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Influences of water chemistry on the acute toxicity of lead to *Pimephales promelas* and *Ceriodaphnia dubia*

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ABSTRACT

The acute toxicity of lead (Pb) was examined for fathead minnows (*Pimephales promelas*; 96-h) and daphnids (*Ceriodaphnia dubia*; 48-h) in waters modified for hardness (as $CaSO_4$), dissolved organic carbon (DOC; as Aldrich humic acid) and alkalinity (as $NaHCO_3$) for parameterization of an acute freshwater biotic ligand model (BLM). Additionally, acute (96-h) and chronic (30-d) bioassays were performed for *P. promelas* to more clearly define the influence of pH (5.5–8.3) on Pb toxicity as modified by addition of HCl or NaOH using an automated titration system. Results indicate that Ca^{2+} is protective against acute Pb toxicity to *P. promelas* but not *C. dubia*. Strong protection was afforded by DOC and $NaHCO_3$ against acute Pb toxicity to *P. promelas*, whereas milder protection was observed for *C. dubia* with both parameters. Dissolved Pb LC50s from the *P. promelas* pH bioassays revealed a complex effect of pH on Pb toxicity, likely explained in part by Pb speciation and the competitive interaction of H^+ with ionic Pb^{2+}. Chronic pH bioassays also demonstrated that 30-d growth is not impaired in fathead minnows at relevant Pb concentrations. The findings reported herein suggest that development of separate BLMs for *P. promelas* and *C. dubia* should be considered.

1. Introduction

Lead (Pb) is a nonessential metal that has gained much notoriety from its past use as an additive in gasoline and from Pb-based paints. Although such applications were phased out beginning in the 1970s in the United States, Pb remains an environmental concern today primarily as a consequence of anthropogenic sources such as those related to Pb mining and industrial processing (World Health Organization, 1995). Establishing safe environmental regulations for Pb, as with other metals, is a challenging endeavor due to natural variability in receiving water chemistry that can differentially impact its chemical speciation, and therefore bioavailability and toxicity. Factors important in determining metal toxicity include pH, concentration and quality of dissolved organic carbon (DOC), water hardness (Ca^{2+}, Mg^{2+}) and inorganic complexing agents (e.g. carbonates, sulfides, and chlorides) (Pagenkopf, 1983; Richards et al., 2001). Until recently, however, the USEPA has only recommended site-specific adjustments to water quality criteria (WQC) based on water hardness for several trace metals including Pb.

The biotic ligand model (BLM) represents a culmination of multiple efforts aimed at simultaneously addressing a wide array of water chemistry parameters that may influence a metal's toxicity. Under an assumption of chemical equilibrium, the BLM relates metal accumulation at the biotic ligand (e.g. gill) to a toxic effect level by considering the influences of cations that compete with the metal for binding at the site of action, and complexation with DOC or other inorganic species that render the metal unavailable for uptake, while at the same time incorporating a physiological basis for toxicity (Paquin et al., 2002). Because of its flexibility and strong potential for application to a variety of metals and test organisms, a BLM-based approach to establishing environmental standards for metals is being pursued in many countries. At present, a freshwater BLM has been implemented only for copper (Cu) within the United States (USEPA, 2007) and regional risk assessments in Europe have employed BLMs for metals such as Cu, nickel and zinc (Bodar et al., 2005). Additional BLMs for other high priority metals such as aluminum, cobalt, iron, silver and Pb are likely to follow in the coming years as each are currently in various stages of development.

At a minimum, data sets for BLM development should include multiple measurements of the same toxicological endpoint (e.g. LC50) and well characterized water chemistry over a range of relevant conditions. Furthermore, sensitive test organisms should be used for toxicity testing if the results are to have ecological relevance. The fathead minnow (*Pimephales promelas*) and daphnid (*Ceriodaphnia dubia*) are two of the most commonly used test organisms for

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establishing WQC and both have documented sensitivity to Pb (Pickering and Henderson, 1966; Spehar and Fiandt, 1986; Schubauer-Berigan et al., 1993; Grosell et al., 2006; Mager et al., 2008, 2010). Specifically, our lab recently investigated the influences of water chemistry on chronic Pb toxicity to fathead minnows during 30, 150 and 300-d flow-through exposures (Grosell et al., 2006; Mager et al., 2008, 2010). While the 30-d fathead minnow studies were performed to evaluate the chronic toxicity of Pb, the tests also allowed for derivation of 96-h LC50s; however, obtaining acute effect levels in this manner is in contrast to the established test protocol for acute toxicity studies (Stephan et al., 1985) as the fish were fed daily throughout. Additionally, Schubauer-Berigan et al. (1993) investigated the importance of pH to acute Pb toxicity in very hard water to several freshwater organisms including fathead minnows and *C. dubia*. From these studies, as well as others using rainbow trout and common carp (Davies et al., 1976; Hodson et al., 1978; Stouthart et al., 1994; Macdonald et al., 2002), it has become evident that Ca^{2+}, DOC, pH and alkalinity are the primary parameters influencing Pb toxicity. Still, a comprehensive analysis of acute Pb toxicity sufficient for BLM parameterization has yet to reach fruition for either of these organisms.

We therefore undertook the present study with the specific goal of obtaining a data set for parameterization of an acute freshwater BLM for Pb. To this end, we evaluated the acute toxicity of Pb to fathead minnows and *C. dubia* using standard bioassays adjusted for parameters known to influence Pb toxicity (Ca^{2+}, DOC, pH and alkalinity). From a recent study by our lab (Grosell et al., 2006), it was shown that pH manipulations using organic buffers (MOPS) are potentially problematic as the buffers themselves may alter the physiology of the test organisms, thereby rendering them more sensitive to Pb. Thus, to more effectively assess the influence of pH on the toxicity of Pb to fathead minnows, we conducted both acute and chronic (30-d) pH bioassays using an automated titration system. Finally, we evaluated the influence of feeding on acute Pb toxicity by comparing the 96-h LC50s from fish that were not fed during tests, as per standard methodology, versus 96-h LC50s obtained from fish that were fed daily throughout.

2. Materials and methods

2.1. Experimental design

Acute toxicity of Pb was evaluated for larval fathead minnows and *C. dubia* neonates using 96-h flow-through and 48-h static-renewal survival tests, respectively, performed in accordance with standard USEPA guidelines (USEPA, 2002). To examine the influence of feeding during fathead minnow acute toxicity tests, two bioassays were first performed in the base water, one in which fish were fed daily and another in which fish were not fed. While not conclusive, the results indicated a potential influence of feeding on the acute toxicity of Pb (see Section 3.1). Therefore, all subsequent acute tests were conducted without feeding to ensure adherence to USEPA standards (Stephan et al., 1985). For both organisms, water chemistry was manipulated to investigate the influence of Ca^{2+} (as $CaSO_4$), DOC (as Aldrich humic acid (HA)), and alkalinity (as $NaHCO_3$) on the acute toxicity of Pb (as $PbNO_3$). A NaCl test was also performed for each organism to distinguish any potential protective contribution from the Na^+ component of the $NaHCO_3$ salt. Additionally, acute and chronic (30-d) pH tests were performed for fathead minnows as described below. The complete lists of tests are provided in Tables 1 and 2. All tests included a treatment-matched control without Pb. Both test organisms were purchased from Aquatic BioSystems, Inc. (Fort Collins, CO, USA). Fathead minnows were <24 h post-hatch on arrival, and an in-house culture of *C. dubia* was maintained to provide <24 h old neonates for testing. All chemicals used for modifying test waters were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Toxicity tests – P. promelas

Acute (96-h) and chronic (30-d) bioassays were administered with 2:1 deionized water:dechlorinated Virginia Key tap water using a gravity flow-through approach as previously described (Grosell et al., 2006). Except for pH, modified water chemistry parameters and Pb concentrations were adjusted by a constant drip of concentrated stock solution delivered by Mariotte bottle or peristaltic pump (2.5 mM $CaSO_4$). Addition of Pb was initiated at least 24 h prior to the introduction of fish to allow for equilibration. To modify pH, an automated titration system was used to more accurately assess the influence of pH and to minimize concurrent changes in the carbonate buffering system. In brief, a MasterFlex peristaltic pump automated through a pump controller (Cole-Parmer, Vernon Hills, IL, USA) with feedback from a pH electrode (sc-100, Hach Co., Loveland, CO, USA) in the primary mixing chamber was used to maintain constant pH values targeting 5.5, 6.4, and 8.3 by addition of HCl (pH ≤ 6.4) or NaOH (pH 8.3). The pH values of the exposure chambers were assessed independently using a combination glass electrode coupled to a PHM201 pH meter (Radiometer, Copenhagen, Denmark), which was calibrated daily using IUPAC standards (Radiometer). The entire system was allowed to operate for at least 3 days prior to the introduction of animals to ensure pH stabilization. An additional base water control test of pH 7.5, unmodified by acid/base addition, was performed alongside the first of these tests (pH 6.4).

