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DEVELOPMENT AND VALIDATION OF A BIOTIC LIGAND MODEL FOR PREDICTING CHRONIC TOXICITY OF LEAD TO *CERIODAPHNIA DUBIA*

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Abstract: While it is increasingly being recognized that biotic ligand models (BLMs) are valuable in the risk assessment of metals in aquatic systems, the development of chronic BLMs has been less advanced for lead than for other metals. The authors investigated the univariate effects of Ca and pH on the chronic reproductive toxicity of Pb to *Ceriodaphnia dubia* at 4 levels. Calcium influenced chronic Pb toxicity to *C. dubia* only to a relatively small extent, whereas a high pH (8.2) provided strong protection against Pb toxicity (compared with lower pH levels). Based on this data set, a chronic Pb BLM for *C. dubia* was developed. The effect of pH was modeled as a single biotic ligand site competition by H^+ with a log stability constant for binding of H^+ to the biotic ligand (K_{HBL}) of 7.6, while no other competitive constants were needed. The developed BLM was shown, in an independent validation with 3 other data sets, to be capable of predicting chronic Pb toxicity to different clones of *C. dubia* by an error of less than a factor of 2 in most synthetic and natural waters considered. The results add to the growing evidence that BLM–based risk assessment or water-quality criteria for Pb are likely to be more appropriate relative to hardness-based assessments or criteria. *Environ Toxicol Chem* 2014;33:394–403. © 2013 SETAC

Keywords: Metal bioavailability Biotic ligand model Risk assessment Water-quality criteria *Daphnia*

INTRODUCTION

Lead is a nonessential metal that can be harmful to aquatic organisms even at low concentrations [1–3]. As with many other metals, the bioavailability and consequently the toxicity of Pb to freshwater organisms is dependent on the physicochemical characteristics of the water [3–5]. The BLM provides a framework to account for the influence of water chemistry on metal toxicity. The BLM concept starts from the principle that the toxicity of a metal is dependent on the concentration of the metal bound to the biotic ligand, that is, a receptor at the cell surface, and the activity of certain cations (e.g., H^+, Ca^{2+}), which compete with the metal ion for binding sites at the biotic ligand [6]. When the concentration of metal bound to the biotic ligand transcends a certain critical concentration, a toxic effect will occur.

In the last decade, chronic BLMs were developed for several metals including Cu [7], Zn [8], and Ni [9]. Furthermore, BLMs are becoming more integrated into risk-assessment procedures in regions such as Europe [10] and the United States [11], but efforts to develop chronic Pb BLMs were until now limited. Recently, a preliminary chronic Pb BLM for *Ceriodaphnia dubia* was developed [2]. This preliminary BLM was based on the data of Mager et al. [3] and a study conducted by Parametrix [12]. However, these studies could not unambiguously establish the individual effects of Ca and pH as the effects differed between them. First, Mager et al. [3] did not find a protective effect of $CaSO_4$ additions on chronic Pb toxicity to *C. dubia*. They also found that pH affected Pb toxicity, but they noted that their results may have been confounded by the addition of the pH buffer 3-(*N*-morpholino)propanesulfonic acid (MOPS) [13]. In contrast, Parametrix [12] reported that increased ambient Ca reduced chronic Pb toxicity to *C. dubia*. However, this increase of Ca was accompanied by increases of pH and alkalinity. Furthermore, speciation calculations predicted colloidal Pb precipitation in 3 of the 4 Ca treatments. Hence, it could not be unambiguously concluded if the effect observed was caused by increased Ca. In addition, the same study reported highest toxicity at intermediate pH (between 7 and 8) and lower toxicity at pH 6 and pH 8.5. Considering all results together, there is no unifying explanation for the differences observed between these 2 studies.

Given these uncertainties, it is unclear whether the preliminary Pb BLM accurately depicts the bioavailability processes concerning Pb. This hinders the development of a definitive chronic BLM for *Ceriodaphnia*, which has been used as a model species for all invertebrates for the normalization of toxicity data in risk-assessment processes [14]. The purpose of the present study was to more clearly define the individual effects of Ca and pH on chronic Pb toxicity to *C. dubia*. We performed chronic reproduction tests with *C. dubia* wherein Ca and pH were modified independently of one another. Furthermore, all tests within a test series were run simultaneously to avoid temporal shifts in toxicity. Based on the obtained data, a final chronic Pb BLM for *C. dubia* was developed. Finally, the developed BLM was validated with several available data sets originating from chronic Pb toxicity studies with *C. dubia* in both synthetic and field-collected natural waters [2,3,12,15].

All Supplemental Data may be found in the online version of this article.
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METHODS

Collection and preparation of test media

All toxicity tests were conducted in modified natural water. The natural water was collected from L'Ourthe Orientale in Brisy, Belgium. This unpolluted water has previously been used successfully for ecotoxicity testing in our lab [9] and has a low hardness and dissolved organic carbon (DOC) concentration. The natural water was filtered on site through a 0.45-μm filter and stored at 4 °C in total darkness in 10 L acid-washed polyethylene barrels until use. The pH of the Brisy water was 6.7 at the time of sampling. The Ca, Mg, Na, Cl, and SO_4 concentrations were 0.25 mM, 0.16 mM, 0.28 mM, 0.41 mM, and 0.10 mM, respectively. The DOC concentration was 3.2 mg/L.

The individual effects of Ca and pH on chronic Pb toxicity were investigated in 2 univariate test series, each consisting of 4 treatments (i.e., 4 exposure media). In the Ca series, Ca concentration was varied by adding $CaCl_2$ to the Brisy water. Four Ca concentrations were investigated: 0.25 mM (unmodified Brisy water), 1 mM (addition of 0.75 mM $CaCl_2$), 1.75 mM (addition of 1.5 mM $CaCl_2$), and 2.5 mM (addition of 2.25 mM $CaCl_2$). All media in the Ca series were adjusted to pH 7 by adding dilute NaOH. In the pH series 4 pH levels were investigated: 6.4, 7.0, 7.6, and 8.2. To all media in the pH series 0.5 mM $CaCl_2$ was added. Initially, pH was adjusted by adding 0.4 and 2.6 mM $NaHCO_3$ to attain pH levels of 7.6 and 8.2, respectively. Levels of Na were set equal between pH treatments by adding Na_2SO_4. All media were aerated for 3 d to allow equilibration with the ambient atmosphere. Subsequently, pH was adjusted to the required pH level by adding dilute HCl or NaOH before dividing the medium in aliquots of 1.6 L. The ultimate physicochemical composition of the different test media is shown in Table 1.

Concentration series in each Ca and pH treatment contained a control and 6 Pb concentrations, which were prepared by adding $PbCl_2$. For the Ca test series the nominal Pb concentrations were 50 μg Pb/L, 100 μg Pb/L, 150 μg Pb/L, 220 μg Pb/L, 320 μg Pb/L, and 400 μg Pb/L. For the pH series the nominal Pb concentrations were 80 μg Pb/L, 110 μg Pb/L, 140 μg Pb/L, 170 μg Pb/L, 220 μg Pb/L, and 320 μg Pb/L for pH levels 6.4, 7.0, and 7.6 and 100 μg Pb/L, 160 μg Pb/L, 220 μg Pb/L, 280 μg Pb/L, 340 μg Pb/L, and 400 μg Pb/L for pH level 8.2. All chemicals were purchased from VWR International.

