Comparative Biochemistry and Physiology, Part C 155 (2012) 423–431

Contents lists available at SciVerse ScienceDirect

Comparative Biochemistry and Physiology, Part C

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

Multi-linear regression analysis, preliminary biotic ligand modeling, and cross species comparison of the effects of water chemistry on chronic lead toxicity in invertebrates

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ARTICLE INFO

Article history:
Received 23 August 2011
Received in revised form 17 November 2011
Accepted 18 November 2011
Available online 26 November 2011

Keywords:
Pb
Dissolved organic carbon
Snail
Rotifer
Water quality
Ceriodaphnia dubia

ABSTRACT

The current study examined the chronic toxicity of lead (Pb) to three invertebrate species: the cladoceran *Ceriodaphnia dubia*, the snail *Lymnaea stagnalis* and the rotifer *Philodina rapida*. The test media consisted of natural waters from across North America, varying in pertinent water chemistry parameters including dissolved organic carbon (DOC), calcium, pH and total CO_2. Chronic toxicity was assessed using reproductive endpoints for *C. dubia* and *P. rapida* while growth was assessed for *L. stagnalis*, with chronic toxicity varying markedly according to water chemistry. A multi-linear regression (MLR) approach was used to identify the relative importance of individual water chemistry components in predicting chronic Pb toxicity for each species. DOC was an integral component of MLR models for *C. dubia* and *L. stagnalis*, but surprisingly had no predictive impact on chronic Pb toxicity for *P. rapida*. Furthermore, sodium and total CO_2 were also identified as important factors affecting *C. dubia* toxicity; no other factors were predictive for *L. stagnalis*. The Pb toxicity of *P. rapida* was predicted by calcium and pH. The predictive power of the *C. dubia* and *L. stagnalis* MLR models was generally similar to that of the current *C. dubia* BLM, with R^2 values of 0.55 and 0.82 for the respective MLR models, compared to 0.45 and 0.79 for the respective BLMs. In contrast the BLM poorly predicted *P. rapida* toxicity ($R^2 = 0.19$), as compared to the MLR ($R^2 = 0.92$). The cross species variability in the effects of water chemistry, especially with respect to rotifers, suggests that cross species modeling of invertebrate chronic Pb toxicity using a *C. dubia* model may not always be appropriate.

1. Introduction

Lead (Pb) is a non-essential metal and persistent environmental toxicant (WHO, 1995). Although anthropogenic Pb pollution has diminished in many areas because of reduced use (e.g., lead based paint and gasoline), it remains an environmental concern for many reasons including mining, industrial processes and water run-off from shooting ranges (Mager, 2012). Current water quality criteria in the United States, Canada and Europe allow for abiotic factors (e.g. pH, hardness, and/or DOC) in an aquatic environment to influence the acceptable levels of metals in ambient waters. This is common practise for many metals as abiotic factors can alter chemical speciation and toxicity of metal ions in water. In both Canada and the United States the water quality criteria for Pb are based on water hardness (USEPA, 1985; CCREM, 1987), with increased hardness allowing higher acceptable levels of Pb in a given environment. More recently it has been shown that the relationship between water hardness and Pb toxicity is dependent solely on the calcium component of hardness, and that even this relationship is not consistent between taxa (Macdonald et al., 2002; Grosell et al., 2006a; Esbaugh et al., 2011; Mager et al., 2011a,b). As such, it seems that a re-evaluation of water quality criteria for Pb seems necessary.

The primary water chemistry components currently thought to affect Pb toxicity include calcium, dissolved organic carbon (DOC), pH and alkalinity (Davies et al., 1976; Schubauer-Berigan et al., 1993; Grosell et al., 2006a; Mager et al., 2010; Esbaugh et al., 2011; Mager et al., 2011a,b). The mechanism by which DOC and alkalinity are generally thought to affect Pb toxicity is via chemical complexation, which limits the concentration of the toxic ionic Pb^{2+} species (Campbell and Evans, 1987; Prosi, 1989). Alternatively, calcium appears to compete with Pb^{2+} for binding sites at the biotic ligand (Macdonald et al., 2002; Rogers et al., 2003; Rogers and Wood, 2004), while pH can both affect speciation and compete with Pb^{2+} for binding sites. Although all of the above parameters have been implicated in protection against Pb toxicity, the degree of protection for each parameter seems to vary depending on the species and toxicological endpoint. For instance, calcium has been implicated in protecting against acute Pb toxicity in the fathead minnow *Pimephales*

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doi:10.1016/j.cbpc.2011.11.005

promelas (Grosell et al., 2006a; Mager et al., 2011b) and rainbow trout (Macdonald et al., 2002), however, no protective effect of calcium was shown for acute or chronic toxicity for the invertebrate species *Ceriodaphnia dubia* (Mager et al., 2011a,b), while hardness was protective against acute toxicity in the invertebrate *Hyalella azteca* (Besser et al., 2005). For this reason it seems important to model the effects of water chemistry on acute and chronic toxicity of Pb in a number of different phyla.

One of the more commonly used models to predict the effects of abiotic factors on metal toxicity is the biotic ligand model (BLM), which systematically combines the effects of water chemistry components on metal speciation and competitive interactions between metal ions and other cations (e.g. Ca^{2+}) at the biological surface (Di Toro et al., 2001; Santore et al., 2001). However, the BLM is only one of many methods available for ascertaining the role of water chemistry on toxicity. While the European Union (EU) has adopted various BLMs for risk assessment and derivation of water quality criteria (WQC), the implementation of BLMs in the United States has met with some resistance. Although the USEPA has adopted the copper BLM for establishing WQC, no State has fully adopted this criterion for copper, nor for any of the additional metals for which a BLM has been developed. Similarly, Canadian water quality standards are still set by hardness equations (CCREM, 1987). One alternative to the BLM is a regression approach, which is the underlying technique initially used to develop hardness-based water quality equations, and has also been evaluated as a tool to predict copper and ammonia toxicity (Erickson et al., 1996; USEPA, 1999; Rogevich et al., 2008), and was also recently applied to acute Pb toxicity for *C. dubia* and *P. promelas* (Esbaugh et al., 2011).

