EF1α-F	AY643400	GACCACTGAGGTTAAATCTGT	142
EF1α-R		GTCGTTCTTGCTGTCTCCAG	
Ferritin-F	EF628371[a]	GAACGTCAATCAGGCTCTGC	163
Ferritin-R		GTTGCCAGCATCCATCTTGG	
G6PD-F	EF628372[a]	CAATGCATGAGCACCAAAGG	124
G6PD-R		GGTAAATCTGTTCCTCGGTG	
GST-F	EF628373[a]	GACGTTCATCTTCTGGAAGC	152
GST-R		GAGGCTTTCTCGCACTGC	
β-globin-F	EF628375[a]	CTATGCTGAACTGAGTGTGC	129
β-globin-R		TGGACTGCAGGTGTGAATG	
ALA-D-F	EF628376[a]	GCACCCTACTCATCCACTG	114
ALA-D-R		GTCATAGCCTCCATAACTGC	
ECaC-F	EF628374[a]	GTAATACCATCCTGCATCTGC	125
ECaC-R		GTTGGGAATCATGTCTAGTGG	
DMT1-F	AF190773	CCATCGCTTTCAATCTGCTG	113
DMT1-R		CTTCAGACCGTACTTGTCCAG	
dALA-D-F[b]	N/A	TTCMGAGAYGCTGCHCAGTC	326
dALA-D-R[b]	N/A	ATGATGATRTCAGCWCCTGC	
dECaC-F[b]	N/A	CTCATCAAYGAGCCCATGAC	1480
dECaC-R[b]	N/A	GYGTCCTCCAGAGCTCGTC	
7B20-GSP[c]	N/A	AACAGAGGGATGTGGCATGTCATACC	2526
8N17-GSP[c]	N/A	CTAAGGGTGTCAATCATTCTGACCATGAC	1245
8J9-GSP[c]	N/A	AACATCAGATGATCCTCTGTGCATAAGCAG	593

Abbreviations: Forward primer (F); reverse primer (R); gene-specific primer (GSP).
[a] Sequences obtained during the course of this study.
[b] Degenerate primers designed from conserved nucleotide regions across multiple fish species were used to obtain fathead minnow ALA-D (*Danio rerio* (BC092804), *Coryphaenoides armatus* (AJ609236), *Tetraodon nigroviridis* (CR668921)) and ECaC (*Oncorhynchus mykiss* (AY256348) and *Danio rerio* (AY325807)) sequences.
[c] Primers used for 5′ RACE designed from sequences identified by microarray analysis. Product sizes include 45 bp corresponding to Clontech Universal Primer A sequence at 5′ end.

2.8. Quantitative PCR

Experiments were carried out in the MX4000 instrument (Stratagene, La Jolla, CA) with AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA) and SYBR Green I dye (Sigma, St. Louis, MO) for detection. A preliminary experiment was performed using the geNorm approach (Vandesompele et al., 2002) to examine the suitability of several "housekeeping" genes for normalization purposes, namely 18S ribosomal RNA, elongation factor 1α (EF1α) and ribosomal protein L8. EF1α proved the strongest candidate, exhibiting stable expression across randomly selected sub-samples from all treatments and developmental time points (data not shown). Because this gene also adhered best to the criteria of amplifying at a similar cycle (i.e. <10) to those of the genes of interest we chose to use EF1α as our target for normalization. All reactions were optimized to establish PCR efficiencies of >95%. Amplicon identities were confirmed by sequencing and a single corresponding melting peak was verified following all amplifications. Cycling was as follows: 95 °C for 5 min followed by 40 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. Six reactions per treatment representing duplicate runs of three biological replicates were performed. Because fish were no longer segregated into replicate beakers beyond 30 days, biological replicates for 150 days consisted of cDNA pools from randomly selected fish. Fold changes were calculated from the log-transformed C_T values and expressed relative to the treatment-matched no Pb controls using a modification of the delta–delta–C_T method (Livak and Schmittgen, 2001) to account for amplification efficiencies as previously described (Vandesompele et al., 2002).

2.9. Statistical analysis

Except for microarray analysis, differences were deemed statistically significant at $P < 0.05$ by two way analysis of variance (ANOVA) using pairwise multi-sample comparison corrections (Bonferroni *t*-test) as appropriate. Data are presented as means ± 1 standard error of the mean (SEM). An α-outlier-generating model approach was employed for determination of statistically significant changes in mRNA expression by microarray analysis as described above.

