Ecotoxicology and Environmental Safety 74 (2011) 238–243

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Effects of water chemistry on the chronic toxicity of lead to the cladoceran, *Ceriodaphnia dubia*

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ARTICLE INFO

Article history:
Received 22 March 2010
Received in revised form
5 November 2010
Accepted 21 November 2010
Available online 8 December 2010

Keywords:
Biotic ligand model
Pb
Alkalinity
Dissolved organic matter
Hardness
Water quality

ABSTRACT

As the first step toward parameterization of a chronic lead (Pb) biotic ligand model (BLM) for *Ceriodaphnia dubia*, 7-d toxicity tests were performed in waters modified to evaluate the influences of hardness, DOM (as Suwannee River NOM and Aldrich humic acid (HA)), pH (buffered with 4 mM MOPS) and alkalinity on the chronic toxicity of Pb. Calculated EC_{20}s for the control base water test and each of the most extreme modified test waters were as follows in $\mu g\ L^{-1}$ Pb (95% confidence interval): base water control=45 (14–53), 5 mM $CaSO_4$=22 (12–30), 32 mg L^{-1} DOM=523 (388–573), 2.5 mM $NaHCO_3$=73 (21–120) and pH 6.4 buffered with MOPS=3.9 $\mu g\ L^{-1}$ Pb (1–5). Results indicate that hardness does not protect against chronic toxicity of Pb to *C. dubia*, whereas HA does protect at the highest concentration tested (597 μM). Additionally, our findings suggest that low pH increases the chronic toxicity of Pb whereas increased alkalinity is protective. The findings reported herein support the need for a chronic Pb BLM as an alternative approach to hardness-based regulations.

1. Introduction

Lead (Pb) is a non-essential metal that persists as a major pollutant of concern world-wide despite significant reductions in its use as a fuel and paint additive (World Health Organization, 1995). Presently, the main routes of entry for Pb to the aquatic environment are largely anthropogenic due to Pb mining and various industrial processes, although natural sources also contribute (e.g. volcanic activity, erosion of Pb ores). Under the current regulatory frameworks of North America and elsewhere the amount of Pb permitted for a local receiving water, as with other metals, can typically be modified based on the water's hardness (ANZECC, 2000; CCME, 2008; USEPA, 1985). Yet, recent evidence indicates that hardness may not be important for chronic Pb exposures and that other parameters (e.g. dissolved organic matter (DOM)) may exert greater influence on toxicity (Macdonald et al., 2002; Grosell et al., 2006; Mager et al., 2008). Thus a re-evaluation as to how site-specific regulations for Pb are adjusted seems warranted.

Although regulatory agencies such as the USEPA have long recognized the importance of water chemistry in mediating metal bioavailability and toxicity (USEPA, 1985), it was not until the advent of the biotic ligand model (BLM) that a tool became available for adequately assessing a wide range of the major parameters that can influence metal bioavailability and toxicity (Paquin et al., 2002). The most bioavailable and toxic form of a metal is assumed to be the free metal ion, although other species (e.g. hydroxides) may also be incorporated into the model if deemed appropriate. In assessing a metal's toxicity, the BLM considers both the mitigating effects of competition with other cations (e.g. Ca^{2+}, Na^{+}) and protons for binding to the biotic ligand and complexation of the metal with free anionic species (e.g. HCO_3^-) and DOM. The USEPA recently incorporated the BLM into the Framework for Metal Risk Assessment (USEPA, 2007a), a clear endorsement of the utility of the model, and issued the first criteria document employing the BLM in 2007 for copper (Cu) (USEPA, 2007b). Additionally, the BLM has been used to conduct regional risk assessments in Europe for several metals including Cu and zinc (Zn) (Bodar et al., 2005).

The cladoceran, *Ceriodaphnia dubia*, is one of several daphnid test species commonly used for biomonitoring and is typically included among analyses of species sensitivity distributions used in establishing water quality criteria (WQC). This animal is particularly well suited for assessment of chronic toxicity due to its ease of culture within the lab and short (7 d) test for survival and reproductive impairment. While recent studies have been directed toward developing chronic daphnid BLMs for Cu (De Schamphelaere and Janssen, 2004; Schwartz and Vigneault, 2007), nickel (Ni) (Keithly et al., 2004)

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doi:10.1016/j.ecoenv.2010.11.005

and Zn (Heijerick et al., 2005), similar efforts for Pb have not been reported. Previous investigations have examined the chronic toxicity of Pb to *C. dubia,* both in isolation and in combination with other metals (Spehar and Fiandt, 1986; Cooper et al., 2009), but the influences of water chemistry in this regard have not been systematically examined. As such, a comprehensive data set for parameterization of a chronic Pb BLM for *C. dubia* is currently unavailable. The purpose of this study was to investigate the chronic toxicity of Pb to *C. dubia* in test waters modified for key water chemistry parameters known to commonly influence metal toxicity, namely hardness, DOM, pH and alkalinity.

