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Multi-linear regression models predict the effects of water chemistry on acute lead toxicity to *Ceriodaphnia dubia* and *Pimephales promelas*

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A B S T R A C T

The current study examined the acute toxicity of lead (Pb) to *Ceriodaphnia dubia* and *Pimephales promelas* in a variety of natural waters. The natural waters were selected to range in pertinent water chemistry parameters such as calcium, pH, total CO_2 and dissolved organic carbon (DOC). Acute toxicity was determined for *C. dubia* and *P. promelas* using standard 48 h and 96 h protocols, respectively. For both organisms acute toxicity varied markedly according to water chemistry, with *C. dubia* LC50s ranging from 29 to 180 µg/L and *P. promelas* LC50s ranging from 41 to 3598 µg/L. Additionally, no Pb toxicity was observed for *P. promelas* in three alkaline natural waters. With respect to water chemistry parameters, DOC had the strongest protective impact for both organisms. A multi-linear regression (MLR) approach combining previous lab data and the current data was used to identify the relative importance of individual water chemistry components in predicting acute Pb toxicity for both species. As anticipated, the *P. promelas* best-fit MLR model combined DOC, calcium and pH. Unexpectedly, in the *C. dubia*MLR model the importance of pH, TCO_2 and calcium was minimal while DOC and ionic strength were the controlling water quality variables. Adjusted R^2 values of 0.82 and 0.64 for the *P. promelas* and *C. dubia* models, respectively, are comparable to previously developed biotic ligand models for other metals.

1. Introduction

For many aquatic organisms, toxicity to metals varies with water chemistry. This variance in toxicity is primarily the result of cations (H^+, Ca^{2+}, Na^+) competing with metal ions for active binding sites, and/or anions (OH^-, Cl^-, HCO_3^-) and other material (DOC, NOM) complexing with metal ions thereby limiting metal bioavailability. These types of interactions have been shown for many metals – including zinc (De Schamphelaere and Janssen, 2004), copper (Pagenkopf, 1983; Welsh et al., 1993; Erickson et al., 1996), cadmium (Meinelt et al., 2001) and silver(Bury, 1998; Erickson et al., 1998; Nichols et al., 2006; Bielmyer et al., 2008). More recently a great deal of effort has been invested in examining the effects of water chemistry on lead (Pb) toxicity in aquatic environments(Grosell et al., 2006; Mager et al., 2008, 2010b, 2011). Lead is a common environmental toxicant (WHO, 1995) and although many of the practises typically associated with anthropogenic Pb (i.e. lead based paint and gasoline) were phased out in the 1970s, Pb remains an environmental concern primarily due to mining and industrial processing.

Available evidence suggests that the primary water chemistry components affecting acute Pb toxicity are calcium (Ca^{2+}), carbonate alkalinity, pH and dissolved organic carbon (DOC).Both DOC and carbonates – whose presence is influenced by pH – are the primary parameters likely to complex with Pb in an aquatic environment and therefore alter Pb speciation away from the toxic ionic Pb^{2+}species (Grosell et al., 2006; Mager et al., 2008, 2010a,b). Conversely, calcium represents the primary competitor with bioavailable Pb species for active binding sites, or idealized biotic ligands (Jones, 1938; Macdonald et al., 2002; Rogers et al., 2003; Rogers and Wood, 2004). In fact, acute toxicity in rainbow trout (*Oncorhyncus mykiss*) is the result of hypocalcemia (Rogers et al., 2003). Additionally, H^+ has also been shown to interact competitively with Pb^{2+} in low pH waters (Mager et al., 2011). A recent study by Mager et al. (2011) systematically examined the effects of each of these constituents (i.e. calcium, alkalinity, pH and DOC) on acute toxicity in *Ceriodaphnia dubia* and *Pimephales promelas* for the purposes of BLM development, with all four parameters providing protection against toxicity to *P. promelas*. Furthermore, similar results were also obtained in 30 day *P. promelas* tests (Grosell et al., 2006; Mager et al., 2011). Interestingly, *C. dubia* exhibited a slightly different protection profile than *P. promelas*, with Ca^{2+} not affording any protection while pH, alkalinity and DOC all provided protection against Pb toxicity. As such the authors suggested it may not be appropriate to use a single freshwater Pb BLM for all aquatic organisms, owing to the potentially different mechanisms of toxicity between species (Mager et al., 2011).

The overall purpose of the recent work on Pb toxicity has been the development of a freshwater biotic ligand model (BLM), which determines the protective impact of an environment by systematically

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combining the complexation and competitive properties of water chemistry components (Di Toro et al., 2001; Paquin et al., 2002). However, the BLM is only one of many tools available for assessing impacts of water chemistry and in fact has met with some resistance, at least within the United States. While the BLM-based Cu water quality criteria (WQC) has been promulgated by the U.S. Environmental Protection Agency (USEPA, 2007), at this time no State has fully adopted this criteria as a water quality standard (WQS). One alternative to the BLM is a regression approach, which was originally used to develop hardness-based WQC for a number of divalent metals by USEPA and subsequently adopted as WQS by all States. While hardness is certainly an important water quality parameter with respect to predicting metal toxicity, as discussed above, a number of other water quality parameters are also of equal or greater importance. Development of multi-linear regression (MLR) models considering multiple water quality variables to predict metal toxicity has been previously evaluated for Cu (Erickson et al., 1996; De Schamphelaere et al., 2002; Rogevich et al., 2008) and USEPA has developed a multi-variable WQC for ammonia that includes temperature and pH as variables (USEPA, 1999). The current study was therefore undertaken in an effort to evaluate this approach for acute freshwater Pb toxicity to two model organisms, *P. promelas* and *C. dubia* using a combination of natural water data sets and previously generated single parameter manipulation data sets.

2. Materials and methods

2.1. Collection of natural waters

Natural waters were collected from 6 different sources from across North America (Table 1). The collection procedures varied by site; however, in all cases 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 was transferred to 100–200 L containers stored in a dark 4 °C room. This allowed for a homogenization of the chemical components within the individual 20 L aliquots of water. The natural waters were collected from the following locations: Green Cove Springs (Jacksonville, Florida, USA), Sweetwater Strand (Everglades, Florida, USA), French Lake (Thunder Bay District, Ontario, Canada), Edisto River (Colleton County, South Carolina, USA), Basin Creek (Wilkes County, North Carolina, USA), well water from the United States Geological Survey (USGS) Environmental Research Laboratory (Columbia, Missouri, USA). Additionally, a low pH water was made by diluting Columbia well water 20-fold 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* and *P. promelas* tests.

2.2. Water chemistry analysis

Analysis for Pb was performed using a graphite furnace atomic absorption spectrophotometer (AAS; Varian), with all samples being passed through a 0.45 μm Versapor membrane filter and acidified (1% HNO_3) prior to analysis. The detection limit for Pb using standard procedures was 1 μg/L. For nominal Pb concentrations less than 3 μg/L, a series of multiple injections were used to concentrate analyte mass. 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 and ionic strength was calculated as the sum of the charge equivalents based on molar concentrations. DOC specific absorbance and F (quality factor) were both calculated according to Richards et al. (2001), with absorbance measured at 350 nm using a plate spectrophotometer (Molecular Devices).

