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Influence of bicarbonate and humic acid on effects of chronic waterborne lead exposure to the fathead minnow (*Pimephales promelas*)

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ABSTRACT

Historically, the USEPA has only considered water hardness when establishing acute and chronic water quality criteria (WQC) for lead (Pb) in freshwater. Yet, recent evidence suggests that hardness may not be protective during chronic Pb exposure and that other factors (e.g., dissolved organic carbon (DOC) and alkalinity) influence toxicity. In fact, we have recently shown that Ca^{2+} (as $CaSO_4$) does not protect against Pb accumulation in fathead minnows (*Pimephales promelas*) during chronic exposures whereas DOC as humic acid (HA) clearly does. To more clearly define the water chemistry parameters mediating chronic Pb toxicity we carried out 300 d exposures to study the influence of DOC and alkalinity on Pb accumulation and toxicity to fathead minnows at 2 different Pb concentrations (170 and 580 nM (35 and 120 μg/L)). Alkalinity was adjusted by addition of 500 μM $NaHCO_3$ and DOC by addition of 4 mg/L HA. Fish were collected at 4, 30, 150 and 300 d of exposure to measure growth and Pb accumulation. Breeding assays (21 d) were performed at the end of these exposures to assess reproductive and larval behavioral endpoints. To determine whether effects were acute or chronic, switched breeding exposures were performed in which control breeders were transferred to either high or low Pb conditions and Pb-exposed breeders transferred to tap water without Pb. Mortality and growth effects were observed primarily in the high Pb treatments and within the first 10 d of exposure. Strong protection against Pb accumulation was afforded by increased DOC at both Pb concentrations. Increased alkalinity also appeared to moderately reduce Pb accumulation although not to the level of statistical significance. Tissue distribution of Pb was analyzed at 300 d and was found to accumulate mostly in bone, gill, intestine and kidney. Unexpectedly, high Pb reduced total reproductive output and increased average egg mass in the HCO_3^- and DOC treatments but not in the control water (+Pb) treatments. No statistically significant differences in egg hatchability or egg Pb accumulation were observed. Results from switched exposures suggest that embryo Pb accumulation arose from acute exposure to embryos rather than parental transfer. Finally, prey capture assays revealed potential Pb-induced motor/behavioral impairment in 10-d-old F1 larvae exposed to high Pb in all water treatments.

1. Introduction

Lead (Pb), a non-essential metal, is of primary interest to the USEPA (Fairbrother et al., 2007) ranking behind only copper (Cu) as one of the most highly reported causes of metal impairment to water quality (Reiley, 2007). As with other metals, the toxicity of Pb can vary greatly depending on effects that differences in local water quality may have on its speciation. Previous studies have shown that acute toxicity of Pb decreases with increasing hardness or alkalinity/pH (Davies et al., 1976; Schubauer-Berigan et al., 1993; Stouthart et al., 1994). For hardness, the protective effect is most likely due to antagonistic binding of Ca^{2+} to a shared channel for Pb at the gill (Busselberg et al., 1991; Rogers and Wood, 2004). Alkalinity, on the other hand, affords protection by the formation of Pb carbonate complexes that sequester free ionic Pb, presumably rendering it unavailable for uptake:

$$Pb^{2+} + CO_3^{2-} \rightarrow PbCO_3; \quad (1)$$

$$Pb^{2+} + 2CO_3^{2-} \rightarrow Pb(CO_3)_2^{2-}. \quad (2)$$

More recently, it has become clear that other influences, such as complexation by dissolved organic carbon (DOC) or other organic/inorganic species, may also be important in determining Pb toxicity (Macdonald et al., 2002). In the case of DOC, protection is attributed to the high number of binding sites (carboxyl, phenolic, amino and sulfhydryl groups) that chelate metals and other cations from the water (Filella and Town, 2000).

The influence of water chemistry on chronic Pb toxicity is less clear than for acute toxicity due to the relative paucity of chronic

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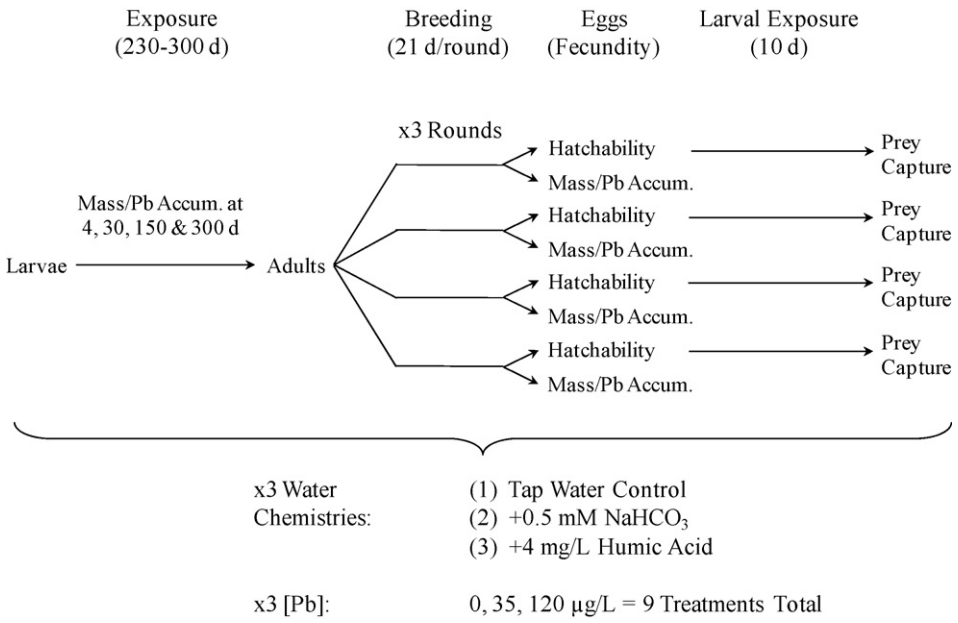

Fig. 1. Flow chart of overall experimental design highlighting the major endpoints of the study. Fathead minnows were exposed to 0, 145 nM Pb (30 µg Pb/L) or 530 nM Pb (110 µg Pb/L) in different water chemistries to investigate the influence of alkalinity and DOC on chronic Pb toxicity. Lead exposures were administered to 8-d-old fathead minnow larvae for 230–300 d and through 3 subsequent rounds of 21 d breeding assays. Eggs were counted daily (fecundity) and collected for hatchability or determination of mass and Pb accumulation. Eggs collected for hatchability were also exposed to Pb through 10 d post-hatch after which larvae were used in prey capture assays to evaluate behavior/motor impairment.

studies. Consequently, acute toxicity data is heavily relied upon for establishing chronic water quality criteria (WQC) leading to potentially uncertain and/or inappropriate levels of environmental protection. From the limited studies available it would seem that hardness and increased pH/alkalinity are protective against chronic Pb toxicity in fish (Davies et al., 1976; Hodson et al., 1978). However, since $CaCO_3$ contributes significantly to both hardness and alkalinity, and changes in these parameters commonly co-vary in natural waters and laboratory experiments, there remains uncertainty as to the protective contribution of each.

One of the main reasons that chronic studies evaluating reproductive toxicity in fish are lacking is the time and effort required to perform such experiments. Hence, we previously conducted short-term exposures to identify the likely key water chemistry parameters influencing chronic Pb toxicity prior to undertaking exposures through reproductive maturity. These efforts demonstrated protective effects by Ca^{2+} (as $CaSO_4$) and DOC (as Aldrich humic acid (HA)) against acute Pb toxicity (Grosell et al., 2006), as well as against chronic Pb accumulation by HA but not Ca^{2+} in fathead minnows (Mager et al., 2008). Reproduction was not evaluated in the latter study and, aside from Pb-induced transcriptional responses, no other toxic effects were observed under the conditions examined. Still, these findings shed some doubt as to the protective influence of Ca^{2+} on chronic Pb toxicity while further supporting DOC as an important protective component that warrants greater consideration. However, because $CaSO_4$ was used to explicitly study the effects of increased hardness without increasing alkalinity in these experiments, the influence of alkalinity alone on chronic Pb accumulation and toxicity remains unclear.