Ecotoxicity testing

The chronic Pb toxicity tests were conducted following the US Environmental Protection Agency (USEPA) protocol [16]. Juvenile *C. dubia* originated from an in-house isoclonal lab culture, which has been maintained for more than 20 yr at 25 °C in carbon-filtered Ghent city tap water to which selenium (1 μg Se/L) and vitamins (75 μg/L thiamine, 1 μg/L cyanocobalamin, 0.75 μg/L biotin) are added. Daphnids were acclimated to test media in aquaria containing 1.5 L of the control test medium (no Pb) for 2 generations prior to test initiation (2 wk). Media were refreshed twice a week. During the acclimation and the testing period, daphnids were fed with *Pseudokirchneriella subcapitata* algae (2×10^5 cells/mL) and Yeast-Urtica-Trout Chow (mixture of 12 mg solids/L). The 4 ecotoxicity tests per test series were all run simultaneously to exclude any possible interference with later data interpretation as a result of temporal sensitivity variation. Tests were initiated with juveniles of the second generation from mothers that produced at least 8 juveniles in a single brood [16]. Juveniles (<24 h old, 1 per replicate) were randomly distributed among 10 replicates for each control and Pb concentration. Tests were conducted in polyethylene cups containing 20 mL of test medium at 25 °C under a light cycle of 16 h light and 8 h dark. Test media were completely renewed daily. Before renewal, fresh test media were adjusted to the required pH by adding dilute HCl or NaOH. Mortality and number of juveniles were scored daily. The toxicity tests were ended when 60% of the control animals had produced 3 broods (7–8 d).

Analytical chemistry

During the test period, samples of fresh (sample of new medium just before transfer of daphnids to the cup) and old (sample taken of medium just after transfer of daphnids to a new cup) test media were collected regularly for analysis of total and filtered Pb, Ca, organic carbon, and inorganic carbon. Total and filtered (0.45 μM, Acrodisc; PALL Life Sciences) samples of fresh media were taken on days 0, 3, and 6. Filtered samples of old media were taken on days 1, 4, and 7. Samples for Pb and Ca measurements were acidified to 0.14 mol/L HNO_3 (Normatom quality; VWR Prolabo). Concentrations of Pb were measured using graphite furnace atomic absorption spectrophotometry (GFAAS Furnace Autosampler; Thermo Fisher Scientific). Concentrations of Ca were measured using flame atomic absorption spectrophotometry (SpectrAA100;

Table 1. Main physicochemical characteristics of the test media used for biotic ligand model development: Values are arithmetic means of all measurements ± standard deviation

			Major ions (mM)						
Test medium	pH	Dissolved organic carbon (mg/L)	Ca	Mg[a]	Na[a]	SO_4[a]	Cl[a]	Dissolved inorganic carbon	Hardness[b] (mg $CaCO_3$/L)
Ca 0.25 mM	7.04 ± 0.06	4.0 ± 1.1	0.24 ± 0.01	0.16	0.32	0.10	0.41	0.20 ± 0.04	40.0
Ca 1.0 mM	7.01 ± 0.05	3.9 ± 1.0	0.89 ± 0.18	0.16	0.32	0.10	1.91	0.17 ± 0.04	105
Ca 1.75 mM	7.04 ± 0.06	3.8 ± 1.0	1.26 ± 0.09	0.16	0.31	0.10	3.41	0.18 ± 0.03	142
Ca 2.5 mM	7.07 ± 0.09	3.9 ± 0.8	1.80 ± 0.06	0.16	0.30	0.10	4.91	0.19 ± 0.04	196
pH 6.4	6.35 ± 0.26	3.3 ± 0.5	0.75 ± 0.01	0.16	2.89	1.40	2.03	0.07 ± 0.04	91.1
pH 7.0	6.94 ± 0.10	3.3 ± 0.5	0.77 ± 0.02	0.16	2.90	1.40	1.93	0.17 ± 0.02	93.1
pH 7.6	7.56 ± 0.10	3.3 ± 0.6	0.74 ± 0.01	0.16	2.96	1.20	1.91	0.53 ± 0.03	90.1
pH 8.2	8.14 ± 0.04	3.2 ± 0.3	0.74 ± 0.02	0.16	2.93	0.10	1.95	2.33 ± 0.08	90.1

[a] No measurements were conducted for Mg, Na, SO_4, and Cl. Baseline concentrations measured in the Brisy water at the time of sampling are reported. For Na, SO_4, and Cl, additions of $NaHCO_3$, $NaSO_4$, NaOH, and HCl were taken into account (see *Materials and Methods*).
[b] Water hardness was calculated from measured Ca and Mg (at time of sampling) concentrations.

Varian). Dissolved organic and inorganic carbon levels were measured with a total organic carbon analyzer (TOC-5000; Shimadzu). The pH values of fresh and old media were measured daily with a pH glass electrode (P407; Consort).

Concentration–response analysis

Effective concentrations (no-observed-effect-concentration [NOEC], lowest-observed-effect-concentration [LOEC], and 10%, 20%, and median effective concentrations [EC10, EC20, and EC50, respectively]) were calculated based on average measured filtered Pb concentrations in fresh and old test media. Total reproduction (number of juveniles per female) relative to the mean control reproduction was used as the end point. The NOECs and LOECs were determined with the Mann-Whitney *U*-test with Bonferroni-Holm correction in the software package SPSS 20 (SPSS). Values of $EC50_{Pbfilt}$, $EC20_{Pbfilt}$, and $EC10_{Pbfilt}$ and their corresponding confidence intervals were determined with the drc package in R 2.14.1 (R Development Core Team) with a log-logistic concentration–response model with 2 parameters

$$y = \frac{100}{1 + exp^{b(\log x - \log EC x)}} \quad (1)$$

where y is predicted reproduction (number of offspring per female) relative to the average of the controls (%), b is a slope parameter, x is the filtered Pb concentration (μg/L), and ECx is the effect (10%, 20%, or 50%) concentration (μg filtered Pb/L). Only $EC50_{Pbfilt}$ values are used for BLM development since EC50s give more precise estimates (smaller confidence intervals).

Chemical speciation calculations

Activities of Pb^{2+}, Ca^{2+}, and H^+ were calculated using Visual Minteq 3.0 (KTH). Speciation was calculated at the $EC50_{Pbfilt}$ and $EC20_{Pbfilt}$. Complexation of Pb with dissolved organic matter, that is, with humic acid and fulvic acid, was modeled according to the NICA-Donnan formulation (as embedded in Visual Minteq 3.0). All default parameter values as described in Milne et al. [17] were used. In all cases we assumed that dissolved organic matter contains 50% carbon on a weight basis. For dissolved organic matter from natural sources (e.g., tap water, surface water) we assumed that 65% of the dissolved organic matter is reactive and behaves as isolated fulvic acid. Previous research has shown that assumptions between 60% and 70% active fulvic acid typically work best for predicting metal toxicity in natural waters [18]. Accordingly, the measured DOC content (mg/L) for natural sources was multiplied by a factor of 1.3 to obtain the amount of fulvic acid (mg/L) to be used as the modeling input. For 4 tap waters to which isolated Aldrich humic acid was added (i.e., media in Mager et al. [3]) we assumed that this humic acid behaved as 100% reactive humic acid. However, it was taken into account that there was a natural background dissolved organic matter concentration present in the tap water. Thus, in these cases, the humic acid concentration (mg/L) was estimated to be 2-fold (measured DOC – measured background DOC) and the fulvic acid concentration to be 1.3-fold the background DOC concentration.

It needs to be mentioned that the publically available Visual Minteq 3.0 contains an error in the speciation calculation code which results in errors in the calculation of Pb speciation when 2 or more different sources of organic matter are present in a medium (in this case fulvic acid and humic acid) in the input file. Speciation calculations in the present study were therefore made in an adapted version obtained after personal communication with J.P. Gustafsson (KTH, Stockholm, Sweden), which is not yet available online.