2. Materials and Methods

2.1. Experimental animals

A starter culture of *Ceriodaphnia dubia* was obtained from Aquatic BioSystems, Inc. (Fort Collins, CO) and gradually acclimated to new culture media over 2–3 d. This media consisted of dechlorinated Virginia Key tap water supplemented with 1 $\mu g\,L^{-1}$ Se (as Na_2SeO_4). Selenium was added to ensure adequate nutrition, as previous studies have revealed that Se is an essential nutrient for daphnids (Keating and Dagbusan, 1984; Winner, 1984). Stock culture media was prepared and stored in a 20 L carboy that was continuously aerated with an air stone. Mass cultures were maintained in 1 L glass dishes (without aeration) with renewal of culture media from the aerated carboy stock 3 times a week and fed a daily diet of 5 mL L^{-1} each of a yeast, Cerophyll®, trout chow mixture (YCT) and algae (*Pseudokirchneriella subcapitata*). All cultures and toxicity tests were carried out in a controlled environmental chamber at 25 °C with a photoperiod of 16 h light and 8 h darkness.

2.2. 7-d survival and reproduction tests

Chronic toxicity tests were performed following static-renewal procedures in accordance with standard USEPA guidelines (USEPA, 2002) to determine the effects of hardness (as $CaSO_4$ and $MgSO_4$), alkalinity (as $NaHCO_3$), pH and DOM. Several concentrations for each parameter were selected to span ranges encompassing concentrations typically found in nature, with the exception of $MgSO_4$ which was tested at a single concentration (1.2 mM). Two sources of DOM were used: Aldrich humic acid (HA), a commonly used commercial source, as well as a naturally derived source in the form of Suwannee River natural organic matter (NOM). The influence of DOM was evaluated by measuring the concentration of dissolved organic carbon (DOC), a surrogate measure of the amount of DOM present. For the pH tests, HCl and NaOH were used to adjust pH to 7.2 and 6.4, and solutions were buffered by adding 3-*N* morpholino propane sulfonic acid (MOPS) to a final concentration of 4 mM. Additional tests with Na_2SO_4, K_2SO_4 and NaCl were performed to account for any influences of the Na^+ and SO_4^- conjugate ions. After completion of all tests it was observed that the base water control test pH was high (pH 7.9; data not shown) compared to all other tests where pH should not have been impacted by targeted modification (pH 7.1–7.4). Thus, to eliminate pH as a potential confounding variable for direct comparison with the other tests we repeated the base water control test by titrating pH to a more representative value of 7.2 with trace-metal grade HCl (Fisher Scientific, Pittsburgh, PA) and NaOH (Sigma-Aldrich, St. Louis, MO).

Concentrations for each of the parameters indicated in Table 1 were achieved by modifying a base water of 2:1 dechlorinated Miami-Dade tap water:deionized water supplemented with 1 $\mu g\,L^{-1}$ Se at least 48 h in advance of testing. Due to the low ionic strength of this base water, separate mass cultures were acclimated to test media (without Pb) for 5–7 d prior to test initiation. Isolated adults were cultured in 30 mL medicine cups containing 15 mL test media with daily water renewals and addition of 0.1 mL each of YCT and algae until a sufficient number of <24-h-old neonates were obtained for testing. Fresh solutions for water renewals were prepared each day by spiking 200 mL aliquots (except controls) with a $PbNO_3$ stock solution over a range of 5–6 Pb concentrations and allowed to equilibrate overnight for use the following day. Algae and YCT (1.33 mL each) were added to each of the equilibrated test media 1 h before water renewals. Exposures were initiated by placing individual neonates into each of 10 cups per Pb concentration containing 15 mL of test media and then monitored daily over 7 d for adult survival and reproductive output (total number of neonates produced per female). All chemicals used to modify test conditions were obtained from Sigma-Aldrich (St. Louis, MO) except for those with NOM (International Humic Substances Society, St. Paul, MN).

2.3. Water chemistry

Dissolved oxygen, temperature and pH were monitored daily in each test. Dissolved Pb concentrations were measured in triplicate at a minimum of 3 different time points including days 1 (initial) and 7 (final). Concentrations of DOC and major ions were measured from a single batch of water that was used for the entire duration of each test. Water samples were passed through a 0.45 μm cellulose syringe filter (Pall LifeSciences, MI) and acidified to 1% trace-metal grade HNO_3 (Fisher Scientific, Pittsburgh, PA) prior to measurement of dissolved Pb concentrations by atomic absorption spectroscopy (Varian 200Z graphite furnace, Australia). Concentrations of Na^+, K^+, Ca^{2+} and Mg^{2+} were measured by flame atomic absorption spectroscopy (Varian 220FS, Australia) and Cl^- and SO_4^{2-} concentrations by anion chromatography (DIONEX DX120, CA). Dissolved oxygen was measured using a handheld dissolved oxygen meter (WTW Oxi 340i, Weilheim, Germany). Measurements of pH were obtained using a PHM201 meter fitted with a combination glass electrode (Radiometer, Copenhagen, Denmark) and total CO_2 (a measure of the total dissolved inorganic carbon) was determined using a Corning 962 carbon dioxide analyzer (UK). Water samples for DOM analysis were passed through a glass microfiber filter (Whatman, Piscataway, NJ) syringe assembly and DOC concentrations measured using a Shimadzu total organic carbon-VCSH analyzer (Kyoto, Japan) (Hansell and Carlson, 2001).

