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Experimentally derived acute and chronic copper Biotic Ligand Models for rainbow trout

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ABSTRACT

We evaluated the effects of varying water chemistry ($[Ca^{2+}]$ = 0.2–3 mM, $[Mg^{2+}]$ = 0.05–3 mM, dissolved organic matter (DOM, natural, from maple leaves) = 0.3–10 mg of C L^{-1}, pH = 5.0–8.5) on the acute (96-h, unfed fish) and chronic (30-d, fed fish) toxicity of waterborne Cu to juvenile rainbow trout (*Oncorhynchus mykiss*) exposed in flow-through conditions. Acute and chronic Biotic Ligand Models (BLMs) were developed from the obtained toxicity data-sets, using the Visual MINTEQ software. Our results indicate that Cu is predominantly an acute toxicant to rainbow trout, as there were no observable growth effects and the 96-h and 30-d LC50 values were similar, with mortality mostly occurring within the first few days of exposure. Calcium and DOM were greatly protective against both acute and chronic Cu toxicity, but Mg seemed to only protect against chronic toxicity. Additional protection by pH 5.0 in acute exposure and by pH 8.5 in chronic exposure occurred. In the range of conditions tested, the observed 96-h LC50 and 30-d LC20 values varied by a factor of 39 and 27 respectively. The newly developed acute and chronic BLMs explained these variations reasonably well (*i.e.* within a 2-fold error), except at pH ≥ 8 where the high observed acute toxicity could not be explained, even by considering an equal contribution of $CuOH^+$ and Cu^{2+} to the overall Cu toxicity. The 96-h LC50 values of 59% of 90 toxicity tests from 19 independent studies in the literature were reasonably well predicted by the new acute BLM. The LC20 predictions from the new chronic BLM were reasonable for 7 out of 14 toxicity tests from 6 independent chronic studies (with variable exposure durations). The observed deviations from BLM predictions may be due to uncertainties in the water chemistry in these literature studies and/or to differences in fish sensitivity. A residual pH effect was also observed for both the acute and the chronic data-sets, as the ratio of predicted *vs.* observed LC values generally increased with the pH. Additional mechanistic studies are required to understand the influence of pH, Na, and Mg on Cu toxicity to trout. The present study presents the first experimentally developed chronic Cu BLM for the rainbow trout. To the best of our knowledge, it also presents the first acute Cu BLM that is based on a published data-set for trout. These newly developed BLMs should contribute to improving the risk assessment of Cu to fish in freshwater.

1. Introduction

Over 40 years of research have brought unequivocal evidence that water chemistry, such as the pH, the hardness and the concentration of dissolved organic matter (DOM), strongly modulates metal bioavailability and hence toxicity to aquatic organisms (Sunda and Guillard, 1976; Allen et al., 1980; Pagenkopf, 1983). The Biotic Ligand Model (BLM), developed from these observations, proposes a conceptual framework describing the relationship between water chemistry and metal bioavailability to aquatic organisms (Bergman and Doward-King, 1997; Di Toro et al., 2001; Santore et al., 2001; Paquin et al., 2002; Niyogi and Wood, 2004). This model is based on the simplifying premise that metal toxicity is proportional to the amount of metal bound onto sensitive biological sites (called biotic ligands (BL), *e.g.* ion channels at the gill epithelium), which is reduced by (i) the presence of aqueous cations (*e.g.* Ca^{2+} and Mg^{2+}) competing with the metal for binding to the BL and by (ii) the presence of aqueous ligands (*e.g.* DOM, OH^-, SO_4^{2-}) competing with the BL for binding to the metal. Thus, for a given metal M, water chemistry and biological species, a concentration of M leading to a certain effect level (*e.g.* LC50: median lethal concentration) can be determined by knowing the affinity constants describing the above chemical reactions (*e.g.* K_{MBL}, K_{HBL}, K_{MSO4}, K_{MOH}, *etc.*) as well as the

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species-specific concentration of M-BL associated with this effect level (*e.g.* LA50: median lethal accumulation). A BLM corresponds to a set of these parameters and is usually specific to a metal, an organism and an exposure regime (*e.g.* acute Cu BLM for *Daphnia magna*, De Schamphelaere and Janssen, 2002).