The model development and validation were always based on (average) measured filtered concentrations of Pb, operationally defined as the Pb passing through a 0.45-μm filter. Yet, it is possible that not all filtered Pb is truly dissolved because colloidal precipitates of Pb minerals may be present in the test solutions. However, in the absence of any measured data on the presence of such colloidal precipitates, we assumed for modeling purposes (i.e., model development and validation) that all filtered Pb was truly dissolved and could interact with dissolved ligands (e.g., DOC) in the medium. Thus, the average of measured filtered Pb concentrations of new and old media was used as both the input and the output of the BLM for both development and validation. We explored also the potential importance of colloidal Pb precipitation in reducing Pb bioavailability within the filtered Pb fraction. This was achieved by performing additional speciation calculations where in Visual Minteq 3.0 the formation of the Pb minerals cerrusite, hydrocerrusite (2 Pb-carbonate minerals), and $Pb(OH)_2(s)$ was allowed. In all test media investigated, no other Pb minerals were ever predicted to form. We anticipated that these additional calculations could provide insight into the role colloidal Pb mineral formation may play in the observed variation of measured filtered EC50 among test media.

BLM development

Biotic ligand model development was based on 7-d $EC50_{Pb2+}$ since the EC50 gives more precise estimates (smaller confidence intervals). The effects of Ca and pH on chronic Pb toxicity to *C. dubia* were modeled as a single-site BLM–type competition effect according to the method described by De Schamphelaere and Janssen [19]. Biotic ligand model parameters (stability constants for binding of H^+ and Ca^{2+} to the biotic ligand [K_{HBL} and K_{CaBL}, respectively]) were determined based on regression equations of the linear relationship between 7-d $EC50_{Pb2+}$ and H^+ and Ca^{2+} activity, respectively. The linear relationships were evaluated by an F test using a significance level of 5% with R 2.14.1 (R Development Core Team). Estimated intercept and slope parameters with corresponding standard errors are reported. The 95% confidence intervals (CIs) on the BLM parameters were calculated based on the standard error of the ratio between the slope and the intercept of the regression equation. The lower and upper limits of the 95% CIs were calculated as $K_{HBL} \pm 1.96 \times$ standard error, respectively.

The BLM equation including a Ca^{2+} and a H^+ competition effect can be written as follows

$$EC50_{Pb^{2+},i,predicted} = EC50^{*}_{Pb^{2+}} \times (1 + K_{HBL} \times [H^+]_i + K_{CaBL} \times [Ca^{2+}]_i) \quad (2)$$

In this model $EC50_{Pb2+,i,predicted}$ is the predicted Pb^{2+} activity for the median effective concentration in test solution i and $\{H^+\}_i$ and $\{Ca^{2+}\}_i$ are the chemical activities of H^+ and Ca^{2+} in test solution i, respectively; $EC50^{*}_{Pb2+}$ is the intrinsic sensitivity of *C. dubia*, which can be regarded as the $EC50_{Pb2+}$ of *C. dubia* in a solution where all H^+ and Ca^{2+} competition effects are absent [19]. With the above model $EC50_{Pb2+}$ of any test solution can be predicted if K_{HBL} and K_{CaBL} are known. The intrinsic sensitivity can be estimated from observations of EC50 in a range of waters as follows

$$EC50^{*}_{Pb^{2+}} = \prod_{i}^{n} \left(\frac{EC50_{Pb^{2+},i,\text{observed}}}{1 + K_{HBL} \times [H^{+}]_i + K_{CaBL} \times [Ca^{2+}]_i} \right)^{\frac{1}{n}} \quad (3)$$

In this equation, $EC50_{Pb2+,i,\text{ observed}}$ is the Pb^{2+} activity in test solution i and n is the number of test solutions considered.

The predicted 7-d $EC50_{Pb2+}$ values were translated to 7-d $EC50_{Pbfilt,pred}$ with Visual Minteq 3.0 and compared with the observed 7-d $EC50_{Pbfilt,obs}$. The developed BLM was also validated with the 7-d $EC10_{Pbfilt}$ and 7-d $EC20_{Pbfilt}$, following the procedure described above but calculating a specific intrinsic sensitivity for $EC10_{Pbfilt}$ and $EC20_{Pbfilt}$.

Independent BLM validation

The developed chronic Pb BLM was validated with Pb toxicity data from the following 4 independent data sets obtained in earlier studies. Mager et al. [3] investigated the effect of modifications of major cation concentrations (Ca, Mg, Na, K), pH (using 3-[*N*-morpholino]propanesulfonic acid–buffered media), alkalinity ($NaHCO_3$ additions), and DOC (added as natural organic matter or as Aldrich humic acid). A total of 21 test media were investigated. The water chemistry and corresponding 7-d $EC50_{Pbfilt}$ and 7-d $EC20_{Pbfilt}$ were taken from their Table 1 [3]. Esbaugh et al. [2] reported on the chronic toxicity of Pb in 5 spiked natural surface waters. The water chemistry and corresponding 7-d $EC50_{Pbfilt}$ and 7-d $EC20_{Pbfilt}$ were taken from their Tables 1 and 2, respectively. Two additional data sets were used. Parametrix [12] investigated the effects of pH and Ca. Chemical compositions of the test media were taken from their Tables 3-1 and 3-2. The 7-d $EC50_{Pbfilt}$ and 7-d $EC20_{Pbfilt}$ were taken from their Tables 3-9 and 3-10. AquaTox [15] retested the French lake natural water, which was identified as an outlier of a previous preliminary BLM study [2]. In addition, a synthetic lab water and a reconstituted French lake water were used to investigate the effect of Pb to *C. dubia*. Chemical characteristics were taken from their Table 3. The 7-d $EC50_{Pbfilt}$ and the 7-d $EC20_{Pbfilt}$ were taken from their Table 19. An overview of the chemical composition of the different test media used for model validation is given in the Supplemental Data, Table S1.

The BLM was validated in 2 different ways. First, validation was performed by calculating a single mean intrinsic sensitivity ($EC50^{*}_{Pb2+}$ and $EC20^{*}_{Pb2+}$) for all validation data sets and the data reported in the present study with Equation 3, hereafter called the "overall intrinsic sensitivity". A second validation was made by calculating mean intrinsic sensitivity ($EC50^{*}_{Pb2+}$ and $EC20^{*}_{Pb2+}$) for each *C. dubia* clone separately with Equation 3, hereafter called the "clone-specific intrinsic sensitivity." Mager et al. [3] and Esbaugh et al. [2] tested Pb toxicity on the same *C. dubia* clone, hereafter called the "UMiami clone." Therefore a clone-specific intrinsic sensitivity was used for these 2 data sets together.

In the calculation of the overall intrinsic sensitivity, the 0.25-mM Ca treatment of the present study was excluded. The following data points were excluded in the calculation of the intrinsic sensitivity in both validation analysis: for the data set of Parametrix [12] the 4 data points for which speciation calculations predicted colloidal precipitation (See *Results-Independent model validation* section) were excluded from the calculation of the intrinsic sensitivities. For Mager et al. [3] the synthetic waters with added humic acid and those buffered with MOPS were excluded from the calculation of the intrinsic sensitivities because such additions represent conditions that are less relevant for natural waters. Furthermore, the French lake water was also excluded from the calculations because Esbaugh et al. [2] showed that Pb toxicity in the French lake water was underestimated by 12-fold using a preliminary *C. dubia* BLM. For the AquaTox [15] data set, the intrinsic sensitivity was calibrated based on all 3 tested waters. The French lake water was tested by both Esbaugh et al. [2] and AquaTox [15]. Hence, for clarity, the French lake water tested by Esbaugh et al. [2] and AquaTox [15] will be called "French lake 1" and "French lake 2," respectively.