Table 1
Water quality and toxicity data for chronic *Ceriodaphnia dubia* tests. All water chemistry data represent mean values.

Test	Concentration ($\mu mol\,L^{-1}$)								Hardness ($mg\,L^{-1}$)	Ionic strength ($mmol\,L^{-1}$)	pH	Temp. (°C)	EC20 [$\mu g\,Pb\,L^{-1}$] (95% CI)	EC50 [$\mu g\,Pb\,L^{-1}$] (95% CI)
	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	SO_4^{2-}	Total CO_2	DOC						
Base water	721	54	236	110	954	73	122	182	35	6.57	7.2	25	45 (14–53)	64 (54–74)
0.5 mM $CaSO_4$	378	27	463	49	464	351	400	100	52	6.47	7.6	25	46 (13–51)	66 (53–70)
1.6 mM $CaSO_4$	385	30	1623	55	459	1147	400	100	172	9.87	7.3	25	17 (17–18)	30 (27–32)
3.5 mM $CaSO_4$	523	37	3465	68	742	2790	400	100	362	15.51	7.5	25	12 (11–12)	17 (16–17)
5.0 mM $CaSO_4$	559	34	5048	60	529	4878	400	100	524	20.49	7.5	25	22 (12–30)	40 (34–45)
2 mg L^{-1} NOM	472	27	239	57	411	22	400	210	30	6.62	7.5	25	71 (66–75)	99 (89–111)
4 mg L^{-1} NOM	496	37	234	60	507	61	400	450	30	9.51	7.4	25	176 (21–201)	253 (212–280)
2 mg L^{-1} HA	347	25	176	43	370	48	400	170	22	5.78	7.4	25	96 (41–128)	150 (120–169)
4 mg L^{-1} HA	397	26	207	51	372	38	400	210	26	6.31	7.3	25	85 (56–129)	242 (172–337)
8 mg L^{-1} HA	469	26	188	49	421	47	400	410	24	8.76	7.4	25	53 (42–106)	198 (130–248)
32 mg L^{-1} HA	881	56	251	114	1070	83	235	597	37	11.83	7.3	25	523 (388–573)	685 (647–717)
pH 6.4	1079	29	171	49	433	44	400	NM	22	4.08	6.4	25	3.9 (1.0–5.2)	7.9 (6.7–8.7)
pH 7.2	2827	31	176	55	NM	NM	400	NM	24	6.16	7.3	25	5.1 (3.1–6.4)	7.6 (6.8–8.4)
1 mM $MgSO_4$	361	24	185	1161	366	1011	400	100	135	8.96	7.4	25	26 (15–37)	45 (29–52)
0.5 mM K_2SO4	411	1180	206	52	417	566	400	100	26	6.35	7.1	25	21 (6–24)	31 (27–32)
1.5 mM NaCl	1650	28	185	47	1427	45	400	100	24	5.97	7.2	25	19 (10–23)	27 (23–36)
0.7 mM $NaHCO_3$	711	25	197	51	444	51	700	100	25	5.63	7.7	25	40 (31–58)	70 (46–82)
1.9 mM $NaHCO_3$	1781	28	200	50	468	52	1900	100	26	6.86	7.9	25	69 (49–85)	110 (90–153)
2.5 mM $NaHCO_3$	2441	30	206	51	574	57	2500	100	26	7.68	8.2	25	73 (21–120)	161 (131–191)
0.5 mM Na_2SO_4	1623	27	181	47	374	557	400	100	23	6.45	7.3	25	16 (11–19)	24 (20–31)

Abbreviations: HA=humic acid; NM=not measured; NOM=natural organic matter.
Notes: Total CO_2 and DOC values of 400 and 100, respectively, represent a single measurement on a large batch of base water. All other DOC and total CO_2 values represent measurements for individual tests. DOC was not measured in the two pH/MOPS treatments as MOPS contaminates the DOC measurements (contains organic carbon). Cl^- and SO_4^{2-} were not measured in the pH 7.2 test due to loss of sample.