On this background, the current study was performed to enhance our understanding of the effects of water chemistry parameters on chronic Pb toxicity in invertebrate species. Chronic toxicity to the cladoceran *C. dubia* was assessed in natural waters from across North America. In combination with existing data sets on *C. dubia* this information was used to assess both the BLM and multi-linear regression (MLR) analysis as methods to predict chronic toxicity. Furthermore, two additional invertebrate species (*Philodina rapida* and *Lymnaea stagnalis*) from the phyla rotifera and mollusca, respectively, were used to assess potential cross-species variation in the effects of abiotic factors.

2. Materials and methods

2.1. Collection of natural waters

Natural waters were collected from 6 different sources from across North America (Table 1). During collection care was taken not to disturb the sediment and to limit particulate material. After collection, water was stored in 20 L polyethylene containers, packed in coolers, and shipped to the University of Miami. Upon arrival in Miami, the water from the respective sites was placed in single 100–200 L containers and stored in a dark 4 °C room. This prevented any discrepancy in chemistry between the 20 L aliquots of water. The natural waters were collected from the following locations: Green Cove Springs (Jacksonville, FL, USA), Sweetwater Strand (Everglades, FL, USA), French Lake (ON, Canada), Edisto River (South Carolina, USA), Basin Creek (Wilkes County, NC, USA), and well water from the United States Geological Survey (USGS) Environmental Research Laboratory (Columbia, MO, USA). Additionally, a low pH water was made by diluting Columbia well water 20-fold with Milli-Q water and then acidifying with HCl to a final pH of 5.5. Dechlorinated Miami-Dade tap water was used as a reference, with a 40% concentration (diluted with Milli-Q water) used for *C. dubia* tests, while full strength tap water was used for *L. stagnalis* tests. Reference water for *P. rapida* tests was generated in the lab according to the EPA recipe for moderately hard water (USEPA, 2002).

2.2. Water chemistry analysis

Analysis for Pb was performed using a graphite furnace atomic absorption spectrophotometer (AAS; Varian), with all samples being acidified (1% HNO_3) and passed through a 0.45 μm filter prior to analysis. The detection limit for Pb using standard procedures was 0.5 μg/

Table 1
Water chemistry parameters measured in chronic toxicity tests for three invertebrate species. Sampling procedures are described in the Materials and methods section and all values are arithmetic mean ± S.E.M.

Site water	pH	μM						
		Na^+	Ca^{2+}	Mg^{2+}	Cl^-	SO_4^{2-}	TCO_2	DOC
L. stagnalis								
Reference	8.03 ± 0.04	1672 ± 66	558 ± 47	162 ± 6	1287 ± 80	299 ± 19	850 ± 100	527 ± 69
Green Cove	8.30 ± 0.01	205 ± 5	731 ± 57	686 ± 10	152 ± 5	466 ± 11	1388 ± 79	96 ± 7
Sweetwater Strand	8.61 ± 0.01	369 ± 6	1861 ± 7	94 ± 2	292 ± 6	14 ± 1	3049 ± 249	576 ± 3
Edisto River	7.27 ± 0.01	203 ± 4	174 ± 10	58 ± 1	214 ± 5	27	166 ± 20	1314 ± 20
French Lake	7.10 ± 0.01	108 ± 2	75 ± 2	47 ± 1	75 ± 1	19 ± 1	83 ± 25	553 ± 2
pH 5.5[a]	5.79 ± 0.05	133 ± 2	96 ± 1	57 ± 1	352 ± 6	21	2 ± 2	36 ± 7
Basin Creek	6.82 ± 0.05	72 ± 4	24 ± 3	23 ± 2	28	17	64 ± 25	119 ± 7
C. dubia								
Reference	7.91 ± 0.03	558 ± 6	252 ± 1	73	643 ± 24	59 ± 2	372 ± 26	222 ± 9
Green Cove	8.05 ± 0.03	250 ± 14	837 ± 20	735 ± 30	195 ± 7	533 ± 23	1756 ± 32	153 ± 11
Sweetwater Strand	8.47 ± 0.01	427 ± 2	2141 ± 3	93 ± 1	368 ± 1	16	3768 ± 24	801 ± 7
Edisto River	7.31 ± 0.02	246 ± 14	216 ± 7	59 ± 1	229 ± 9	27 ± 1	223 ± 10	1443 ± 2
French Lake	7.07 ± 0.02	157 ± 23	114 ± 21	49	128 ± 38	22 ± 2	177 ± 9	686 ± 16
pH 5.5[a]	6.51 ± 0.03	165 ± 7	162 ± 4	62 ± 3	586 ± 27	29 ± 1	28 ± 3	114 ± 19
Basin Creek	7.00 ± 0.02	108 ± 3	246 ± 4	29 ± 1	535 ± 6	25	138 ± 5	214 ± 12
P. rapida								
Reference	8.15 ± 0.01	1071	346	527	59	678	1051	79
Green Cove	8.22 ± 0.01	186	695	632	134	425	1265	94
Sweetwater Strand	8.44 ± 0.02	249	1403	61	221	0	2374	889
French Lake	7.23 ± 0.02	109	77	43	79	21	184	674
Basin Creek	7.33 ± 0.01	75	27	22	33	17	111	283
Edisto River	7.32 ± 0.01	204	202	60	199	28	238	1405

[a] USGS, Columbia water diluted 20 times with Milli-Q water and acidified with HCl.

L, which equalled 3 times the standard deviation of ten measurements. For nominal Pb concentrations less than 3 μg/L, a series of multiple injections was used to concentrate analyte mass and ensure values were above the detection limit. The pertinent cation (Na^+, K^+, Ca^{2+}, Mg^{2+}) concentrations were analyzed using a flame AAS (Varian), while the anions (Cl^-, SO_4^{2-}, NO_3^-) were analyzed using ion chromatography (Dionex, DX-120). Levels of bicarbonate/carbonate were assessed using a double endpoint titration procedure (Hill, 1973) as previously described (Genz et al., 2008). Sample pH was measured using a combination glass electrode coupled to a PHM220 pH meter. DOC concentrations were determined by first filtering water samples through 0.45 μM GF/F 25 mm Whatman filter paper, then analyzing the filtrate for total organic carbon using high temperature catalytic oxidation (Shimadzu total organic carbon-VCSH). Water hardness was calculated using the measured magnesium and calcium concentrations. The DOC quality was assessed for natural waters using an F-index metric, with a higher value indicating a more protective DOC type (Richards et al., 2001). This metric was calculated as previously described (Richards et al., 2001), using a microplate spectrometer. These tests were only performed on filtered pre-test water samples.