Upon arrival, fish were gradually acclimated to modified test water for 7 d prior to Pb exposures and were thus 8 days old at the onset of toxicity testing. Acclimation to test waters is important as it has been demonstrated to affect gill-metal binding in fathead minnows (Bielmyer et al., 2008). Fish were raised on a daily diet of *Artemia* spp. nauplii (fed *ad libitum*). For the acute bioassays, food was withheld 24 h before and during Pb exposures except for the test examining the effect of feeding, in which case fish were fed daily. Chronic bioassays to examine the effect of pH on Pb toxicity were performed as for the acute tests except that in all cases fish were fed daily and on the final day of exposure, all surviving fish were collected and weighed to analyze potential growth effects. Initial sample sizes comprised a minimum of 10 larvae per each of 3 replicate, 1 L plastic beakers per Pb concentration. Mortality was recorded and uneaten food and fecal matter were siphoned from the exposure beakers daily. All procedures were approved by the University of Miami Animal Care and Use Committee.

2.3. Toxicity tests – C. dubia

An in-house culture of *C. dubia* was maintained in full strength dechlorinated Virginia Key tap water. This stock culture was used to supply separate cultures for acclimation of daphnids to test water for at least 5–7 d prior to testing. Neonates were collected for bioassays within 24 h of hatching from the acclimated cultures. As previous studies have demonstrated that selenium (Se) is an essential nutrient for daphnids (Keating and Dagbusan, 1984; Winner, 1984), all stock cultures and test waters were supplemented with 1 μg/L Se (as Na_2SeO_4) to ensure adequate nutrition. Test waters for 48-h *C. dubia* bioassays were initially prepared with 2:1 dechlorinated Virginia Key tap water:deionized water (Base H_2O A). However, as it became difficult to maintain cultures under these low ionic strength conditions, later tests were conducted with full strength dechlorinated tap water (Base H_2O B). Control tests were performed for both ionic strength base waters. Because the switch to full strength base water occurred in the middle of the HA series, duplicate tests were performed for the 8 mg/L HA treatment using each of the different ionic strength base waters. Lead was added to aliquots of test water the day before testing to allow for equilibration. For each bioassay, 5 replicate 30 mL polypropylene cups each containing 20 mL of test solution and 5 *C. dubia* neonates were used for each Pb treatment. All

Table 1
Water quality data for acute and chronic *P. promelas* bioassays (mean ± SEM) are provided in the last column. The ranges of dissolved Pb test concentrations (excluding no Pb controls) are provided in the last column.

Test	Concentration (in μM)							DOC (μmol C/L)	Hardness (mg/L)	Ionic str. (mM)	pH	Temp. (°C)	Test range (μg/L Pb)
	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	SO_4^{2-}	Total CO_2						
Acute (96 h)													
Unfed base H_2O	519 ± 6	25 ± 0	131 ± 3	54 ± 0	431 ± 3	40 ± 1	182 ± 24	79 ± 2	19 ± 0	4.73	7.58 ± 0.04	22.7 ± 0.2	49–901
Fed base H_2O	579 ± 10	25 ± 0	159 ± 4	60 ± 1	511 ± 2	45 ± 1	267 ± 7	88 ± 2	22 ± 1	4.94	7.43 ± 0.07	23,1 ± 0.2	58–838
0.5 mM $CaSO_4$	624 ± 8	26 ± 0	258 ± 3	62 ± 1	567 ± 6	153 ± 1	236 ± 24	97 ± 2	33 ± 1	5.60	7.46 ± 0.05	244 ± 0.2	103–661
1.0 mM $CaSo_4$	643 ± 2	25 ± 0	445 ± 6	70 ± 1	590 ± 7	334 ± 8	202 ± 3	94 ± 3	53 ± 1	6.41	7.55 ± 0.02	23.6 ± 0.2	300–1993
1.5 mM $CaSO_4$ A	658 ± 4	25 ± 0	590 ± 13	68 ± 2	616 ± 4	507 ± 10	192 ± 9	90 ± 2	67 ± 2	6.92	742 ± 0.03	24.5 ± 0.2	580–4154
1.5 mM $CaSO_4$ B	962 ± 28	27 ± 3	550 ± 46	95 ± 1	732 ± 31	377 ± 15	422 ± 27	95 ± 1	66 ± 5	7.18	7.71 ± 0.09	19.0 ± 0	460–3438
2.5 mM $CaSO_4$	633 ± 9	20 ± 0	2950 ± 132	62 ± 2	462 ± 10	2019 ± 69	265 ± 21	88 ± 7	309 ± 14	13.38	747 ± 0.04	17.5 ± 0.5	1019–8031
2 mg/L HA	600 ± 2	26 ± 0	137 ± 2	59 ± 1	598 ± 4	54 ± 1	167 ± 27	115 ± 1	20 ± 0	5.34	7.61 ± 0.03	23.8 ± 0.1	177–1158
4 mg/L HA	605 ± 9	25 ± 0	154 ± 10	60 ± 2	606 ± 4	52 ± 1	185 ± 15	138 ± 5	22 ± 1	5.65	7.59 ± 0.04	24.0 ± 0.2	109–1252
8 mg/L HA	616 ± 3	26 ± 0	152 ± 4	63 ± 1	609 ± 4	52 ± 1	176 ± 29	216 ± 6	22 ± 1	6.53	7.51 ± 0.09	24.9 ± 0.1	231–3671
16 mg/L HA	630 ± 7	26 ± 0	148 ± 2	61 ± 1	618 ± 2	55 ± 1	248 ± 6	420 ± 23	21 ± 0	9.04	7.61 ± 0.02	24.6 ± 0.2	884–6481
0.5 mM $NaHCO_3$	853 ± 10	28.±1	157 ± 9	66 ± 3	736 ± 7	63 ± 1	428 ± 14	88 ± 1	23 ± 1	5.36	7.52 ± 0.05	22.2 ± 0.2	145–1929
1.0 mM $NaHCO_3$	1089 ± 15	28 ± 0	160 ± 5	66 ± 1	722 ± 3	63 ± 1	744 ± 19	91 ± 1	23 ± 1	5.90	7.87 ± 0.01	22.1 ± 04	173–1679
1.5 mM $NaHCO_3$	1353 ± 8	33 ± 0	149 ± 5	70 ± 1	592 ± 57	42 ± 1	1002 ± 28	96 ± 0	22 ± 0	6.16	7.99 ± 0.01	22.3 ± 0.1	636–1859
2.0 mM $NaHCO_3$	2073 ± 21	29 ± 0	156 ± 8	68 ± 2	746 ± 1	64 ± 1	1662 ± 44	93 ± 1	23 ± 1	7.04	8.30 ± 0.03	21.5 ± 0	665–1210
1.5 mM NaCL	1361 ± 30	29 ± 1	165 ± 10	69 ± 4	1956 ± 9	62 ± 1	258 ± 25	92 ± 3	24 ± 1	6.23	7.50 ± 0.02	22.5 ± 0.1	58–1065
pH 5.5	716 ± 19	12 ± 0	218 ± 10	60 ± 1	1473 ± 458	52 ± 2	21 ± 15	134 ± 1	28 ± 1	3.78	5.35 ± 0.07	19.8 ± 0.3	60–1109
pH 6.4	561 ± 9	10 ± 0	226 ± 15	61 ± 1	903 ± 8	41 ± 2	24 ± 12	118 ± 1	29 ± 2	4.36	6.34 ± 0.04	20.5 ± 0	69–1207
pH 7.5	579 ± 6	10 ± 0	234 ± 14	*63 ± 1*	415 ± 7	43 ± 0	514 ± 58	104 ± 2	30 ± 2	5.49	7.50 ± 0.02	20.5 ± 0	86–1723
pH 8.3	1050 ± 114	10 ± 0	186 ± 5	60 ± 2	577 ± 16	48 ± 1	667 ± 25	123 ± 1	25 ± 1	6.31	8.26 ± 0.06	20.0 ± 0	231–1949
Chronic (30 d)													
pH 5.5	718 ± 6	11 ± 0	257 ± 48	66 ± 5	1443 ± 135	55 ± 1	19 ± 13	136 ± 13	22 ± 5	4.21	5.64 ± 0.11	20.5 ± 0.5	30–447
pH 6.4	555 ± 12	9 ± 0	203 ± 27	53 ± 1	934 ± 57	42 ± 1	49 ± 16	115 ± 4	26 ± 3	4.31	6.41 ± 0.03	20.2 ± 0.4	29–1348
pH 7.5	569 ± 16	11 ± 0	202 ± 29	54 ± 1	698 ± 92	47 ± 2	543 ± 69	108 ± 4	26 ± 3	5.58	7.50 ± 0.03	20.2 ± 0.4	32–1828
pH 8.3	1215 ± 133	13 ± 0	180 ± 12	58 ± 2	648 ± 35	60 ± 3	798 ± 66	123 ± 5	24 ± 1	6.48	8.22 ± 0.03	18.3 ± 0.3	25–1049