2.3. Toxicity testing with *P. promelas*

All tests and acclimations were performed in a temperature controlled environmental chamber (26 °C) with a 16:8 light/dark cycle. *P. promelas* (<24 h old) were obtained from Aquatic Biosystems (Fort Collins, CO, USA). All tests were based on the standard USEPA methods for acute toxicity testing using *P. promelas* (USEPA, 2002). For tests involving moderate/hard water, animals were immediately introduced to field waters, while a gradual acclimation was adopted for soft waters due to the greater degree of physiological stress. In all cases, animals were acclimated to full strength field water for at least 3 days prior to toxicity testing. Due to the limited volume of field water, a static renewal protocol was used for Pb toxicity testing for *P. promelas*. Prior to testing, individual Pb concentrations were made by spiking field/reference water with varying amounts of $Pb(NO_3)_2$ (CAS 10099-74-8), after which the solutions were allowed to equilibrate for approximately 24 h. A single replicate consisted of 10 fish in 500 ml of test solution held in a 1 L plastic beaker, with three replicates per treatment. Animals were fed brine shrimp for 1 h after 48 h of exposure, which was followed by an almost complete water change (≥95%). Previous work has shown feeding helps maintain control survival for static renewal field validation tests, especially with respect to low ionic

Table 1
Water chemistry composition of seven North American field waters sampled and two lab generated reference waters used for acute Pb toxicity testing on *Ceriodaphnia dubia* and *Pimephales promelas*. Natural organic material quality factors are described using the F value.

Site water			μM								Hardness
	pH	F	Na^+	Ca^{2+}	Mg^{2+}	K^+	Cl^-	SO_4^{2-}	TCO_2	DOC	(mg L^{-1})
Reference – *C. dubia*	7.69	0.72	517	230	67	35	592	45	560	133	30
Reference – *P. promelas*	7.51	0.68	606	244	77	37	492	49	338	146	32
Green Cove Spring, FL.	8.24	0.80	216	736	636	40	159	460	1338	73	137
Sweetwater Strand, FL.	8.72	0.40	373	1851	53	29	325	12	3750	577	191
Edisto River, SC	7.00	0.40	207	203	60	40	194	25	225	1244	26
French Lake, Ontario	7.30	0.42	103	76	40	15	64	18	145	583	12
USGS, Columbia, MO.	8.35	0.82	1177	1934	1044	72	774	417	5043	45	298
pH 5.5[a]	5.48	0.78	71	94	51	14	352	20	53	36	15
Basin Creek, NC.	6.69	0.73	63	24	19	7	21	15	59	100	4

[a] USGS, Columbia, MO diluted 20-fold and acidified with HCl.

strength site waters (Bielmyer et al., 2007) and the above method limited food in the test system to only 1 h during the test. Immediately prior to feeding, the brine shrimp were rinsed with deionised water to prevent the introduction of additional chemical components. Lead samples as well as pH and dissolved O_2(dO_2) measurements were taken at 0 and 96 h, as well as before and after the water change at 48 h. Samples for water chemistry were taken prior to feeding at 48 h, and after 96 h. Temperature was measured everyday and mortality was assessed at 96 h. Tests were deemed unreliable if control survival was less than 90%, and as such were repeated.

2.4. 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. *Ceriodaphnia dubia* were obtained from an in-house culture with a strain originally from Aquatic Biosystems (Ft. Collins, CO) and maintained in dechlorinated Miami-Dade tap water at 26 °C. All tests were based on the standard USEPA methods for acute toxicity testing using *C. dubia*(USEPA, 2002). For all tests, a water specific mass culture was started and maintained for 3 days. All mass cultures were fed a combination of yeast-trout chow-Cerophyl (YTC) and *Selenastrum capricornutum* (5 ml of each) daily. Individual Pb concentrations ($Pb_2(NO_2)_3$) were prepared 24 h in advance for acute testing. Acute test replicates comprised 5 neonates (<24 h old) held in 20 ml of solution, with a complete water change at 24 h. Each concentration consisted of 4 replicates, with mortality monitored daily for 48 h. Animals were not fed during acute testing. Lead samples as well as dO_2 and pH measurements were collected at the beginning and end of the test. Water samples were collected prior to the 24 h water change and temperature was monitored daily.

2.5. Data analyses

All statistical analyses were performed according to EPA guidelines for acute toxicity testing (USEPA, 2002). Curve fitting and hypothesis testing for response data were performed using ToxCalc software (version 5.0). Briefly, each test was assessed for no observable effective concentration (NOEC) and lowest observable effective concentration (LOEC) using either parametric or non-parametric analysis of variance (ANOVA) methods. All tests were subsequently assessed for LC50 values using either Maximum Likelihood Probit (preferred) or Trimmed Spearman–Karber analyses. Details regarding the exact statistical methods used for each test are stated in Table 3. Lead speciation calculations were performed using the freeware program Visual MINTEQ 3.0. All default settings were used and dissolved organic material (DOM) was modelled using the NICA-Donnan option (Milne et al., 2001, 2003). In all cases, DOM was assumed to contain 50% carbon (DOC) by mass, with a 65% fulvic acid composition. This is based on previous modelling studies on natural waters that showed assumptions of 60–70% fulvic acid composition were best (Tipping, 2002). For regression analyses data were log-transformed, with the exception of pH data. Toxicity endpoints were then regressed against all water chemistry parameters using step-wise, best subset, multi-linear regression approaches (SigmaStat 3.5). The best fit model was based upon adjusted R^2, with care taken to limit co-linearity of water chemistry parameters as judged by the variance inflation factor.

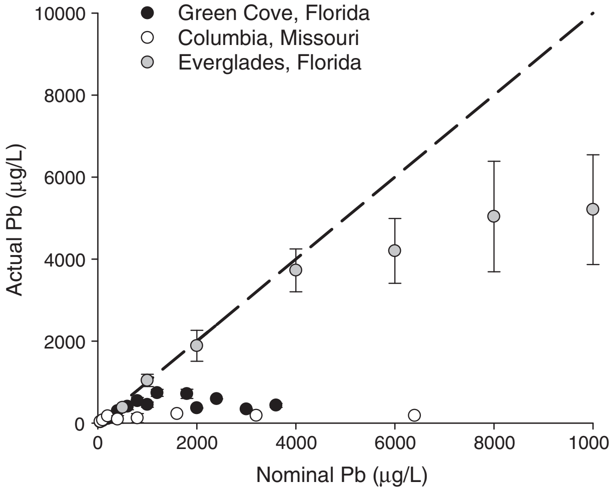

Fig. 1. The relationship between the dissolved and nominal Pb concentrations in three natural waters where acute dissolved Pb toxicity was not observed for *Pimephales promelas*. Values are mean values of 4–6 measurements over a 96 hour bioassay.