3. Results

3.1. Influence of water chemistry on Pb toxicity

Measured water chemistries were in accordance with our target nominal values and were consistently maintained across treatments (Table 1). Lead concentrations were stable within treatments, but differed slightly between with mean values ranging from 157 ± 5 nM (33 ± 1 μg/L) Pb in base water to 177 ± 7 nM (37 ± 1 μg/L) and 187 ± 7 nM (39 ± 1 μg/L) Pb in $CaSO_4$- or HA-supplemented water, respectively. Lead concentrations in control treatments remained below the detection

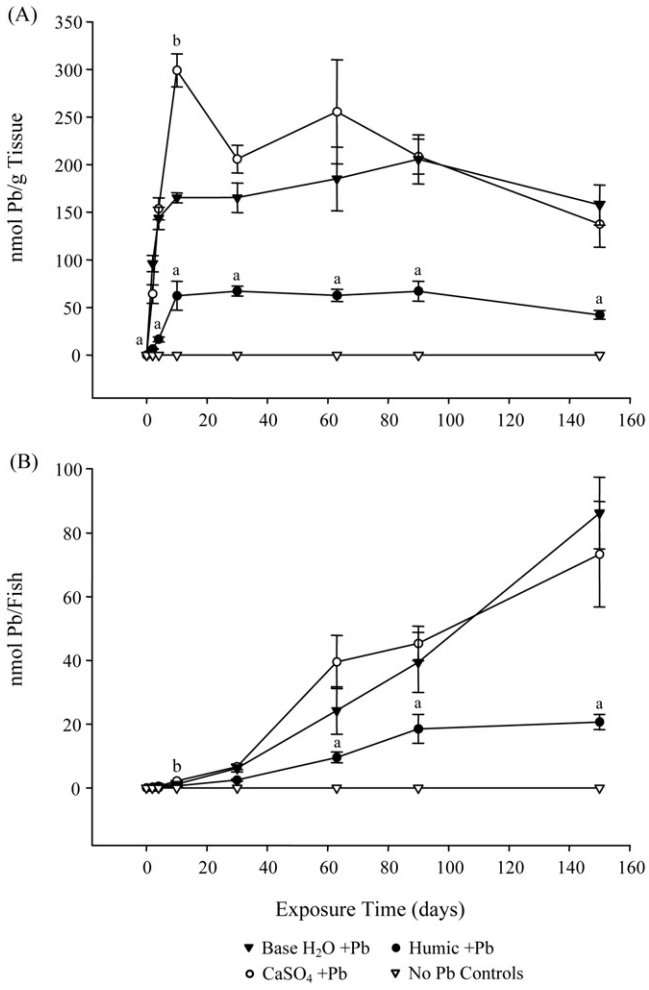

Fig. 1. Influence of water chemistry on whole body Pb accumulation by fathead minnows during 150 days exposures. Lead accumulation is expressed per wet weight (A) and per fish (B). All treatments +Pb exhibited statistically significant differences from corresponding control except for 2 and 4 days time points in (B). Statistically significant difference for HA + Pb (a) or $CaSO_4$ + Pb (b) compared to base water + Pb.

limit (0.5 μg/L). Strong protection against Pb accumulation was clearly afforded by the increase in DOC concentration with fish typically exhibiting about half the amount accumulated by fish from base water (Fig. 1A), whereas elevation in $CaSO_4$ failed to prevent Pb accumulation. These results generally held true with respect to the influence of water chemistry when the same data were plotted as a function of Pb content per fish to account for an apparent growth dilution effect initiated around 10 days (Fig. 1B). Lead continued to accumulate as the fish grew, though again to a lesser degree in the presence of HA.

Whole body ion concentrations remained relatively stable across test media irrespective of Pb addition (Table 3). Ontogenic changes in whole body ion concentrations independent of Pb were examined by comparing overall means for each time point. There were two immediately apparent observations: a spike in 10 days concentrations (129.4 ± 3.8 μmol/g Ca^{2+}, 105.7 ± 2.5 μmol/g K^+, 62.3 ± 1.4 μmol/g Na^+) and opposite trends displayed by Ca^{2+} and K^+ concentrations over time. Whereas Ca^{2+} increased from 90.4 ± 3.5 μmol/g at 2 days to 125.6 ± 2.8 μmol/g at 150 days (mean 112.0 ± 1.4 μmol/g), K^+ decreased from 110.4 ± 3.7 to 85.5 ± 1.4 μmol/g (mean 89.1 ± 1.0 μmol/g) over the same period. Conversely, Na^+ remained stable with a mean concentration of 49.4 ± 0.8 μmol/g (excluding 10 days data). Taken together, however, the results indicate a lack of substantial ionoregulatory disturbance in response to either Pb or water chemistry.

3.2. Morphological and growth effects

We regularly monitored fish for development of spinal curvature and/or black discoloration and peripheral erosion of the caudal fin as these endpoints were previously reported in rainbow trout chronically exposed to Pb (Davies et al., 1976; Holcombe et al., 1976). Arbitrary scoring of spinal curvature by eye could not be correlated to Pb exposure or test media (data not shown). Furthermore, at no time was convincing evidence of caudal fin abnormalities apparent. There was no indication of Pb-induced mortality; a few minor, transitory growth effects due to Pb or water chemistry were observed (632 ± 41 mg overall mean weight at 150 days, Table 4).