mass cultures and bioassays were maintained in a controlled environmental chamber at 25 °C.

2.4. Water chemistry

For the acute fathead minnow tests, dissolved Pb was measured daily and general water chemistry was measured at least twice during the 96-h exposure period. For the chronic tests, dissolved Pb was measured daily for the first 4–5 days and then at least twice per week thereafter and general water chemistry was typically measured once per week ($n = 3$–4). For the *C. dubia* bioassays, Pb concentrations and general water chemistry were measured on day 0 and day 2. A Varian 200Z graphite furnace (Australia) was used to measure dissolved Pb concentrations by atomic absorption spectroscopy. Samples were first filtered through a 0.45 μm syringe filter (Pall LifeSciences, MI, USA) and acidified to 1% HNO_3 (trace metal grade, Fisher Scientific, PA, USA). Concentrations of Na^+, K^+, Ca^{2+}, and Mg^{2+} were determined by flame atomic absorption spectroscopy (Varian 220FS, Australia) and Cl^- and SO_4^{2-} concentrations were measured by anion chromatography (DIONEX DX120, CA, USA). Total CO_2 (dissolved inorganic carbon) was measured using a Corning 962 carbon dioxide analyzer (UK) except for pH and $NaHCO_3$ treatments which were measured by manual double endpoint titrations with HCl and NaOH using a 2 mL Gilmont micrometer burette (Cole-Parmer, Vernon Hills, IL, USA). The fine-scale manual dispensing of fluid by the burette (± 0.5% accuracy) allowed for higher resolution measurements over those obtained by the CO_2 analyzer. High temperature catalytic oxidations using a Shimadzu total organic carbon-VCSH analyzer (Kyoto, Japan) were

Table 2
Water quality data for acute *C. dubia* bioassays (mean ± SEM). The ranges of dissolved Pb test concentrations (excluding no Pb controls) are provided in the last column.

Test	Concentration (in μM)							DOC (μmol C/L)	Hardness (mg/L)	Ionic str. (mM)	pH	Temp (°C)	Test range (μg/L Pb)
	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	SO_4^{2-}	Total CO_2						
Base H_2O A	1260 ± 79	64 ± 4	315 ± 22	146 ± 14	1063 ± 78	85 ± 5	506 ± 23	213 ± 27	47 ± 4	8.02	7.58 ± 0.06	25	180–907
Base H_2O B	2369 ± 103	109 ± 5	828 ± 77	309 ± 18	2120 ± 110	205 ± 12	1085 ± 59	334 ± 16	116 ± 10	12.54	8.01 ± 0.01	25	107–544
0.5 mM $NaHCO_4$	735 ± 20	50 ± 3	614 ± 39	*130*	*1110*	*90*	*587*	*242*	*76*	*11.42*	7.44	25	355–1851
1.0 mM $NaHCO_4$	792 ± 30	51 ± 9	1088 ± 23	*130*	*1110*	*90*	*587*	*242*	*125*	*12.29*	*7.44*	25	261–1707
1.5 mM $NaHCO_4$	1272 ± 11	60 ± 1	1653 ± 4	130 ± 1	964 ± 8	1037 ± 1	489 ± 12	282	183 ± 1	12.86	7.44 ± 0.01	25	164–523
2.0 mM $NaHCO_4$	1411 ± 128	67 ± 1	2302 ± 9	142 ± 1	1078 ± 8	1528 ± 18	555 ± 23	321	250 ± 1	15.35	7.43 ± 0.02	25	157–440
2 mg/L HA	1179 ± 78	58 ± 1	256 ± 17	149 ± 1	887 ± 7	63 ± 3	714 ± 20	228	41 ± 2	8.09	7.80 ± 0.01	25	219–1303
4 mg/L HA	1216 ± 97	66 ± 1	361 ± 2	170 ± 1	1012 ± 26	75 ± 11	793 ± 23	266	54 ± 1	8.96	7.80 ± 0.01	25	491–1695
8 mg/L HA A	1256 ± 164	55 ± 1	221 ± 1	134 ± 1	861 ± 5	63 ± 14	621 ± 25	171	36 ± 1	7.11	749 ± 0.01	25	*203–1569*
8 mg/L HA B	2477 ± 250	110 ± 10	701 ± 46	297 ± 23	2198 ± 193	204 ± 14	1125 ± 99	415 ± 21	102 ± 7	13.33	7.89 ± 0.01	25	101–802
32 mg/L HA	2535 ± 193	110 ± 11	673 ± 59	302 ± 34	2152 ± 209	215 ± 29	1184 ± 118	897 ± 32	99 ± 9	19.11	8.15 ± 0.01	25	239–1439
64 mg/L HA	4034 ± 167	282 ± 11	796 ± 28	242 ± 12	3391 ± 268	368 ± 29	1250 ± 66	1298 ± 41	106 ± 4	25.77	8.15 ± 0.02	25	271–5026
0.5 mM $NaHCO_3$	2168 ± 193	74 ± 1	400 ± 2	104 ± 2	1385 ± 5	133 ± 1	857 ± 13	214 ± 12	51 ± 1	9.13	7.96 ± 0.11	25	279–1344
1.0 mM $NaHCO_3$	2823 ± 76	74 ± 2	396 ± 7	104 ± 3	1485 ± 52	96 ± 2	1326 ± 50	233 ± 7	51 ± 1	9.93	8.19 ± 0.02	25	416–1547
1.5 mM $NaHCO_3$	2937 ± 209	69 ± 6	295 ± 20	142 ± 11	1445 ± 132	108 ± 8	1715 ± 137	228 ± 33	44 ± 3	9.96	8.06 ± 0.04	25	389–1876
2.0 mM $NaHCO_3$	2944 ± 99	58 ± 2	289 ± 10	105 ± 3	928 ± 41	70 ± 2	2175 ± 87	199 ± 13	40 ± 1	9.46	8.23 ± 0.04	25	355–1890
1.5 mM $NaHCO_3$	2164 ± 179	59 ± 3	216 ± 45	106 ± 5	2066 ± 183	119 ± 19	428 ± 51	216 ± 30	33 ± 5	8.61	748 ± 0.03	25	345–1732

Notes: values in italics were not measured due to loss of samples but were estimated from averages of measurements on comparable test solutions for speciation calculations. All regressions were tested using Maximum Likelihood-Probit.