2.3. Toxicity testing with *C. dubia*

All tests and acclimations were performed in a temperature controlled environmental chamber (26 °C) with a 16:8 light/dark cycle. *C. dubia* were obtained from an in-house culture originally from Aquatic Biosystems (Ft. Collins, CO, USA) and maintained in dechlorinated Miami-Dade tap water at 26 °C. All tests were based on the standard USEPA methods for chronic toxicity testing using *C. dubia* (USEPA, 2002), and specifically matched to the methods of Mager et al. (2011a). For all tests, a water specific mass culture (1 L) of relatively low density (30–50 individuals) was started and maintained for 3 days. All mass cultures were fed a combination of a commercially available YTC solution (5 mL; Aquatic Biosystems) and *Pseudokirchneriella subcapitata* (5 mL of 3×10^7 cells/mL stock; Aquatic Biosystems) daily. Prior to testing, individual Pb concentrations were prepared by spiking test waters with varying amounts of $Pb(NO_3)_2$ (CAS 10099-74-8), 1.33 mL of YTC suspension and equilibrated at 26 °C for approximately 24 h. Each concentration was spiked with 1.33 mL of *P. subcapitata* suspension 1 h prior to test set-up. This allowed equilibration of Pb to both the water and food to ensure that a combination of dietary and aqueous exposure was tested. Chronic test replicates comprised a single neonate (<24 h old) held in 15 mL of solution, with daily changes of test solution. Each test consisted of 6 concentrations with each containing 10 replicates that were monitored daily for survival and reproduction for 7 days. Temperature of the test solutions was monitored daily, while pO_2 (data not reported) and pH were measured 4 times throughout. Two water samples for ion and total CO_2 analysis were taken throughout.

2.4. Toxicity testing with *L. stagnalis*

All tests and acclimations were performed in a temperature controlled environmental chamber (26 °C) with a 16:8 light/dark cycle. *L. stagnalis* were obtained from an in-house culture maintained in dechlorinated Miami-Dade tap water. Toxicity testing was performed using a variation of the method of Grosell et al. (2006b). Egg masses were harvested from the mass culture and immediately placed in approximately 500 mL of aerated field/reference water until snails hatched (approximately 7 days) and began feeding on solid food (lettuce ad libitum; 7–10 days after hatching). Prior to testing, individual Pb concentrations were prepared by spiking test waters with varying amounts of $Pb(NO_3)_2$ and equilibrated at test temperature for approximately 24 h. Test replicates comprised of individual snails held in 100 mL of solution within a 150 mL plastic container with lid. Gentle aeration was provided to test containers throughout the experiment. Snails were fed lettuce ad libitum at a rate sufficient to ensure food was always available. Water changes and food replacement (lettuce) were performed every 48 h for a duration of 14 days. Each test consisted of 6 concentrations and each concentration contained 10 replicates. At the completion of the test, each individual snail was gently blotted dry with a Kimwipe and the wet weight was recorded. Pb samples were taken from three replicates (2 replicates for <3 μg/L Pb) before and after each water change. Temperature was measured at every water change, while dO_2 (data not reported) and pH measurements were taken at various intervals throughout the tests. Samples for water ionic composition and DOC concentration were taken at days 4, 10 and 14.

2.5. Toxicity testing with *P. rapida*

All animal housing and toxicity tests were performed in a temperature (26 °C) and light (16:8 light/dark cycle) controlled room. Individual *P. rapida* were obtained from an in-house culture (originally provided by T. Snell, Georgia Tech), maintained in EPA moderately hard water and fed commercial rotifer feed (Sparkle, INVE Aquaculture). Test waters were prepared 24 h in advance, and consisted of 55 mL of site water, 330 μL of Sparkle feed (10 g/L) and spiked with various concentrations of $Pb(NO_3)_2$. A duplicate control water solution (no added Pb) was prepared for analysis of anion, cation and DOC concentrations, which was necessary due to the large volumes needed for analysis as compared to the small toxicity test volumes. Each test consisted of 6 concentrations, and 4 replicates per concentration. Each test replicate consisted of 10 mL of test solution in a single cell of a 6-cell culture plate, with 4 test replicates and 1 water chemistry replicate per treatment. The water chemistry replicate was used only to test final Pb concentrations and pH. A total of 6 adult rotifers were added to each cell and left for 4 days in a 26 °C temperature controlled room. After 4 days the test replicates were fixed using a 5% buffered formalin solution with rose Bengal, and the water chemistry plate was sampled for final Pb concentrations and pH. The replicates were left to fix for 24 h after which the individuals in each replicate were counted using a dissecting microscope (10–25× magnification). Population growth rate was calculated according to Snell and Moffat (1992). Briefly, the natural log of the final population size was subtracted by the natural log of the initial population size and divided by the duration of exposure in days.

2.6. Data analyses

All statistical analyses were performed according to EPA guidelines for chronic toxicity testing (USEPA, 2002). Each test was assessed for no observable effect concentration (NOEC) and lowest observable effect concentration (LOEC) using either parametric or non-parametric hypothesis testing methods performed by ToxCalc software (version 5.0). The EC50 for each test was estimated using a log-logistic model, while EC20 values were calculated using linear interpolation methods. Pb speciation calculations were performed using the freeware program Visual MINTEQ 3.0. All default settings were used and binding of metal ions to dissolved organic matter (DOM) was modeled using the NICA-Donnan formulation (Milne et al., 2001; Milne et al., 2003). In all cases, DOM was assumed to contain 50% carbon (DOC) by mass, and to have an assumed composition of 65% active fulvic acid. In other words, a measured DOC of 1 mg/L would have an input of fulvic acid into the speciation model of 1.3 mg/L. This is based on previous modeling studies on natural waters that showed assumptions of 60–70% fulvic acid composition were best for modeling metal speciation (Tipping, 2002).