3.3. Identification of Pb-responsive genes using microarrays

Analysis of expression profiles revealed four candidate genes exhibiting significant responses to Pb (GEO accession no. GSE8404). The cDNAs were identified either directly or after cloning the corresponding full-length transcripts by 5′ RACE as ferritin heavy chain, glutathione-*S*-transferase alpha (GST), glucose-6-phosphate dehydrogenase (G6PD), and β-globin (Table 2). Except for β-globin all exhibited increased transcription in response to Pb that remained fairly consistent over time (Fig. 2A). The greatest magnitude of change was displayed by GST at all time points which exhibited a pattern that was closely mirrored by G6PD expression. Ferritin was the most stably expressed for the entire duration but was induced to a lesser extent than GST and, except at 30 days, G6PD as well. The only gene that showed decreased levels due to Pb was β-globin, evident most profoundly at 30 days with minor reductions or no change occurring at earlier time points.

3.4. Influence of water chemistry on Pb-induced mRNA expression

Quantitative PCR was employed to verify microarray results and to determine if the Pb-induced mRNA expression responses reflected the influence of water chemistry on whole body Pb accumulation. Results obtained by qPCR and microarray were in strong agreement as evident by regression analysis ($r^2 = 0.82$, slope = 1.28; Fig. 2B). For the most part, expression responses examined by qPCR strongly paralleled the influence of water chemistry on Pb accumulation (Fig. 3). Ferritin, G6PD, and GST all showed decreased induction in fish from the HA treatments which displayed lower Pb accumulation. Conversely, as was the case with respect to Pb accumulation, $CaSO_4$ typically did not afford a protective effect against transcriptional changes.

Table 3
Fathead minnow whole body ions concentrations (μmol g^{-1})

	Exposure time (days)[a]						
	2 (18)	4 (18)	10 (12)	30 (12)	63 (10)	90 (7)	150 (10)
Ca^{2+} (A)							
Base water − Pb	87 ± 3	108 ± 4	134 ± 5	97 ± 4	101 ± 10	108 ± 9	126 ± 5
Base water + Pb	110 ± 5	105 ± 4	126 ± 4	99 ± 3	104 ± 4	118 ± 7	124 ± 4
$CaSO_4$ − Pb	96 ± 8	122 ± 4	125 ± 6	104 ± 4	103 ± 8	N/A	137 ± 8
$CaSO_4$ + Pb	95 ± 4	104 ± 2	130 ± 7	108 ± 3	88 ± 5	118 ± 2	145 ± 11
Humic − Pb	77 ± 4	93 ± 6	121 ± 17	106 ± 3	96 ± 3	125 ± 6	122 ± 6
Humic + Pb	78 ± 10	94 ± 4	140 ± 11	94 ± 4	96 ± 5	110 ± 7	113 ± 7
K^+ (B)							
Base water − Pb	112 ± 8	108 ± 5	114 ± 3	83 ± 2	75 ± 1	69 ± 4	85 ± 2
Base water + Pb	127 ± 6[b]	96 ± 3	101 ± 5[b]	80 ± 1	75 ± 1	76 ± 2	83 ± 1
$CaSO_4$ − Pb	114 ± 9	111 ± 4	104 ± 3[c]	86 ± 1	74 ± 1	N/A	83 ± 3
$CaSO_4$ + Pb	117 ± 6	108 ± 3	101 ± 5	88 ± 1	73 ± 1	76 ± 2	73 ± 8
Humic − Pb	98 ± 5	96 ± 7	105 ± 5[c]	84 ± 1	77 ± 2	83 ± 2	88 ± 2
Humic + Pb	94 ± 9	96 ± 2	109 ± 11	87 ± 1	74 ± 1	78 ± 2	95 ± 3
Na^+ (C)							
Base water − Pb	50 ± 1	49 ± 1	66 ± 2	48 ± 2	50 ± 1	39 ± 3	45 ± 1
Base water + Pb	52 ± 3	47 ± 2	62 ± 2	50 ± 1	53 ± 2	54 ± 4[b]	55 ± 1[b]
$CaSO_4$ − Pb	51 ± 2	53 ± 1	61 ± 2	52 ± 1	47 ± 1	N/A	50 ± 3
$CaSO_4$ + Pb	49 ± 1	48 ± 2	60 ± 3	50 ± 1	49 ± 2	50 ± 2	52 ± 6
Humic − Pb	47 ± 3	46 ± 1	62 ± 4	57 ± 5[c]	49 ± 1	46 ± 1	54 ± 4
Humic + Pb	50 ± 1	47 ± 1	62 ± 6	50 ± 1	46 ± 1	48 ± 3	50 ± 3

[a] Numbers in parenthesis denote *n*-numbers except for 150 days $CaSO_4$ ± Pb ($n = 6$).
[b] Denotes $P < 0.05$ vs. treatment-matched −Pb control at same time point.
[c] Denotes $P < 0.05$ vs. base water − Pb at same time point.

The expression pattern for β-globin was not reflective of the Pb accumulation data.