Having narrowed the field for potential key water chemistry parameters, we proceeded with the present study aimed primarily at investigating water chemistry influences on Pb-induced reproductive effects. Specifically, we again evaluated the influence of HA to determine whether the protection against whole body Pb accumulation observed previously translated into protection against full-term reproductive effects. We also investigated the effect of increased alkalinity (as $NaHCO_3$) in lieu of Ca^{2+} given its previous failure to protect against chronic Pb accumulation. The reproductive endpoints of fecundity, hatchability, egg mass, egg Pb accumulation and attachment of eggs to breeding substrate were monitored. Additionally, growth, Pb accumulation and potential Pb-induced neurological impairment in larval offspring were assessed.

2. Materials and methods

2.1. Experimental design

The main goal of this study was to examine the influence of DOC (as HA) and alkalinity (as $NaHCO_3$) on the reproductive toxicity of chronic Pb exposure to fathead minnows (Fig. 1). To this end, Pb exposures were administered in 3 different laboratory waters (described below) to 8-d-old fathead minnow larvae for 230–300 d and subsequently through 3 sequential rounds of 21 d breeding assays. During these breeding assays eggs were counted daily to assess fecundity and collected for hatchability or determination of egg mass and Pb accumulation. As Pb is a known neurotoxin (White et al., 2007), we also sought to evaluate potential Pb-induced behavioral/motor impairment in F1 offspring hatched from eggs produced during the breeding assays. Therefore, eggs collected for hatchability were exposed to Pb for 10 d post-hatch after which larvae were analyzed using a prey capture assay. Whole body and tissue Pb burdens were also measured to determine whether the influences of water chemistry on Pb accumulation were predictive and consistent with observed chronic toxicity. For completion, standard endpoints of mortality and growth were also monitored.

2.2. Experimental animals

Fathead minnow (*Pimephales promelas*) embryos were obtained from Aquatic BioSystems, Inc. (Fort Collins, CO; <24 h post-hatch on arrival), distributed evenly among 1 L plastic beakers and placed under a 5 mL/min flow-through water supply (~75–85 fish per

Table 1
Chemistry of test media (mean ± SEM in μM except for hardness (Hard.) which is expressed as mg/L as calculated by APHA Standard Methods; $n = 22$ except for pH $n = 29$, CO_2 $n = 34$, and DOC $n = 25$). Water temperature mean ± SEM throughout the exposures was 22 ± 1 °C.

	$[Na^+]$	$[K^+]$	$[Ca^{2+}]$	$[Mg^{2+}]$	$[Cl^-]$	$[SO_4^{2-}]$	$[CO_2]$	[DOC]	Hard.	pH
Control	2528	100	619	274	2280	180	701	257	91	8.1
Tap H_2O	±180	±5	±28	±16	±133	±18	±11	±8	±4	±0.1
+500 μM	3066	102	639	275	2206	169	1191	257	93	8.3
$NaHCO_3$	±184	±4	±23	±13	±153	±16	±23	±12	±3	±0.1
+4 mg/L	2573	104	636	278	2249	167	690	318	93	8.0
Humic	±176	±4	±22	±13	±145	±18	±23	±8	±3	±0.1

beaker, 3 replicates per treatment). After the first 20 d of exposure 1 L beakers were replaced with 21 L aquaria and flows increased to 70 mL/min for the remainder of the experiment. Fish were fed once daily an *ad libitum* diet of freshly hatched *Artemia* nauplii for the first month followed by *Artemia* and Tetramin flake food for 1 week and then flake food only thereafter. Leftover food, feces and dead fish (if any) were removed daily prior to feeding. Fish were maintained on a 16 h:8 h light:dark photoperiod with an average water temperature of 22 ± 1 °C.

2.3. Chronic Pb exposures

Dechlorinated Virginia Key tap water was modified by nominal addition of 500 μM $NaHCO_3$ or 4 mg/L HA using a gravity flow-through approach as previously described (Grosell et al., 2006; Table 1). Larvae were gradually acclimated to different water chemistries for one week prior to initiation of Pb exposures at 8 d of age. Concentrated $PbNO_3$ solutions were dispensed at 1 mL/min via Mariotte bottles into 2 separate mixing chambers for each water chemistry targeting final "low" Pb concentrations of ~170 nM (35 μg/L) and "high" Pb concentrations of ~580 nM (120 μg/L). Using a hardness representative of the present study (92 mg/L) both Pb concentrations exceeded current hardness-based calculations for chronic Pb WQC (11 nM (2.3 μg/L)) and our high Pb exposure also exceeded that calculated for acute Pb WQC (285 nM (59 μg/L)) (USEPA, 2002). In sum, 9 different treatments were tested including controls (Fig. 1). High Pb exposures were initiated 100 d following that of low Pb exposures for reasons discussed in Section 3 (see Section 3.1). All chemicals used for maintaining test conditions were obtained from Sigma–Aldrich (St. Louis, MO).

Whole body lead accumulation was determined from acid-digested fish collected at 4, 30, 150 and 300 d of exposure as previously described (Grosell et al., 2006); $n = 18$ (3 pools of 6 fish/tube), 12, 12, and 6, respectively. For the 300 d time point, whole body Pb burdens were estimated by summing the Pb accumulated in individual tissues harvested for determination of internal Pb distribution and dividing by the total body mass of origin. Tissues were collected from 3 males and 3 females and included gills, brain, testes, ovaries, anterior intestine, liver, kidneys and the remaining carcass.

2.4. 21 day breeding assays

Breeding pairs were selected randomly and evenly from replicate tanks toward the end of the exposures and maintained in flow-through conditions matching water chemistries and Pb concentrations to those leading up to reproductive assays. Aquaria (21 L) were partitioned in half with a screen divider to accommodate two separate breeding pairs per tank. Due to space limitations these assays were conducted in 3 sequential rounds beginning at approximately 210, 250 and 280 d of exposure. Two replicate tanks (4 breeding pairs) per treatment were used in each round for a total of 12 breeding assays per treatment. Each pair was provided with a breeding substrate modeled after previously described methods (Thorpe et al., 2007).

Breeding substrates were removed daily for egg counts/collection and replaced with clean substrates. Eggs were counted as either "attached" (adhered to PVC substrate) or "detached" (found in lower collection tray) and summed to obtain total daily output (designated as a single clutch). Eggs were collected for either mass/Pb accumulation or hatchability/prey capture (Fig. 1). Those collected for mass/Pb accumulation were briefly rinsed in deionized water, dried by decanting and siphoning off surrounding water, counted and gently transferred to pre-weighed eppendorf tubes. Acid digests and Pb measurements were performed as above. Eggs collected for hatchability and prey capture were transferred to hatching chambers containing 1.9 L of treatment water and vigorously aerated.

Following the initial 3 rounds of reproductive assays additional experiments were arranged to determine whether any observations were due to chronic Pb exposure to adults or acute Pb exposure to embryos. To this end, breeding assays were conducted as previously (tap water only) with the exception that breeding pairs were switched to opposing exposure conditions (i.e. breeding fish from control conditions transferred to low or high Pb tap water and low or high Pb-exposed breeders transferred to control tap water).

2.5. Prey capture assays

To examine potential neurological effects on offspring, eggs from the breeding assays were allowed to hatch. Following hatch, larvae were maintained for 10 d in ~1 L treatment-matched water (daily static-renewal) and fed freshly hatched *Artemia* nauplii daily until 48 h prior to the prey capture assays. The assay was initiated by placing a single fathead minnow larva into a small weight boat (4 cm L × 4 cm W × 0.8 cm H) containing 5 mL of treatment water. Ten *Artemia* nauplii were then introduced and the number ingested within a 5 min period was scored. Times were recorded at the 1st, 5th and 10th ingestions if completed. For those ingesting less than 5 of the *Artemia*, the number remaining at the end of 5 min was noted. A total of 8–14 larvae were tested from each of 2–3 replicate beakers.

Table 2
Summary of nominal and mean ± SEM measured waterborne Pb concentrations over full duration of study (in nM and (μg/L), $n = 71$ for controls and 75–78 for +Pb).