For regression analyses, data were first natural log transformed, with the exception of pH data. Toxicity endpoints also were natural log transformed and were then regressed against various combinations of water chemistry parameters (major cations, pH, DOC, TCO_2) using best-fit step-wise multi-linear regression approaches (SigmaStat 3.5). All water chemistry variables were included in multi-linear regression analyses and the best fit models were based upon adjusted R^2, with care taken to limit co-linearity of water chemistry parameters as judged by the variance inflation factor. The relationship between the primary independent regression variables, as well as the variance inflation factors of the best-fit model, is provided in the supplemental material (Table 2–5; supplemental material). As a point of comparison, toxicity predictions were performed using a preliminary chronic BLM for Pb built using data previously reported for *C. dubia* (Mager et al., 2011a), as well as a single unpublished data set regarding the effects of pH on *C. dubia* toxicity (Stubblefield, personal communication). This unpublished dataset consisted of 4 data points from pH 6–8.5. The log(EC50)–pH regression resulted in a slope of -0.73 ($R^2=0.96$). No competitive effects of major cations were modeled, in line with the findings of Mager et al. (2011a, 2011b). Pb speciation was calculated as described above using Visual MINTEQ 3.0. As such, the preliminary BLM consists of the following equation to predict the EC50 on the basis of the free Pb^{2+} ion activity as a function of pH, expressing a 'competitive' effect of H^+ ions on chronic Pb^{2+} toxicity:

$$EC50_{Pb2+} = 10^{Q50,CD-S_{pH}\times pH} \tag{1}$$

with $S_{pH}=0.73$, and Q50,CD a parameter representing the intrinsic sensitivity of *C. dubia* ($=-3.06$ for the University of Miami culture, based on data in Mager et al., 2011a, b). This equation is linked to Visual MINTEQ 3.0 to convert predictions of $EC50_{Pb2+}$ to $EC50_{dissolved}$, using the same assumptions as for the speciation calculations, as explained above. Calculation of the intrinsic sensitivity factors for *L. stagnalis* (Q50,LS $=-4.11$) and *P. rapida* (Q50,PR $=-3.74$ for the population size endpoint) was based on the data presented in the current study as described previously (Di Toro et al., 2001; Santore et al., 2001; De Schamphelaere and Janssen, 2004a; De Schamphelaere et al., 2006; De Schamphelaere and Janssen, 2010; Schlekat et al., 2010).

3. Results and discussion

3.1. Water chemistry

A relatively diverse array of water chemistries were observed in the suite of natural waters. Based on pre-test chemistry (supplemental information), broad variability was observed with regard to pertinent chemical parameters related to Pb toxicity, such as DOC (36–1244 μM), hardness (4–298 mg/L), total CO_2 (53–5043 μM), calcium (24–1934 μM) and pH (5.48–8.72). The exact water chemistries measured during the toxicity tests are shown in Table 1, with little deviation from pre-test measurements. The water chemistry values in Table 1 were used for all subsequent models and analysis.

3.2. *C. dubia* Pb toxicity

Successful toxicity tests were performed in 5 of 8 waters using *C. dubia* (Table 2), and in all of these cases reproduction was found to be a more sensitive endpoint than mortality (data not shown). No significant response was observed for either mortality or reproduction in the USGS, Columbia water using nominal Pb concentrations up to 200 μg/L. This nominal concentration was likely above the solubility limit for Pb in this particular water, as suggested by the measured Pb concentrations of 77 ± 8 μg/L. Previous work on acute toxicity of *C. dubia* showed similar solubility problems in water from this particular site, with little difference in measured dissolved Pb concentrations despite a nominal concentration range of 200–6400 μg/L, and highlights the effects of carbonates (total CO_2) at limiting dissolved Pb species. The chronic Pb toxicity of *C. dubia* in Basin Creek water gave a non-monotonous response (poor fitting dose response), and as such reliable toxicity estimates could not be calculated. Furthermore, suitable control reproductive output could not be obtained over the 7-d test in the pH 5.5 water (mean neonates = 12.8 per female). Of the successful toxicity tests, *C. dubia* 7d-EC50s for reproduction ranged from 20 to 573 μg/L, with the highest toxicity in the soft water from French Lake; the most protective water was the high DOC Edisto River water.

To more fully ascertain the influence of water chemistry on chronic Pb toxicity in *C. dubia* the current results were combined with a previously generated data set investigating Pb toxicity in these

Table 2
Chronic Pb toxicity estimates for three invertebrate species in seven natural waters, and one reference water. Represented data are for waters where a successful test was performed. Only five natural waters were used for *P. rapida* toxicity tests.

Organism	Endpoint	Site water	Pb (μg/L)					
			EC20	95% CI	EC50	95% CI	NOEC	LOEC
C. dubia	Reproduction	Reference	35.4	10–40.1	56.3	47.1–67.2	47.5	68.4
		Green Cove Spring	22.6	0.9–35.46	94.8	45.5–197.8	16.4	39.9
		Sweetwater Strand	96.7	17.1–230.2	301.1	220.8–410.6	26.7	146.1
		Edisto River	223.3	45.1–363.6	573.4	491.3–669.1	110.6	243.9
		French Lake	12.1	10.7–14.4	20.1	n.a.	7.6	20.2
L. stagnalis	Individual growth	Reference	4.3	3–15.5	18.1	9.2–35.6	10.9	27.8
		Green Cove Spring	1.5	0.3–6.2	7.3	1.5–36.6	5.8	11.9
		Sweetwater Strand	49.5	18.6–67.9	131.9	68.5–254.1	46.4	106.7
		Edisto River	32.4	14.8–246.5	244.6	160.6–372.4	234.3	387.5
		French Lake	15.8	10.4–18.8	27.8	19.6–39.5	24	37.6
		pH 5.5[a]	2.1	0.8–2.7	3.6	1.8–7	3.8	9.3
		Basin Creek	1.5	0.3–4.3	9.4	3.9–22.6	5.9	12.3
P. rapida	Population growth	Reference	13.4	9.2–20.4	19.9	11.9–33.1	7.6	23.9
		Green Cove Spring	23.2	8.8–84.5	135	57.2–318.7	26.7	61
		Sweetwater Strand	103.3	0–265.2	135.2	90–203.2	203.2	419.6
		Edisto River	39.8	24.9–156.7	154.9	68.5–350.6	139.6	261.3
		French Lake	46.8	0–59.8	77.9	63.7–95.2	43.5	86.9
		Basin Creek	3.2	2.5–4.4	10.6	7.3–15.4	2.2	4.2