Table 3
96-h LC50s (95% CI) for acute *P. promelas* bioassays.

Test	[μg/L Pb]	[nmol/L Pb]
Unfed base H_2O	178 (151–209)	859 (729–1009)
Fed base H_2O	439 (214–961)	2119 (1033–4638)
0.5 mM $CaSO_4$	744 (629–1031)	3591 (3036–4976)
1.0 mM $CaSO_4$	1015 (910–1111)	4899 (4392–5362)
1.5 mM $CaSO_4$ A	1068 (919–1227)	5154 (4435–5922)
1.5 mM $CaSO_4$ B	1148 (962–1342)	5541 (4643–6477)
2.5 mM $CaSO_4$	1719 (1487–1948)	8296 (7177–9402)
2 mg/L HA	608 (553–662)	2934 (2669–3195)
4 mg/L HA	1075 (973–1219)	5188 (4696–5883)
8 mg/L HA	1356 (1162–1534)	6544 (5608–7403)
16 mg/L HA	3249 (3004–3500)	15681 (14498–16892)
0.5 mM $NaHCO_3$	816 (713–927)	3938 (3441–4474)
1.0 mM $NaHCO_3$	996 (864–1155)	4807 (4170–5574)
1.5 mM $NaHCO_3$	698 (340–905)[a]	3369 (1641–4368)[a]
2.0 mM $NaHCO_3$	N/A[c]	N/A[c]
1.5 mM NaCl	370 (130–1067)	1786 (627–5150)
pH 5.5	162 (118–224)[b]	782 (569–1081)[b]
pH 6.4	265 (149–416)	1279 (719–2008)
pH 7.5	624 (444–891)	3012 (2143–4300)
pH 8.3	340 (282–410)[b]	1641 (1361–1979)[b]

[a–b]All LC50s were obtained using Maximum Likelihood-Probit unless otherwise indicated as follows: [a]Maximum Likelihood-Logit; [b]Trimmed Spearman–Karber. [c]Lack of mortality prevented calculation of an LC50.

used to measure dissolved organic carbon (DOC) concentrations (Hansell and Carlson, 2001).

2.5. Calculations and statistical analyses

All statistical tests were chosen following USEPA guidelines (USEPA, 2002) and performed using ToxCalc v. 5.0 (Tidepool Scientific Software). LC50s were estimated using Maximum Likelihood-Probit (preferred), Trimmed Spearman–Karber or Maximum Likelihood-Logit analyses. The LC20s and LC10s for all chronic tests were estimated using Linear Interpolation (survival was more sensitive than growth in all tests). See Tables 3–5 for the exact statistical method used for each test. Calculations of Pb speciation were performed using the Biotic Ligand Model v. 2.3.3 (HydroQual, Inc.; http://www.hydroqual.com/wr_blm.html). As the BLM assumes a default DOC background composition of 10% HA (Santore et al., 2001), this percentage was used for speciation calculations in all cases except for tests in which HA was added. For the HA tests, the %HA values were estimated proportionally relative to the base water control by assuming all additional DOC was attributable to 100% HA. Measured values from the respective tests were used for all other parameters, except when unavailable, in which cases measured control values were used.

3. Results and discussion

3.1. Acute Pb toxicity to P. promelas – influence of feeding

A comparison of LC50s from the base water tests during which fish were either fed or unfed initially suggested that providing food during Pb exposure affords significant (2.5 fold) protection against the acute toxicity of Pb (Table 3). This result indicated that 96-h LC50s estimated from our previous 30-d chronic studies (Grosell et al., 2006), during which fish were fed, may be inappropriate for acute BLM parameterization. However, it is important to note that the present acute fed and unfed bioassays were conducted at different times and that the water chemistry varied between tests. Specifically, the base water in the fed bioassay had proportionately higher concentrations (typically 10–20%) in all water chemistry parameters measured except K^+ and pH than in the unfed bioassay (Table 1). In fact, these differences in water chemistry were sufficient to substantially alter Pb speciation, as ionic Pb^{2+} concentrations at the dissolved Pb LC50 were 8-fold higher from the feeding bioassay than from the unfed bioassay (Table 6). Thus, some of the protection during the feeding tests was likely due to other factors (e.g. Ca^{2+} and DOC), particularly in light of the results from the acute bioassays examining the influences of water chemistry described below. Nonetheless, to eliminate any potential influence of feeding and to ensure adherence to USEPA standards food was withheld during all subsequent bioassays.

Further insight into the effect of feeding was gained from the 96-h LC50s calculated upon completion of the acute and chronic pH bioassays, as fish were fed during the latter but not the former. It should be noted that these were the last of all tests performed, and therefore were unavailable for our initial assessment of a feeding effect. Comparing the LC50s from these two data sets, a consistent trend indicating an influence of feeding on the acute toxicity of Pb was not apparent (Tables 3 and 4), thus further supporting that the possible feeding effect suggested by the initial bioassays was likely due to differences in water chemistry as opposed to feeding. However, as mentioned previously, the fact that fish were not fed during the subsequent acute bioassays evaluating the influences of water chemistry eliminates any concerns for future regulatory adoption of a Pb BLM based on the results reported herein.

Table 5
48-h LC50s (95% CI) for acute *C. dubia* bioassays.

Test	[μg/L Pb]	[nmol/L Pb]
Base H_2O A	395 (368–416)	1906 (1776–2008)
Base H_2O B	387 (336–418)	1868 (1622–2017)
0.5 mM $CaSO_4$	433 (375–498)	2090 (1810–2403)
1.0 mM $CaSO_4$	597 (503–682)	2881 (2428–3292)
1.5 mM $CaSO_4$	385 (348–432)	1858 (1680–2085)
2.0 mM $CaSO_4$	319 (285–358)	1540 (1375–1728)
2 mg/L HA	425 (337–507)	2051 (1626–2447)
4 mg/L HA	546 (441–631)	2635 (2128–3045)
8 mg/L HA A	591 (461–682)	2852 (2225–3292)
8 mg/L HA B	532 (473–604)	2568 (2283–2915)
32 mg/L HA	964 (376–2606)	4653 (1815–12577)
64 mg/L HA	3116 (2457–3611)	15039 (11858–17428)
0.5 mM $NaHCO_3$	384 (341–428)	1853 (1646–2066)
1.0 mM $NaHCO_3$	779 (697–840)	3760 (3364–4054)
1.5 mM $NaHCO_3$	571 (473–663)	2756 (2283–3200)
2.0 mM $NaHCO_3$	765 (665–856)	3692 (3209–4131)
1.5 mM NaCl	446 (377–515)	2153 (1819–2486)

Notes: all LC50s were obtained using Maximum Likelihood-Probit.

Table 4
Acute and chronic toxicity data for *P. promelas* from 30-d pH bioassays.

	96-h LC50 (95% CI)		30-d LC20 (95% CI)		30-d LC10 (95% CI)	
Test	[μg/L Pb]	[nmol/L Pb]	[μg/L Pb]	[nmol/L Pb]	[μg/L Pb]	[nmol/L Pb]
pH 5.5	188 (181–195)[a]	907 (874–941)[a]	N/A[b]	N/A[b]	N/A[b]	N/A[b]
pH 6.4	169 (149–192)[a]	816 (719–927)[a]	41 (37–48)	198 (179–232)	33 (29–38)	159 (140–184)
pH 7.5	293 (124–331)	1414 (598–1597)	74 (4.6–128)	357 (22–618)	44 (0–81)	212 (0–391)
pH 8.3	790 (651–1022)	3813 (3142–4932)	149 (8.5–243)	719 (41–1173)	99 (4.1–181)	478 (20–874)

[a] LC50 was obtained using Trimmed Spearman–Karber. All other LC50s were obtained using Maximum Likelihood-Probit; LC20s and LC10s were obtained using Linear Interpolation.
[b] All fish had perished prior to completion of 30-d test.