	Nominal	Tap	HCO_3^-	Humic
Control		0.8 ± 0.3 (0.2 ± 0.1)	1.3 ± 0.3 (0.3 ± 0.1)	1.4 ± 0.4 (0.3 ± 0.1)
Low Pb	170 (35)	137 ± 5 (28 ± 1.1)	148 ± 6 (31 ± 1.2)	144 ± 7 (30 ± 1.4)
High Pb	580 (120)	506 ± 23 (105 ± 4.8)	547 ± 22 (113 ± 4.6)	542 ± 22 (112 ± 4.5)

2.6. Water chemistry

Dissolved Pb concentrations were measured from water samples passed through a 0.45 μm cellulose syringe filter (Acrodisc, Pall Life Sciences, MI) and acidified to 1% HNO_3 (Fisher Scientific, trace metal grade) via graphite furnace atomic absorption spectroscopy (Varian 200Z, Varian, Australia). Concentrations of major cations (Na^+, K^+, Ca^{2+}, and Mg^{2+}) and anions (Cl^- and SO_4^{2-}) were determined by flame atomic absorption spectroscopy (Varian 220FS, Varian, Australia) and anion chromatography (DIONEX DX120, CA), respectively, total CO_2 by a Corning 962 carbon dioxide analyzer (UK) and DOC by high temperature catalytic oxidations using a Shimadzu total organic carbon-VCSH analyzer (Kyoto, Japan) (Hansell and Carlson, 2001). Water pH was measured using a PHM201 meter (Radiometer, Copenhagen, Denmark) fitted with a combination glass electrode. Water chemistry and temperature was typically analyzed on a bi-weekly schedule (Table 1) and dissolved Pb was measured once or twice a week (Table 2).

2.7. Calculations and statistical analysis

Data are presented as means ± 1 standard error of the mean (SEM). Analysis of variance (ANOVA) followed by Student's *t*-test of individual means with multi-sample comparison corrections (Bonferonni) as appropriate was used for statistical analysis as indicated in tables and legends. In all cases, differences were deemed statistically significant at $P \leq 0.05$. Lead speciation calculations were performed using the biotic ligand model (HydroQual, Inc., Version 2.3.3).

3. Results and discussion

3.1. Water chemistry

In a previous 150 d study (Mager et al., 2008) we used a 2:1 deionized water:dechlorinated tap water mixture to achieve a moderately soft base water for investigating hardness and DOC effects on Pb toxicity. Due to the much larger scale and duration of this study, we chose instead to use full strength tap water to eliminate the difficulties associated with higher flow demands of deionized water. Accordingly, this led to a base water with approximately 3-fold higher concentrations of all water constituents and higher pH. Overall, water chemistry measurements were consistent with our target nominal values and were stable across treatments except for the modified parameters (Table 1).

We chose to study the effects of Pb at 2 different concentrations, one "low" concentration similar to that used in our previous study (~170 nM, 35 μg/L Pb) and a "high" concentration to maximize the likelihood of observing toxicity. For the latter, we aimed for the highest Pb concentration that could be achieved without inducing mortality to such an extent that the number of fish remaining would be insufficient for sample collection throughout the course of the experiment. The first 2 attempts using measured dissolved Pb concentrations of 1690 nM (350 μg/L) and 1110 nM (230 μg/L) both resulted in excessive mortality within the first 10 d of exposure. Due to the time invested for these initial attempts our final high Pb exposure of 530 nM (110 μg/L) trailed behind our low and control exposures by 100 d. However, no effect of exposure starting time was discernable on growth or reproduction by two-way ANOVA. Final mean Pb concentrations for both low and high exposures were moderately lower than targeted, but comparable across treatments (Table 2).

Table 3A
Summary of mean ± SEM body masses (mg; *n* are denoted in parentheses) during first 150 days of exposure ± low and high concentrations of Pb in different test treatments.

	Exposure time in days		
Treatment	4 (18)	30 (12)	150 (12)
Tap control	4.2 ± 0.5	46 ± 3	1334 ± 146
Tap + low Pb	3.5 ± 0.2	33 ± 3	1426 ± 99
Tap + high Pb	2.5 ± 0.3[a]	43 ± 7	1344 ± 133
HCO_3^- control	3.8 ± 0.1	37 ± 4	1082 ± 156
HCO_3^- + low Pb	3.9 ± 0.3	34 ± 4	1276 ± 126
HCO_3^- + high Pb	2.8 ± 0.1[a]	56 ± 7[a]	1274 ± 148
Humic control	4.1 ± 0.3	34 ± 5	1341 ± 164
Humic + low Pb	3.9 ± 0.1	37 ± 3	1399 ± 100
Humic + high Pb	2.8 ± 0.3[a]	50 ± 7	1317 ± 161

[a] Statistically significant difference vs. corresponding control and low Pb at same time point as determined by two-way ANOVA.

3.2. Mortality and growth

Most of the mortality (≥95%) occurred during the first 10 d of exposure, primarily within the high Pb treatments. Humic acid and HCO_3^- protected against 10 d Pb-induced mortality to nearly

Table 3B
Summary of mean ± SEM body masses (g) following each of 3 rounds of 21 d reproduction assays in different test media (*n* = 4 unless noted otherwise in parentheses) and final sex ratios (male:female, *n* = 3) at conclusion of study (includes pairs used for reproduction).

		Round (approximate age)			
Treatment	Sex	1 (230 d)	2 (270 d)	3 (300 d)	Ratio
Tap control	♂	4.23 ± 0.47	4.60 ± 0.47	4.78 ± 0.41	0.99 ± 0.12
	♀	1.40 ± 0.21 (3)	1.77 ± 0.07 (3)	1.40 ± 0.06 (3)	
Tap + low Pb	♂	3.13 ± 0.42	4.95 ± 0.15	5.43 ± 0.50	0.95 ± 0.08
	♀	1.53 ± 0.08	1.55 ± 0.15	1.87 ± 0.03 (3)	
Tap + high Pb	♂	3.70 ± 0.25 (3)	2.83 ± 0.47 (3)	4.40 ± 0.46	1.00 ± 0.04
	♀	1.48 ± 0.19	2.15 ± 0.57	1.45 ± 0.14	
HCO_3^- control	♂	4.00 ± 0.48	4.23 ± 0.34	4.03 ± 0.09 (3)	0.96 ± 0.20
	♀	1.55 ± 0.16	2.80 (1)	1.78 ± 0.21	
HCO_3^- + low Pb	♂	4.28 ± 0.30	5.05 ± 0.31	5.73 ± 0.68	1.41 ± 0.19
	♀	1.67 ± 0.03 (3)	1.80 ± 0.50	1.75 ± 0.23	
HCO_3^- + high Pb	♂	3.55 ± 0.25	3.53 ± 0.49	4.33 ± 0.55	1.37 ± 0.19
	♀	1.40 ± 0.11	1.78 ± 0.33	1.65 ± 0.10	
Humic control	♂	3.38 ± 0.25	4.95 ± 0.42	4.88 ± 0.43	0.76 ± 0.05
	♀	1.63 ± 0.19	2.00 ± 0.21	2.03 ± 0.14	
Humic + low Pb	♂	4.08 ± 0.35	4.35 ± 0.21	4.10 ± 0.07	0.90 ± 0.11
	♀	1.80 ± 0.06	1.88 ± 0.12	1.77 ± 0.03 (3)	
Humic + high Pb	♂	3.55 ± 0.29	4.75 ± 0.36	3.80 ± 0.27	1.10 ± 0.10
	♀	1.53 ± 0.34 (3)	1.73 ± 0.13	1.57 ± 0.32 (3)	

No statistically significant differences in mass within sex were determined by two-way ANOVA.

the same extent where cumulative percent mortalities were 28.9% (tap water), 20.0% (HCO_3^-) and 19.3% (HA), respectively. Additionally, some adult mortality of note occurred during the breeding assays from which 14 of 108 fish died prior to completion of the 21 d. Female breeders accounted for 11 of these deaths due most likely to male aggression (attacks against females were commonly observed) whereas males likely succumbed to natural mortality after spawning (Andrews and Flickinger, 1973).