The BLM was not validated with $EC10_{Pbfilt}$ values as these were not available for all data sets. Furthermore, EC10 values are usually less precise than EC20 or EC50 values (larger confidence intervals).

RESULTS

Effect of Ca and pH on Pb toxicity

No colloidal Pb precipitation was predicted in the Ca or pH test series. Control survival was 100% in all Ca treatments. Control reproduction in the Ca test series was on average 14.0 (± 4.7) juveniles per female (± standard deviation; Table 2). In the controls of the 3 highest Ca concentrations (1.00–1.75 mM),

Table 2. General biological characteristics of the toxicity tests

Test medium	Control reproduction[a]	Percentage of males in control	EC50 (μg Pb_{filt}/L)[b]	EC20 (μg Pb_{filt}/L)[b]	EC10 (μg Pb_{filt}/L)[b]	NOEC (μg Pb_{filt}/L)[c]	LOEC (μg Pb_{filt}/L)[c]
Ca 0.25 mM	14.1 ± 4.6	30%	81.2 (66.6–95.8)	64 (42–86)	55 (30–81)	83 (–33%)	127 (–100%)
Ca 1.0 mM	14.4 ± 4.2	10%	104[d] (86.5–132)	[d]	[d]	86 (–10%)	132 (–100%)
Ca 1.75 mM	13.9 ± 4.4	0%	130 (110–150)	112 (80–144)	102 (63–142)	93 (–0%)	140 (–62%)
Ca 2.5 mM	13.6 ± 6.3	10%	115[d] (93.7–135)	[d]	[d]	94 (–0%)	135 (–100%)
pH 6.4	21.3 ± 7.5	0%	99.8 (87.6–112)	79 (60–98)	70 (48–92)	50 (–7%)	75 (–26%)
pH 7	22.4 ± 5.0	0%	106 (94.7–118)	81 (61–101)	69 (45–93)	46 (–17%)	72 (–26%)
pH 7.6	23.9 ± 5.4	0%	110 (95.8–124)	80 (58–102)	67 (41–92)	44 (–13%)	63 (–28%)
pH 8.2	25.0 ± 5.8	0%	320 (242–398)	153 (87–129)	99 (34–165)	164 (–7%)	201 (–42%)

[a] Number of juveniles per mother animal ± standard deviation; percentage of males in control; EC10, EC20, and EC50; NOEC; and LOEC.
[b] For EC50s, EC20s, and EC10s, 95% confidence intervals are reported in parentheses.
[c] For NOECs and LOECs, reduction in reproduction relative to the control (%) is reported in parentheses.
[d] Because of the steepness of the concentration response, no reliable EC50, EC10, and EC20 could be calculated for the 1 mM and 2.5 mM Ca treatment with the log-logistic dose response. The EC50s were derived from the regression between the observed effect (%) at the 2 concentrations encompassing the 50% effect level and the log filtered concentration. The reported confidence limits for these tests are the 2 concentrations that encompass the 50% effective levels.
EC10 = 10% effective concentration; EC20 = 20% effective concentration; EC50 = median effective concentration; NOEC = no-observed-effect-concentration; LOEC = lowest-observed-effect-concentration.

we observed ≤10% males, while 30% males were observed in the control of the 0.25-mM Ca treatment. The concentration–response data of the Ca test series are shown in Figure 1A. No reliable log-logistic concentration–response curve could be fitted for the 1.0-mM and 2.5-mM Ca treatments. The $EC50_{Pbfilt}$ values for these treatments were therefore estimated by linear regression between the observed effect (%) at the 2 concentrations encompassing the median effective levels and the log filtered concentration (micrograms of Pb per liter). The $EC50_{Pbfilt}$ values were similar at the 3 highest Ca concentrations (Table 2), 104 μg/L to 130 μg/L, but were 1.3- to 1.6-fold lower at the 0.25 mM Ca concentration. Figure 2A shows the positive linear relationship between 7-d $EC50_{Pb2+}$ and Ca^{2+} activity ($p = 0.22$, $r = 0.78$). The estimated slope of this relationship was 4.79×10^{-6} ($\pm 2.70 \times 10^{-6}$). The intercept of the relation was 4.23×10^{-9} mol/L ($\pm 2.27 \times 10^{-9}$).

Control females in the pH test series produced on average 23.2 ± 5.5 juveniles (mean ± standard deviation; Table 2). No mortality or males were observed in the controls of all pH treatments. The concentration–response data of the pH test series are shown in Figure 1B. The Pb toxicity, expressed as the average filtered concentration in the new and old media, was similar between pH 6.4 and pH 7.6. At pH 8.2 Pb toxicity was clearly lower. The 7-d $EC50_{Pbfilt}$ varied at most 1.1-fold between pH 6.4 and 7.6 (i.e., 100–110 μg/L), while a pH change from 7.6 to 8.2 resulted in a 2.9-fold increase of the 7-d $EC50_{Pbfilt}$ (Table 2). Figure 2B shows the positive linear relationship between 7-d $EC50_{Pb2+}$ and H^+ activity ($p = 0.003$, $r = 0.99$). The estimated slope of this relationship was 6.25×10^{-2} ($\pm 3.25 \times 10^{-3}$). The intercept of the relation was 1.62×10^{-9} mol/L ($\pm 7.58 \times 10^{-10}$).

The NOEC and LOEC values are provided in Table 2. The NOECs and LOECs at 0.25 mM Ca are less reliable, as already

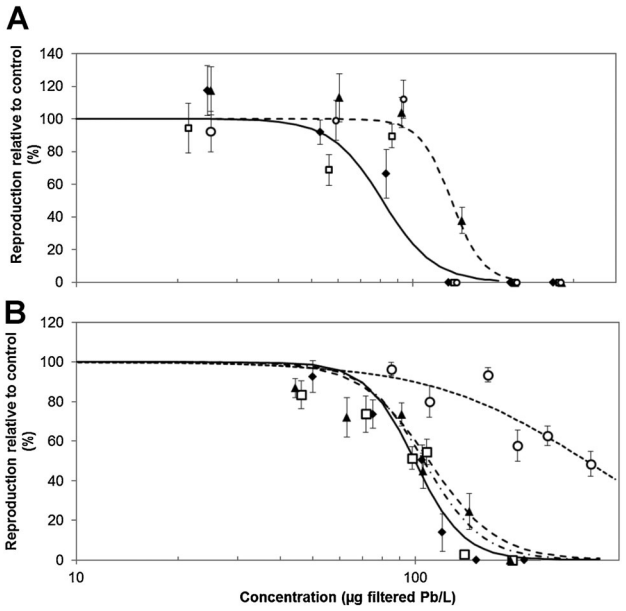

Figure 1. Concentration–response curves of Pb toxicity to *Ceriodaphnia dubia* for the Ca test series (**A**) and the pH test series (**B**). Data points are the mean reproduction relative to the control reproduction: Ca 0.25 mM (◆), Ca 1 mM (□), Ca 1.75 mM (▲), and Ca 2.5 mM (◯); pH 6.4 (◆), pH 7.0 (□), pH 7.6 (▲), and pH 8.2 (◯). Plotted error bars are standard errors. Fitted curves are log-logistic concentration–response curves: Ca 0.25 mM and pH 6.4 (full line) or pH 7 (dashed–dotted line), Ca 1.75 mM and pH 7.6 (dashed line) or pH 8.2 (dotted line). No reliable log-logistic concentration–response curve could be fitted for the 1.0-mM and 2.5-mM Ca treatments.