The BLM has been recognized as a mechanistic, cost-effective and flexible tool to develop site-specific water quality criteria (Campbell et al., 2006; Van Sprang et al., 2009, 2016; Peters et al., 2011, 2016; Hoppe et al., 2015) and several legislations now use BLM-based approaches for the regulation of different metals (*e.g.* the freshwater Cu criteria of the United States and the European Union; USEPA, 2007; ECHA, 2008; ECI, 2008; Lathouri and Korre, 2015). Over the past decades, toxicity data collected in different waters have been used to develop BLMs. However, while water chemistry effects on acute (short-term) metal toxicity are relatively well documented for certain metals and organisms, they remain largely unknown for chronic (long-term) toxicity. The collection of chronic toxicity data-sets (exposure typically > 2–3 weeks) is inherently more time-consuming and costly than the collection of acute toxicity data-sets (typically 48–96 h of exposure). So far, a few chronic BLMs have been developed, but mostly for invertebrates (Cu: De Schamphelaere and Janssen, 2004a; Zn: Heijerick et al., 2005; Ni: Deleebeeck et al., 2008; Schlekat et al., 2010; Pb: Nys et al., 2014; De Schamphelaere et al., 2014). For fish, only a chronic Zn BLM for rainbow trout (De Schamphelaere and Janssen, 2004b) and a chronic Pb BLM for fathead minnow (Van Sprang et al., 2016) have been proposed. Therefore, chronic water quality criteria often rely on simplifying approaches that differ from one jurisdiction to another. In the case of the EU, chronic Cu toxicity to fish is based on the acute Cu BLM originally developed for *Daphnia magna* (De Schamphelaere and Janssen, 2008; Delbeke et al., 2010). In the case of the US, chronic Cu toxicity is based on acute toxicity, calculated by applying an Acute-to-Chronic Ratio (ACR) to the output of an acute BLM (USEPA, 2007). Currently, there is one available acute Cu BLM for rainbow trout, which is hosted on the website of Windward Environmental (formerly hosted by HDR-Hydroqual) and based on an unpublished data-set (personal communication with Robert C. Santore and Robert Dwyer).

In the present study, we generated new acute and chronic Cu toxicity data-sets for juvenile rainbow trout under varying pH and concentrations of Ca, Mg and DOM (in a baseline soft water) using a flow-through exposure system. Most of the pH data were published earlier (Ng et al., 2010), but all other data are reported here for the first time. For this fish species, a few studies have specifically considered the effects of water chemistry parameters (pH, alkalinity, hardness, DOC concentration) on acute Cu toxicity (*e.g.* Cusimano et al., 1986; Howarth and Sprague, 1978; Mudge et al., 1993; Marr et al., 1999; Welsh et al., 2000; Naddy et al., 2002) and fewer studies have specifically looked at these effects on chronic Cu toxicity (Waiwood and Beamish, 1978a,b; Mudge et al., 1993; Taylor et al., 2000; McGeer et al., 2002). Our main goals were (i) to develop new acute and chronic BLMs for juvenile rainbow trout from our own experimental data presented here, (ii) to evaluate the performance of these new BLMs at predicting Cu toxicity to rainbow trout in independent studies, and (iii) to compare the predictions from these new BLMs with the existing BLM-based approaches in the USA and in the EU. To the best of our knowledge, our new acute and chronic data-sets allowed development of the first experimentally derived chronic Cu BLM for the rainbow trout, as well as the first acute Cu BLM that is based on a published data-set for this species.