Protection was less evident for growth which was inhibited to a similar extent at 4 d by high Pb regardless of water treatment (Table 3A). Growth had recovered in all cases by 30 d and no further differences were observed at any time point beyond. Average fish masses at 30 and 150 d of exposure were 41 ± 2 mg and 1311 ± 45 mg, respectively ($n = 108$). Masses were also recorded by sex after each round of reproduction at 230 d, 270 d and 300 d of exposure (Table 3B). Mean values were 3766 ± 123, 4489 ± 137 and 4623 ± 167 mg for males, respectively ($n = 32$–35); 1547 ± 53, 1889 ± 105 and 1700 ± 59 mg for females ($n = 28$–35), respectively. No statistically significant growth differences were observed at any age due to water chemistry alone.

Sex ratios were determined at the end of the study from all fish remaining after the 150 d time point (Table 3B). In all of the tap water exposures as well as the HCO_3^- controls ratios were close to 1. Sex ratios trended towards higher numbers of males in HCO_3^- and HA treatments with Pb, though the differences were not statistically significant. Also, the apparent shift in the HA treatments with Pb were in reference to a lower baseline of males:females in the HA control water when compared to tap water controls.

3.3. Whole body Pb accumulation

As in our 150 d study, we investigated the potential mitigating effects of DOC on Pb accumulation by the nominal addition of 4 mg/L HA. This resulted in a similar base water increase of 60 μM DOC as previously (Table 1). Assuming a background DOC comprised of 10% HA (Santore et al., 2001), a 60 μM measured increase (assumed to be 100% HA) would translate into a 3.4-fold increase in the humic acid component of the DOC. However, it should be noted that addition of 60 μM DOC in the present study is a smaller proportional increase than that in our previous 150 d study due to the higher starting background levels in undiluted tap water vs. 33% tap water (257 ± 8 μM DOC vs. 82 ± 1 μM DOC, respectively). Nevertheless, this increase was sufficient to provide strong protection against Pb accumulation for both low and high Pb concentrations when compared to tap water controls (Fig. 2A). Thus, these findings further illustrate the substantial protective effect of relatively small increases in DOC against whole body Pb accumulation by fathead minnows. This is perhaps best explained by the much higher calculated affinity (~250-fold) of Pb for organic carbon over the gill (Macdonald et al., 2002).

In the alkalinity treatment we increased the HCO_3^- concentration by 500 μM using $NaHCO_3$ which raised the total CO_2 concentration from 701 ± 11 μM to 1191 ± 23 μM and pH from 8.1 ± 0.1 to 8.3 ± 0.1 relative to the tap water control (Table 1). At many of the time points examined alkalinity appeared to reduce whole body Pb burdens at both Pb concentrations, though only at 300 d in the high concentration was this difference statistically significant (Fig. 2A). Furthermore, these reductions were typically not to the extent of that observed with HA. Differences between the protective effects of HA and alkalinity may be resolved by the greater complexing capacity of HA due to the high number of strong Pb binding sites available per mg of carbon (Macdonald et al., 2002). Conversely, Pb is removed in a nearly linear fashion by increased carbonate complexation at higher alkalinities (Davies et al., 1976; Macdonald et al., 2002). Thus, one might expect that a nearly 1.7-fold increase in total CO_2 at a pH close to 8 as reported herein would lead to a proportional decrease in Pb accumulation. However, this was not observed, suggesting that some inorganic forms of Pb, which increase with increasing alkalinity/pH, might be available for uptake (see Section 3.5). Whatever the mechanism, clearly an increase in HCO_3^- concentration does not necessarily lead to a proportional protective effect against Pb accumulation in fathead minnows.

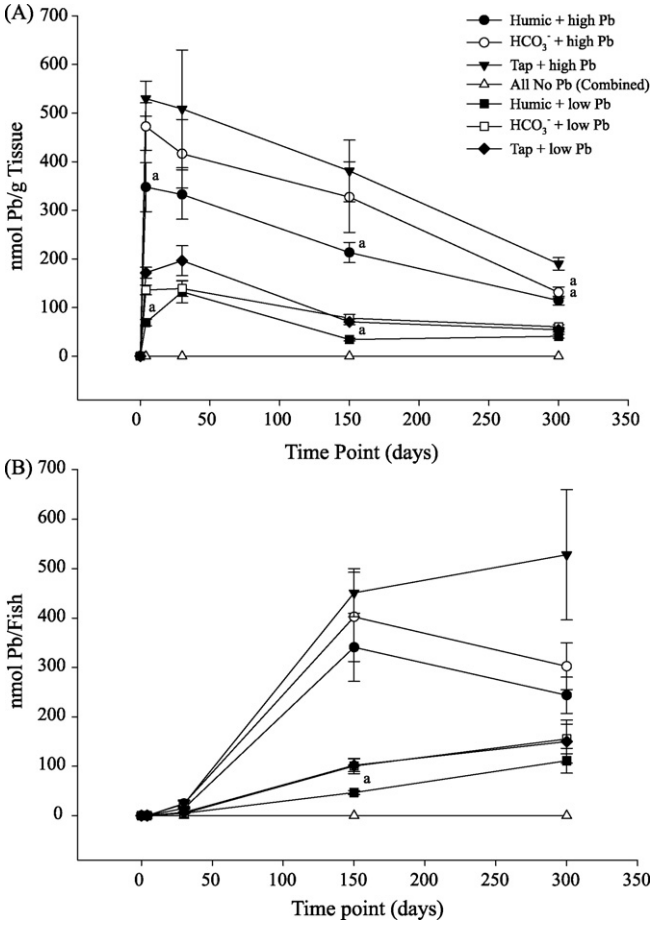

Fig. 2. Influence of HCO_3^- and HA on whole body Pb accumulation in fathead minnows exposed to either low (145 nmol/L) or high (530 nmol/L) Pb concentrations. Lead burdens are reported per wet weight of tissue (A) and per fish to illustrate an apparent growth dilution that effect persists to 300 d in all cases except for HCO_3^- and HA high Pb treatments (B). Mean ± SEM, $n = 3$ for 4 d, $n = 12$ for 30 and 150 d, and $n = 6$ for 300 d. Statistically significant difference from [a]tap water + Pb at same time point and Pb concentration determined by a two-way ANOVA with time and water chemistry as variables followed by Bonferroni-corrected Student's *t*-test. All Pb-exposed fish at all time points accumulated significantly more Pb than unexposed controls (not indicated by symbols).

The apparent decrease in Pb accumulated on a per mass basis with age beyond the 30 d time point, as evident in Fig. 2A, can likely be attributed to a growth dilution effect when the same data are plotted per number of fish as shown in Fig. 2B. These results suggest that the mass of tissues not accumulating Pb likely grow at a rate greater than those tissues that do accumulate Pb. This is not surprising given that Pb primarily targets bone (see below) which will account for less proportional mass as the fish grows.