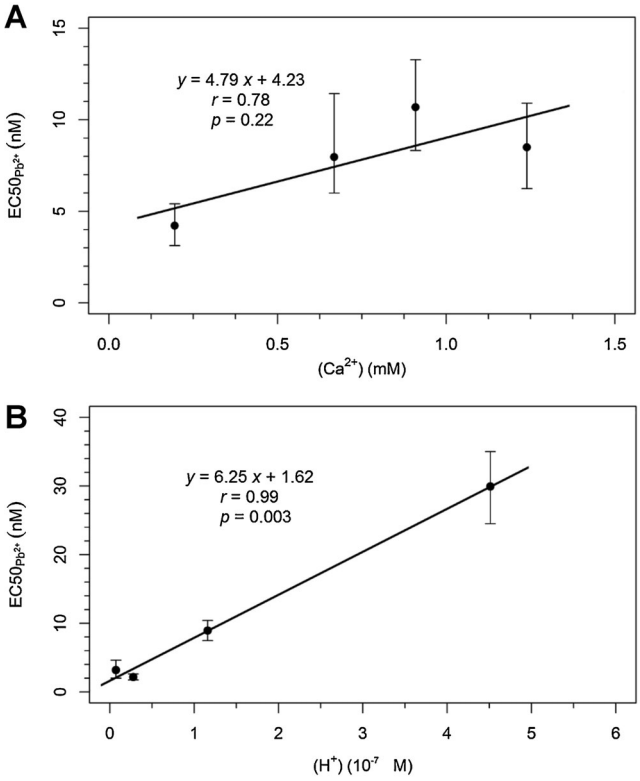

Figure 2. Median effective concentration ($EC50_{Pb2+}$, expressed as Pb^{2+} activity in nanomolar) as a function of Ca^{2+} activity (millimolar; **A**) and H^+ activity (10^{-7} M; **B**). The regression line, equation, and p value are given. Plotted error bars are 95% confidence intervals on the $EC50_{Pb2+}$.

33% effect was observed at the NOEC. The NOECs and LOECs of the other treatments show the same trends as the regression-based effect concentrations, with higher NOEC and LOEC values at high pH and no effect of Ca.

Model development

Based on the results from the univariate pH and Ca test series, a chronic Pb BLM was developed. A stability constant for Ca ($\log K_{CaBL}$) was not incorporated into the BLM for reasons discussed below (see *Discussion*). In the absence of other competing ions, K_{HBL} can be estimated by the ratio of the slope to the intercept of $EC50_{Pb2+}$ as a function of H^+ activity [19]. Log K_{HBL} was calculated to be 7.6 (95% CI 6.5–7.9). Without the nonsignificant Ca competition term the BLM can be written as follows

$$EC50_{Pb^{2+},i,predicted} = EC50^{*}_{Pb^{2+}} \times (1 + K_{HBL} \times [H^+]_i) \quad (4)$$

An autovalidation was done to investigate how well the model predicts Pb toxicity for the data set used for BLM development. Using Equation 3 mean clone-specific intrinsic sensitivities ($EC50^{*}_{Pb2+}$, $EC20^{*}_{Pb2+}$, and $EC10^{*}_{Pb2+}$) of 1.73 nM, 1.01 nM, and 0.75 nM, respectively, were calculated based on all data points (except that for 0.25 mM Ca, for reasons discussed below). For the $EC50_{Pbfilt}$, all predicted values were within 1.5-fold of those observed, except for the 0.25-mM Ca treatment (Figure 3). For the $EC20_{Pbfilt}$, all predicted values were within 1.5-fold of those observed. Finally, for the $EC10_{Pbfilt}$, all predicted values were within 2-fold of the observed values (Supplemental Data, Table S3).

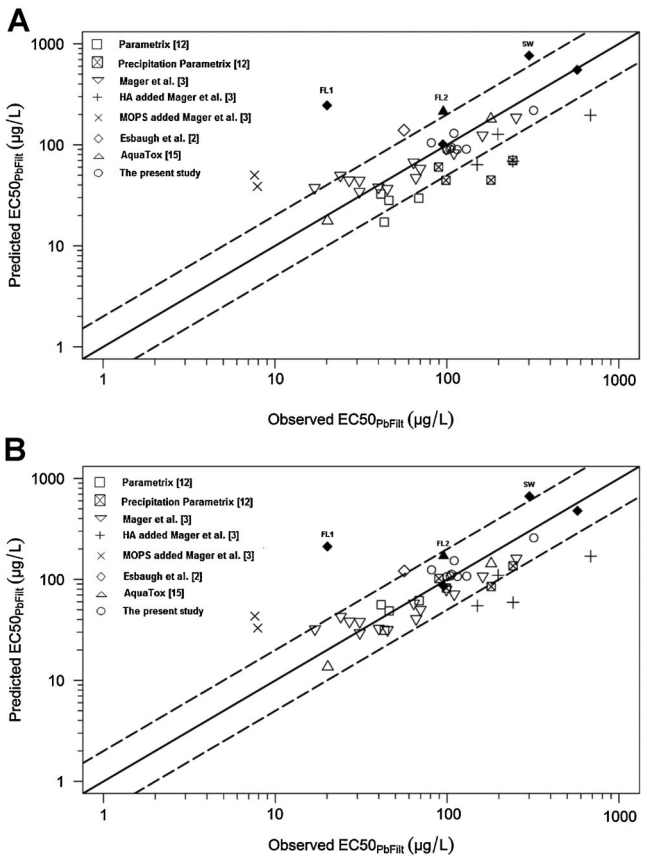

Figure 3. Predicted versus observed median effective concentration (EC50, expressed as µg filtered Pb/L) for the developed biotic ligand model (BLM) calibrated with the overall intrinsic sensitivity (**A**) and the developed BLM calibrated with the clone-specific intrinsic sensitivities (**B**): predictions for the data used for the BLM development and the validation. Predictions were made using Equation 4 linked to Visual Minteq. Dashed line represents a difference of a factor of 2 between the observed and predicted data. Full line represents a perfect fit between observed and predicted data. Open data points are from synthetic media, and filled points are from natural waters. Crossed symbols represent data points where precipitation is predicted by speciation calculations. Symbols are as follows: □ Parametrix [12], ⊠ waters where precipitation was predicted [12], ▽ Mager et al. [3], + humic acid added to media [3], 3-(*N*-morpholino)propanesulfonic acid added to media [3], ◇ Esbaugh et al. [2], Δ AquaTox [15], ○ data from the present study. FL1 = French lake 1 [2]; FL2 = French lake 2 [15]; SW = Sweetwater Strand [2].

Independent model validation

The overall intrinsic sensitivities were estimated at 1.33 nM and 0.54 nM for $EC50^{*}_{Pb2+}$ and $EC20^{*}_{Pb2+}$, respectively. Using these overall intrinsic sensitivities, 7-d $EC50_{Pbfilt}$ values in synthetic waters were predicted with an average error of 2.11 (prediction error range, 1.00–6.61; Figure 3A), while 7-d $EC20_{Pbfilt}$ values in synthetic waters were predicted with an average error of 2.14 (prediction error range, 1.01–5.44). The $EC50^{*}_{Pb2+}$ clone-specific intrinsic sensitivities were estimated at 3.06 nM, 1.06 nM, and 0.94 nM for the Parametrix, UMiami, and AquaTox clone, respectively, while the $EC20^{*}_{Pb2+}$ values were 1.40 nM, 0.44 nM, and 0.17 nM, respectively. Using these clone-specific intrinsic sensitivities, both 7-d $EC50_{Pbfilt}$ and 7-d $EC20_{Pbfilt}$ in the synthetic waters were predicted with an average error of 1.89 (prediction error range, 1.05–5.70 and 1.01–5.45, respectively; Figure 3B). All overall and clone-dependent intrinsic sensitivities and average prediction errors of the 7-d $EC50_{Pbfilt}$ and 7-d $EC20_{Pbfilt}$ values for the different types of waters are summarized in the Supplemental Data, Tables S2). The predicted 7-d $EC50_{Pbfilt}$ and 7-d $EC20_{Pbfilt}$ values for all waters are provided in the Supplemental Data, Table S3.