2. Materials and methods

2.1. Experimental fish and acclimation to baseline conditions

Juvenile rainbow trout (*Oncorhynchus mykiss*; initially approximately 2 g) were purchased from Humber Springs Trout Farm (Orangeville, ON, Canada) and were acclimated to laboratory conditions in aerated flow-through tanks supplied with dechlorinated hard water (HW) from the City of Hamilton. This water originates from Lake Ontario and its composition was (in $\mu mol\ L^{-1}$): [Ca] ~ 1000; [Mg] ~ 150; [Na] ~ 600; [Cl] ~ 700; [titratable alkalinity] ~ 1800; with [DOC] ~ 2 $mg\ L^{-1}$. Fish were then gradually acclimated to the soft water used as the basis for the toxicity tests (noted RW, for "Reference Water"). This was done by decreasing the flow rate of dechlorinated HW and increasing the flow rate of ion-poor water produced by reverse osmosis (Anderson Water Systems, Dundas, ON, Canada) up to a ratio of 15:85 (*i.e.* 6.7-fold dilution) reached in 8 days. The resultant RW composition was (in $\mu mol\ L^{-1}$): [Ca] ~ 150; [Mg] ~ 50; [Na] ~ 70; [Cl] ~ 140; [titratable alkalinity] ~ 270; with pH ~ 7, [Cu] ~ 1 $\mu g\ L^{-1}$, temperature ~ 13 °C and [DOC] ~ 0.5 $mg\ L^{-1}$ (see Section 3.1 for more details on DOC concentration).

Fish were acclimated to these new RW conditions for at least 22 days prior to the experiments. They were fed with commercial trout pellets (Martin Feed Mills, Elmira, ON, Canada) at 2% body weight per day and maintained under a 12-h light/12-h dark photoperiod throughout the study. Procedures conformed to the guidelines of the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board (AUP 06-01-05).

2.2. Preparation of test solutions

The RW described above was used as basis for the toxicity tests at varying pH and concentrations of Ca, Mg and DOC. Note that aqueous Na concentration is usually a parameter of interest with respect to Cu toxicity to aquatic organisms (Laurén and McDonald, 1985; Grosell et al., 2002; Grosell, 2012). However in our study, in repeated tests where the Na concentration was raised to the 0.5–5 mM range by the addition of NaCl (Reagent grade, BioShop Canada Inc, Burlington, ON, Canada), increased fish mortality occurred in the control tanks (30–90%). While the effect is unexplained, this suggests that elevation of NaCl level may in itself be acutely toxic to rainbow trout acclimated to very soft, ion-poor water. This high control mortality prevented the incorporation of Na into the response matrix of this study, and is a matter for future investigation.

Copper was added in RW as $CuSO_4$ (pentahydrate, 99% pure, Sigma Aldrich, St. Louis, MO, USA) at seven concentrations ranging from 0 to 250 $\mu g\ L^{-1}$. For the pH-set, adjustment of water pH to 5.0, 6.0, 7.0, 8.0 and 8.5 was performed with 1 M HCl or 1 M NaOH (ACS grade, Fisher Scientific, Toronto, ON, Canada) using a pH titrator (TTT90 titrator, Radiometer, Copenhagen, Denmark), as detailed in Ng et al. (2010). For the Ca-set, Ca concentration was adjusted to 0.5, 1.2 and 3 mM by addition of $CaCl_2$ (Reagent grade, General Chemical Canada Ltd, Mississauga, ON, Canada). For the Mg-set, Mg concentration was adjusted to 1.2 and 3 mM by addition of $MgCl_2$ (ACS grade, Fisher Scientific). For the DOC-set, natural organic matter (NOM) was added at 1, 2, 5 and 10 mg of $C\ L^{-1}$. This NOM was produced in our laboratory, from maple leaves (maple tree species: *Acer rubrum*). Approximately 20 kg of maple leaves (mostly reddish in color) were collected during early fall and washed thoroughly with de-ionized water. For each batch, approximately 5 kg of leaves were incubated per 100 L of reverse osmosis water in 200-L tanks for 3 weeks. During incubation, aeration was provided and the leaves were periodically stirred. After formation of a dark brown color in the aqueous phase, the leaves were removed and the water was passed through 1-µm glass-fiber filters (144-mm diameter filters; Geotech Environmental Equipment, Denver, CO, USA). The NOM (pH = 5–6) was then resinated with cation Resin USF C-211 (H) (Siemens Water Technologies, Ancaster, ON, Canada) to remove metals. The resin was slowly added to the stirred NOM solution (1 L resin per 10 L NOM solution). After 24 h, the NOM solution (pH = 2.6) was decanted and transferred to 200-L aerated polyethylene tanks for ageing (to mimic the natural process). The DOC concentration was monitored in filtered samples (0.45 µm Acrodisc® syringe filters, Pall Corporation, Ann Arbor, MI, USA)