3.4. Internal distribution of Pb

The internal distribution of Pb at 300 d was consistent with previous findings by our lab in fathead minnows (Grosell et al., 2006) and by others in salmonids (Holcombe et al., 1976; Hodson et

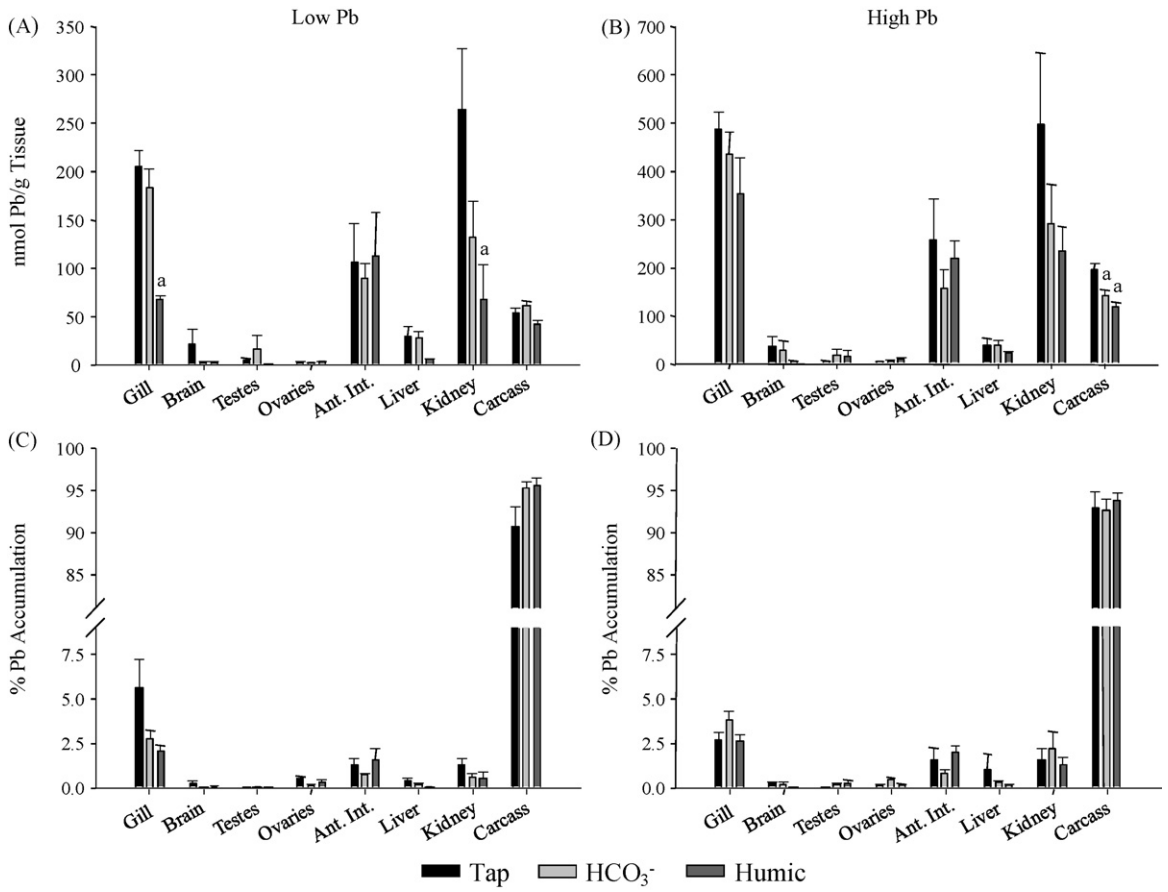

Fig. 3. Influence of HCO_3^- and HA on Pb accumulation (A and B) and internal distribution (C and D) of selected fathead minnow tissues following 300 d exposures to either low (145 nmol Pb/L; left side) or high (530 nmol Pb/L; right side) Pb concentrations. Mean ± SEM, $n = 6$, except for testes and ovaries in lower panels $n = 3$. Statistically significant difference by Student's t-test from [a]tap water + Pb at same Pb concentration.

al., 1978; Varanasi and Gmur, 1978). On a Pb concentration basis, Pb accumulated mostly in the gill, kidney, anterior intestine and carcass with far less found in the brain and liver (Fig. 3A and B). In contrast, the relative contribution of total Pb accumulated was accounted for mostly by the carcass, representing 95% (Fig. 3C and D), which previous work by our lab has shown is predominantly a reflection of skeletal accumulation (Grosell et al., 2006). While whole body Pb burdens did not differ across water treatments at 300 d in low Pb, the gill and kidney individually accumulated less Pb in the HA treatment when compared to the tap water control (Fig. 3A). In the high Pb treatments, Pb accumulation in the carcass mirrored that observed at the whole body level with significantly less found in HCO_3^- and HA treatments (Fig. 3B).

High accumulation by the intestine is somewhat surprising and suggests that some dietary Pb exposure likely occurred. It is conceivable that Pb was ingested following rapid adsorption to food. Mount et al. (1994) found increased Pb accumulation in rainbow trout fed a diet of *Artemia* previously exposed to waterborne Pb for 24 h and others have clearly demonstrated that dietary Pb can be taken up and accumulated by the intestine (Alves and Wood, 2006; Ojo and Wood, 2007). Alternatively, secretions into the intestine from other organs (e.g., bile from the liver) may have resulted in apparent intestinal Pb accumulation.

Because sex was easily discernable at 300 d, equal numbers of each ($n = 3$; 6 total) were collected to examine differential Pb accumulation between males and females. No statistically significant differences with respect to sex were found at the whole body level, although females did accumulate more Pb in intestine, kidney and brain in the high Pb treatments, but not low Pb treatments, when tested by one-way ANOVA (data not shown).

Table 4
Concentrations (in nM) for major Pb species within each test media as predicted by the biotic ligand model. Values for $Pb(OH)_2$, $Pb(OH)_3^-$, $PbSO_4$, $PbCl^+$ and $PbCl_2$ were <0.03 nM (<0.01%) in all cases. Relative abundances expressed as the percentage of total dissolved Pb are provided in parentheses.

	Pb^{2+}	$PbOH^+$	$PbCO_3$	$Pb(CO_3)_2^{2-}$	Total organic Pb
Tap control	4.9×10^{-4} (0.061)	7.6×10^{-4} (0.095)	0.020 (2.53)	1.9×10^{-4} (0.023)	0.78 (97.3)
Tap low Pb	0.091 (0.066)	0.14 (0.103)	3.8 (2.74)	0.035 (0.025)	133 (97.1)
Tap high Pb	0.42 (0. 084)	0.66 (0. 131)	18 (3.46)	0.16 (0. 032)	487 (96.3)
HCO_3^- control	5.3×10^{-4} (0.040)	1.3×10^{-3} (0.099)	0.058 (4.43)	1.4×10^{-3} (0.111)	1.2 (95.3)
HCO_3^- low Pb	0.065 (0.044)	0.16 (0.108)	7.1 (4.81)	0.18 (0.120)	140 (94.9)
HCO_3^- high Pb	0.30 (0.056)	0.75 (0. 136)	33 (6.09)	0.83 (0. 152)	512 (93.6)
Humic control	6.4×10^{-4} (0.046)	7.9×10^{-4} (0.057)	0.021 (1.47)	1.5×10^{-4} (0.011)	1.4 (98.4)
Humic low Pb	0.072 (0.048)	0.089 (0.060)	2.3 (1.56)	0.017 (0.011)	146 (98.3)
Humic high Pb	0.31 (0.058)	0.39 (0. 071)	10 (1.86)	0.072 (0.013)	531 (98.0)

Table 5
Fathead minnow 21 d reproductive output per breeding pair (mean ± SEM). Parentheses indicate total values for 3 rounds combined except for switched exposures (2 rounds combined).

	Avg. tot. # eggs laid	Avg. # clutches	Avg. clutch size
Tap control	334 ± 68 (4006)	3.5 ± 0.5 (42)	95.4 ± 9.6
Tap + low Pb	416 ± 62 (4991)	4.5 ± 0.4 (54)	90.8 ± 8.3
Tap + high Pb	320 ± 44 (3834)	3.0 ± 0.4 (36)	109.3 ± 11.9
Tap (low Pb breeders)[c]	349 ± 44 (2789)	4.3 ± 0.5 (34)	82.03 ± 10.34
Tap (high Pb breeders)[c]	134 ± 47 (1073)[b]	1.9 ± 0.4 (15)[b]	71.5 ± 11.1
Tap + low Pb (cont. breeders)[c]	149 ± 38 (1195)	2.8 ± 0.4 (22)	52.9 ± 9.9[b]
Tap + high Pb (cont. breeders)[c]	97 ± 41 (774)[b]	1.6 ± 0.7 (13)[b]	59.5 ± 13.9
HCO_3^- control	546 ± 68 (6557)[b]	4.1 ± 0.3 (49)	134.0 ± 10.4[b]
HCO_3^- + low Pb	345 ± 69 (4140)[a]	3.2 ± 0.5 (38)	112.2 ± 11.6
HCO_3^- + high Pb	256 ± 42 (3067)[a]	3.0 ± 0.3 (36)	85.2 ± 8.8[a]
Humic control	467 ± 77 (5600)	4.1 ± 0.5 (49)	114.3 ± 9.7
Humic + low Pb	413 ± 67 (4950)	3.0 ± 0.5 (36)	137.7 ± 9.6
Humic + high Pb	195 ± 49 (2336)[a]	2.1 ± 0.4 (25)[a]	92.6 ± 12.7

[a,b]Statistically significant differences as determined by Student's *t*-test as follows: vs. [a]treatment-matched control; [b]vs. tap control. [c]Switched breeding exposures in which control breeders were transferred to either high or low Pb conditions and Pb-exposed breeders transferred to tap without Pb.