Values of $EC50_{Pbfilt}$ and $EC20_{Pbfilt}$ in media where MOPS buffer or humic acid was added were mostly overestimated and underestimated with more than 2-fold error, respectively. When these synthetic media were excluded mean prediction errors using the overall intrinsic sensitivity were 1.72 (range, 1.00–4.03) and 1.79 (range, 1.01–4.34) for $EC50_{Pbfilt}$ and $EC20_{Pbfilt}$, respectively. The mean prediction errors for the same waters using the clone-specific intrinsic sensitivities were 1.44 (range, 1.05–2.16) and 1.48 (range, 1.01–2.17) for $EC50_{Pbfilt}$ and $EC20_{Pbfilt}$, respectively. Colloidal Pb precipitation of hydrocerrusite and lead hydroxide was predicted by Minteq in 4 waters, all of which were from the Parametrix data set [12]. The Pb toxicity was almost always overestimated in these 4 waters but was mostly within 2-fold error. The 7-d $EC50_{Pbfilt}$ and 7-d $EC20_{Pbfilt}$ values of the 5 field waters [3,15] were predicted using the overall intrinsic sensitivity with mean prediction errors of 3.83 (prediction error range, 1.03–12.26) and 4.37 (range, 1.38–11.25), respectively. Using the clone-specific intrinsic sensitivity, the 7-d $EC50_{Pbfilt}$ and 7-d $EC20_{Pbfilt}$ values of the 5 field waters were predicted with mean prediction errors of 3.37 (prediction error range, 1.07–10.6) and 3.65 (range, 1.03–9.86), respectively. However, toxicity in the French lake 1 field water from the Esbaugh et al. [2] data set was underestimated by at least 10-fold. When this water was excluded, 7-d $EC50_{Pbfilt}$ values were predicted with average prediction errors of 1.72 (prediction error range, 1.03–2.54) and 1.57 (prediction error range, 1.09–2.22) using the mean and clone-specific intrinsic sensitivity, respectively.

DISCUSSION

Effects of Ca and pH on Pb toxicity

While 2 studies had previously investigated the effects of water chemistry on chronic Pb toxicity to *C. dubia* [3,12], the present study refines the roles of Ca and pH on chronic Pb toxicity to *C. dubia*, obtained using tests performed simultaneously and completely in parallel.

Although the control reproduction in the Ca test series (14 juveniles/female) was slightly lower than required for test validity (15 juveniles/female) according to the USEPA [16], effective concentrations were highly reproducible. Indeed, the 1-mM Ca treatment in the Ca series and the pH-7 treatment in the pH series are highly comparable in terms of chemical composition and in terms of their measured $EC50_{Pbfilt}$ (i.e., 104 µg filtered Pb/L and 106 µg filtered Pb/L, respectively). A log-logistic concentration–response curve could not be fitted to the 1.0-mM and 2.5-mM Ca treatments, presumably because of the steep concentration response, that is, reproduction going from almost 100% to almost 0% in a narrow concentration interval (i.e., 50 µg/L). Therefore, $EC50_{Pbfilt}$ values for these treatments were calculated with a linear regression function. As mentioned above, the $EC50_{Pbfilt}$ values of the 1.0-mM Ca treatment and the pH-7 treatment, which were highly comparable in chemical composition, were almost identical. Therefore, it is reasonable to assume that the EC50 values calculated by linear regression for this test are reliable.

Observed $EC50_{Pbfilt}$ values in the present study ranged 4-fold between 81 µg/L and 320 µg/L (Table 2). The highest toxicity

was observed at the lowest Ca concentration, while the lowest toxicity was observed at the highest pH level. A 1.3-fold to 1.6-fold lower $EC50_{Pbfilt}$ was observed at the lowest Ca concentration compared to the other Ca treatments. This observation appears to be in contradiction with Mager et al. [3,4], who found no evidence of a protective Ca effect on chronic or acute Pb toxicity to *C. dubia*. Parametrix [12], on the contrary, reported that an increase in Ca reduced chronic Pb toxicity to *C. dubia*. However, increased Ca concentrations in their study were accompanied by correlated increases of pH and alkalinity. In natural waters, Ca would seldom increase without covariations in, among others, alkalinity and pH. However, the concurrent increases in Ca and pH hampered unambiguous conclusions about the effects of Ca based on the Parametrix study [12]. The higher toxicity at low Ca concentration observed in the present study may suggest a potential competitive effect of Ca on the Pb biotic ligand site. However, a second possible explanation of this observation is that the combination of both low Ca and Pb stress invoked a higher Pb sensitivity than at higher Ca concentrations. It has indeed been shown that some daphnid species have high Ca requirements to support their regular moulting [20]. Despite the absence of an effect on control reproduction, the *C. dubia* from the 0.25-mM Ca treatment did indeed appear to be more stressed than others, based on the higher percentage of males observed in the control (Table 2). In contrast, no such signals of low Ca stress were observed in the Ca test series of Mager et al. [3]. Regardless of what the explanation is for the observed effect of increasing Ca in the present study, the magnitude of the effect is relatively small compared with effects reported earlier for fish [4,5].

Generally, Ca has a protective effect against Pb-induced mortality to fish during acute (96 h) and chronic (30 d) exposure [4,5], although mortality occurred mainly during the first week of the chronic exposure [5]. Commonly, Pb is regarded as a Ca analogue [21]. Previous research demonstrated that at high Pb concentrations Pb uptake in fish is linked with the Ca^{2+} transport mechanism at the cell surface [22]. An increase in Ca concentration results in stronger competition between Pb^{2+} and Ca^{2+} for uptake, which could explain the lower toxicity of Pb. However, at low Pb concentrations more relevant for the chronically very sensitive species *C. dubia*, this competitive uptake mechanism may be less relevant [4]. Furthermore, Ca did not protect against Pb accumulation and transcriptional responses in *Pimephales promelas* (fathead minnow) during chronic exposure at similar low Pb concentrations [23]. Uptake of Pb^{2+} under these conditions may instead occur by mimicry of a different ion through a channel or transporter with low Ca^{2+} affinity and high Pb^{2+} affinity [4], although this requires further experimental validation.

In agreement with previous research [3,12], high pH was found to have a strong protective effect against the chronic Pb toxicity to *C. dubia*. Parametrix [12] observed in a pH interval from 7 to 8.5 a similar but less pronounced pH effect. However, they also reported a protective effect at pH 6. Mager et al. [3] found higher Pb toxicity at low pH but noted that their results were probably confounded by the use of MOPS buffer in the test solutions, which resulted in an increase in Pb sensitivity compared with tests that were not MOPS buffered. More recent studies have demonstrated that MOPS and CO_2 atmospheres are inappropriate methods of pH adjustment in Pb toxicity testing [13]. The toxicity of Pb, expressed as filtered Pb, may be influenced by pH in 2 ways. First, H^+ ions can compete with Pb^{2+} for binding sites at the biotic ligand, an effect of increasing importance at lower pH [24]. Second, at high pH, the complexation of Pb^{2+} with OH^-, CO_3^{2-}, and DOC becomes increasingly important, resulting in a higher fraction of Pb complexes, which are less bioavailable than the free Pb^{2+} ion and therefore also less toxic [25].