Table 6
BLM-predicted concentrations (in μg/L) for major Pb species at the 96-h LC50 for acute *P. promelas* bioassays. For $Pb(OH)_2$, $Pb(OH)_3^-$ and $PbCl_2$ values were <1 μg/L (<1%) in all cases. The relative abundances of each species expressed as the percentage of total dissolved Pb are shown in parentheses.

Test	Pb^{2+}	$PbOH^+$	$PbCO_3$	$(PbCO_3)_2^{2-}$	$PbSO_4$	$PbCl^+$	Total organic Pb
Unfed base H_2O	4.0 (2.3)	2.2 (1.3)	16 (8.8)	0.01 (0.010)	0.067 (0.038)	0.056 (0.031)	156 (88)
Fed base H_2O	32 (7.4)	13 (3.0)	126 (29)	0.084 (0.019)	0.59 (0.13)	0.53 (0.12)	267 (61)
0.5 mM $CaSO_4$	75 (10)	35 (4.7)	278 (37)	0.18 (0.025)	4.4 (0.59)	1.4 (0.18)	350 (47)
1.0 mM $CaSO_4$	121 (12)	64 (6.3)	444 (44)	0.31 (0.031)	14 (1.4)	2.2 (0.21)	370 (36)
1.5 mM $CaSO_4$ A	173 (16)	71 (6.7)	435 (41)	0.22 (0.020)	29 (2.7)	3.2 (0.30)	356 (33)
1.5 mM $CaSO_4$ B	70 (6.1)	37 (3.2)	697 (61)	1.39 (0.12)	8.7 (0.76)	1.4 (0.12)	333 (29)
2.5 mM $CaSO_4$	312 (18)	75 (4.4)	861 (50)	0.63 (0.037)	137 (7.9)	3.2 (0.19)	330 (19)
2 mg/L HA	28 (4.7)	18 (3.0)	109 (18)	0.071 (0.012)	0.62 (0.10)	0.55 (0.090)	452 (74)
4 mg/L HA	66 (6.2)	41 (3.8)	268 (25)	0.19 (0.017)	1.4 (0.13)	1.3 (0.12)	697 (65)
8 mg/L HA	49 (3.6)	27 (2.0)	159 (12)	0.088 (0.010)	1.0 (0.076)	1.0 (0.073)	1119 (83)
16 mg/L HA	74 (2.3)	50 (1.6)	425 (13)	0.42 (0.013)	1.6 (0.05)	1.5 (0.046)	2698 (83)
0.5 mM $NaHCO_3$	56 (6.9)	25 (3.1)	418 (51)	0.55 (0.067)	1.4 (0.17)	1.3 (0.16)	313 (38)
1.0 mM $NaHCO_3$	22 (2.2)	22 (2.2)	651 (65)	3.5 (0.35)	0.53 (0.053)	0.48 (0.048)	296 (30)
1.5 mM $NaHCO_3$	8.0 (1.1)	11 (1.5)	417 (59)	4.0 (0.57)	0.125 (0.018)	0.14 (0.020)	264 (38)
2.0 mM $NaHCO_3$	No LC50	No LC50	No LC50	No LC50	No LC50	No LC50	No LC50
1.5 mM NaCl	21 (5.7)	9.2 (2.5)	87 (24)	0.068 (0.018)	0.48 (0.13)	1.2 (0.33)	250 (68)
pH 5.5	55 (34)	0.26 (0.16)	0.016 (0.010)	8.1×10^{-10} (<0.01)	1.1 (0.67)	2.8 (1.7)	103 (63)
pH 6.4	41 (15)	2.0 (0.76)	0.72 (0.27)	2.2×10^{-6} (<0.01)	0.65 (0.25)	1.3 (0.50)	219 (83)
pH 7.5	27 (4.3)	21 (3.3)	271 (43)	0.48 (0.076)	0.45 (0.072)	0.41 (0.066)	304 (49)
pH 8.3	1.1 (0.33)	3.4 (0.99)	78 (23)	0.98 (0.29)	0.021 (0.010)	0.021 (0.010)	256 (75)

3.2. Acute Pb toxicity to P. promelas and C. dubia – influences of Ca^{2+}, DOC and alkalinity

Water chemistry measurements and Pb toxicity results for *P. promelas* and *C. dubia* bioassays are summarized in Tables 1–5. For the fathead minnow tests, measured Ca^{2+} concentrations were consistently lower than targeted nominal concentrations (including two attempts at 1.5 mM) due to the inherent difficulty associated with the solubility limit of $CaSO_4$ in water and therefore large stock solution volumes needed to adjust concentrations during flow-through exposures. An exception was the 2.5 mM $CaSO_4$ test for which delivery by peristaltic pump rather than Mariotte bottle helped address this issue. In any event, a range of concentrations useful for evaluation of a Ca^{2+} effect was still achieved. Ambient Ca^{2+} elicited a clear protective effect against the acute toxicity of Pb to fathead minnows, exhibiting an apparent saturation pattern (Fig. 1). Water samples from the 0.5 and 1.0 mM nominal $CaSO_4$ tests for *C. dubia* were lost prior to completion of water chemistry analysis, although Ca^{2+} was one of the parameters measured prior to loss. Nevertheless, it is clear that unlike for fathead minnows there was no appreciable effect of ambient Ca^{2+} on the acute toxicity of Pb to *C. dubia* (Fig. 1).

The results for fathead minnows and *C. dubia* with respect to Ca^{2+} suggest different and potentially complex interactions between water chemistry and the organisms. It has been shown in rainbow trout that at relatively high Pb concentrations (as used in acute fathead minnow tests) Pb enters via a Ca^{2+} channel and that competition between Ca^{2+} and Pb^{2+} for uptake at this site results in reduced Pb bioavailability (Rogers and Wood, 2004). This mechanism is supported by the Pb speciation data as ionic Pb^{2+} concentrations at the dissolved Pb LC50 increase with increasing Ca^{2+} concentrations suggesting that Pb^{2+} and Ca^{2+} are indeed competing for binding to the biotic ligand (Table 6). Conversely, this trend was not well supported for *C. dubia* (Table 7). Thus, at the lower Pb concentrations used in the *C. dubia* tests, Pb^{2+} may be entering by another mechanism, for example through a high affinity divalent metal transporter (DMT1) that has low affinity for Ca^{2+} (Gunshin et al., 1997; Bury and Grosell, 2003). As a result, competitive interactions between Ca^{2+} and Pb^{2+} are possibly minimized under these conditions and Ca^{2+} does therefore not reduce Pb toxicity. Interestingly, Komjarova and Blust (2009a,b) used a stable Pb isotope to show that Pb uptake rates in *Danio rerio* and *Daphnia magna* were both inhibited in water with 2.5 mM Ca^{2+}, but not in water with 0.5 mM Ca^{2+}. These experiments were performed at a low Pb concentration (5 μg/L dissolved Pb), comparable to exposure levels in the *C. dubia* toxicity tests, and thus suggest that the lack of Ca^{2+} protection at low Pb concentrations may not apply uniformly or that Pb accumulation and acute toxicity are not strictly linked. Additional studies to address this issue are clearly needed.

Dissolved organic carbon (added as HA) offered robust protection against acute Pb toxicity for both *C. dubia* and fathead minnows (Tables 3 and 5). However, linear regressions of the LC50s indicate that HA affords stronger protection for fathead minnows than for *C. dubia* as evident by a higher slope for the former (Fig. 2). Interestingly, in contrast to the clearly linear response of fathead minnows, the response of *C. dubia* to increased DOC may in fact be non-linear (e.g. sigmoidal) and suggests an apparent threshold concentration below which HA had no effect on Pb toxicity. The underlying mechanism for these different response relationships is unclear at this time.