3.5. Role of Pb speciation

Since metal bioavailability in general is considered to be strongly tied to speciation, calculations were performed to determine concentrations and relative abundances of the major Pb species within each of the different water treatments using the biotic ligand model (HydroQual, Inc.; Paquin et al., 2002) and are summarized in Table 4. Clearly, the vast majority of Pb (>90%) was organically bound regardless of water treatment, although as Pb concentrations increased the proportions of the major inorganic and free ion species also increased. Not surprisingly, the treatments exhibiting the largest percentages of free Pb^{2+} ion were the tap water treatments while the HCO_3^- treatments had the highest percentages of Pb–carbonate complexes. The latter would also account for the highest overall percentages of inorganic Pb species observed in the HCO_3^- treatments.

Interestingly, HA reduced all inorganic forms of Pb to the greatest extent except for the free Pb^{2+} ion which was reduced to a similar extent as that achieved with HCO_3^-. Given that fish appeared to accumulate more Pb from the HCO_3^- than from the HA treatments suggests that some of the inorganic Pb other than the free Pb^{2+} ion was bioavailable. For example, Pb could be similar to Cu, for which the monohydroxide form is believed to be bioavailable (USEPA, 2007). Thus, $PbOH^+$ which increases with increasing alkalinity/pH above ~pH 6.5 in fresh water (Stumm and Morgan, 1996) might similarly contribute to Pb accumulation. Finally, in light of our reproductive and behavioral results, our speciation data suggests that inorganically and organically complexed Pb exerts chronic toxicity in a manner not reflected by whole body Pb accumulation, though it is unclear at this time how this might occur.

3.6. Fecundity

Unexpectedly, Pb effects on fecundity were observed in the HCO_3^- and HA treatments but not in the tap water controls (Table 5). For the HCO_3^- treatments, low and high Pb concentrations reduced 21 d total reproductive output. This effect appeared related to both reduced clutch size and reduced number of clutches produced when compared to treatment-matched controls, although only the former difference was statistically significant (high Pb only). It should be noted, however, that addition of HCO_3^- alone actually increased reproductive output, suggesting that increased alkalinity may promote greater fecundity but also greater sensitivity to Pb. Surprisingly, HA reduced total reproductive output at the high Pb concentration. This effect was apparently due more to a reduced number of clutches laid than to clutch size. Because one member of the breeding pair occasionally perished before 21 d, total reproductive output was also analyzed as eggs per female per day to eliminate any potential artifact or bias in the data due to mortality. This correction had little influence, however, as differences were similar to those previously described (Supplementary Fig. 1A).

To determine whether effects were acute or chronic, switched breeding exposures were carried out in which control breeders were transferred to either high or low Pb conditions and Pb-exposed breeders transferred to tap water without Pb. These experiments were not performed in the high alkalinity and DOC waters, as we anticipated Pb effects far more likely to occur in tap water. Since this was not the case it is difficult to conclude whether the effects observed in the HCO_3^- and HA treatments were due to acute or chronic Pb exposure. However, the findings with switched exposures in tap water were intriguing in that fecundity was often reduced acutely regardless of direction of transfer (Table 5; Supplementary Fig. 1B). These data suggest that fathead minnows may experience reduced reproductive output, at least temporarily, with abrupt fluctuations in Pb exposure. From the present study it cannot be concluded whether any of the observations on fecundity are the result of physiology or behavior; nevertheless, results from the switching experiments may hold particular relevance since in natural environments pollutant loads are often pulsatile.

Overall, fecundity in our control treatments (Supplementary Fig. 1) was low compared to that of Tyler and colleagues (Harries et al., 2000; Thorpe et al., 2007) who reported mean values ranging from 43 to 112 eggs/female/d, but comparable to more commonly reported mean values approximating 20 eggs/female/d by others (for examples see (Jensen et al., 2001; Sellin and Kolok, 2006; Watanabe et al., 2007). Thorpe et al. (2007) suggested that higher fecundity observations might be accounted for by the use of screened trays placed beneath the breeding substrates to collect detached eggs. Although our counts were certainly higher than would have been without a collection tray, our daily reproductive output per female was still less than in the studies with higher fecundity. Water chemistry or other environmental factors and inter-facility variation may represent important factors in explaining these discrepancies.

Indeed, as we have shown in the present study, simple differences in water chemistry can influence reproductive output. This was evident in our HCO_3^- treatment which led to significantly higher fecundity when compared to the control tap water treatment (Table 5). It may be that the higher buffering capacity of the HCO_3^- treatment confers greater overall reproductive fitness, or possibly that higher alkalinity leads to greater sperm motility

Table 6
Effect of Pb and water chemistry on fathead minnow egg mass (n = 2–6 clutches) and egg Pb accumulation (n = 4–12 clutches) and attachment to PVC breeding substrate[f] (n = 13–54) (mean ± SEM).

	Egg mass (mg)	Pb accum. (nmol/g)	% Detached
Tap control	1.52 ± 0.09	0.26 ± 0.15	80.8 ± 3.3
Tap + low Pb	1.55 ± 0.07	75.9 ± 28.1[a]	87.0 ± 3.0[b]
Tap + high Pb	1.59 ± 0.10	92.6 ± 17.8[a]	72.7 ± 4.3
Tap (low Pb breeders)[e]	1.41 ± 0.10	0.58 ± 0.41	41.3 ± 4.5[b]
Tap (high Pb breeders)[e]	1.64 ± 0.10	1.21 ± 1.54	66.2 ± 5.8[b]
Tap + low Pb (cont. breeders)[e]	1.46 ± 0.12	35.9 ± 8.9[b]	73.3 ± 5.7[d]
Tap + high Pb (cont. breeders)[e]	1.53 ± 0.02	39.2 ± 10.9[b,c]	61.6 ± 10.0
HCO_3^- Control	1.61 ± 0.08	0.99 ± 1.16	74.4 ± 3.3
HCO_3^- + low Pb	1.50 ± 0.07	59.5 ± 22.2[a]	83.4 ± 2.8
HCO_3^- + high Pb	1.81 ± 0.12[a]	93.0 ± 33.1[a]	63.5 ± 4.7
Humic control	1.52 ± 0.07	0.36 ± 0.14	51.4 ± 4.6[b]
Humic + low Pb	1.47 ± 0.08	72.7 ± 21.9[a]	50.7 ± 4.4
Humic + high Pb	1.99 ± 0.10[a]	85.9 ± 12.2[a]	44.7 ± 5.3

[a]Statistically significant differences from treatment-matched control as determined by two-way ANOVA with Pb concentration and water chemistry as variables. [b,c]Statistically significant differences as determined by Student's t-test as follows: [b]vs. control tap water; [c]vs. tap + high Pb; [d]vs. tap + low Pb. [e]Switched breeding exposures in which control breeders were transferred to either high or low Pb conditions and Pb-exposed breeders transferred to tap without Pb. [f]Proportions of detached eggs per clutch were arcsine transformed to obtain normal distribution of data.

and therefore greater fertilization and water hardening of eggs. Of course the latter would be reflected in the number of eggs counted only if a loss of unfertilized eggs (by disintegration or paternal ingestion, for example) occurred prior to counting. However, the higher base-line fecundity in the HCO_3^- treatment was decreased by addition of Pb perhaps by countering in some manner any such potential beneficial effects of HCO_3^- to egg fertilization and water hardening.