CI = confidence interval.
[a] USGS, Columbia water diluted 20 times with Milli-Q water and acidified with HCl.

organisms (Esbaugh et al., 2011; Mager et al., 2011a). Using a multi-linear regression approach three different water chemistry components were found to significantly affect chronic Pb toxicity – DOC, total CO_2 and sodium (Fig. 1A) – which largely agrees with previous findings regarding chronic *C. dubia* toxicity (Mager et al., 2011a). DOC is well known to be the primary water characteristic affecting Pb speciation (Campbell and Evans, 1987; Prosi, 1989), as demonstrated by the vast majority of dissolved Pb bound to DOC in the present toxicity tests (Table 3). It has also previously been shown to protect against both acute and chronic Pb toxicity to *P. promelas* (Grosell et al., 2006a; Mager et al., 2010; Esbaugh et al., 2011; Mager et al., 2011b) and *C. dubia* (Esbaugh et al., 2011; Mager et al., 2011a,b) and is a major protective component against freshwater toxicity of many other metals including copper (De Schamphelaere and Janssen, 2004b; Ryan et al., 2004; Ryan et al., 2009), zinc (Heijerick et al., 2002; Heijerick et al., 2005), nickel (Kozlova et al., 2009) and silver (Bielmyer et al., 2007).

Total CO_2 and pH are naturally co-variant in water due to the acid–base properties of carbon dioxide, so assessing the separate role of these factors in predicting Pb toxicity is difficult. It is well known that carbonates affect Pb speciation and therefore limit the concentration of toxic ionic Pb^{2+} species (Campbell and Evans, 1987; Prosi, 1989), while pH can similarly affect speciation through its affect on carbonates. However, pH can also act as a competitive parameter via H^+ competition, which has previously been suggested as a mechanism of protection against acute Pb toxicity in low pH waters for *P. promelas* (Mager et al., 2011b) and rainbow trout (Macdonald et al., 2002). The competitive effects of pH can be isolated by examining only the ionic Pb^{2+} EC50s, which correlate poorly with pH for *C. dubia* ($R^2=0.005$, $P=0.75$; regression not shown), and as such the available evidence points toward a role of pH/total CO_2 affecting Pb speciation, as opposed to competition with ionic Pb for ligand binding sites. In further support of this assertion, the best-fit MLR model (Fig. 1A) was equally effective when total CO_2 was replaced with pH. It is interesting to note that the preliminary BLM structure currently incorporates a competitive component for pH (see Eq. (1) in Materials and methods). The implications of this component of the BLM structure will be discussed further below, but it seems prudent for further studies to examine the separate effect of pH on chronic Pb toxicity across a range of pH values to more fully address this issue.

A third component of the MLR model is sodium. It is currently unclear what role sodium plays in Pb toxicity of *C. dubia*, as sodium is not known to directly compete with Pb for binding sites. However, recent studies on acute Pb toxicity in *C. dubia* revealed that the inclusion of ionic strength improved the predictive power of a model designed using a similar MLR approach (Esbaugh et al., 2011). Similarly effects of ionic strength have also been suggested for silver and copper, where the respective BLMs routinely lose their predictive power in very low ionic strength waters (Bielmyer et al., 2007; Ryan et al., 2009; Ng et al., 2010). Since sodium is a primary component of ionic strength these effects may be attributed to the same mechanism, the basis of which is likely osmoregulatory. Organisms in freshwater environments constantly lose ions to the more dilute environment, which they must replace through uptake. Diffusive ion loss occurs at higher rates in smaller organisms, such as *C. dubia*, because of the higher surface area to volume ratios (Grosell et al., 2002). It seems likely that the apparent effects of sodium are simply a product of varying physiological stress imposed on the organism as a consequence of the environment, and that organisms that are under greater physiological stress may also be more sensitive to Pb toxicity. This interpretation is supported by the fact that sodium can be replaced with chloride, another major freshwater osmolyte, as an MLR parameter with little effect on the overall model performance.

Table 3
Predicted concentrations of major lead species at the EC50 in natural water toxicity tests for three invertebrate species, as calculated using Visual MINTEQ 3.0. Values in parenthesis are the percentage of total dissolved Pb species.

		Pb^{2+} (μg/L)	Total organic (μg/L)[a]
C. dubia	Reference	0.12	55.5 (99)
	Green Cove Spring	0.46	82.9 (87)
	Sweetwater Strand	0.099	288 (96)
	Edisto River	0.98	571 (99)
	French Lake	0.032	20.0 (99)
L. stagnalis	Reference	0.0055	18.0 (99)
	Green Cove Spring	0.011	6.88 (94)
	Sweetwater Strand	0.033	126 (96)
	Edisto River	0.32	244 (99)
	French Lake	0.062	27.7 (99)
	pH 5.5[b]	1.7	1.85 (52)
	Basin Creek	0.18	9.13 (98)
P. rapida	Reference	0.067	18.3 (92)
	Green Cove Spring	1.1	105 (78)
	Sweetwater Strand	0.027	133 (98)
	French Lake	0.16	77.6 (99)
	Basin Creek	0.018	10.6 (99)
	Edisto River	0.14	155 (99)

[a] Total organic equals the sum of all calculated organic Pb species.
[b] USGS, Columbia water diluted 20 times with Milli-Q water and acidified with HCl.