Recent studies have shown that the effect of DOC on metal toxicity is not strictly the result of complexation with bioavailable metal species. Additionally, DOC interacts directly with exposed respiratory surfaces leading to alterations of transepithelial potentials in fish (Galvez et al., 2008). Specific to daphnids, it has been shown that DOC,

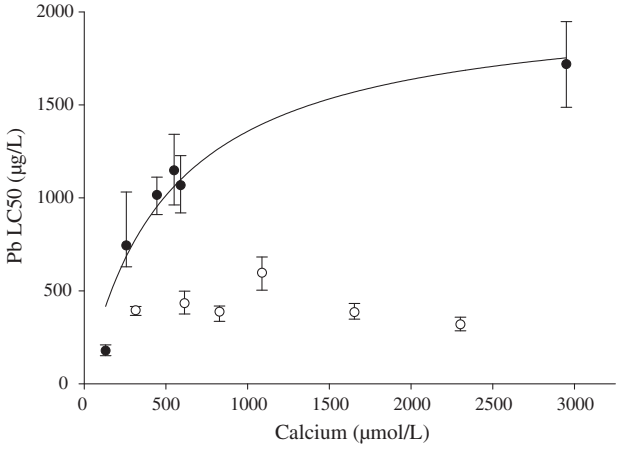

Fig. 1. Influence of calcium on acute Pb-induced mortality (LC50 ± 95% CI) from 96-h larval fathead minnow (filled circles; $y = 2060.5927 * x/(517.5896 + x)$; $r^2 = 0.9426$; $P = 0.0013$) or 48-h *C. dubia* (open circles) bioassays.

Table 7
BLM-predicted concentrations (in μg/L) for major Pb species at the 48-h LC50 for acute *C. dubia* bioassays. For $Pb(OH)_2$, $Pb(OH)_3^-$ and $PbCl_2$ values were <1 μg/L (<1%) in all cases. The relative abundances of each species expressed as the percentage of total dissolved Pb are shown in parentheses.

Test	Pb^{2+}	$PbOH^+$	$PbCO_3$	$(PbCO_3)_2^{2-}$	$PbSO_4$	$PbCl^+$	Total organic Pb
Base H_2O A	3.3 (0.84)	2.1 (0.53)	34 (8.6)	0.067 (0.017)	0.099 (0.025)	0.11 (0.028)	355 (90)
Base H_2O B	0.57 (0.15)	0.90 (0.23)	31 (7.9)	0.38 (0.098)	0.032 (0.010)	0.035 (0.010)	354 (92)
0.5 mM $CaSO_4$	3.1 (0.71)	1.3 (0.30)	21 (4.8)	0.032 (0.010)	1.2 (0.27)	0.091 (0.021)	406 (94)
1.0 mM $CaSO_4$	8.1 (1.3)	3.4 (0.57)	54 (9.0)	0.082 (0.014)	2.8 (0.47)	0.23 (0.039)	529 (89)
1.5 mM $CaSO_4$	3.5 (0.92)	1.5 (0.38)	21 (5.5)	0.031 (0.010)	0.92 (0.24)	0.095 (0.025)	358 (93)
2.0 mM $CaSO_4$	2.1 (0.66)	0.83 (0.26)	13 (4.2)	0.022 (0.010)	0.72 (0.23)	0.061 (0.019)	302 (95)
2 mg/L HA	1.5 (0.36)	1.6 (0.38)	38 (9.0)	0.18 (0.042)	0.035 (0.010)	0.043 (0.010)	383 (90)
4 mg/L HA	1.6 (0.30)	1.7 (0.31)	43 (8.0)	0.23 (0.042)	0.041 (0.010)	0.051 (0.010)	500 (91)
8 mg/L HAA	15 (2.5)	7.7 (1.3)	153 (26)	0.30 (0.050)	0.34 (0.057)	0.41 (0.069)	414 (70)
8 mg/L HA B	0.61 (0.11)	0.73 (0.14)	26 (4.9)	0.25 (0.047)	0.035 (0.010)	0.038 (0.010)	504 (95)
32 mg/L HA	0.14 (0.014)	0.30 (0.031)	11 (1.2)	0.21 (0.022)	0.010 (<0.010)	0.010 (<0.010)	952 (99)
64 mg/L HA	0.69 (0.022)	1.5 (0.047)	57 (1.8)	1.2 (0.037)	0.066 (<0.010)	0.065 (<0.010)	3056 (98)
0.5 mM $NaHCO_3$	1.2 (0.31)	1.7 (0.45)	49 (13)	0.41 (0.11)	0.051 (0.013)	0.050 (0.013)	332 (86)
1.0 mM $NaHCO_3$	2.4 (0.30)	5.8 (0.74)	250 (32)	5.6 (0.72)	0.071 (0.010)	0.10 (0.013)	516 (66)
1.5 mM $NaHCO_3$	1.5 (0.26)	2.7 (0.48)	152 (27)	3.3 (0.58)	0.051 (0.010)	0.064 (0.011)	412 (72)
2.0 mM $NaHCO_3$	1.7 (0.22)	4.6 (0.60)	322 (42)	13 (1.7)	0.038 (0.010)	0.047 (0.010)	423 (55)
1.5 mM NaCl	5.7 (1.3)	2.8 (0.62)	37 (8.4)	0.050 (0.011)	0.23 (0.051)	0.36 (0.080)	400 (90)

as HA, stimulates Na^+ uptake and that this stimulation is dependent on both ambient Ca^{2+} and pH (Glover et al., 2005; Glover and Wood, 2005). This stimulatory action is believed to be the result of the same epithelial hyperpolarization that occurs in fish, as daphnids possibly take up Na^+ via an electrogenic $2Na^+$:H^+ exchanger (Bianchini and Wood, 2008). An interaction between Pb^{2+} and Ca^{2+} might be involved because Ca^{2+} can substitute for Na^+ at this exchanger (Ahearn et al., 2001). Hence, the interactions among DOC, Ca^{2+}, and Na^+ homeostasis in daphnids is likely to be quite different from that observed in fish which lack this transporter. These differences may explain the observed discrepancies in DOC protection against Pb toxicity in fish versus daphnids, although the complexity of the interactions between these parameters remains to be fully characterized.

Alkalinity adjusted by $NaHCO_3$ addition also afforded protection against acute Pb toxicity, but as for the DOC effect, it appears that alkalinity adjustments are more potent in altering acute Pb toxicity for fathead minnows than for *C. dubia* (Tables 3 and 5). A lack of mortality prevented the calculation of an LC50 for fathead minnows from the 2.0 mM $NaHCO_3$ test (Table 3). This lack of Pb-induced mortality was a product of, on one hand, the protective effect of alkalinity (as HCO_3^-), and on the other hand, the influence of alkalinity on Pb solubility. At high total CO_2 concentrations, obtaining enough Pb in solution to cause mortality was not possible. It should be noted that alkalinity (as $NaHCO_3$) adjustments automatically result in pH changes and that alkalinity (total CO_2) and pH therefore co-vary. Furthermore, no effects of NaCl addition were observed confirming that effects from $NaHCO_3$ additions were due to HCO_3^- and associated pH changes rather than Na^+.

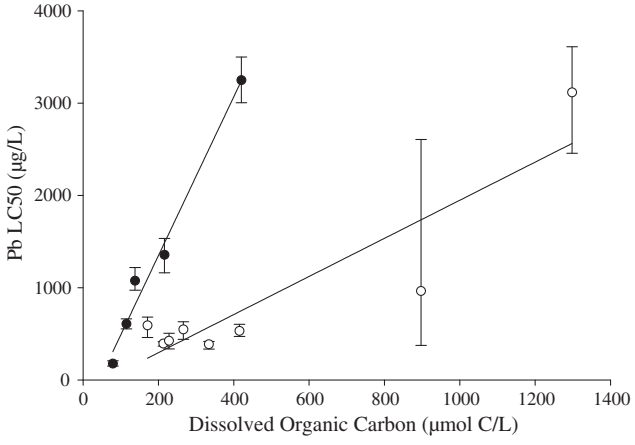

Fig. 2. Influence of DOC (as Aldrich humic acid) on acute Pb-induced mortality (LC50 ± 95% CI) from 96-h larval fathead minnow (filled circles; $y = 8.6026^*x - 372.2428$); $r^2 = 0.9819$; $P = 0.0010$) or 48-h *C. dubia* (open circles; $y = 2.0645^*x - 116.5652$); $r^2 = 0.8122$; $P = 0.0022$) bioassays.