3. Results

3.1. Water chemistry

A relatively diverse array of water chemistries were used in the current study (Table 1), with primary chemical components of protection –

Table 2

Water chemistry parameters measured in lead toxicity tests on *Pimephales promelas* and *Ceriodaphnia dubia*. Values for *P. promelas* are mean ± SEM of four samples taken throughout the test, while *C. dubia* values ion values are single sample measurements and pH is mean ± SEM of initial and final measurements.

Site water		µM						
	pH	Na^+	Ca^{2+}	Mg^{2+}	Cl^-	SO_4^{2-}	TCO_2	DOC
P. promelas								
Reference	7.55 ± .02	667 ± 68	251 ± 12	75 ± 2	704 ± 143	52 ± 2	352	182
Green Cove	8.2 ± .02	230 ± 12	776 ± 30	679 ± 37	133 ± 22	468 ± 22	1526 ± 82	73 ± 9
Sweetwater Strand	8.53 ± 0.01	378 ± 16	1891 ± 39	78 ± 4	274 ± 22	13 ± 1	3662 ± 368	599 ± 17
Edisto River	7.08 ± 0.02	268 ± 38	195 ± 2	65 ± 3	194 ± 2	25 ± 1	212 ± 29	1321 ± 11
USGS, Columbia	8.44 ± 0.03	1253 ± 80	1020 ± 290	1185 ± 55	920 ± 7	487 ± 11	2920 ± 412	64 ± 7
pH 5.5	5.67 ± 0.09	165 ± 32	101 ± 5	58 ± 2	429 ± 57	25 ± 3	5 ± 5	53 ± 2
Basin Creek	6.71 ± 0.02	98 ± 30	23 ± 1	25 ± 4	64 ± 33	20 ± 3	75 ± 8	105 ± 1
C. dubia								
Reference	7.27 ± 0.02	640	295	86	767	71	418	219
Green Cove	8.20 ± 0.02	264	888	782	240	606	1724	112
Sweetwater Strand	8.58 ± 0.01	401	2157	98	393	22	3824	653
Edisto River	7.32 ± 0.04	230	218	60	250	25	270	1467
French Lake	7.18 ± 0.01	108	99	58	115	20	160	622
USGS, Columbia	8.36 ± 0.02	1475	1152	1126	936	492	3525	137
pH 5.5	5.71 ± 0.05	78	106	58	408	21	0	50
Basin Creek	6.78 ± 0.03	81	32	26	72	19	68	104

Table 3
Ceriodaphnia dubia and *Pimephales promelas* toxicity values (μg/L dissolved lead) and water effect ratios (WER) for test waters. Statistical tests are denoted in the table footnote.

Organism	Site water	Pb (μg/L)				LC50 WER
		LC50	95% CI	NOEC	LOEC	
C. dubia	Reference[a, e]	100.1	82.1–122	41.1	100.7	1
	Green Cove Spring[a, e]	435.4	389.4–486.9	295.1	540.5	4.35
	Sweetwater Strand[a, d]	995.6	747.5–1258.1	378.4	1039.1	9.94
	Edisto River[c, e]	1179.6	968.2–1437.1	<984.8	984.8	11.28
	French Lake[b, d]	290.3	253.4–314.1	<281.7	281.7	2.90
	USGS, Columbia[b, d]	107.6	73.1–180.6	34.9	71.8	1.08
	pH 5.5[b, e]	73.5	45.1–119.1	36.2	75.8	0.73
	Basin Creek[a, d]	28.8	23.1–36.6	18.1	52.2	0.29
P. promelas	Reference[a, d]	622.3	530.6–709.2	<388.6	388.6	1
	Edisto River[b, e]	3597.9	3261.7–3968.8	2271	5476.5	5.78
	pH 5.5[b, e]	40.7	32.1–48.2	13.6	42.2	0.07
	Basin Creek[b, d]	68.1	58.7–79.1	35.9	77.2	0.11

[a] Steel's many-one rank hypothesis test.
[b] Dunnett's hypothesis test.
[c] Heteroscedastic *t*-test.
[d] Maximum likelihood Probit point estimate.
[e] Trimmed Spearman–Karber point estimate.

calcium, pH, total CO_2, and DOC – ranging from 24–1934 μM, 5.5–8.7, 53–5043 μM and 36–1244 μM, respectively. The calculated hardness of the various waters ranged from 4 to 298 mg/L. The quality of the natural organic material of which DOC comprises in the natural waters was assessed by specific absorbance (F, Table 1), with a range from 0.4 to 0.82. Interestingly the natural waters with higher concentrations of DOC also had lower quality DOC with respect to aromaticity and metal binding capabilities, as indicated by a lower F value (Richards et al., 1999). The exact values measured in toxicity tests for both organisms are shown in Table 2, with little deviation from pre-test measurements. The water chemistry values in Table 2 were used for all subsequent models and analysis.

3.2. Effects of Pb exposure – *P. promelas*

Acute Pb toxicity tests were successfully completed in 4 of 8 waters. A successful test could not be performed in water from French Lake due to persistent control group mortality. Three waters (Green Cove Springs, Sweetwater Strand and Columbia) showed no mortality as a consequence of acute exposure up to the dissolved Pb solubility limits which are depicted in Fig. 1. Calculated acute dissolved Pb LC50s, NOECs, LOECs and water effect ratios (WERs) for *P. promelas* are shown in Table 3, while acute LC50 Pb speciation estimates in the respective waters are shown in Table 4. The acute dissolved Pb LC50 ranged from 40 to 3597 μg/L, with the lowest LC50 occurring in the pH 5.5 water and the highest LC50 occurring in the water from the Edisto River. When primary water chemistry components were evaluated individually, only DOC concentration correlated well with acute dissolved Pb toxicity (Fig. 2a–d). When LC50s were expressed on the basis of Pb^{2+}, calcium concentration also correlated acute toxicity (Fig. 2e, f).

The protective effects of calcium on acute dissolved Pb toxicity were more fully examined by combining the natural water data set with previously collected lab data sets for acute Pb toxicity in *P. promelas* (Mager et al., 2011). Interestingly, there was a strong relationship between calcium and Pb^{2+} LC50 when only acidic or neutral pH environments were included, as depicted by the observed versus predicted plot in Fig. 3. The lone available alkaline data point did not conform to the regression, with an observed acute Pb^{2+} LC50 approximately 30-times higher than would be predicted based on data collected from neutral/acidic environments.