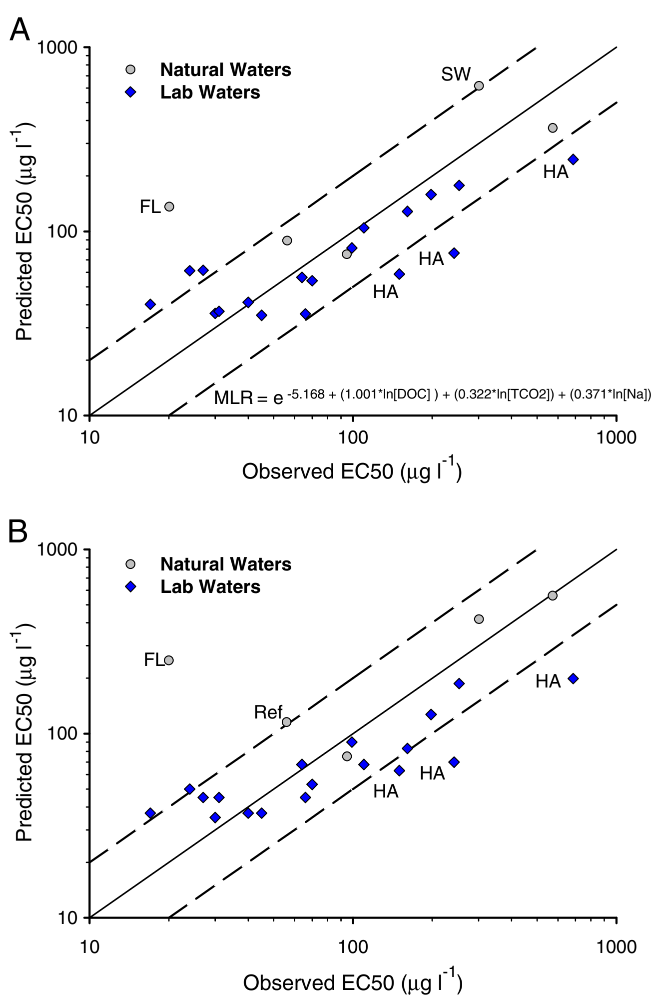

Fig. 1. An observed versus predicted 7d-EC50 regression plot for chronic *Ceriodaphnia dubia* Pb toxicity as predicted by (A) multi-linear regression (MLR) analysis and (B) the preliminary *Ceriodaphnia dubia* biotic ligand model. The solid line represents equal observed versus predicted toxicity, while the dotted lines represent ±2-fold observed versus predicted toxicity. The lab generated data (diamond) is from Mager et al. (2011a). Those tests referred to in the text are designated as follows: FL = French Lake, SW = Sweetwater Strand, Ref = reference, and HA = humic acid.

3.3. L. stagnalis and P. rapida chronic Pb toxicity

Successful chronic Pb toxicity tests were performed in 7 of 8 natural waters for *L. stagnalis* (Table 3), with no toxic effects of Pb in the USGS, Columbia water. This is similar to tests performed with *C. dubia* and is again likely due to the low Pb solubility in this particular water. In the case of the *L. stagnalis* test, the highest amount of measured Pb was only 3.01 ± 0.77 μg/L at a nominal concentration of 50 μg/L. Successful toxicity tests were performed in all 6 test waters for *P. rapida* (Table 3), although control population growth rate in Sweetwater Strand water was half that of the other waters.

L. stagnalis is the most sensitive known organism to dissolved Pb exposure, with a growth 30d-EC20 of <4 μg/L (Grosell et al., 2006b). The results obtained in the current study are similar in range with growth EC20s and EC50s ranging from 1.5 to 49.5 and 3.7 to 181 μg/L dissolved Pb, respectively. The greatest toxicity occurred in low DOC waters (Tables 1 and 2), such as Green Cove Springs, Basin Creek and the pH 5.5 water. In fact, use of an MLR approach to isolate determinants of chronic Pb toxicity yielded only DOC as a significant factor (Fig. 2). Interestingly the other parameters known to affect speciation – total CO_2 and pH – had no predictive value for *L. stagnalis* chronic Pb toxicity (on the basis of dissolved Pb), nor did any competitive parameters such as calcium. In contrast, an examination of the ionic Pb^{2+} EC50 revealed a significant negative correlation of log ($EC50_{Pb2+}$) with pH (Fig. 3) suggesting that H^+ competition does occur. The slope of this relationship for *L. stagnalis* (S_{pH}) equals 0.78, which is similar to the slope for *C. dubia* used in the BLM (S_{pH} = 0.73; see Materials and methods), suggesting a similar competitive pH effect in these two species. However, the importance of this for predicting EC50s with the MLR seems to be minimal relative to DOC. It is surprising that calcium had no predictive impacts for chronic *L. stagnalis* toxicity because previous work has suggested that interference of the high calcium uptake rates in juvenile snails is the controlling factor for their hypersensitivity (Grosell and Brix, 2009). The lack of any calcium effect on chronic Pb toxicity to *L. stagnalis* contradicts this suggestion; however, it is noteworthy that pH and calcium are highly correlated in the natural water data set where low pH waters are also low in calcium (R^2 = 0.68; supplemental information). This could result in a stronger pH relationship masking the competitive effects of calcium, though as with pH any relationship would be of minimal importance from a standpoint of predicting EC50s with the MLR.

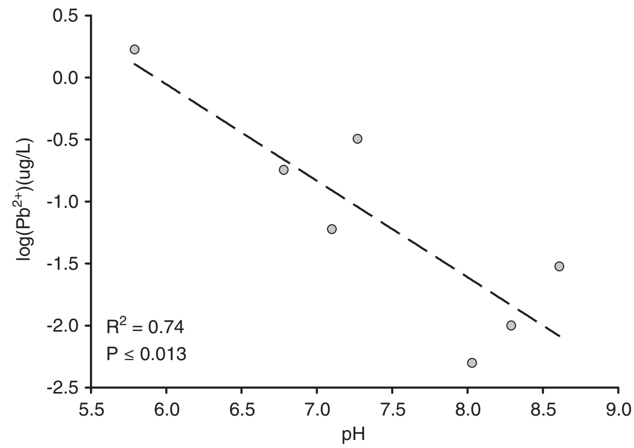

Fig. 3. Linear regression analysis showing the relationship between environmental pH and the log chronic Pb^{2+} EC50 (μg/L) for *Lymnaea stagnalis*.