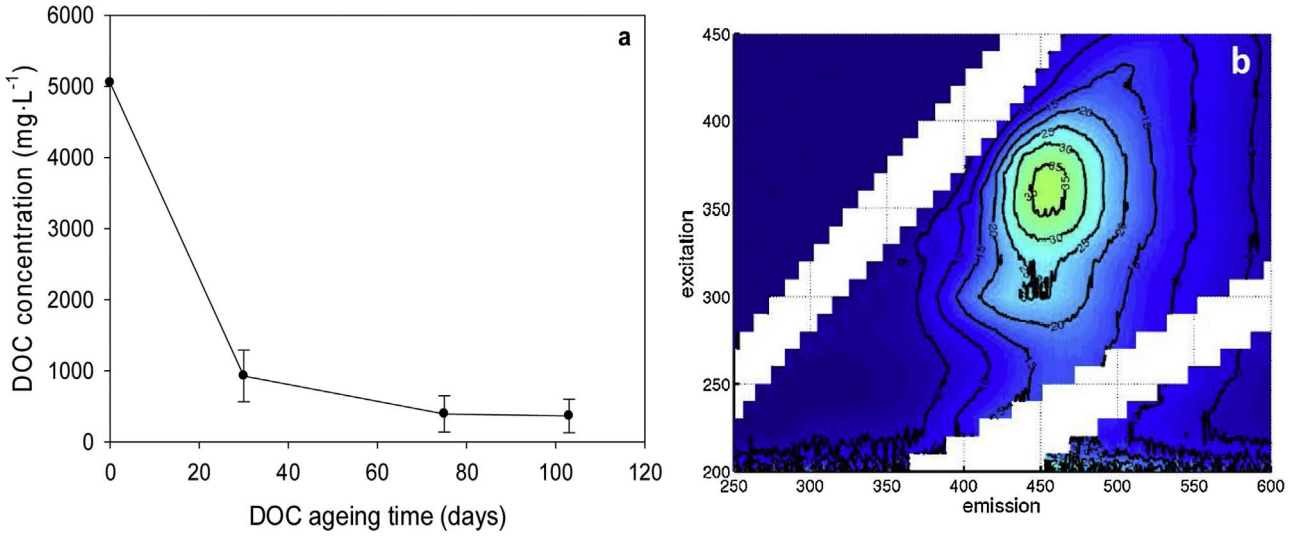

Fig. 1. Maple leaves DOC: a) decrease in concentration with ageing time, b) excitation/emission spectrum (wavelength in nm) at 100 days of ageing.

and the ageing process was stopped when this concentration stabilized, *i.e.* after approximately 3 months (Fig. 1a).

2.3. Acute and chronic toxicity tests

For each toxicity test, fish (n = 17 for the acute tests and n = 14–15 for the chronic tests) were transferred to 20-L flow-through aerated tanks served with baseline RW (250 mL min^{-1}) from a head tank *via* separate mixing chambers. Thus, this system provided 18 vol renewals per day and had a residence time of 80 min. For the pH-set, fish were acclimated to a gradual change of pH for 1 day in the experimental tanks (Ng et al., 2010). On the next day (0 h of toxicity test), Cu was added to each mixing chamber using a Mariotte bottle (0.5 mL min^{-1}) to achieve the desired final concentration in the experimental tank. For Ca, Mg and DOM, stock solutions were directly metered at the rate of 2–3 mL min^{-1} to the head tank used for mixing of HW and reverse osmosis water to produce RW. Drip rates of Cu, ratio of HW and reverse osmosis water, and pH in experimental tanks were monitored daily.

For the acute tests, feeding was withheld 24 h before beginning the exposure and throughout the 96-h test. The average fish wet weight for these tests was 6.4 ± 1.1 g. Fish in each tank were monitored every 3–5 h and dying fish (overturned) were removed immediately and euthanized with an overdose of tricaine methanesulfonate (MS-222; Syndel Laboratories, Qualicum Beach, BC, Canada). In chronic tests, fish grew on average from 6.7 ± 1.6 to 10.2 ± 2.5 g wet weight over the 30-d tests. For these tests, fish were fed a 2% wet weight body ration each day and weighed in bulk at 0 h, 96 h, 10 d, 20 d and 30 d to calculate the biomass and adjust the food accordingly during the exposure. Dying fish were removed and euthanized as described above and faeces were siphoned daily.