3.3. Effects of Pb exposure – *C. dubia*

Successful acute Pb toxicity tests were performed in all 8 natural waters for *C dubia*. Calculated acute dissolved Pb LC50s, NOECs, LOECs and WERs for *C. dubia* are shown in Table 3, while acute LC50 Pb speciation estimates in the respective waters are shown in Table 4. The acute dissolved Pb LC50s ranged from 28 to 1129 μg/L, with the lowest LC50 occurring in the Basin Creek water and the highest occurring in the water from the Edisto River. Of the primary water chemistry components thought to affect acute dissolved Pb toxicity, only DOC seemed to correlate well with acute Pb toxicity (Fig. 4), and no obvious correlation was apparent between calcium concentration and ionic Pb^{2+} toxicity (Fig. 4e, f).

3.4. Multi-linear regressions

A series of multi-linear regression (MLR) analyses were performed in an effort to more fully examine the effects of varying water chemistry on acute dissolved Pb toxicity. For these analyses, the data sets combined the field water data with previously generated lab data (Mager et al., 2011). The data sets for each species were assessed independently to allow for examination of likely physiological differences and possible disparities in toxicity mechanisms between species. For *P. promelas*, the best-fit MLR model combined three of the four factors previously shown to affect acute Pb toxicity – calcium, pH and DOC (Fig. 5A). The best-fit MLR model had relatively high predictive power (adjusted $R^2 = 0.82$), with all three parameters significantly contributing to the model ($P \leq 0.001$). Also note that toxicity predictions based on this model would have under protected in only one of 23 test waters, based on commonly used ±2-fold error

Table 4
Predicted concentration (μg/L) of major lead chemical species in natural water bioassays for *Ceriodaphnia dubia* and *Pimephales promelas*. Percent of total dissolved lead abundance denoted in parenthesis. Total organic fraction indicates the sum of humic acid and fulvic acid organic species.

		Pb^{2+}	$PbOH^+$	$PbCO_3^{2-}$	$PbCO_3$	Total organic
C. dubia	Reference	1.0 (1.1)	0.4 (0.4)	0 (0)	1.0 (1.1)	97 (97)
	Green Cove Spring	4.6 (1.1)	14.8 (3.4)	5.1 (1.4)	130.1 (29.9)	274 (63)
	Sweetwater Strand	0.6 (0.06)	4.7 (0.5)	16.7 (1.7)	84.6 (8.5)	887 (89)
	Edisto River	2.1 (0.19)	1.0 (0.09)	0 (0)	1.5 (0.14)	1123 (99)
	French Lake	1.0 (0.36)	0.3 (0.13)	0 (0)	0.3 (0.12)	264 (91)
	USGS, Columbia	0.1 (0.21)	0.7 (0.9)	1.4 (1.8)	12.3 (15.7)	63 (80)
	pH 5.5	40.4 (54.8)	0.4 (0.65)	0 (0)	0 (0)	31 (43)
	Basin Creek	0.7 (2.7)	0.1 (0.39)	0 (0)	0.1 (0.16)	27 (96)
P. promelas	Reference	17.4 (2.8)	13.7 (2.2)	0 (0)	27.4 (4.4)	558 (89)
	Edisto River	35.5 (0.98)	9.8 (0.27)	0 (0)	12.2 (0.34)	3534 (98)
	pH 5.5	19.4 (47.6)	0.2 (0.51)	0 (0)	0.01 (0.01)	20 (50)
	Basin Creek	3 (4.4)	0.3 (0.54)	0 (0)	0.1 (0.24)	64 (94)

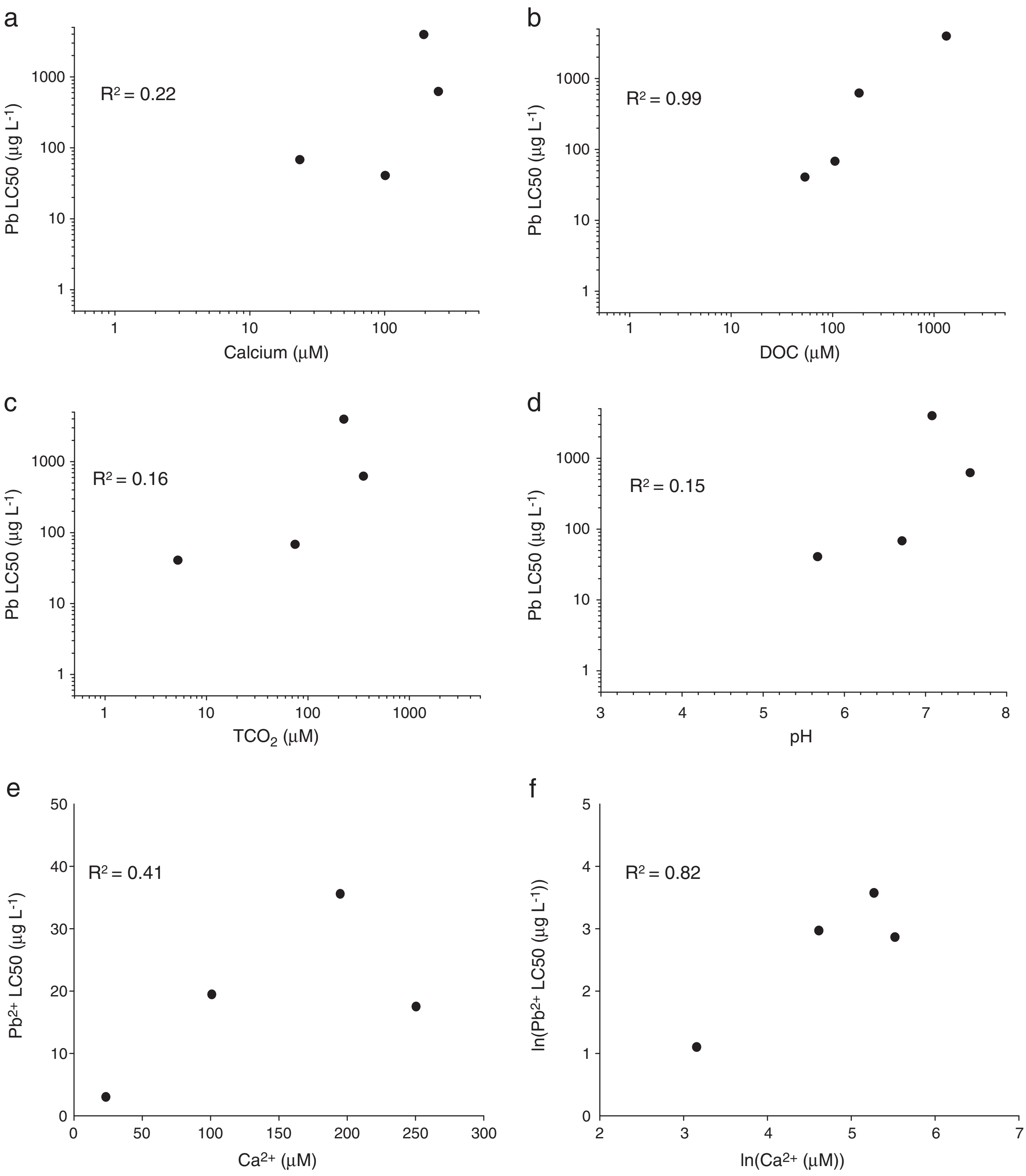

Fig. 2. Regression analyses representing the relationship of varying natural water chemistry parameters and acute dissolved Pb/Pb^{2+} toxicity in *Pimephales promelas* ($N=4$). a) calcium, b) dissolved organic carbon, c) total CO_2, d) pH, e) ionic Pb^{2+} versus calcium, f) natural log transformation of ionic Pb^{2+} versus calcium.

criterion (Di Toro et al., 2001). Replacing pH with TCO_2 in the best-fit model resulted in a slight decrease in adjusted R^2 from 0.82 to 0.81 (data not shown). This is consistent with the high degree of covariance in these two parameters as a consequence of the buffering properties of CO_3^{2-}/HCO_3^-.