In addition to the genes identified by microarray analysis, two others were selected for qPCR analysis based on their putative roles in Ca^{2+} transport (Shahsavarani et al., 2006) and route of Pb entry in mammals (Gunshin et al., 1997; Bressler et al., 2004) (ECaC and divalent metal transporter-1 (DMT1), respectively). Expression of ALA-D mRNA was also examined because Pb is known to inhibit activity of this enzyme (Hodson, 1976; Warren et al., 1998). An increase in ECaC transcription level was evident in response to Pb only within the $CaSO_4$ treatments and only at 10 days and beyond (Fig. 3). Analysis of DMT1 and ALA-D mRNA revealed no statistically significant changes irrespective of Pb exposure, and no gene examined in this study demonstrated a change due to water chemistry alone (data not shown).

4. Discussion

4.1. Chronic Pb toxicity and influence of Ca^{2+} and DOC

While Ca^{2+} did not protect against Pb accumulation when compared to moderately soft base water, HA clearly did. These results are in agreement with previous Pb gill binding experiments using rainbow trout and may be explained by the ≥2 orders of magnitude differences in calculated Pb binding affinities for organic matter (log *K* 8.4) vs. gill (log *K* 6.0) and vs. Ca^{2+} gill binding (log *K* 4.0) (Macdonald et al., 2002). These are important findings given that current WQC are hardness-based (USEPA, 2006) and provide further support that a re-evaluation of the means by which chronic Pb criteria are established is warranted.

Table 4
Summary of mean ± SEM waterborne Pb concentrations (nM) and body mass (mg) during 150 days exposures ±Pb in different test media

Medium	[Pb]	Exposure time (days)[a]						
		2 (18)	4 (18)	10 (12)	30 (12)	63 (10)	90 (7)	150 (10)
Base water − Pb	−1.2 ± 1.6[b]	3.1 ± 0.1	2.9 ± 0.2	7.2 ± 0.7	41 ± 2	151 ± 24	243 ± 27	735 ± 114
Base water + Pb	157 ± 5	2.8 ± 0.1	3.7 ± 0.4	7.2 ± 0.8	50 ± 1	116 ± 22	178 ± 29	605 ± 95
$CaSO_4$ − Pb	−1.0 ± 1.7[b]	2.5 ± 0.4	4.0 ± 0.4	6.6 ± 0.8	52 ± 1	144 ± 19	N/A	783 ± 75
$CaSO_4$ + Pb	177 ± 7	3.6 ± 0.2	3.2 ± 0.5	7.7 ± 0.9	50 ± 1	155 ± 19	227 ± 34	606 ± 172
Humic − Pb	−0.4 ± 1.7[b]	2.2 ± 0.3	2.8 ± 0.3	6.9 ± 0.9	57 ± 5	87 ± 12	234 ± 44	588 ± 82
Humic + Pb	186 ± 7	3.2 ± 0.5	3.5 ± 0.1	9.8 ± 1.0[c]	50 ± 1	149 ± 22[c]	256 ± 39	526 ± 75

[a] Numbers in parenthesis denote *n*-numbers except for 150 days $CaSO_4$ ± Pb ($n = 6$).
[b] Measured values are below detection limit.
[c] Denotes $P < 0.05$ vs. corresponding control at same time point.

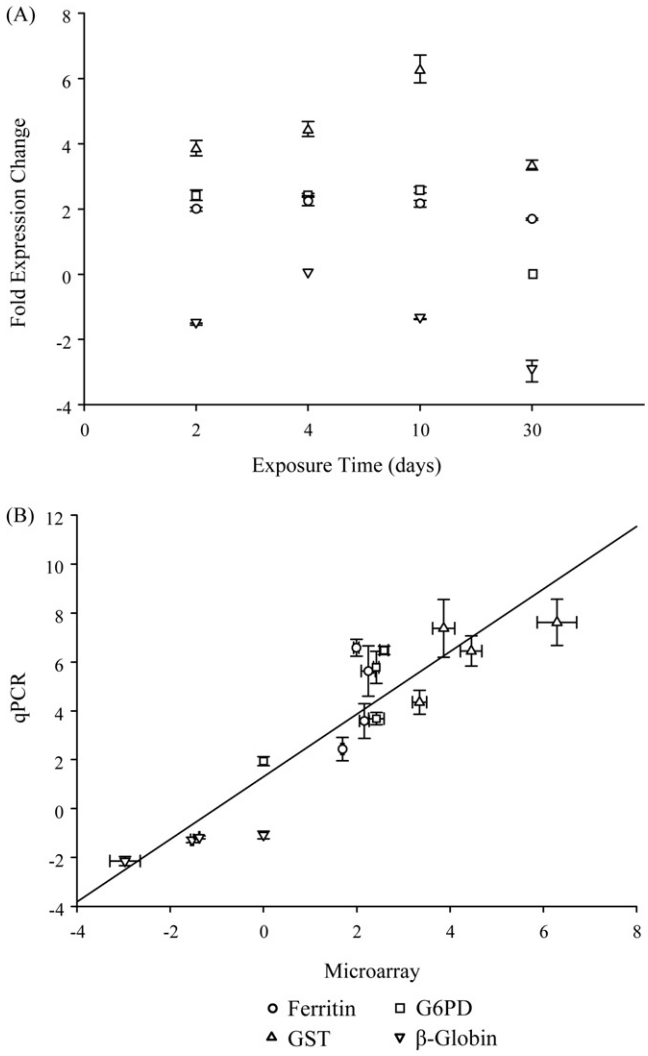

Fig. 2. Time course of fold changes in mRNA expression ± SEM by microarray using fathead minnows exposed to 157 ± 5 nM (33 ± 1 μg/L) Pb vs. no Pb controls in base water (A). Correlation of microarray and qPCR data comparing fold changes in mRNA expression from base water ± Pb samples for all time points out to 30 days ($r^2 = 0.82$; slope = 1.28) (B).