3.3. Acute and chronic Pb toxicity to P. promelas – influence of pH

3.3.1. 96-h mortality

To more accurately assess the direct influence of pH on Pb toxicity to fathead minnows, acute and chronic toxicity tests were performed at four different pH values (5.5, 6.4, 7.5 and 8.3) using an automated titration system. Measured pH values were stable and within ~0.1 unit of our target nominal values in all cases (Table 1). Comparing total CO_2 concentrations from the pH series with those from the $NaHCO_3$ series confirms that this approach allows for a more independent analysis of pH without concurrent alkalinity adjustments owing to changes in carbonate/bicarbonate concentrations. This is well illustrated by comparing the 2.0 mM nominal $NaHCO_3$ test with the pH 8.3 acute and chronic tests. Mean total CO_2 concentration during the $NaHCO_3$ test was 1662 μM compared to 667 μM and 798 μM for the acute and chronic pH tests, respectively, despite a pH range of only 8.22–8.30 across the 3 tests (Table 1). Thus, while changes in alkalinity due to equilibration with atmospheric CO_2 could not be eliminated, the effect appeared to be minimized.

To the best of our knowledge, this is the first analysis of the influence of pH on the toxicity of Pb to fathead minnows using HCl and NaOH addition during a flow-through exposure. Schubauer-Berigan et al. (1993) used static exposures to fathead minnows performed by adjusting the pH of very hard water (300–320 mg/L $CaCO_3$) by addition of HCl and capping exposure chambers to control pH fluctuations. Their results demonstrated increased Pb toxicity at low pH (6.3), but distinct LC50s could not be obtained at pH 7.3 and 8.3 likely owing to the high alkalinity and hardness of the test waters, which can influence Pb solubility. Stouthart et al. (1994) observed a similar influence of low pH (5.6) on Pb toxicity to the egg and larval stages of common carp (*Cyprinus carpio*) using a pH-stat flow-through system. While our findings were consistent with an increase in Pb toxicity at low pH, a clear relationship between 96-h dissolved Pb LC50s and pH was not immediately apparent (Tables 3 and 4). However, a closer analysis of the speciation data for all pH tests helps to explain the observed effects. Fig. 3A illustrates a plot of 96-h ionic Pb^{2+} LC50s as a function of H^+ concentration using data from both acute and chronic pH bioassays (Tables 6 and 8). A regression analysis of the data indicates a curvilinear relationship, suggesting that the

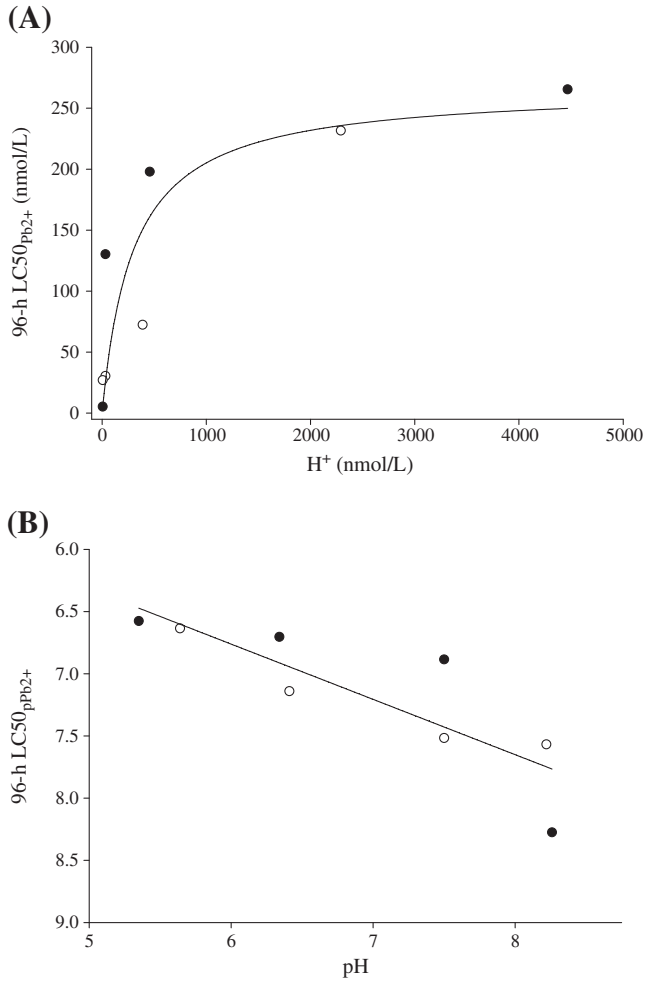

Fig. 3. Influence of pH on acute (96-h) Pb toxicity to fathead minnows. A plot of ionic Pb^{2+} LC50s as a function of H^+ concentration reveals a curvilinear relationship ($y = 266.5813^*x/(299.5607 + x)$; $r^2 = 0.7325$; $P = 0.0067$) in (A). A linear regression of 96-h LC50s (expressed as pPb^{2+}) as a function of pH yields an $r^2 = 0.7176$ ($y = 0.4445^*x + 4.0946$; $P = 0.0079$). Filled circles indicate data from acute (unfed) bioassays and open circles indicate data from chronic (fed) bioassays.

influence of pH on the toxicity of Pb to fathead minnows is not strictly a function of proton competition for binding at the gill. By performing negative log transformations, the same data can be presented alternatively as a plot of the pPb^{2+} LC50 as a function of pH (Fig. 3B). These relationships are similar to those observed with pH and nickel toxicity to rainbow trout (Deleebeeck et al., 2007). The authors of that study suggested several potential pH-induced physiological changes at the gill that may also hold relevance for Pb toxicity, including alterations in ion transport properties and membrane permeability, as well as increased mucus secretion and its effects on the chemistry of the gill microenvironment (Deleebeeck et al., 2007). In the end, while the influence of pH on Pb toxicity may be partly explained as a function of the counteracting effects of H^+ on Pb speciation and competition between H^+ and Pb^{2+} for binding at the gill, it appears that varying physiological responses to changes in pH likely also contribute to Pb sensitivity.

3.3.2. 30-d mortality and growth

Detailed mortality profiles for each of the 30-d bioassays are shown in Fig. 4. Chronic toxicity could not be assessed at pH 5.5 as complete mortality in the control treatment had occurred by day 9. This is likely because fathead minnows cannot survive low pH water for prolonged periods; an observation that is supported by a previous study which documented complete mortality within 15 d of older (and thus presumably less sensitive) fathead minnows subjected to pH 4.8 (Leino et al., 1987). Most of the mortality in the remaining three tests occurred within the first 10 d of Pb exposure with the exception of two concentrations in the pH 6.4 test. These findings were consistent with our previous studies of Pb-exposed fathead minnows (Grosell et al., 2006; Mager et al., 2010b), but contrasted with the results of another study using rainbow trout which revealed that most mortality occurred after approximately 30 d of Pb exposure, corresponding to about 10–15 days following swim-up (Mebane et al., 2008). Differences in the developmental stage at which the exposures were initiated (eggs for the rainbow trout study versus 8-d old larvae in the fathead minnow studies) and/or species-specific differences could account for the inconsistent effects.

Body masses were measured for the surviving fish at the end of 30 d revealing no discernable Pb effects on growth in all but one of the chronic pH tests. In the lone case in which Pb appeared to reduce growth (pH 8.3 at 434 μg/L), only 2 surviving fish were available for comparison with the control (Table 9). However, since the concentration at which this apparent growth inhibition occurred was well in excess of the LC20, the results indicate that chronic Pb exposure has little effect on fathead minnow growth at environmentally relevant concentrations.

4. Conclusions

We have characterized the influences of alkalinity, hardness, and DOC on the acute toxicity of Pb to fathead minnows and *C. dubia*, and the influence of pH on the acute and chronic toxicity of Pb to the fathead minnow. Our findings revealed substantial differences between these two species with respect to Pb sensitivity as well as the influences of water chemistry on the acute toxicity of Pb. Such differences are likely to reflect variations in the physiological responses of these organisms to

Table 8
BLM-predicted concentrations (in μg/L) for major Pb species at the 96-h LC50s and 30-d LC20s for chronic *P. promelas* bioassays. For $Pb(OH)_2$, $Pb(OH)_3^-$ and $PbCl_2$ values were <1 μg/L (<1%) in all cases. The relative abundances of each species expressed as the percentage of total dissolved Pb are shown in parentheses.