2.4. Calculations and data analysis

Toxicity data for *C. dubia* were analyzed by the Linear Interpolation Method (USEPA, 2002) to estimate effect levels for each test using mean measured dissolved Pb concentrations and the statistical software ToxCalc 5.0 (Tidepool Scientific Software). Reproduction (as number of offspring per initial female) was more sensitive than survival in all tests and subsequent analysis focused on this biological endpoint. Regression analyses were used to investigate relationships between ambient water chemistry and toxicity of Pb using SigmaPlot 11.0 (Systat Software, Inc.). Lead speciation estimates were calculated using the BLM (HydroQual, Inc., Version 2.3.3). It should be noted that the influence of the DOM (as DOC) entered as an input is actively used by the BLM, with a percentage specified as HA, and the remaining amount assumed to behave as fulvic acid. A DOM composition of 10% HA was used for speciation calculations in all cases except for tests in which HA was added. This value represents the typical percentage of HA present in natural DOM (Thurman, 1985) and is used as the default background value for the BLM (Santore et al., 2001). For the tests modified by addition of HA, the %HA input values were estimated proportionally relative to the base water control by assuming that all additional DOM was attributable to 100% HA. All other input parameters were based on measured values for the respective tests, except when unavailable, in which cases measured control values were used.

3. Results

3.1. Influences of water chemistry on Pb speciation

Concentrations and relative abundances of the major dissolved Pb species were calculated at the estimated EC_{20}s for each of the test conditions in Table 1 using the BLM (Table 2). As expected, greater Pb–DOC and Pb–carbonate complexation was observed with increasing DOC and $NaHCO_3$ concentrations, respectively, while increasing Ca^{2+} concentrations mildly increased ionic Pb^{2+} concentrations associated with dissolved Pb EC_{20}s, likely due in part to Ca^{2+} displacing Pb^{2+} from DOC. Nevertheless, overall ionic Pb^{2+} concentrations at the EC_{20}s were within a similar range across all tests. Hence, speciation estimates by the BLM performed well at normalizing the effects of water chemistry. In fact, the coefficient of variation (CV) in EC_{20}s across all tests based on dissolved Pb was 161%, but only 52% based on ionic Pb^{2+}. Performing the same evaluation based on Pb^{2+} and $PbOH^+$ combined results in a CV of 72%, suggesting $PbOH^+$ is not contributing significantly to toxicity. Given the CV for the test method (determined using reference toxicants and standardized effluents) has been estimated at 35–67% (Ausley, 1997; USEPA, 2002) it would appear that ionic Pb^{2+} explains the vast majority of variation in response across all tests beyond that inherent to the test method.

3.2. Influences of water chemistry on the chronic toxicity of Pb to C. dubia.

The main objective of this study was to investigate the influences of hardness, DOC, pH and alkalinity on the chronic toxicity of Pb to *C. dubia*. Control tests designed to assess the influences of the conjugate Na^+ and SO_4^- ions (i.e. Na_2SO_4, K_2SO_4 and NaCl) from the salts used in the hardness and alkalinity tests revealed lower Pb effect levels than obtained with the base water control (Table 1). However, the greater toxicity observed in these tests is contrary to an expected effect of protection, or no effect, with the addition of potentially competing cations (i.e. Na^+ and K^+), and is likely explained by the nearly twofold decrease in DOM (measured as DOC) when compared to the base water control. We therefore chose to include the mean EC_{20} or EC_{50} from these 3 tests (18.7 and 27.3 μg Pb L^{-1}, respectively) as an additional control in Figs. 1 and 2, primarily to avoid introducing disproportionate weight in the regression analyses by using effect levels from each test individually.

As adjustments to WQC are currently based on water hardness, the influences of Ca^{2+} (as $CaSO_4$) and Mg^{2+} (as $MgSO_4$) on the chronic toxicity of Pb to *C. dubia* were examined. Importantly, results from both $CaSO_4$ and $MgSO_4$ bioassays failed to reveal a protective influence of water hardness when compared to the base water control. In fact, estimated dissolved Pb EC_{50}s indicate an increase in the toxicity of Pb at elevated Ca^{2+} and Mg^{2+} concentrations, with greatest toxicity from the $CaSO_4$ bioassays evident at the 3.5 mM concentration (Table 1). However, if the conjugate ion control tests (i.e. Na_2SO_4, K_2SO_4 and NaCl) are used as a basis for comparison, the effect levels from the $MgSO_4$ test shared overlapping CIs with each of these tests, suggesting no effect of Mg^{2+} on the chronic toxicity of Pb to *C. dubia*. On the other hand, an increase in toxicity due to Ca^{2+} was still evident at the 3.5 mM Ca^{2+} concentration, whereas a concentration of 0.5 mM Ca^{2+} was protective (as evaluated by EC_{50}s), when compared to the conjugate ion control tests.

The parameter demonstrating the greatest protection against the toxicity of Pb was DOM (as evaluated by measured DOC concentrations), although from a regression of EC_{20}s this effect appeared relatively minor at concentrations < 450 μM DOC (Fig. 1A). However, a regression of EC_{50}s revealed a linear protective effect of DOM (Fig. 1B). We were only able to increase Suwannee

Table 2
BLM-predicted concentrations (in μg L^{-1}) for major Pb species within each test media at the calculated dissolved Pb EC_{20}. Relative abundances, expressed as % of total dissolved Pb, are provided in parentheses. Speciation calculations for $Pb(OH)_2$, $Pb(OH)_3^-$, $PbCl^+$ and $PbCl_2$ are not listed (each represented $< 0.05\%$).