The best-fit MLR (adjusted $R^2=0.63$) for *C. dubia* was based on DOC and ionic strength, with both factors contributing significantly to the predictive power of the model ($P \leq 0.003$; Fig. 5b). Overall the *C. dubia* MLR was not as strong as that of *P. promelas*, and would under protect in 3 of 25 test waters. The relationship between ionic strength and acute dissolved Pb toxicity is shown in Fig. 6.

4. Discussion

Generally *P. promelas* was more resistant than *C. dubia* to acute Pb exposure in the various test waters, which is in agreement with previous studies (Mager et al., 2011). The absence of *P. promelas* mortality in all of the test waters was also of interest. Specifically, no mortality was observed in test water from Sweetwater Strand, Green Cove Spring and USGS, Columbia. This is likely due to the limited solubility of Pb in these waters (Fig. 1) versus the amount of protective water chemistry components. These three waters have the highest measured levels of TCO_2 and pH – the primary factors that

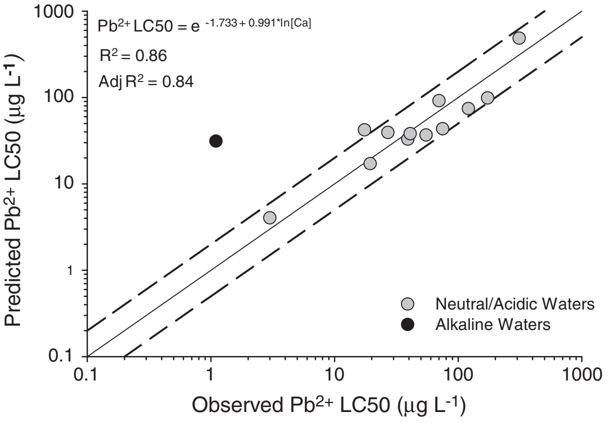

Fig. 3. Observed versus predicted effects plot depicting the relationship between calcium concentration and acute ionic Pb^{2+} toxicity as a consequence of environmental pH in *Pimephales promelas* (acidic /neutral pH, $N=12$; alkaline pH, $N=1$). The predicted effects were determined based on linear regression analysis of calcium and acute ionic Pb^{2+} toxicity in acidic/neutral pH environments.

affect Pb solubility – while also having high concentrations of DOC and/or calcium. Additionally, a successful test could not be performed in the French Lake water due to repeatedly low control survival; likely a product of physiological stress relating to the low ion content combined with high DOC.

Of the three field waters where LC50s could be calculated for *P. promelas*, only one (Edisto River) was protective relative to the reference water (Table 3), while in the others waters LC50s were approximately 10-times lower. When the primary water chemistry components are examined individually (Fig. 2a–d) only DOC seems to correlate with acute dissolved Pb toxicity; however, the variance in multiple parameters within a single test water and the small sample size make it difficult to draw any conclusions in this regard. To more properly address the potential protective effects of calcium – a Pb competitor for ligands – speciation calculations were performed for each bioassay to estimate the amounts of ionic Pb^{2+} at the LC50 for each test (Table 4).Regression analyses of the speciation data support a competition based protection of calcium against Pb^{2+} (Fig. 2e,f). This is further highlighted when the current data are combined with previous data sets examining the effects of calcium and pH on acute dissolved Pb toxicity of *P. promelas* (Mager et al., 2011). A well supported regression of calcium and acute Pb^{2+} toxicity ($R^2=0.86$) was obtained when combining all the bioassays performed in physiologically neutral or acidic waters (Fig. 3). Interestingly, the only data available from alkaline environments did not conform to this regression, with 30-fold higher sensitivity than predicted. It should be noted that all the lab data, including this alkaline point, is generated from the same base water. When calcium and pH were combined using a multi-linear approach this alkaline data point was not reconciled. Within the acidic/neutral water data set pH had no predictive impact, either alone or in combination with calcium (data not shown). It should also be noted that the presence of $PbOH^+$ species does not explain the higher toxicity in the alkaline water bioassay, as several bioassays have higher estimated amounts of $PbOH^+$. These data suggest that calcium may only be protective in physiologically neutral/acidic environments, or at the very least conforms to a different protective profile than in alkaline environments. Furthermore these analyses provide no evidence for competitive protection by protons, which was suggested by Mager et al. (2011). This is not to say that proton competition does not occur, but that any actual effect is minimized versus the competitive effects of calcium.

As expected, *C. dubia* was the more sensitive organism to acute dissolved Pb exposure, and sensitivity varied greatly depending on water chemistry (Table 3). With respect to individual water chemistry components, only DOC correlated well with protection against acute Pb exposure in *C. dubia* ($R^2=0.76$; Fig. 4b). Of the four test waters that provided relative protection against acute dissolved Pb exposure versus the base water, three contained considerably more DOC than the reference water, while the two less protective waters both contained less DOC than the reference water. This agrees with previous data showing that increasing environmental DOC leads to an increase in organic Pb species while decreasing the concentration of Pb^{2+} (Mager et al., 2010a, 2011); a result corroborated by Pb speciation estimates from the natural waters (Table 4). The other main chemical components used by the BLM to predict acute Pb toxicity – calcium, total CO_2 and pH – appear to show no correlation with acute dissolved Pb toxicity (Fig. 4a,c,d); however, simultaneous variability in multiple parameters makes it difficult to make any conclusions in this regard. Additionally, there is no protective effect of calcium on Pb^{2+} toxicity (Fig. 4e,f). This is in contrast to *P. promelas*, but supports previous work on the effects of calcium on acute Pb toxicity in *C. dubia* (Mager et al., 2011). As such it seems likely that the mechanism of acute toxicity in *C. dubia* is not hypocalcemia as found for freshwater fish (Rogers et al., 2003); however this remains to be tested.