4.2. Pb-responsive genes identified by microarray

The results from our custom fathead minnow arrays elicited four Pb-responsive genes that displayed clear patterns of transcriptional changes that could be verified by qPCR. Several factors may have contributed to the small number of pronounced Pb-induced responses. First, we specifically chose to expose fish to low Pb concentrations to target the most sensitive responses. Thus, the concentrations used may have approached levels that induce changes in mRNA expression that reflect lower threshold responses. Secondly, the cDNA library was of limited complexity (some redundancy) leading to an array composition of much less than 5000 unique cDNAs (Wintz et al., 2006). Thirdly, RNA samples were pooled from multiple fish leading to a possible masking of subtle responses to Pb by native inter-individual variability. Additionally, whole fish were used out of practicality and to examine global transcription responses. However, this may have imposed a greater challenge to detecting relatively small toxic responses occurring in specific organs. Admittedly, these are potential drawbacks, but the genes garnered by such an approach of presumably reduced sensitivity are more likely to serve as robust indicators of low-level chronic exposure and effects.

What is most striking about the present findings is that the transcriptional responses strongly reflected the influence of water chemistry on toxicity and that the genes fit together well in a biochemical scheme (see below). The changes in ferritin, G6PD, and GST expression all mirrored the Pb accumulation data indicating these genes may hold potential as sensitive indicators of Pb accumulation, though lack of a clear toxicological link points to the need for further study. Nevertheless, given that the genes can all be linked within specific pathways suggests that when used together they may offer indication of a Pb-specific response.

4.3. Toxic mechanisms: biochemical pathways implicated in Pb toxicity

The four genes revealed by microarray analysis link pathways previously implicated in anemia (Fig. 4). Induction of G6PD and GST indicates recruitment of the pentose phosphate shunt commonly employed by erythrocytes to combat oxidative stress (Lachant et al., 1984). Deficiency in G6PD, a common genetic disorder in humans, results in hemolytic anemia due to an inability to defend against oxidative stress (Beutler, 1994). The condition arises because NADPH, as required for GST-mediated glutathione conjugation (and which would otherwise be available from normal levels of G6PD production), is not sufficiently maintained to detoxify harmful free radicals. Our microarray experiments also revealed decreased β-globin mRNA levels in response to Pb in base water at 30 days, perhaps further supporting a reactive oxygen species (ROS)-mediated hemolytic loss of erythrocytes. A parallel to these findings may be found with a previous observation of hematocrit reduction followed by recovery occurring near the same times of exposure as in our experiments (Hodson et al., 1978) (Fig. 3).

The inhibitory effect of Pb on ALA-D, a key enzyme in the heme synthesis pathway, has been well documented (Hodson, 1976; Warren et al., 1998; Schmitt et al., 2002) and studies have linked this inhibition to ferritin Fe^{2+} release (Oteiza et al., 1995; Rocha et al., 2003). However, species-specific inconsistencies have been reported and clear links to higher order effects have proved elusive (Hodson et al., 1977; Schmitt et al., 2002). The absence of an ALA-D response by qPCR in the present study is in agreement with a 29 days time course analysis using juvenile rainbow trout exposed to Pb concentrations bracketing those employed herein which revealed no change in whole body enzymatic activity (Burden et al., 1998). This, in addition to increased ferritin expression, a possible feedback response to free Fe^{2+} displaced by accumulating ALA substrate and/or released during hemolysis, is perhaps expected given these findings (Fig. 4). Lead is also known to bind ferritin directly displacing reactive iron in the process (Kelada et al., 2001). Therefore, it is likely that at least two major pathways may lead to anemia as a result of