	Pb^{2+}	PbOH	$PbCO_3$	$Pb(CO_3)_2$	$PbSO_4$	Total organic Pb
Base water	0.16 (0.37)	0.044 (0.098)	0.16 (0.37)	3.0×10^{-5} (6.6×10^{-5})	0.0045 (0.010)	45 (99.2)
0.5 mM $CaSO_4$	0.19 (0.42)	0.13 (0.28)	1.7 (3.6)	0.0027 (0.0059)	0.024 (0.052)	44 (95.6)
1.6 mM $CaSO_4$	0.17 (1.0)	0.053 (0.31)	0.62 (3.6)	5.1×10^{-4} (0.0030)	0.052 (0.31)	16 (94.7)
3.5 mM $CaSO_4$	0.12 (0.97)	0.053 (0.44)	0.59 (4.9)	8.6×10^{-4} (0.0072)	0.064 (0.53)	11 (93.1)
5.0 mM $CaSO_4$	0.27 (1.2)	0.12 (0.54)	1.3 (5.8)	0.0019 (0.0087)	0.22 (1.0)	20 (91.4)
2 mg L^{-1} NOM	0.12 (0.17)	0.066 (0.092)	0.87 (1.2)	0.0011 (0.0015)	0.0011 (0.0015)	70 (98.5)
4 mg L^{-1} NOM	0.18 (0.10)	0.079 (0.045)	1.0 (0.59)	0.0010 (5.7×10^{-4})	0.0044 (0.0025)	175 (99.3)
2 mg L^{-1} HA	0.089 (0.091)	0.038 (0.039)	0.50 (0.52)	4.8×10^{-4} (5.0×10^{-4})	0.0017 (0.0018)	95 (99.4)
4 mg L^{-1} HA	0.057 (0.067)	0.020 (0.023)	0.25 (0.29)	1.9×10^{-4} (2.2×10^{-4})	8.7×10^{-4} (0.0010)	85 (99.6)
8 mg L^{-1} HA	0.0086 (0.016)	0.0037 (0.0070)	0.049 (0.092)	4.7×10^{-5} (8.8×10^{-5})	1.6×10^{-4} (3.1×10^{-4})	53 (99.9)
32 mg L^{-1} HA	0.17 (0.032)	0.056 (0.011)	0.41 (0.079)	1.9×10^{-4} (3.6×10^{-5})	0.0051 (0.0010)	522 (99.9)
pH 6.4	0.099 (2.5)	0.0042 (0.11)	0.032 (0.82)	1.8×10^{-6} (4.7×10^{-5})	0.0017 (0.044)	3.8 (96.5)
pH 7.2	0.019 (0.37)	0.0063 (0.12)	0.078 (1.5)	6.1×10^{-5} (0.0012)	3.0×10^{-4} (0.0059)	5.0 (98.0)
1 mM $MgSO_4$	0.19 (0.74)	0.076 (0.29)	0.91 (3.5)	9.6×10^{-4} (0.0037)	0.054 (0.21)	25 (95.3)
0.5 mM K_2SO_4	0.16 (0.76)	0.033 (0.16)	0.38 (1.8)	1.8×10^{-4} (8.6×10^{-4})	0.031 (0.15)	20 (97.1)
1.5 mM NaCl	0.11 (0.56)	0.028 (0.15)	0.34 (1.8)	2.1×10^{-4} (0.0011)	0.0018 (0.0092)	19 (97.5)
0.7 mM $NaHCO_3$	0.093 (0.23)	0.080 (0.20)	1.87 (4.7)	0.0066 (0.017)	0.0018 (0.0046)	38 (94.9)
1.9 mM $NaHCO_3$	0.13 (0.19)	0.17 (0.24)	11 (15)	0.17 (0.25)	0.0024 (0.0034)	58 (84.0)
2.5 mM $NaHCO_3$	0.070 (0.10)	0.18 (0.24)	15 (20)	0.63 (0.86)	0.0013 (0.0018)	58 (78.8)
0.5 mM Na_2SO_4	0.071 (0.44)	0.023 (0.14)	0.29 (1.8)	2.2×10^{-4} (0.0014)	0.014 (0.087)	16 (97.5)