Interestingly, C. *dubia* were more resistant to acute Pb toxicity than *P. promelas* in the pH 5.5 water, both with respect to dissolved and ionic Pb (Tables 3 and 4). Although an explanation for this is not immediately clear, it is possible that the low pH water represented a greater physiological stress to *P. promelas*. While pH 5.5 does not induce mortality in *P. promelas* in 96 h, it has been previously demonstrated that 9 d exposure at this pH results in 100% mortality (Mager et al., 2011). This stress may lead to greater sensitivity to other stresses, such as acute Pb exposure. This finding may also be indicative of different mechanisms of toxicity between organisms, leading to different relative Pb sensitivities as a factor of water chemistry. Finally, protons may also provide a greater degree of competitive protection for *C. dubia* than for *P. promelas*. Unfortunately, the current data set is not suited to test this possibility because of the strong directing effect that the single low pH point has on any regression. Since the available data for *C. dubia* skew largely toward neutral/alkaline waters, future efforts should be directed at examining the effects of low pH on acute dissolved/ionic Pb toxicity to ensure proper modelling predictions in all environments.

A final set of analyses used a multi-linear regression (MLR) approach on a data set that combines natural water and laboratory water data for both *C. dubia* and *P. promelas.* Relatively well supported MLR models were obtained for both species (Fig. 5a, b). As expected, the best-fit MLR model for *P. promelas* consisted of pH, DOC and calcium, and would protect in 22 of 23 tested waters. In this model DOC and pH represent parameters that act to limit Pb^{2+}, while calcium directly competes with these Pb^{2+} species. Interestingly, this model is able to overcome the pH dependent calcium protection profiles discussed above, likely due to the emphasis placed on the complexation components. In fact, the competitive interactions of calcium account for only 16–32% of the protective effects of water chemistry in the modelled test waters, as calculated from the multi-linear regression model formula.

In contrast to *P. promelas*, the *C. dubia* model did not conform to expectation with regard to TCO_2 and pH, nor did it perform as well as that for *P. promelas* as evidenced by the lower R^2 (Fig. 5b). The relatively poor performance of the *C. dubia* MLR model may be in part due to the centralized distribution of the data, with only one third of the data falling outside of the central group. Nonetheless, the best-fit MLR model for *C. dubia* would protect in 22 of the 25 modelled test waters. The water chemistry components isolated by the best-fit MLR model for *C. dubia* were DOC andionic strength, with DOC responsible for 47–63% of the protection from water chemistry in the modelled waters, as calculated from the multi-linear regression model. This

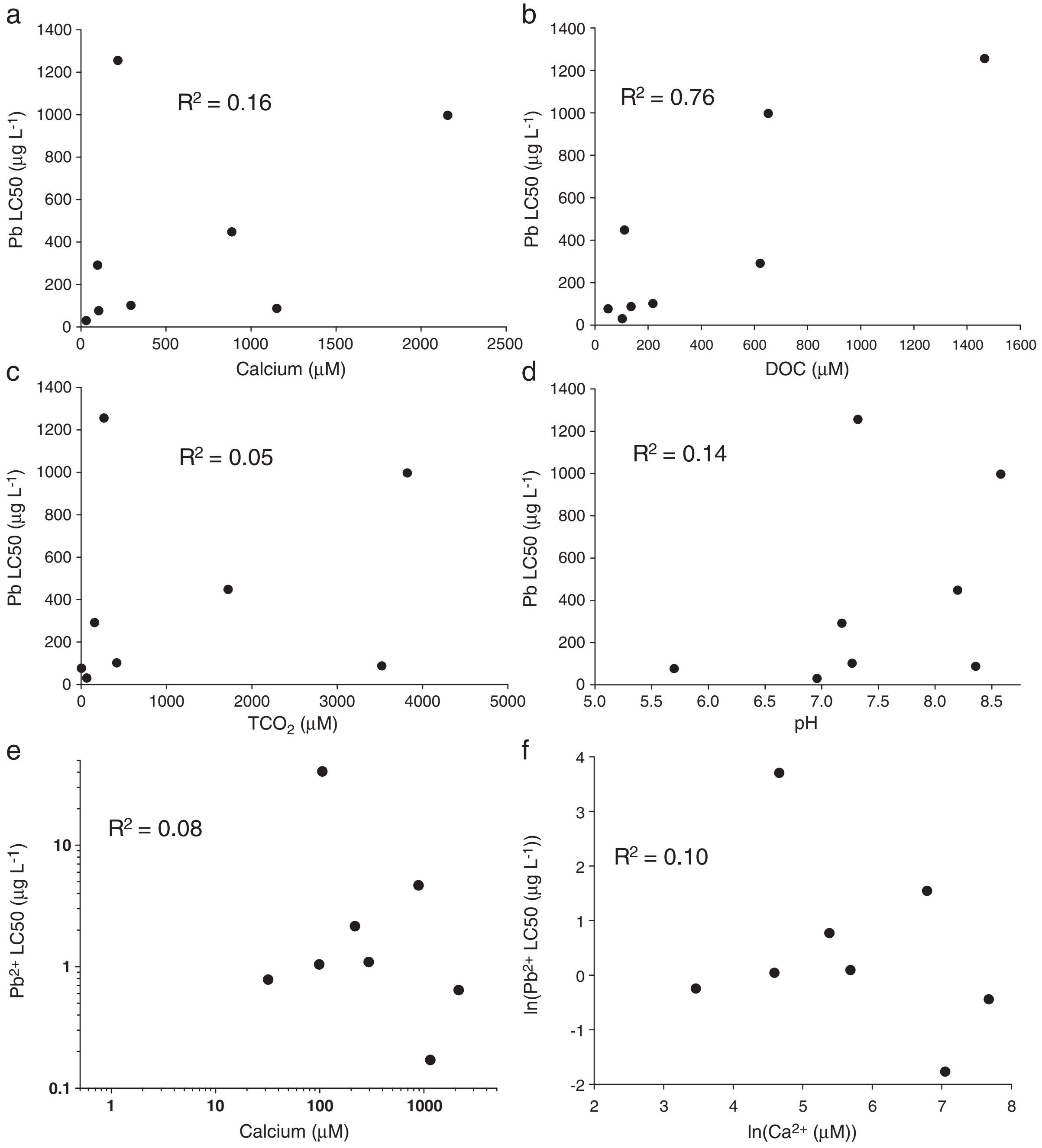

Fig. 4. Regression analyses representing the relationship of varying natural water chemistry parameters and acute dissolved Pb/Pb^{2+} toxicity in *Ceriodaphnia dubia* ($N = 8$). a) calcium, b) dissolved organic carbon, c) total CO_2, d) pH, e) ionic Pb^{2+} versus calcium, f) natural log transformation of ionic Pb^{2+} versus calcium.

suggests that much of the protection from water chemistry is based simply on the overall ion richness of the environment. Interestingly, ionic strength alone shows a much better correlation ($P \leq 0.005$) with acute Pb toxicity than any individual ion parameter (Fig. 6), and is only outperformed by DOC.