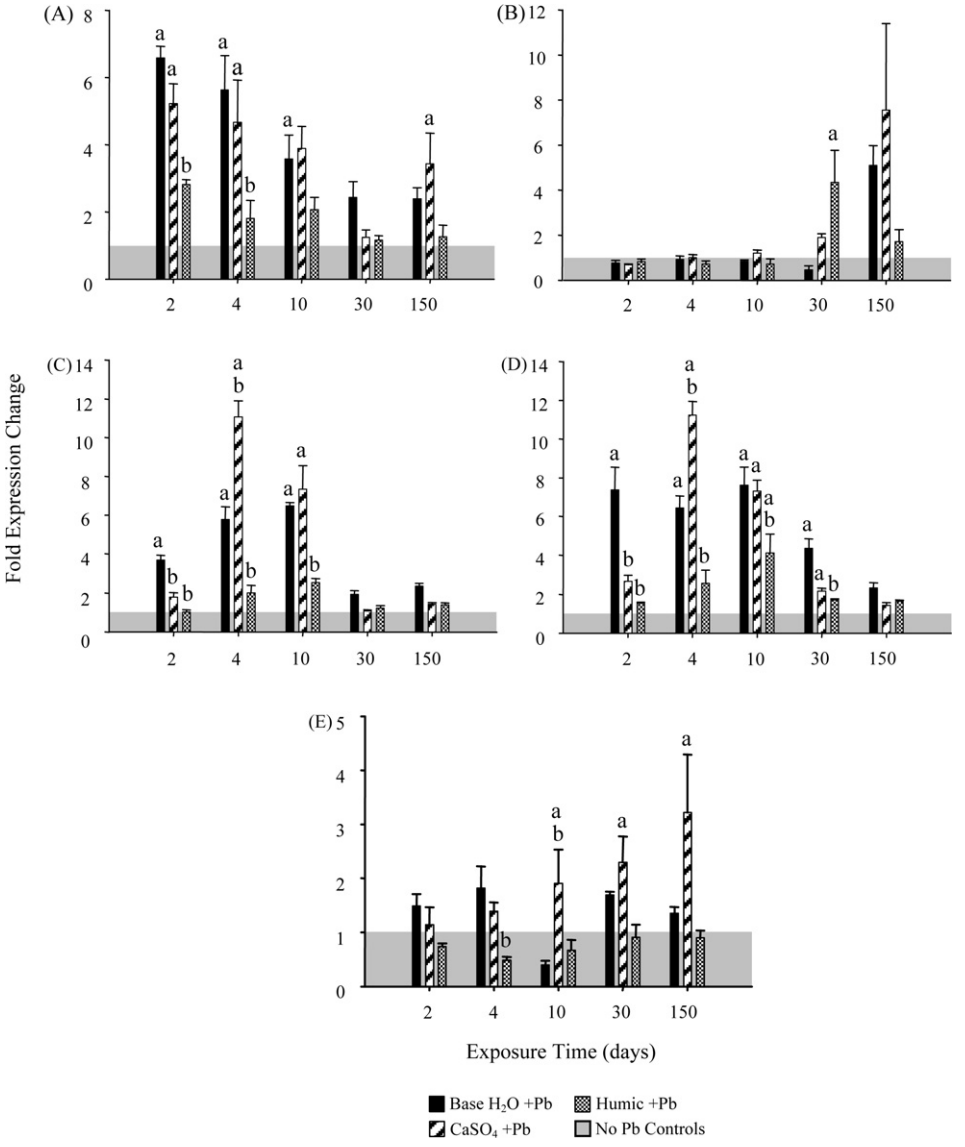

Fig. 3. qPCR analysis of chronic Pb-induced mRNA expression changes in different water chemistries. Data normalized to EF1α is expressed relative to treatment-matched controls. Ferritin (A), β-globin (B), G6PD (C), GST (D), ECaC (E). Statistically significant difference from corresponding control (a) or from base water + Pb (b).

Pb exposure in the fathead minnow: (1) ROS-induced hemolysis and (2) reduced heme synthesis due to ALA-D inhibition.

Ferritin Fe^{2+} displacement and increased ROS production may play a role in neurological impairment as well. Evidence suggests a role for ferritin Fe^{2+} dysregulation in human neurological disorders (Ke and Ming Qian, 2003; Berg and Hochstrasser, 2006) and that ferritin may represent a dominant means of Fe^{2+} delivery to the brains of mammals (Fisher et al., 2007). Thus, it may be interesting to further investigate the significance of Fe^{2+} dysregulation in the neurological effects commonly observed in Pb-exposed fish.

Traditional toxic biomarkers (e.g. metallothionein) often lack specificity and thus may provide little in the way of relevance, mechanistic insight, or ability to predict long-term outcomes of ecological significance. As Pb appears to be rather unique among metals with respect to its role in anemia, it would seem the genes identified by our microarrays may narrow the focus for targeting additional genes in associated pathways. Although the above results more clearly defined the influences of Ca^{2+} and HA on chronic Pb accumulation, the lack of an observed physiological or morphological effect points to the need for additional study if relevant diagnostic capabilities for ecological assessment are to be achieved. Additionally, it will be of interest to further evaluate the influence of other water chemistry parameters such as pH and alkalinity. In any event, the role of DOC in mediating toxicity should garner greater consideration in the development of a chronic BLM for Pb. The results reported herein point to the power of microarray technologies in helping elucidate underlying toxic mechanisms and for further revealing the means by which Pb may elicit detrimental effects to fish and potentially higher organisms as well.