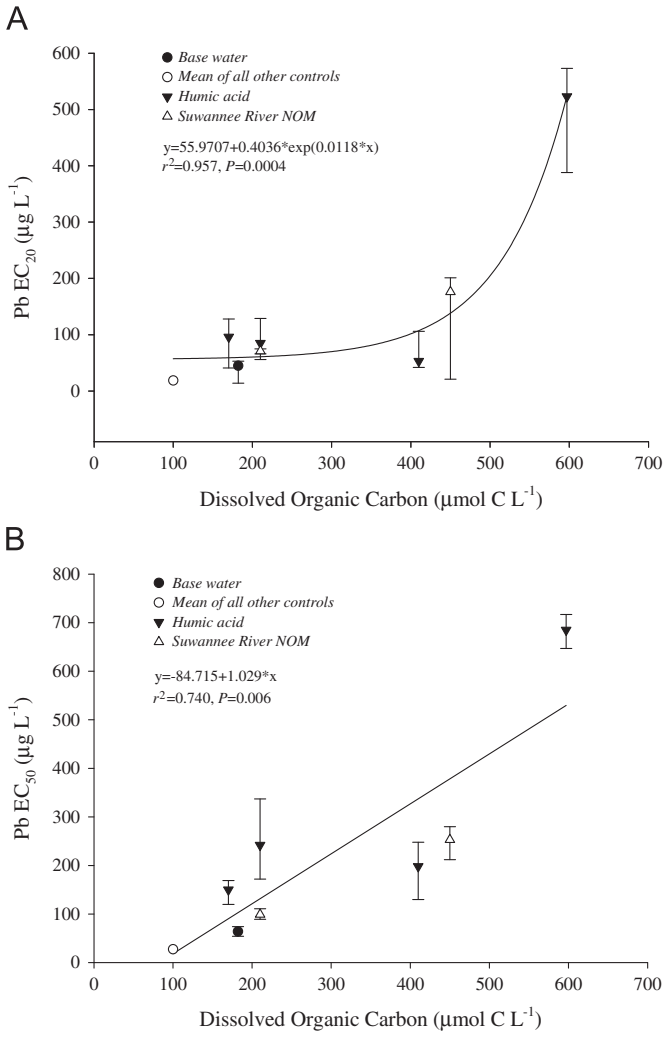

Fig. 1. The effect of DOC (as Aldrich humic acid and Suwannee River NOM) on the chronic toxicity of Pb to *Ceriodaphnia dubia* expressed as the dissolved Pb concentration resulting in (A) 20% reduced reproductive output over 7 d (EC_{20}) and (B) 50% reduced reproductive output over 7 d (EC_{50}). Error bars represent 95% confidence intervals. The means of all other controls were calculated from the Na_2SO_4, K_2SO_4 and NaCl tests.

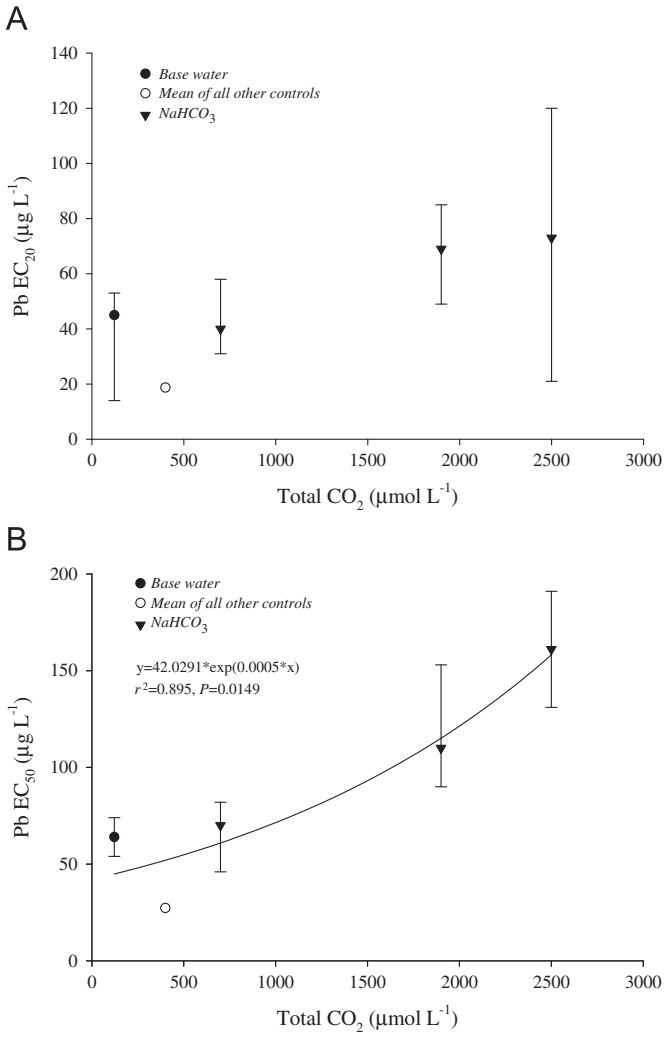

Fig. 2. The effect of alkalinity on the chronic toxicity of Pb to *Ceriodaphnia dubia* expressed as the dissolved Pb concentration resulting in (A) 20% reduced reproductive output over 7 d (EC_{20}) and (B) 50% reduced reproductive output over 7 d (EC_{50}). Error bars represent 95% confidence intervals. The means of all other controls were calculated from the Na_2SO_4, K_2SO_4 and NaCl tests.

River NOM to 450 µM (4 mg L^{-1} nominal) as higher concentrations resulted in direct toxicity to *C. dubia*. This is perhaps not surprising given that addition of NOM to the already low ionic strength base water will cause a further reduction in ionic strength via complexation and thus an ionoregulatory stress on the animals. Such an effect is supported by a previous study of the acute toxicity of Pb in the presence of NOM to rainbow trout (Schwartz et al., 2004). For this reason we used HA as an alternative source of DOC. Tests with HA produced comparable results to that obtained with Suwannee River NOM over the same concentration range.