3.7. Egg mass

Perhaps most surprising from this study was the effect of high Pb on fecundity in the presence of HA and increased alkalinity while no Pb effects were detectable in tap water + Pb only exposures. Consistent with the reduced reproductive output for the HCO_3^- and HA treatments with high Pb was the increase in average egg mass from the same treatments (Table 6). It is tempting to speculate that, when exposed to Pb, more energy may be directed toward producing fewer, higher quality eggs than in similar water conditions without Pb. Given that no effects on hatchability were observed in the HCO_3^- and HA treatments with high Pb (see below), the greater egg masses in these same groups may support this possibility. No differences were observed for any of the tap water experiments including switched exposures aimed at revealing potential acute effects (Table 6).

3.8. Egg Pb accumulation

When exposed to Pb, eggs accumulated similar amounts irrespective of Pb concentration or water chemistry (Table 6). It is possible that Pb accumulated directly from acute waterborne exposure to sperm and/or eggs or, alternatively, was transferred from chronically exposed parents. To evaluate the contributions of each, eggs were analyzed for Pb accumulation from the switched breeding experiments carried out as previously described. Lead accumulated to a great extent (nearly 40 nmol Pb/g) in eggs from control fish transferred to Pb exposure for breeding, indicating that direct acute exposure to the eggs accounts for most of the accumulation rather than parental transfer (Table 6). Nevertheless, a discrepancy seems to exist as egg Pb concentrations were not as high as in the non-switched Pb exposures and the difference seems unlikely accounted for by parental transfer given the low Pb accumulated in eggs from Pb-exposed parents bred in control water. However, the results of Weber (1993) support direct environmental exposure to eggs as the source of Pb accumulation. Furthermore, previous work has shown that waterborne Pb can enter through the zona radiata (Rombough, 1985) though superficial adsorption to the egg surface could also account for the observed accumulation.

3.9. Egg attachment to breeding substrate

It might reasonably be expected that failure of eggs to attach, or remain attached, to a breeding substrate might lead to drifting or other transport away from a defended nest. Such detached eggs may be subjected to greater predation and infection and thus reduced viability in the wild. Therefore, we recorded separately the number of eggs attached to the PVC breeding substrate and those detached to see if Pb and/or water chemistry had any influence. For the most part, egg attachment was low with nearly 70–80% detached in both tap water and HCO_3^- treatments (Table 6). In comparison, HA promoted attachment (to ~50%) and this result was not influenced by addition of Pb. Humic acid is known to bind biological surfaces, thereby potentially facilitating egg attachment to surfaces with HA staining/deposition. At low levels, Pb negatively impacted attachment slightly in tap water control and HCO_3^- treatments, but this effect was not consistent at the high Pb concentrations. Interestingly, switching either Pb-exposed fish to control water or vice versa seemed to improve egg attachment (Table 6).

3.10. Hatchability

No statistically significant differences in egg hatchability as a function of water chemistry or Pb treatment were observed (data not shown). In fact, percent hatchability was high overall with a mean ± SEM of 89.8 ± 1.4%, n = 32 (93.5 ± 1.6%, n = 11 for HA; 87.6 ± 3.2%, n = 10 for HCO_3^-; 88.1 ± 2.1%, n = 11 for tap water controls).

3.11. Behavior/motor function of offspring

The impact of Pb as a neurotoxin in mammals is well recognized and has been the focus of much research. Far less effort has centered on Pb neurotoxicty in fish. Weber examined juvenile fathead minnows feeding on *Daphnia magna* (Weber et al., 1991) and reproductive behavior of adult fathead minnows (1993) during shorter exposures (4 week) to higher Pb concentrations (2415–4831 nM (500–1000 μg/L)) than that of the present study and found various

Table 7
Effect of Pb and water chemistry on ability of 10-d-old fathead minnow larvae to ingest 5 of 10 *Artemia* nauplii in 5 mL of treatment water within 5 min. Number of clutch replicates shown in parentheses.

	% Ingesting half	Fraction
Tap control	87.5	21/24 (2)
Tap + low Pb	84.4	27/32 (3)
Tap + high Pb	43.5	17/33 (3)
Tap + high Pb (switched)[a]	77.8	14/18 (2)
HCO_3^- control	61.5	16/26 (2)
HCO_3^- + low Pb	79.2	19/24 (2)
HCO_3^- + high Pb	70.0	14/20 (2)
Humic control	92.3	24/26 (2)
Humic + low Pb	95.7	22/23 (2)
Humic + high Pb	80.0	26/30 (3)

[a] Switched breeding exposures in which control breeders were transferred to high Pb conditions.

Pb-induced alterations on feeding and behavior. Other neurological effects include lordoscoliosis and black discoloration of the caudal peduncle as observed in species of trout (Davies et al., 1976; Holcombe et al., 1976). Because fish are more sensitive to toxicants and starvation at early life stages, we decided to investigate Pb effects on prey capture ability of 10-d-old larvae. Furthermore, by using F1 offspring we hoped to distinguish effects due to chronic parental Pb exposure from those arising from direct acute waterborne Pb exposure.

Hatched larvae from 21 d breeding experiments were cultured for 10 d by static-renewal in treatments matching those of their respective parents. Initially, the test design was simply to record the time to completion for 5 and 10 ingestions out of 10 possible *Artemia* nauplii in 5 mL of test water within a time limit of 5 min. However, introduction of the *Artemia* typically induced a stress response of frantic swimming followed by a period of rest (typically 1–2 min) during which the larva would remain stationary and unresponsive to *Artemia* that appeared clearly within range of easy and rapid detection. Usually, a presumed recovery to "normal" swimming (i.e. slower, more controlled) would then commence followed shortly thereafter by active foraging behavior, seemingly upon first notice of the *Artemia* after rest. Often, a larva would not ingest all of the 10 *Artemia*, or even half in some cases, within the given 5 min time period. Considering the variability in startle swimming duration and recovery period, and failures of some larvae to complete all 10 ingestions, we decided to use the interval between the 1st and 5th ingestion as likely representing the most reproducible endpoint to assess prey capture ability.

The percentages of larvae ingesting half (5) of the possible *Artemia* are shown in Table 7. Fractions were also included to indicate the number of tests performed per treatment and the number of larvae completing 5 ingestions for the analysis depicted in Fig. 4. High Pb concentrations significantly increased the duration between the 1st and 5th ingestions in both HA and HCO_3^- treatments, but had no effect on this time in the tap water controls (Fig. 4). However, far fewer larvae completed the 5th ingestion in high Pb tap water (44%) compared to the other high Pb treatments indicating protective effects from DOC and HCO_3^- for this endpoint (Table 7). Yet, larvae from control breeders hatched in high Pb tap water were more similar to controls (78% completed), suggesting that the reduced ingestion in high Pb tap water may be due to chronic parental Pb exposure rather than acute larval exposure. Finally, for reasons unknown, larvae seemed to perform faster and ingest more prey in water of higher DOC content (without Pb).

To summarize, high Pb concentrations altered feeding performance in all water treatments, although the influence of water chemistry differed depending on the endpoint examined. Larvae from DOC and HCO_3^- treatments with high Pb required more time to ingest 5 *Artemia* than those from treatment-matched controls.

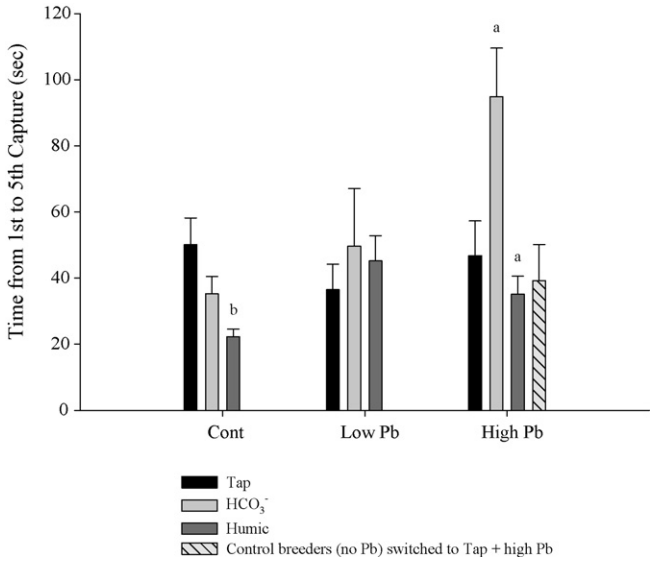

Fig. 4. Lead and water chemistry influence on 10 d larval fathead minnow prey capture ability. Values represent mean ± SEM durations between 1st and 5th ingestions; $n = 13–26$. 8–14 larvae were tested from each of 2–3 replicate beakers. Those not completing 5 ingestions were analyzed separately (see Table 7). Statistically significant difference from [a]treatment-matched control or from [b]tap water control as determined by Student's *t*-test.