BLM development and validation

The developed Pb BLM for *C. dubia* only included a H^+ competition effect. Earlier, Ca, Mg, and Na were shown not to affect chronic Pb toxicity to *C. dubia* [3]. Additional reasons for not including a competitive effect of Ca^{2+}, based on the present study, are discussed below. The possible influence of DOC and other ligands (e.g., CO_3^{2-}, Cl^-, SO_4^{2-}, HCO_3^-) on Pb toxicity was incorporated into the BLM through Pb-complexation effects modeled in Visual Minteq. The effect of pH was modeled in the current Pb BLM as a classic linear H^+ competition term, while in the preliminary *C. dubia* BLM developed based on the Parametrix data set [12] a log-linear pH function was used [2]. Our choice for a linear H^+ competition term was based on the fact that the linear relation between $\log(EC50_{Pb2+})$ and pH ($r = 0.90$, $p = 0.10$, $n = 4$, regression not shown, slope = -0.59) was not better than the linear relation between $EC50_{Pb2+}$ and H^+ ($r = 0.99$, $p = 0.003$, $n = 4$; Figure 2B). Although it could be argued that the regression (and the resulting log K_{HBL}) in Figure 2B could have been influenced by the data point at the lowest pH level (highest H^+ activity), the linear regression based on data with only the 3 lowest H^+ activities ($r = 0.95$, $p = 0.20$, regression not shown) yielded an almost identical log K_{HBL} value (i.e., 7.53) compared with the one based on data from all 4 pH levels (i.e., 7.59). Thus, based on our data set, we saw no reason to deviate from the classic BLM concept to model the effect of pH on toxicity of the Pb^{2+} ion. In addition, the log K_{HBL} based on the same Parametrix data set [12] (from which the preliminary BLM was developed [2]) was 7.50, which is close to the log K_{HBL} of the present study (i.e., 7.59) and clearly within the 95% CI (6.49–7.87). The log K_{HBL} of the *C. dubia* Pb BLM is more than 3 log units higher than the log K_{HBL} of the acute fish BLM, that is, 4 [24], which suggests that the H^+ effect is more important for chronic Pb toxicity to *C. dubia* than for acute Pb toxicity to fish.

The mathematical approach for estimating BLM parameters requires assuming univariate changes in Ca or pH, altering 1 ion while keeping all other water chemistry parameters constant. However, this is in practice not feasible because some chemical elements will always covary when modifying a single physicochemical characteristic. For example, in our Ca series chloride increased concurrently with Ca by approximately 4.5 mM, while in our pH series alkalinity increased concurrently with pH. However, the alkalinity observed in our test series probably had negligible effects on *C. dubia* reproduction [26]. Furthermore, Mager et al. [3] showed that the addition of Cl to test solutions did not influence Pb toxicity. Therefore, we believe that the concurrent changes in Cl and alkalinity in our Ca and pH series had no significant effects on the test outcomes.

The BLM was validated with a set of independent data from 4 different studies [2,3,12,15]. In total, 36 waters were incorporated in the independent validation, including 5 natural waters. Using a single overall intrinsic sensitivity for all data sets, Pb toxicity, expressed as $EC50_{Pbfilt}$, was predicted within 2-fold error for 58% of the synthetic test waters. Calibration of the intrinsic sensitivity for each separate *C. dubia* clone led to clear improvements in the model predictions. Using the clone-specific intrinsic sensitivity, $EC50_{Pbfilt}$ values were predicted within 2-fold error for 77% of the synthetic waters. The clone-specific intrinsic sensitivities differed up to 3-fold between the 4

independent *C. dubia* clones (i.e., 0.95–3.06 nmol/L). The intrinsic sensitivity parameter corresponds to the sensitivity, which is independent of water chemistry [19] and should therefore be similar between different studies. However, the differences in sensitivities seem to be in the normal range because toxicant sensitivities can vary substantially between different lab clones [27,28]. For example, acute Cd toxicity to *Daphnia magna* has been shown to differ up to 2 orders of magnitude between different lab clones [28]. Furthermore, variability in pretreatment culturing and/or differences in testing conditions can result in different sensitivities [27]. In addition, sensitivity within a lab clone can shift with time up to 1 order of magnitude within fewer than 10 generations [29]. Some prominent under- and overestimations can be noted from Figure 3. Toxicity in synthetic media where MOPS was used to buffer the pH was overestimated by at least 4-fold, a finding in agreement with recent reports that MOPS may not always be suited for pH manipulations in toxicity testing [13]. However, this was previously not observed for Cu and Zn toxicity [30]. In addition, $EC50_{Pbfilt}$ values in 3 out of 4 media where Aldrich humic acid was used as a source of dissolved organic matter were underestimated by more than 2-fold. This overestimation of Pb toxicity in media with added Aldrich humic acid may be explained by an underestimation by the NICA-Donnan model in Visual Minteq of the Pb-binding capacity or Pb-binding strength to Aldrich humic acid. The ability of DOC to bind metal ions is dependent on the nature of the DOC, with humic acid generally providing more protection against metal toxicity than fulvic acid [31]. When media containing MOPS or humic acid were excluded, Pb toxicity, expressed as $EC50_{Pbfilt}$, was predicted within 2-fold of those observed for 68% and 92% of the synthetic test waters using the mean and clone-specific intrinsic sensitivity, respectively. Values of $EC50_{Pbfilt}$ were slightly better predicted than those of $EC20_{Pbfilt}$. However, differences in prediction errors were small.

A Ca effect was not included in the final chronic Pb BLM because of a combination of several reasons. First, Mager et al. [3] found no protective effect of Ca on chronic Pb toxicity to *C. dubia*, and the finding that chronic Pb toxicity was slightly higher in the present study at the lowest Ca concentration was somewhat uncertain because of a high number of males recorded in this specific test. Second, the effect of Ca^{2+} on Pb^{2+} toxicity was of less importance than the effect of pH. Only a 3-fold range of $EC50_{Pb2+}$ values was observed between the lowest and the highest Ca concentration (Figure 2A), while a 13-fold range in $EC50_{Pb2+}$ values was observed between the lowest and highest pH (Figure 2B). Third, including a Ca effect did not improve the overall predictive capacity of the BLM. The BLM combining both a single-site H^+ ($\log K_{HBL} = 7.6$) and a single-site Ca^{2+} competition effect ($\log K_{CaBL} = 3.1$, based on the regression in Figure 2A) and the BLM with only a single-site H^+ competition effect ($\log K_{HBL} = 7.6$), both using clone-specific intrinsic sensitivities, predicted Pb toxicity in all synthetic and natural waters (except the test waters containing MOPS or humic acid and the French lake 1 test water) with average prediction errors of 1.42- and 1.40-fold, respectively, and with maximum prediction errors of 3.31- and 2.22-fold, respectively. Both models predicted Pb toxicity for 92% of these test waters within 2-fold error (see Supplemental Data, Tables S2.4 and S3).