An explanation for the ionic strength effect is not immediately apparent; however, it seems likely that it relates to osmoregulatory and water transport physiology. Previous studies with Ag and Cu have also shown ionic strength to be an important water quality metric with respect to predicting metal toxicity. In particular these studies have shown that in very low ionic strength water the various BLMs lose their predictive ability presumably because standard test organisms are under osmoregulatory stress in these waters (Bielmyer et al., 2007; Ryan et al., 2009; Ng et al., 2010). Organisms in freshwater environments constantly loseosmolytes to the surrounding environment, while taking up water. This is enhanced in small organisms, such as *C. dubia*, due to a high surface area to volume ratio. Freshwater organisms therefore have to actively transport ions against their concentration gradient to maintain homeostatic levels. In fact, acute Pb toxicity in freshwater fish is due to a disturbance in calcium homeostasis, hypocalcemia (Rogers et al., 2003), explaining the competitive interactions of calcium and Pb for biotic ligand binding sites in *P. promelas*. Interestingly, the lack of a specific ion effect combined with the protective impact of overall ionic strength suggests that the mechanism of acute Pb toxicity in *C. dubia* is a more general osmoregulatory disturbance that may simultaneously affect multiple ions. It should also be noted that it is difficult to rule out an ionic strength effect on *P. promelas* toxicity due to the lack of

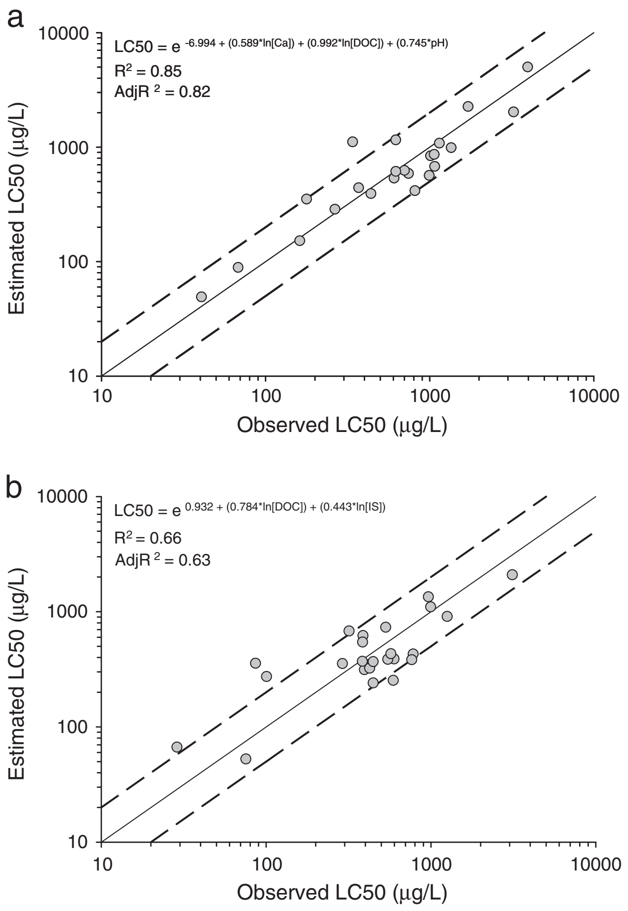

Fig. 5. Observed effect versus predicted effect regression plots for multi-linear regression models of water chemistry and acute Pb toxicity for (a) *Pimephales promelas* ($N = 23$), and (b) *Ceriodaphnia dubia* ($N = 25$). Model formula denoted in the figure. The solid line represents equal observed versus predicted toxicity, while the dotted lines represent ± 2-fold observed versus predicted toxicity.

mortality in high ionic strength waters; however, the strength of the current model and the obvious calcium effects suggest any such effect would be of minimal importance. Further efforts should be dedicated to investigating any possible influence that ionic strength may have on acute *C. dubia* Pb toxicity, and how this information can be used in a BLM framework.

Two findings from this approach should have a direct impact on our understanding of how water chemistry can affect acute Pb toxicity. First, it is apparent from these two models that the effects of water chemistry on acute Pb toxicity differ depending on the organism in question. This point was first raised by Mager et al. (2011) when discussing the lack of protection afforded by increasing levels of calcium in acute *C. dubia* bioassays, and may now also be extended to TCO_2 and possibly pH. The lack of a pH effect in the *C. dubia* MLR is surprising and may be the result of an absence of controlled pH experiments in the *C. dubia* data set, but which is present in the *P. promelas* data set. The obvious effects of pH on Pb speciation would suggest pH must be involved in determining acute Pb toxicity, although, if additional Pb species (e.g., $PbOH^+$) are toxic to *C. dubia* then little or no pH effect on Pb toxicity would be expected. Clearly effort should be dedicated to examining the effects of pH on acute *C. dubia* Pb toxicity. It should be pointed out that the lack of protection afforded by TCO_2 and pH in the current *C. dubia* model pertains only to the toxic effects of dissolved Pb species, since both pH and TCO_2 will affect the solubility of total Pb in aquatic environments. Ultimately, these analyses provide further evidence that the practise of using a single model – MLR, BLM or otherwise – to predict Pb toxicity in all aquatic organisms in a given environment may not be appropriate.

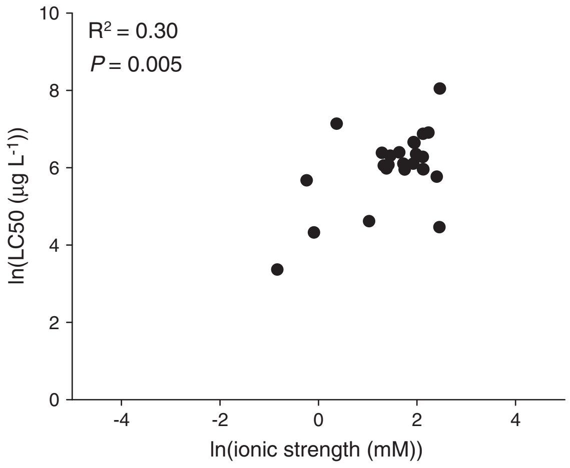

Fig. 6. Regression analysis representing the relationship between ionic strength and acute Pb toxicity in *Ceriodaphnia dubia* ($N = 25$).

Finally, although it is not possible to directly compare the predictive power of the respective MLR models to that of the BLM – as acute Pb BLMs have not yet been developed – the MLRs from the current study are comparable to the predictive capability of acute BLMs developed for other metals (Di Toro et al., 2001; Santore et al., 2002). As such, further development of MLRs to improve our understanding of how water quality variables affect metal toxicity and possibly as an alternative regulatory tool to the BLM shows promise.

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References

Bielmyer, G.K., Brix, K.V., Grosell, M., 2008. Is Cl^- protection against silver toxicity due to chemical speciation? Aquat. Toxicol. 87, 81–87.

Bielmyer, G.K., Grosell, M., Paquin, P.R., Mathews, R., Wu, K.B., Santore, R.C., Brix, K.V., 2007. Validation study of the acute biotic ligand model for silver. Environ. Toxicol. Chem. 26, 2241–2246.

Bury, N.R., 1998. Effects of chloride, calcium, and dissolved organic carbon on silver toxicity: comparison between rainbow trout and fathead minnows. Environ. Toxicol. Chem. 56–62.