However, larvae from these same high Pb treatments more frequently completed 5 ingestions than those from tap water with high Pb. Thus, regardless of the exact nature of the effect, these findings indicate that fathead minnows chronically exposed to Pb may produce offspring with ecologically relevant behavioral impairment. However, clarifying these effects and understanding the role of water chemistry will require more research.

4. Conclusions

We have demonstrated that increased HCO_3^- and DOC (as HA) protect against chronic Pb accumulation by fathead minnows. Yet paradoxically these same parameters appear to augment reproductive toxicity at high Pb concentrations. Indeed, the influences of HCO_3^- and HA on the effects of Pb exposure throughout this study were unexpected and somewhat puzzling. However, these influences were consistent across several of the endpoints examined lending credence to the connectivity of the combined impacts of Pb and water chemistry. Since Pb in either its carbonate- or DOC-bound form is believed to be, for the most part, biologically unavailable the chronic toxicity observed in the HCO_3^- and HA treatments is difficult to explain. Furthermore, in the absence of Pb, addition of HCO_3^- or HA may actually improve various aspects of fathead minnow reproduction. Specifically, HCO_3^- may increase basal fecundity and HA may improve egg attachment to a breeding substrate as well as the ability of hatched larvae to capture prey. In the end, the unexpected nature of the observed Pb effects on reproduction, and their apparent interactions with water chemistry which cannot be explained at present, suggest that more research is needed if we are to understand the true nature of chronic waterborne Pb toxicity to fathead minnows.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2009.10.012.

References

Alves, L.C., Wood, C.M., 2006. The chronic effects of dietary lead in freshwater juvenile rainbow trout (*Oncorhynchus mykiss*) fed elevated calcium diets. Aquat. Toxicol. 78, 217–232.

Andrews, A.K., Flickinger, S.A., 1973. Spawning requirements and characteristics of the fathead minnow. Proc. Southeast Assoc. Game Fish. Comm. 27, 759–766.

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.

Fairbrother, A., Wenstel, R., Sappington, K., Wood, W., 2007. Framework for metals risk assessment. Ecotoxicol. Environ. Saf. 68, 145–227.

Filella, M., Town, R.M., 2000. Determination of metal ion binding parameters for humic substances. Part 1. Application of a simple calculation method for extraction of meaningful parameters from reverse pulse polarograms. J. Electroanal. Chem. 485, 21–33.

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.

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.

Harries, J.E., Runnalls, T., Hill, E., Harris, C.A., Maddix, S., Sumpter, J.P., Tyler, C.R., 2000. Development of a reproductive performance test for endocrine disrupting chemicals using pair-breeding fathead minnows (*Pimephales promelas*). Environ. Sci. Technol. 34, 3003–3011.

Hodson, P.V., Blunt, B.R., Spry, D.J., 1978. pH-induced changes in blood lead of lead-exposed rainbow trout (*Salmo gairdneri*). J. Fish. Res. Board. Can. 35, 437–445.

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.

Jensen, K.M., Korte, J.J., Kahl, M.D., Pasha, M.S., Ankley, G.T., 2001. Aspects of basic reproductive biology and endocrinology in the fathead minnow (*Pimephales promelas*). Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 128, 127–141.

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.

Mager, E.M., Wintz, H., Vulpe, C.D., Brix, K.V., Grosell, M., 2008. Toxicogenomics of water chemistry influence on chronic lead exposure to the fathead minnow (*Pimephales promelas*). Aquat. Toxicol. 87, 200–209.

Mount, D.R., Barth, A.K., Garrison, T.D., Barten, K.A., Hockett, J.R., 1994. Dietary and waterborne exposure of rainbow trout (*Oncorhynchus mykiss*) to copper, cadmium, lead and zinc using a live diet. Environ. Toxicol. Chem. 13, 2031–2041.

Ojo, A.A., Wood, C.M., 2007. *In vitro* analysis of the bioavailability of six metals via the gastro-intestinal tract of the rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 83, 10–23.

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.

Reiley, M.C., 2007. Science, policy, and trends of metals risk assessment at EPA: how understanding metals bioavailability has changed metals risk assessment at USEPA. Aquat. Toxicol. 84, 292–298.

Rombough, P.J., 1985. The influence of the zona radiata on the toxicities of zinc, lead, mercury, copper and silver ions to embryos of steelhead trout *Salmo gairdneri*. Comp. Biochem. Physiol. C 82, 115–117.

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.

Santore, R., Di Toro, D.M., Paquin, P.R., Allen, H.E., Meyer, J.S., 2001. A biotic ligand model of the acute toxicity of metals 2. Application to acute copper toxicity in freshwater fish and Daphnia. Environ. Toxicol. Chem. 20, 2397–2402.

Schubauer-Berigan, M.K., Dierkes, J.R., Monson, P.D., Ankley, G.T., 1993. pH-dependent toxicity of Cd, Cu, Ni, Pb and Zn to *Ceriodaphnia dubia*, *Pimephales promelas Hyalella azteca and Lumbriculus variegatus*. Environ. Toxicol. Chem. 12, 1261–1266.

Sellin, M.K., Kolok, A.S., 2006. Cd exposures in fathead minnows: effects on adult spawning success and reproductive physiology. Arch. Environ. Contam. Toxicol. 51, 594–599.

Stouthart, A.J.H.X., Spanings, F.A.T., Lock, R.A.C., Wendelaar Bonga, S.E., 1994. Effects of low water pH on lead toxicity to early life stages of the common carp (*Cyprinus carpio*). Aquat. Toxicol. 30, 137–151.

Stumm, W., Morgan, J.J., 1996. Aquatic Chemistry. Chemical Equilibria and Rates in Natural Waters, third ed. John Wiley & Sons, Inc., New York, p. 296.

Thorpe, K.L., Benstead, R., Hutchinson, T.H., Tyler, C.R., 2007. An optimised experimental test procedure for measuring chemical effects on reproduction in the fathead minnow, *Pimephales promelas*. Aquat. Toxicol. 81, 90–98.

USEPA, 2002. National Recommended Water Quality Criteria: 2002. Office of Water, Washington, DC.

USEPA, 2007. Aquatic Life Ambient Freshwater Quality Criteria-Copper. Office of Water, Washington, DC.

Varanasi, U., Gmur, D.J., 1978. Influence of water-borne and dietary calcium on uptake and retention of lead by coho salmon (*Oncorhynchus kisutch*). Toxicol. Appl. Pharmacol. 46, 65–75.

Watanabe, K.H., Jensen, K.M., Orlando, E.F., Ankley, G.T., 2007. What is normal? A characterization of the values and variability in reproductive endpoints of the fathead minnow, *Pimephales promelas*. Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 146, 348–356.

Weber, D.N., Russo, A., Seale, D.B., Spieler, R.E., 1991. Waterborne lead affects feeding abilities and neurotransmitter levels of juvenile fathead minnows (*Pimephales promelas*). Aquat. Toxicol. 21, 71–80.

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

White, L.D., Cory-Slechta, D.A., Gilbert, M.E., Tiffany-Castiglioni, E., Zawia, N.H., Virgolini, M., Rossi-George, A., Lasley, S.M., Qian, Y.C., Basha, M.R., 2007. New and evolving concepts in the neurotoxicology of lead. Toxicol. Appl. Pharmacol. 225 (1), 1–27.