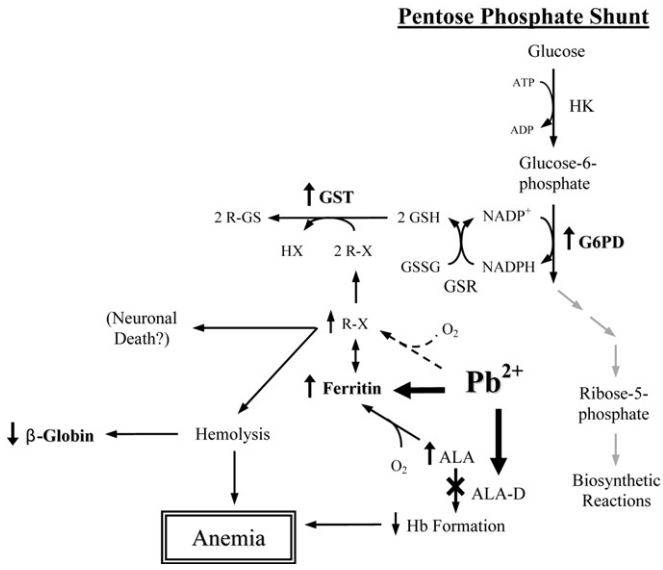

Fig. 4. Pathways likely affected by Pb illustrating potential roles in hematological and neurological dysfunction. Genes identified by microarrays are shown in bold. Inhibition of ALA-D incurs detrimental effects by impaired heme synthesis and increased ALA levels. Binding of Pb and/or ALA to ferritin promotes release of reactive oxygen species (R–X) leading to oxidative stress, hemolysis (and an apparent decrease in β-globin expression due to the loss of erythrocytes) and potentially neuronal death. Ferritin and the pentose phosphate shunt enzymes GST and G6PD increase as compensatory and detoxification responses, respectively. *Abbreviations*: Hexokinase (HK), hemoglobin (Hb).

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References

Ballatori, N., 2002. Transport of toxic metals by molecular mimicry. Environ. Health Perspect. 110 (Suppl. 5), 689–694.

Berg, D., Hochstrasser, H., 2006. Iron metabolism in Parkinsonian syndromes. Mov. Disord. 21, 1299–1310.

Beutler, E., 1994. G6PD deficiency. Blood 84, 3613–3636.

Bressler, J.P., Olivi, L., Cheong, J.H., Kim, Y., Bannona, D., 2004. Divalent metal transporter 1 in lead and cadmium transport. Ann. N.Y. Acad. Sci. 1012, 142–152.

Burden, V.M., Sandheinrich, M.B., Caldwell, C.A., 1998. Effects of lead on the growth and delta-aminolevulinic acid dehydratase activity of juvenile rainbow trout, *Oncorhynchus mykiss*. Environ. Pollut. 101, 285–289.

Busselberg, D., Evans, M.L., Rahmann, H., Carpenter, D.O., 1991. Effects of inorganic and triethyl lead and inorganic mercury on the voltage activated calcium channel of Aplysia neurons. Neurotoxicology 12, 733–744.

Davies, P.H., Goettl, J.P., Sinley, J.R., Smith, N.F., 1976. Acute and chronic toxicity of lead to rainbow trout *Salmo gairdneri*, in hard and soft water. Water Res. 10, 199–206.

Dix, D.J., Gallagher, K., Benson, W.H., Groskinsky, B.L., McClintock, J.T., Dearfield, K.L., Farland, W.H., 2006. A framework for the use of genomics data at the EPA. Nat. Biotechnol. 24, 1108–1111.

Fisher, J.L., Devraj, K., Ingram, J., Slagle-Webb, B., Madhankumar, A., Liu, X., Klinger, M., Simpson, I.A., Connor, J., 2007. Ferritin—a novel mechanism for delivery of iron to the brain and other organs. Am. J. Physiol.-Cell Physiol..

Grosell, M., Gerdes, R., Brix, K.V., 2006. Influence of Ca, humic acid and pH on lead accumulation and toxicity in the fathead minnow during prolonged water-borne lead exposure. Comp. Biochem. Physiol. C-Toxicol. Pharmacol. 143, 473–483.

Gunshin, H., Mackenzie, B., Berger, U.V., Gunshin, Y., Romero, M.F., Boron, W.F., Nussberger, S., Gollan, J.L., Hediger, M.A., 1997. Cloning and characterization of a mammalian proton-coupled metal-ion transporter. Nature 388, 482–488.

Hansell, D.A., Carlson, C.A., 2001. Biogeochemistry of total organic carbon and nitrogen in the Sargasso Sea: control by convective overturn. Deep-Sea Res. Part 2, Top. Stud. Oceanogr. 48, 1649–1667.

Hodson, P.V., 1976. Delta-aminolevulinic acid dehydratase activity of fish blood as an indicator of a harmful exposure to lead. J. Fish. Res. Board. Can. 33, 268–271.

Hodson, P.V., Blunt, B.R., Spry, J., Austen, K., 1977. Evaluation of erythrocyte delta-aminolevulinic acid dehydratase acivity as a short-term indicator in fish of a harmful exposure to lead. J. Fish. Res. Board. Can. 34, 501–508.