Chronic Pb toxicity for *P. rapida* was assessed using a population growth rate endpoint (Table 2), which conformed to classical concentration dependent responses with a chronic EC20 and EC50 range of 3–103 and 10–154 μg/L dissolved Pb, respectively. The only available Pb toxicity data for rotifers is for *Brachionus calyciflorus*, which was more tolerant in a test medium similar to the current reference medium with an EC20 of 307 μg/L for population growth (Grosell et al., 2006b). Of the natural waters tested, *P. rapida* showed the highest sensitivity in the soft Basin Creek water, while the lowest toxicity was observed in the water from the Edisto River.

In contrast to expectation, and to both *C. dubia* and *L. stagnalis*, chronic Pb toxicity in *P. rapida* was not correlated to DOC using MLR analysis (Fig. 4). In fact, DOC alone regressed poorly with EC50 (R^2 = 0.22, $P \leq 0.35$). The best fit model for *P. rapida* instead incorporated only calcium and pH, but the relatively high covariance between calcium and pH in the test waters suggests this model should be viewed with caution. Furthermore, the protective effects of both parameters appear to be more than a competitive effect as regression of ionic Pb^{2+} EC50s and calcium and pH have R^2 values of only 0.09 and 0.04, respectively (supplemental information). Although it is difficult to draw many conclusions from this model, both because of the co-linearity of independent variables within the test solutions and the

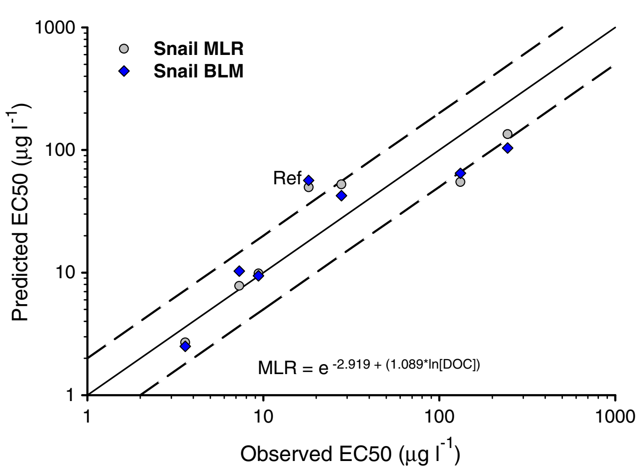

Fig. 2. Chronic *Lymnaea stagnalis* Pb toxicity observed versus estimated effect regression plots for the multi-linear regression model (MLR) and preliminary *Ceriodaphnia dubia* biotic ligand model (BLM). Multi-linear regression model formula denoted. The solid line represents equal observed versus predicted toxicity, while the dotted lines represent ±2-fold observed versus predicted toxicity. The reference (Ref) water referred to in the text is designated.

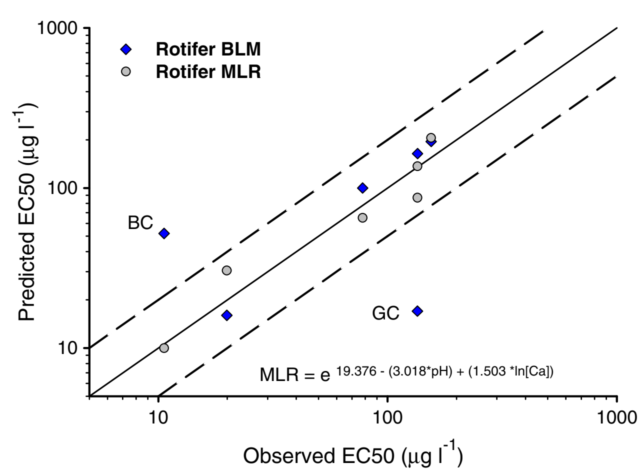

Fig. 4. Chronic *Philodina rapida* Pb toxicity observed versus estimated effect regression plots for the multi-linear regression model (MLR) and preliminary *Ceriodaphnia dubia* biotic ligand model (BLM). Multi-linear regression model formula denoted. The solid line represents equal observed versus predicted toxicity, while the dotted lines represent ±2-fold observed versus predicted toxicity. Those tests referred to in the text are designated as follows: BC = Basin Creek, GC = Green Cove.

sample number of data points, it seems clear that *P. rapida* do not conform to typical invertebrate patterns with respect to the predictive components of Pb toxicity. This is especially with regard to the effects of DOC. One possible explanation for this interesting protective water chemistry pattern is that ionic Pb^{2+} may not be the only toxic Pb species to rotifers. Since a major food source for rotifers is organic detritus, it seems possible that Pb bound to food and ingested may contribute to toxicity. Previous work has also suggested that the combination of dietary and waterborne exposure to the amphipod *H. azteca* may be more toxic than waterborne exposure alone (Besser et al., 2005). It is also possible that DOC may have unknown affects on rotifer physiology that could affect Pb^{2+} uptake, such as membrane permeability or potential. The latter secondary effects have been suggested as the explanation for higher than expected Pb^{2+} uptake rates in green algae (*Chlorella kesslerii*) in the presence of humic acid (Slaveykova et al., 2003; Hassler et al., 2004; Lamelas et al., 2005; Lamelas and Slaveykova, 2007), and DOC concentration has also been shown to affect the electrical properties of gill epithelial in rainbow trout (*Oncorhynchus mykiss*) (Galvez et al., 2008). Nonetheless, it is clear that further work is needed to fully address the effects of water chemistry on Pb toxicity to *P. rapida*.