The influence of alkalinity (modified by $NaHCO_3$) on the chronic toxicity of Pb was also examined. Not surprisingly, pH increased with addition of $NaHCO_3$ culminating in the highest pH of any test at pH 8.2 in the 2.5 mM $NaHCO_3$ treatment (Table 1). A significant relationship could not be established using all of the estimated EC_{20}s (Fig. 2). However, if the base water control is removed from the dataset, and only the mean of all other controls (18.7 µg Pb L^{-1}) is used as a basis for comparison, a Michaelis–Menten type relationship ($r^2 = 0.99$, $P < 0.01$) is observed suggesting bicarbonate has a strong but saturable effect on the chronic toxicity of Pb (data not shown). A regression of EC_{50}s, on the other hand, revealed a curvilinear protective effect of alkalinity (Fig. 2).

To evaluate the influence of low pH on the toxicity of Pb, a bioassay was performed at a pH of 6.4 achieved by titration with HCl and NaOH and maintained by addition of MOPS buffer. Results indicated that toxicity of Pb was increased at pH 6.4 as evident by the lower EC_{20} compared to the base water control as well as the mean of all other controls (18.7 µg L^{-1} Pb; Table 1). An additional test was included at pH 7.2 to control for the combined effects of MOPS and Pb on *C. dubia* toxicity. This test resulted in an EC_{20} that was significantly lower than any other control test (Table 1) indicating increased Pb sensitivity imparted by the MOPS buffer.

4. Discussion

Results from a recent study with *D. magna* indicate that the vast majority of Ca^{2+} uptake by daphnids (97–100%) is from the water (Tan and Wang, 2009). Hence, if Pb (or other metals suspected to compete with Ca^{2+}) is in fact taken up via the Ca^{2+} pathway(s) in daphnids, it would be reasonable to expect that toxicity should decrease with increasing ambient Ca^{2+} concentrations owing to an increase in competitive Ca^{2+}–Pb^{2+} interactions at the biotic ligand. However, our results do not support a shared uptake route for Ca^{2+} and Pb^{2+} as progressive increases in Ca^{2+} concentration failed to

elicit a corresponding decrease in the toxicity of Pb. These findings are consistent with the observed lack of protection by Ca^{2+} against acute toxicity of Pb to *C. dubia* (Mager et al., 2011). The present results therefore provide further support for the notion that at relatively low Pb concentrations (as used in both the present and previous acute toxicity study) entry of Pb^{2+} may occur via a channel or transporter with low affinity for Ca^{2+} (e.g. divalent metal transporter 1 (DMT1)), and therefore little protection is afforded by competition with Ca^{2+} under these conditions (Mager et al., 2011).

While the observed lack of a clear protective effect of Ca^{2+} (or Mg^{2+}) against acute and chronic toxicity of Pb to *C. dubia* is somewhat alarming given that WQC for Pb are currently hardness based, it is not the first example to this effect. In fact, a similar lack of Ca^{2+} protection has been observed during chronic Ni exposures to *C. dubia* (Keithly et al., 2004) and chronic Cu exposures to *Daphnia magna* (De Schamphelaere and Janssen, 2004). Zinc, on the other hand, appears to differ in this regard, as studies have shown that acute and chronic toxicity of Zn to *D. magna* is reduced with increasing hardness concentrations up to 250 mg L^{-1} as $CaCO_3$ (Heijerick et al., 2002, 2005). This difference may be due to the higher concentrations of Zn needed to elicit toxicity compared with Cu, Ni and Pb. At these higher concentrations, the majority of Zn may be moving through a Ca^{2+} channel (and therefore subject to competitive interaction with Ca^{2+}) rather than a low capacity metal transporter such as DMT1.

Consistent with our present results with *C. dubia*, our lab recently demonstrated that Ca^{2+} was also ineffective at protecting against chronic Pb accumulation and Pb-induced gene transcriptional responses in fathead minnows (Mager et al., 2008). Thus, there appears to be mounting evidence that a hardness-based WQC may be inadequate for protecting aquatic life against Pb exposures, as well as potentially other metals, particularly in light of the influences of other water chemistry parameters as reported herein.

In contrast to water hardness, a clear protective effect against chronic toxicity of Pb to *C. dubia* was afforded by DOM (Fig. 1). A regression of EC_{50}s collected from the present study (Fig. 1B) is in agreement with previous investigations of acute toxicity of Pb to *C. dubia* and fathead minnows (Mager et al., 2011) and chronic toxicity of Pb to fathead minnows (Grosell et al., 2006), both of which demonstrated a linear protective effect with increasing DOM (as DOC). Linear protective effects of DOM against the chronic toxicity of Cu and Zn to *D. magna* have also been described (Heijerick et al., 2003; De Schamphelaere and Janssen, 2004). However, in the case of *C. dubia*, results from both acute (Mager et al., 2011) and chronic Pb exposures suggest a potential threshold of DOM concentration below which minimal protection is afforded. Hence, the toxic response of *C. dubia* to Pb in the presence of increasing DOM may in fact be non-linear, a relationship that is more clearly borne out by the curvilinear regression of EC_{20}s (Fig. 1A). Overall, these results demonstrate a protective influence of elevated DOM against chronic toxicity of Pb, although it is important to recognize that protection is minimal, or even absent, at relatively low levels of DOM.