Hodson, P.V., Blunt, B.R., Spry, D.J., 1978. Chronic toxicity of water-borne and dietary lead to rainbow trout (*Salmo gairdneri*) in Lake Ontario water. Water Res. 12, 869–878.

Holcombe, G.W., Benoit, D.A., Leonard, E.N., McKim, J.M., 1976. Long-term effects of lead exposure on three generations of brook trout (*Salvelinus fontinalis*). J. Fish. Res. Board Can. 33, 1731–1741.

Ke, Y., Ming Qian, Z., 2003. Iron misregulation in the brain: a primary cause of neurodegenerative disorders. Lancet Neurol. 2, 246–253.

Kelada, S.N., Shelton, E., Kaufmann, R.B., Khoury, M.J., 2001. Delta-aminolevulinic acid dehydratase genotype and lead toxicity: a HuGE review. Am. J. Epidemiol. 154, 1–13.

Lachant, N.A., Tomoda, A., Tanaka, K.R., 1984. Inhibition of the pentose phosphate shunt by lead: a potential mechanism for hemolysis in lead poisoning. Blood 63, 518–524.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. Methods 25, 402–408.

Loguinov, A.V., Mian, I.S., Vulpe, C.D., 2004. Exploratory differential gene expression analysis in microarray experiments with no or limited replication. Genome Biol. 5, R18.

Macdonald, A., Silk, L., Schwartz, M., Playle, R.C., 2002. A lead-gill binding model to predict acute lead toxicity to rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. C-Toxicol. Pharmacol. 133, 227–242.

Oteiza, P.I., Kleinman, C.G., Demasi, M., Bechara, E.J., 1995. 5-Aminolevulinic acid induces iron release from ferritin. Arch. Biochem. Biophys. 316, 607–611.

Paquin, P.R., Gorsuch, J.W., Apte, S., Batley, G.E., Bowles, K.C., Campbell, P.G., Delos, C.G., Di Toro, D.M., Dwyer, R.L., Galvez, F., Gensemer, R.W., Goss, G.G., Hostrand, C., Janssen, C.R., McGeer, J.C., Naddy, R.B., Playle, R.C., Santore, R.C., Schneider, U., Stubblefield, W.A., Wood, C.M., Wu, K.B., 2002. The biotic ligand model: a historical overview. Comp. Biochem. Physiol. C-Toxicol. Pharmacol. 133, 3–35.

Patel, M., Rogers, J.T., Pane, E.F., Wood, C.M., 2006. Renal responses to acute lead waterborne exposure in the freshwater rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol..

Rocha, M.E., Dutra, F., Bandy, B., Baldini, R.L., Gomes, S.L., Faljoni-Alario, A., Liria, C.W., Miranda, M.T., Bechara, E.J., 2003. Oxidative damage to ferritin by 5-aminolevulinic acid. Arch. Biochem. Biophys. 409, 349–356.

Rogers, J.T., Wood, C.M., 2004. Characterization of branchial lead-calcium interaction in the freshwater rainbow trout *Oncorhynchus mykiss*. J. Exp. Biol. 207, 813–825.

Schmitt, C.J., Caldwell, C.A., Olsen, B., Serdar, D., Coffey, M., 2002. Inhibition of erythrocyte delta-aminolevulinic acid dehydratase (ALAD) activity in fish from waters affected by lead smelters. Environ. Monit. Assess. 77, 99–119.

Shahsavarani, A., McNeill, B., Galvez, F., Wood, C.M., Goss, G.G., Hwang, P.P., Perry, S.F., 2006. Characterization of a branchial epithelial calcium channel

(ECaC) in freshwater rainbow trout (*Oncorhynchus mykiss*). J. Exp. Biol. 209, 1928–1943.

Storey, J.D., Tibshirani, R., 2003. Statistical significance for genomewide studies. Proc. Natl. Acad. Sci. U.S.A. 100, 9440–9445.

USEPA, 2006. National Recommended Water Quality Criteria. Office of Water, Washington, DC.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, Speleman, F.A., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3, RESEARCH0034.

Warren, M.J., Cooper, J.B., Wood, S.P., Shoolingin-Jordan, P.M., 1998. Lead poisoning, haem synthesis and 5-aminolaevulinic acid dehydratase. Trends Biochem. Sci. 23, 217–221.

Weber, D.N., 1993. Exposure to sublethal levels of waterborne lead alters reproductive behavior patterns in fathead minnows (*Pimephales promelas*). Neurotoxicology 14, 347–358.

Wintz, H., Yoo, L.J., Loguinov, A., Wu, Y.Y., Steevens, J.A., Holland, R.D., Beger, R.D., Perkins, E.J., Hughes, O., Vulpe, C.D., 2006. Gene expression profiles in fathead minnow exposed to 2,4-DNT: correlation with toxicity in mammals. Toxicol. Sci. 94, 71–82.

Yang, Y.H., Dudoit, S., Luu, P., Lin, D.M., Peng, V., Ngai, J., Speed, T.P., 2002. Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res. 30, e15.