3.4. Multi-linear models of chronic Pb toxicity and comparison with the BLM

MLR models for all three species were generated (Figs. 1, 2 and 4) with varying levels of robustness. A statistical evaluation of the BLM and various MLR models for the respective species is shown in Table 4. The *C. dubia* MLR has the least predictive power of the respective species MLR models, with an R^2 of 0.55 ($P<0.001$; Fig. 1, Table 4). Of the twenty-four EC50s, five would be overestimated by this model based on the criteria that the predicted EC50 exceeds the observed by more than 2-fold. The most dramatic of these five is the French Lake water which is a soft water with a high level of DOC. Conversely three EC50s were underestimated by the MLR model, all of which were from an Aldrich humic acid manipulated data set (Mager et al., 2011a). An explanation for the error in the model with respect to these outlying points may stem from the DOC sources of the various test waters. It is well established that NOM/DOC are heterogenic parameters with source specific protection profiles, and that Aldrich humic acid in particular provides higher binding capacity than from those of natural aquatic sources (Richards et al., 2001; Glover et al., 2005a; Glover et al., 2005b), which could explain the underestimation of EC50 in these media. Furthermore, sources of DOC with higher F-indexes and/or specific absorbance coefficients, which are metrics describing the DOC quality, provide greater protection against metal toxicity (Richards et al., 2001; De Schamphelaere and Janssen, 2004b). The F-index for the French Lake water was among the lowest of the natural waters tested (0.42; Table 1 supplemental material), and since sodium and total CO_2 are very low in this water, its protection profile is dependent almost entirely on DOC. This may partially explain why the model so dramatically overestimates the EC50. It seems prudent to more fully examine (i) the effects that NOM/DOC source may have on chronic Pb toxicity in these organisms, (ii) the effects of combinations of a low degree of protection by several water quality parameters at the same time, as well as (iii) how to properly incorporate this information into any predictive model.

Table 4
A comparison of R^2 and *P*-values for the species-specific multi-linear regression models, as well as the *Ceriodaphnia dubia* biotic ligand model as applied to each species. All statistics are based on the natural log observed Pb EC50 (μg/L) versus the natural log predicted Pb EC50 (μg/L).

	Multi-linear regression		Biotic ligand model	
	R^2	*P* value	R^2	*P* value
C. dubia	0.55	<0.001	0.45	<0.001
L. stagnalis	0.82	0.005	0.79	0.007
P. rapida	0.92	0.002	0.19	0.23

As a point of comparison the chronic Pb MLR model for *C. dubia* was compared to predictions performed using a preliminary chronic BLM for Pb also developed using *C. dubia* (Fig. 1B). Of particular note is that most points that were over or underpredicted by the MLR model were also incorrectly predicted by the BLM (Fig. 1B). In fact, the overall predictions of both models are generally quite similar, although based on R^2 the MLR performs slightly better (Table 4). This is quite interesting because the basis of the two models is somewhat different, as the current BLM includes pH as a competitive factor while the MLR uses sodium. However, the other two components of the MLR (DOC and total CO_2) are two factors that affect speciation, which is clearly a component of the BLM. The overall similarity of the MLR and BLM models would suggest that DOC and total CO_2 are far more important factors to consider when predicting chronic Pb toxicity in *C. dubia*. Nonetheless, both models would likely be improved by the addition of sodium and pH manipulated data sets, and especially pH manipulated data sets that attempt to limit the potentially confounding effects of total CO_2.

With respect to non-model invertebrate species the MLR models for both *L. stagnalis* and *P. rapida* were considerably more robust than that of *C. dubia* (Figs. 2 and 4) with R^2 values of 0.82 and 0.92, respectively. Some caution must be exercised when making comparative evaluations, however, as the number of data points for these models is much lower than for that of *C. dubia*. Similar to that of *C. dubia*, the MLR model for *L. stagnalis* seems to closely mimic predictions of the BLM, again showing the overall importance of Pb speciation to predicting Pb toxicity in invertebrates. Although the MLR slightly outperforms the BLM, it seems safe to suggest that the BLM developed for *C. dubia* could be effectively applied to *L. stagnalis*. This is important as *L. stagnalis* is the most sensitive known organism to waterborne Pb toxicity (Grosell et al., 2006b; Grosell and Brix, 2009). In contrast, the *C. dubia* BLM poorly predicted Pb toxicity in *P. rapida* (Fig. 4 and Table 4), while the MLR is quite effective at predicting toxicity in these organisms. This is mostly explained by both the importance the BLM places on DOC when predicting *P. rapida* chronic Pb toxicity, as well as the absence of a calcium component for predicting toxicity. Interestingly, the chronic nickel *C. dubia* BLM is equally ineffective at predicting toxicity for rotifers, while effectively predicting toxicity in *L. stagnalis* (Schlekat et al., 2010). The inability of *C. dubia* BLMs to predict chronic toxicity to *P. rapida* while successfully predicting toxicity in *L. stagnalis* is surprising for two reasons. First, both *C. dubia* and *P. rapida* are filter feeding organisms that could similarly be exposed to dietary Pb during testing, which is not the case for *L. stagnalis*. Secondly, both *C. dubia* and *P. rapida* toxicity tests are based on similar reproductive endpoints, while the snail test is a growth endpoint. As such, it would seem more likely that a *C. dubia* BLM would not perform well for snails. Regardless of the explanation, it seems clear that models developed for *C. dubia* – BLM or MLR – may not be effective at extrapolating chronic Pb toxicity to all invertebrate phyla, especially rotifers, and that further work is clearly necessary to understand Pb toxicity in this interesting phylum.

4. Conclusions

The current study used a combination of chronic toxicity testing in natural waters and MLR analysis to elucidate the protective impacts of water chemistry parameters on chronic Pb toxicity in three invertebrate species. These analyses show that chronic toxicity is predicted by different variables for each species, although DOC and Pb speciation are crucial factors for *C. dubia* and *L. stagnalis*. The use of an MLR approach gave largely similar results to the BLM for these two species. In contrast, the MLR and BLM deviated largely for *P. rapida*, with the BLM largely ineffective at predicting toxicity in these organisms. As such, it may not be appropriate to extend the *C. dubia* BLM to all invertebrate species. Furthermore, the proposed MLR models modestly outperform the current iteration of the chronic Pb BLM for freshwater invertebrates when predicting toxicity for *C. dubia* and *L. stagnalis*. However, the proposed MLR models vastly outperform the BLM for *P. rapida*, and as such this technique shows promise as a complement to the BLM approach, especially for problematic species such as rotifers. Finally, these results give further evidence that the current reliance on water hardness alone for setting water quality standards for Pb is inappropriate.

Acknowledgments

Funding for the present work was provided by the International Lead and Zinc Research Organization (ILZRO). We also thank our collaborators who participated in water collection, including David Buchwalter, Greg Pyle, Jamie Hughes and Ning Wang. In addition, we are grateful for technical assistance provided by Charles Farmer. Terry Snell generously provided a starter culture of *Philodina rapida*.

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