De Schamphelaere, K.A., Heijerick, D.G., Janssen, C.R., 2002. Refinement and field validation of a biotic ligand model predicting acute copper toxicity to *Daphnia magna*. Comp. Biochem. Physiol. C 133, 243–258.

De Schamphelaere, K.A.C., Janssen, C.R., 2004. Bioavailability and chronic toxicity of zinc to juvenile rainbow trout (*Oncorhynchus mykiss*): comparison with other fish species and development of a bioticx ligand model. Environ. Sci. Technol. 6201–6209.

Di Toro, D.M., Allen, H.E., Bergman, H.L., Meyer, J.S., Paquin, P.R., Santore, R.C., 2001. Biotic ligand model of the acute toxicity of metals. 1. Technical basis. Environ. Toxicol. Chem. 20, 2383–2396.

Erickson, R.J., Benoit, D.A., Mattson, V.R., Nelson Jr., H.P., Leonard, E.N., 1996. The effects of water chemistry on the toxicity of copper to fathead minnows. Environ. Toxicol. Chem. 181–193.

Erickson, R.J., Brooke, L.T., Kahl, M.D., Venter, F.V., Harting, S.L., Markee, T.P., Spehar, R.L., 1998. Effects of laboratory test conditions on the toxicity of silver to aquatic organisms. Environ. Toxicol. Chem. 572–578.

Genz, J., Taylor, J.R., Grosell, M., 2008. Effects of salinity on intestinal bicarbonate secretion and compensatory regulation of acid-base balance in *Opsanus beta*. J. Exp. Biol. 211, 2327–2335.

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

Hill, A.G., 1973. Acid-base balance; chemistry, physiology, pathophysiology. Williams and Wilkins, Baltimore.

Jones, J.R.E., 1938. The relative toxicity of salts of lead, zinc and copper to the stickleback (*Gasterosteus aculeatus* L.) and the effect of calcium on the toxicity of lead and zinc salts. J. Exp. Biol. 15, 394–407.

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

Mager, E.M., Brix, K.V., Gerdes, R.M., Ryan, A.C., Grosell, M., 2010a. Effects of water chemistry on the chronic toxicity of lead to the cladoceran, *Ceriodaphnia dubia*. Ecotoxicol. Environ. Saf.

Mager, E.M., Brix, K.V., Grosell, M., 2010b. Influence of bicarbonate and humic acid on effects of chronic waterborne lead exposure to the fathead minnow (*Pimephales promelas*). Aquat. Toxicol 96, 135–144.

Mager, E.M., Esbaugh, A.J., Brix, K.V., Ryan, A.C., Grosell, M., 2011. Influences of water chemistry on the acute toxicity of lead to *Pimephales promelas* and *Ceriodaphnia dubia*. Comp. Biochem. Physiol. C 153, 82–90.

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

Meinelt, T., Playle, R.C., Pietrock, M., Burnison, B.K., Wienke, A., Steinberg, C.E.W., 2001. Interaction of cadmium toxicity in embryos and larvae of zebrafish (*Danio rerio*) with calcium and humic substances. Aquat, Toxicol.

Milne, C.J., Kinniburgh, D.G., Tipping, E., 2001. Generic NICA-Donnan model parameters for proton binding by humic substances. Environ. Sci. Technol. 35, 2049–2059.

Milne, C.J., Kinniburgh, D.G., van Riemsdijk, W.H., Tipping, E., 2003. Generic NICA-Donnan model parameters for metal-ion binding by humic substances. Environ. Sci. Technol. 37, 958–971.

Ng, T.Y., Chowdhury, M.J., Wood, C.M., 2010. Can the biotic ligand model predict Cu toxicity across a range of pHs in softwater-acclimated rainbow trout? Environ. Sci. Technol. 44, 6263–6268.

Nichols, J.W., Brown, S., Wood, C.M., Walsh, P.J., Playle, R.C., 2006. Influence of salinity and organic matter on silver accumulation in Gulf toadfish (*Opsanus beta*). Aquat. Toxicol. 78, 253–261.

Pagenkopf, R.K., 1983. Gill surface interaction model for trace-metal toxicity to fishes: role of complexation, pH, and water hardness. Environ. Sci. Technol. 342–347.

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

Richards, J.G., Burnison, B.K., Playle, R.C., 1999. Natural and commercial dissolved organic matter protects against the physiological effects of a combined cadmium and copper exposure on rainbow trout (*Oncorhynchus mykiss*). Can. J. Fish. Aquat. Sci. 56, 407–418.

Richards, J.G., Curtis, P.J., Burnison, B.K., Playle, R.C., 2001. Effects of natural organic matter source on reducing metal toxicity to rainbow trout (Oncorhynchus mykiss) and on metal binding to their gills. Environ. Toxicol. Chem. 20, 1159–1166.

Rogers, J.T., Richards, J.G., Wood, C.M., 2003. Ionoregulatory disruption as the acute toxic mechanism for lead in the rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 64, 215–234.

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

Rogevich, E.C., Hoang, T.C., Rand, G.M., 2008. The effects of water quality and age on the acute toxicity of copper to the Florida apple snail, *Pomacea paludosa*. Arch. Environ. Contam. Toxicol. 54, 690–696.

Ryan, A.C., Tomasso, J.R., Klaine, S.J., 2009. Influence of pH, hardness, dissolved organic carbon concentration, and dissolved organic matter source on the acute toxicity of copper to *Daphnia magna* in soft waters: implications for the biotic ligand model. Environ. Toxicol. Chem. 28, 1663–1670.

Santore, R.C., Mathew, R., Paquin, P.R., DiToro, D., 2002. Application of the biotic ligand model to predicting zinc toxicity to rainbow trout, fathead minnow, and *Daphnia magna*. Comp. Biochem. Physiol. C 133, 271–285.

Tipping, E., 2002. Cation binding by humic substances. In: Campbell, P.G.C., Harrison, R.M., de Mora, S.J. (Eds.), Cambridge Environmental Chemistry Series. Cambridge University Press, New York.

USEPA, 1999. Update of ambient water quality criteria for ammonia. United States Environmental Protection Agency EPA-822-R-99-014.

USEPA, 2002. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms, United States Environmental Protection Agency EPA-821-R-02-0125th ed.

USEPA, 2007. Update of ambient water quality criteria for copper. United States Environmental Protection Agency EPA-822-F-07-001.

Welsh, P.G., Skidmore, J.F., Spry, D.J., Dixon, D.G., Hodson, P.V., Hutchinson, N.J., Hickie, B.E., 1993. Effects of pH and dissolved organic carbon on the toxicity of copper to larval fathead minnow (*Pimephales promelas*) in natural lake waters of low alkalinity. Can. J. Fisher. Aquat, Sci. 50.

WHO, 1995. Environmental Health Criteria 165: International Programme on Chemical Safety. World Health Organization.