Alkalinity also proved protective against chronic toxicity of Pb to *C. dubia*, perhaps not surprising given that Pb is strongly complexed by carbonate and hydroxide species to form presumably non-bioavailable forms of the metal. However, a clear interpretation of the data is somewhat complicated by the base water control test having a slightly higher DOC concentration (182 μM) as compared to the conjugate ion controls and the $NaHCO_3$ test series (100 μM), which may explain the apparent weak effect of $NaHCO_3$ at the EC_{20} when compared to the base water control (Fig. 2A). Yet, if the estimated EC_{50}s are regressed using both the base water control and the mean of all other controls a curvilinear protective effect of $NaHCO_3$ is observed (Fig. 2B). Such evidence in support of a protective effect of increased alkalinity against chronic toxicity of Pb to *C. dubia* is in agreement with a previous study by Chapman et al. (1980) using *D. magna*, although a direct comparison is somewhat difficult as the parameters of water hardness and alkalinity co-varied in the Chapman study. Nevertheless, a nearly tenfold increase in protection against chronic toxicity of Pb was observed over an approximately threefold increase in hardness and alkalinity (Chapman et al., 1980). Given the lack of protection by Ca^{2+} and Mg^{2+} in the present study, these results are likely attributable in large part to the influence of alkalinity. Thus, taking into account the confounding effect of increased DOC in the base water test of the present study, the evidence to date indicates that alkalinity is important in mitigating the chronic toxicity of Pb to daphnids.

De Schamphelaere et al. (2004) have previously shown that Cu and Zn bioavailability to *D. magna* is not affected by MOPS buffer and at concentrations up to 750 mg L^{-1} (~4 mM) does not alter 21-d reproduction in this cladoceran species. However, our results suggest a potential problem with using this buffer for *C. dubia* bioassays as the daphnids from the control pH 7.2 MOPS tests demonstrated elevated Pb sensitivity. This potential MOPS effect is supported by a previous report of fathead minnows exposed to Pb in MOPS-buffered water adjusted to pH 8.3 (Grosell et al., 2006). Increased toxicity of Pb was observed in the pH 8.3-MOPS test compared to control water, a finding inconsistent with previous reports of increased protection against the toxicity of Pb to *C. dubia* and fish with increased pH and alkalinity (Davies et al., 1976; Mager et al., 2011). Although control performance in both tests with MOPS during the present study was very good it is possible that MOPS may be exerting stress on the organisms that enhances their sensitivity to Pb. An effect of low pH on the chronic toxicity of Pb to *C. dubia* is supported by the pH 6.4 MOPS test when compared to the other controls, particularly when analyzed in the context of the Pb speciation data. That is, a greater percentage of ionic Pb^{2+} is present at pH 6.4 such that less total dissolved Pb is required to induce 20% mortality (i.e. 3.9 μg L^{-1} Pb). Nonetheless, given the likely confounding influence of MOPS, results from the pH tests are difficult to interpret in the context of the other data and should be considered with caution. Additional tests examining the influence of MOPS buffer or alternative approaches to modifying pH (e.g. enriched CO_2 atmosphere or flow-through studies with continuous acid/base addition) should help resolve the validity of these tests and further elucidate the influence of pH on the toxicity of Pb to *C. dubia*.

5. Conclusions

This study indicates that the water parameters tested that clearly protect against chronic toxicity of Pb to *C. dubia* are DOM and alkalinity. Additionally, our findings further support the notion that Ca^{2+} and Mg^{2+} offer little protection during chronic Pb exposures and therefore the role of water hardness in the establishment of Pb WQC should be re-evaluated. Finally, our data suggest that the toxicity of Pb is also pH dependent with increasing toxicity at lower pH as might be expected based on Pb speciation. Importantly, however, our results indicate that pH-dependent tests with MOPS may be confounded by physiological and/or chemical interactions with the buffer. Clearly, further study of the most appropriate method of performing pH manipulated experiments with daphnids is needed. Overall, the data reported herein represent a strong initial analysis of the influences of water chemistry on the chronic toxicity of Pb and will further aid in the parameterization of a chronic Pb BLM for *C. dubia*.

Acknowledgments

This work was funded by the International Lead Zinc Research Organization (ILZRO) and the United States Environmental Protection

Agency (USEPA) under the Science to Achieve Results (STAR) Graduate Fellowship Program (awarded to EMM). USEPA has not officially endorsed this publication and the views expressed herein may not reflect the views of the USEPA.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2010.11.005.

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