Figure 4 shows how well the BLM, calibrated with the clone-specific intrinsic sensitivities, predicts the observed Pb toxicity, expressed as $EC50_{Pbfilt}$, in the Parametrix study [12], the Mager et al. study [3], and the present study. The individual effect of pH

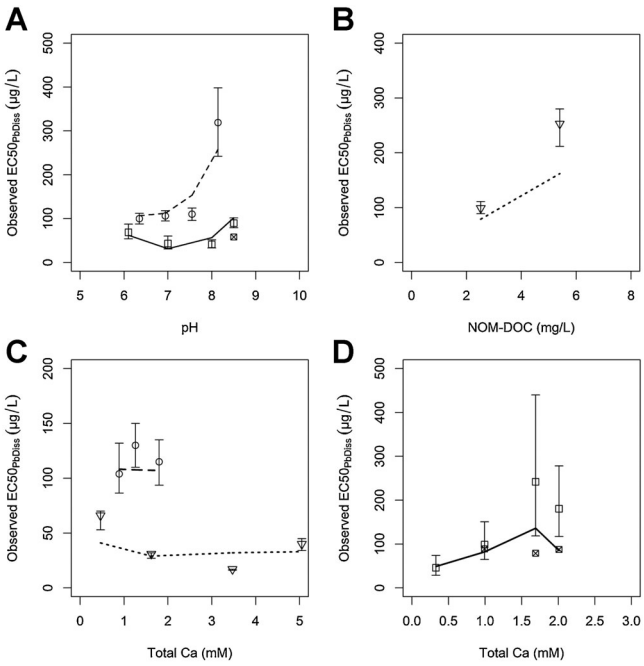

Figure 4. Observed (symbols) and predicted (lines) effects of pH (data from the present study and Parametrix [12]; **A**), dissolved organic carbon (data from Mager et al. [3]; **B**), Ca (data from the present study and from Mager et al. [3]; **C**), and Ca (data from Parametrix [12]; **D**) on the chronic toxicity to *Ceriodaphnia dubia*. Error bars are 95% confidence limits. Symbols are as follows: ◯ data from the present study, □ Parametrix [12], ▽ Mager et al. [3]. Crossed symbols show truly dissolved Pb concentrations as calculated by Visual Minteq, when colloidal Pb precipitation is predicted. Lines are as follows: full line, Parametrix [12]; dashed line, present study; and dotted line, Mager et al. [3]. The data of the 0.25-mM Ca treatment of the present study are not included for reasons mentioned in the text. EC50 = median effective concentration; NOM-DOC = natural organic matter–dissolved organic carbon.

on Pb toxicity tested in the present study and in the Parametrix study [12] (Figure 4A) and the univariate effect of natural organic matter addition (Figure 4B) on Pb toxicity reported by Mager et al. [3] were all accurately predicted by the BLM. Calcium was univariately varied by Mager et al. [3] and in the present study. The absence of an effect of Ca on Pb toxicity in both Ca test series was accurately predicted by the developed BLM (Figure 4C). In contrast, Parametrix [12] reported that increased ambient Ca reduced chronic Pb toxicity to *C. dubia*. However, it should be noted that the Ca effect in that study was not tested in a univariate manner as Ca concentrations covaried with concentrations of other cations, pH, and alkalinity. Despite the absence of a Ca effect parameter in the final BLM, the reported trend of increasing $EC50_{Pbfilt}$ with increasing Ca is predicted reasonably well by the BLM (Figure 4D), suggesting that the trend of decreasing toxicity with increasing Ca in this particular study is explained by the covariation of Ca with other chemical parameters and not by Ca itself. Colloidal Pb precipitation was predicted by Visual Minteq in 4 waters, all of which were from the Parametrix data set. Chronic 7-d Pb toxicity was almost always overestimated in these 4 waters. Predicted $EC50_{Pbfilt}$ values were almost always closer to the calculated true dissolved concentration than to the observed filtered concentration (Figure 4C and D). This suggests that colloidal Pb precipitation indeed may be present in these waters. Taking into account Pb precipitation in speciation calculations can therefore provide more insight into the role that colloidal Pb

mineral formation may play in the observed variation of measured filtered EC50s among test media.

The BLM optimized with the clone-specific intrinsic sensitivity predicted $EC50_{Pbfilt}$ for all field waters within 2-fold error, except for the French lake 1 water and the Sweetwater strand water, where toxicity was underestimated with 11- and 2.2-fold error, respectively (Figure 3B). However, the underestimation of toxicity in the French lake 1 field water was even higher (12-fold) with a preliminary chronic Pb BLM for *C. dubia* [2] (see Supplemental Data, Figure S1 and Table S3). Esbaugh et al. [2] argued that the underestimation of Pb toxicity in French lake 1 water was potentially caused by the nature of the DOC in this field water, which has low protective capacities, and was not taken into account in the BLM structure. Interestingly, retesting of the same French lake natural water by AquaTox [15] but with a sample taken at a later date (i.e., French lake 2) resulted in an $EC50_{Pbfilt}$ which could be predicted within a factor 2 error (Figure 3B), suggesting that the French lake 1 test is likely an outlier for unknown reasons. While the original observed variation in $EC50_{Pbfilt}$ values among the natural waters, not considering the French lake 1 test, was 6-fold (i.e., 94.8–573 μg/L), most of the $EC50_{filt}$ values were predicted with the new BLM within an error of 2-fold (Figure 3). This suggests that the *C. dubia* BLM of the present study can be used to predict Pb toxicity in natural waters.

To evaluate the improvement of the newly developed BLM (present study) compared with the preliminary BLM [2], Pb toxicity, expressed as $EC50_{Pbfilt}$, was predicted for all waters following the same procedure as for the validation of the newly developed BLM with the clone-specific intrinsic sensitivity (see *Materials and Methods*) but using the BLM equation described in Esbaugh et al. [2] instead of Equation 4. Overall, the BLM developed in the present study improved predictions of Pb toxicity in the synthetic and natural media when compared with the preliminary BLM developed by Esbaugh et al. [2] (Supplemental Data, Figure S1). When the media where MOPS or humic acid was added and the French lake 1 field water was not considered, Pb toxicity was predicted by the preliminary BLM with an average of 1.53 (range, 1.01–2.74) and within 2-fold error for 86% of the waters, while the BLM developed in the present study predicted Pb toxicity with an average of 1.40 (range, 1.04–2.22) and for 92% of the synthetic and natural waters within 2-fold error (Supplemental Data, Table S2).

Implications for risk assessment

Our results with Pb add to the growing evidence that water hardness is not always the main water chemistry parameter influencing chronic metal toxicity [3,7,32]. This is worrying because water-quality criteria for several metals, including Pb, are still commonly derived using hardness correction equations [33,34], allowing a higher Pb concentration in surface waters with higher hardness. For example, for a water hardness of 100 mg $CaCO_3$/L, the Canadian water-quality guideline for the protection of aquatic life is 3.18 μg filtered Pb/L [34], which is close to the 30-d EC20 of $\leq$4 μg dissolved Pb/L reported for the most sensitive freshwater organism, *Lymnaea stagnalis*, at this hardness level [1]. This raises questions whether this and other sensitive species are adequately protected by hardness-based water-quality guidelines, especially at high hardness and low DOC concentrations [32]. Biotic ligand models, on the contrary, have proven their usefulness in predicting chronic metal toxicity in natural waters based on a more complete knowledge of bioavailability-influencing water characteristics (such as pH, DOC, Ca, Mg, Na) [7,9]. Therefore, BLM–based water-quality criteria are gaining increased attention in the regulatory field. In 2007, BLM–based water-quality criteria for Cu were incorporated in the ambient water-quality criteria guidelines of the United States [11]. Around the same time, BLMs were also incorporated in the risk assessment of Cu, Ni, and Zn in the European Union [35–37]. The BLM–based Cu water-quality criteria were already shown to be an improvement compared with hardness-based criteria, especially in waters with high hardness levels [38,39]. In addition, BLM–based approaches to calculate freshwater-quality criteria for Zn have been developed recently [10,40]. This illustrates that the incorporation of bioavailability of metals in current water-quality guidelines and risk assessments is indispensable and that further research is needed to develop Pb BLMs (also with other organisms) and to investigate how these can be implemented in the risk-assessment process. In the meantime, based on the present research with *C. dubia*, BLM–based criteria are likely to be more appropriate relative to hardness-based criteria to address the risk of Pb in surface waters.

SUPPLEMENTAL DATA

Table S1 (20 KB XLS).
Table S2 (14 KB XLS).
Table S3 (27 KB XLS).
Figure S1 (285 KB PDF).

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