Test	Pb^{2+}	$PbOH^+$	$PbCO_3$	$(PbCO_3)_2^{2-}$	$PbSO_4$	$PbCl^+$	Total organic Pb
96 h LC50							
pH 5.5	48 (25)	0.27 (0.14)	0.035 (0.018)	4.8×10^{-9} (<0.01)	0.98 (0.52)	2.0 (1.1)	137 (73)
pH 6.4	15 (8.9)	0.69 (0.41)	0.63 (0.37)	4.6×10^{-6} (<0.01)	0.25 (0.15)	0.47 (0.28)	152 (90)
pH 7.5	6.3 (2.2)	3.6 (1.2)	61 (21)	0.11 (0.036)	0.12 (0.039)	0.15 (0.050)	221 (76)
pH 8.3	5.6 (0.71)	14 (1.8)	412 (52)	5.6 (0.70)	0.13 (0.016)	0.11 (0.014)	352 (45)
30 d LC20							
pH 5.5	No LC20	No LC20	No LC20	No LC20	No LC20	No LC20	No LC20
pH 6.4	1.4 (3.4)	0.065 (0.16)	0.059 (0.14)	4.3×10^{-7} (<0.01)	0.023 (0.057)	0.044 (0.11)	39 (96)
pH 7.5	0.33 (0.45)	0.19 (0.25)	3.2 (4.4)	0.010 (0.010)	0.010 (0.010)	0.010 (0.010)	71 (95)
pH 8.3	0.20 (0.13)	0.50 (0.34)	14 (9.7)	0.20 (0.13)	<0.010 (<0.010)	<0.010 (<0.010)	134 (90)

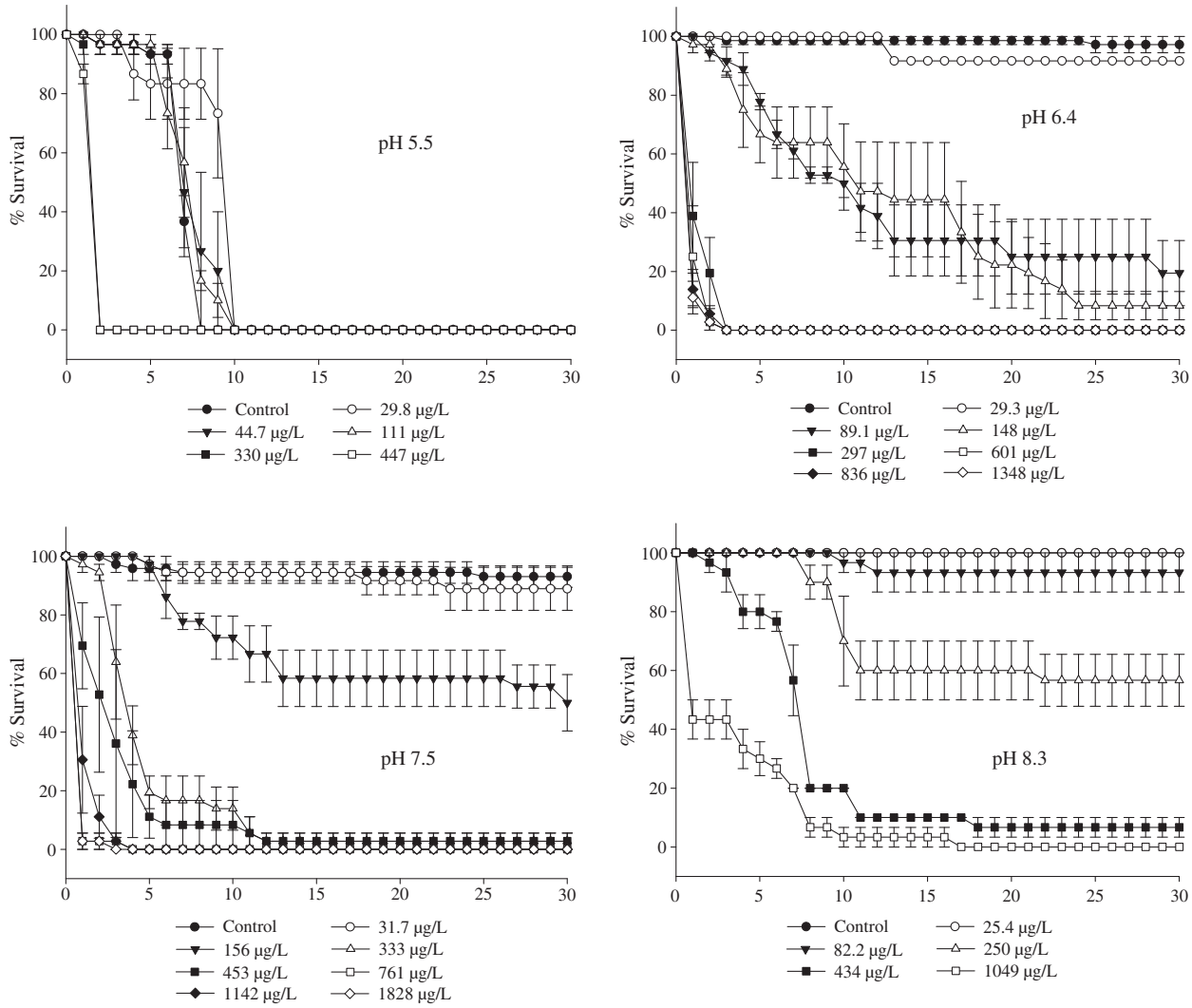

Fig. 4. Cumulative fathead minnow mortality during 30-d Pb exposures in waters of varying pH expressed as mean percent survival ± SEM.

their ambient surroundings, underscoring the notion that, in addition to the physical and chemical environment, physiology is also important to toxicity. Based on the above, it seems likely that different BLMs will be required to ensure adequate environmental protection of both species. While differences in sensitivity to metals among organisms are common and have been dealt with by calibrating BLMs to toxicity data for metals such as Cu, such an approach will likely be insufficient for Pb. Rather, since it appears that water chemistry parameters which protect fathead minnows against Pb are either less effective or without effect on *C. dubia*, particularly with respect to the role of Ca^{2+}, different BLMs (or other model predictions) taking these differences into account will need to be considered. Finally, it is clear that the influences of pH and alkalinity on the toxicity of Pb to fathead minnows are complex, and that controlling alkalinity and pH separately is not trivial, but fortunately these parameters tend to co-vary in nature. Nevertheless, the findings reported herein should help elucidate the respective significance of each, as well as those for other key parameters such as DOC and Ca^{2+}, for the purposes of BLM parameterization.

Table 9
Whole body mass data (wet weight in mg) for surviving *P. promelas* from chronic Pb bioassays (mean ± SEM).

Test	Pb (µg/L)	Mass (*n*)
pH 6.4	Control	75.2 ± 6.0 (35)
	29.3 ± 2.7	98.2 ± 4.3 (34)
	89.1 ± 8.2	94.5 ± 5.2 (34)
	148 ± 13	86.5 ± 13 (7)
	297 ± 27	57.2 ± 20 (3)
pH 7.5	Control	70.2 ± 5.8 (34)
	31.7 ± 4.2	86.1 ± 4.6 (34)
	156 ± 19	93.5 ± 4.8 (32)
	333 ± 22	104 ± 7 (18)
	453 ± 64	134.1 (1)
pH 8.3	Control	94.0 ± 3.7 (30)
	25.4 ± 1.9	83.3 ± 5.0 (30)
	82.2 ± 3.8	86.5 ± 4.5 (27)
	250 ± 20	88.4 ± 6.0 (17)
	434 ± 14	58.2 ± 16[a] (2)

[a] Significantly different from corresponding control as determined by Bonferroni *t*-test ($P \leq 0.05$).

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