Water samples from each tank were passed through 45-µm Acrodisc® syringe filters (Pall Corporation, Ann Arbor, MI, USA) at 24 h and 96 h for the acute tests and at 24 h, 96 h, 10 d, 20 d, and 30 d for the chronic tests for subsequent analyses of Cu, Ca, Mg, Na, Cl, alkalinity and DOC concentrations (see Section 2.4). In addition, for the pH-set only, 7 fish from each tank were euthanized at 24 h (acute and chronic tests) and 30 d (chronic tests) and dissected for Cu and Na analyses of gills and whole bodies (Ng et al., 2010).

The 96-h, 10-d, 20-d and 30-d LC50, LC20 and LC10 estimates (lethal concentration to kill 50%, 20% and 10% of fish respectively), together with their 95% confidence limits, were calculated from filtered water Cu concentrations using the Toxicity Relationship Analysis Program (TRAP) version 1.22 (USEPA, 2002), employing the Tolerance Distribution analysis and the Gaussian distribution model. Fish specific growth rates (SGR, in % per day) in the chronic tests were calculated based on fish wet weight at t = 0 (W_0) and at t = 10 d, 20 d and 30 d (W_t) (SGR = ($\ln(W_0) - \ln(W_t)$)/t 100). Finally, for the pH-set only, the acute and chronic 24-h LA50 (for 96-h and 30-d mortality respectively) were estimated from the measured 24-h Cu gill contents, as detailed in Ng et al. (2010). The LA50 is the gill burden of Cu predictive of 50% mortality at a later timepoint (*e.g.* 96 h or 30 d).

2.4. Water and tissue analyses

Filtered water samples were acidified with 1% trace-metal grade HNO_3 for Cu and Na analyses, and 0.5% $LaCl_3$ was also added for Ca and Mg analyses. Copper concentration was measured by a Graphite Furnace Atomic Absorption Spectrometer (GFAAS, Varian Spectra AA-20 with a GTA-110 furnace, Mulgrave, Australia) and Na, Ca, and Mg concentrations were measured by a Flame Atomic Absorption Spectrometer (FAAS, Varian Spectra-220FS, Mulgrave, Australia). Metal recovery was checked with a certified reference material (TM15, National Water Research Institute, Environment Canada, Burlington, Canada) and was 100 ± 10%. Chloride concentration in the water was measured colorimetrically at 480 nm by the $Hg(SCN)_2$ method (Zall et al., 1956). Water alkalinity was measured by the pH 4 endpoint titration method (McDonald and Wood, 1981). For measurements of DOC concentration, water samples were stored in the dark at 4 °C until analysis with a Total Organic Carbon analyzer (Shimadzu TOC-VCPH, Mandel Scientific Company Inc., Guelph, ON, Canada). Additionally, the distribution of fluorescent components in the maple leaves DOM was determined by excitation–emission matrix (EEM) fluorescence spectroscopy. For each sample (n = 3 replicates), fluorescence scans were conducted using a Varian Cary Eclipse Fluorescence Spectrophotometer (Varian, Mississauga, ON). Excitation wavelengths were between 200 and 450 nm, using 10-nm increments and emission intensities were collected between 250 and 600 nm with approximately 1-nm increments. Often a four-component model is used to determine fluorescent components in NOM: typically humic-like, fulvic-like, tryptophan-like, and tyrosine-like fluorophores are resolved (DePalma et al., 2011; Al-Reasi et al., 2012). These components are determined using parallel factor analysis (PARAFAC, Stedmon and Bro, 2008). For the maple leaves DOM, there was no evidence of proteinaceous fluorophores (Fig. 1b) so a two component humic and fulvic model was used. The composition of the replicate DOM samples was determined using humic acid-like and fulvic acid-like components previously reported by Al-Reasi et al. (2012) for linear calibration. With only one type of DOM studied here a full PARAFAC analysis was not possible, as many diverse

Table 1
Chemical characteristics of the test media used in the acute (96-h) and chronic (30-d) toxicity tests. Values are measured means, except for the % of active DOM and % FA which were assumed (except for the % FA in the DOM-set which was estimated by EEM-PARAFAC). See text for details.

Nominal conditions	Temp (°C)	pH	Ca (mM)	Mg (mM)	Na (mM)	K (mM)	SO_4 (mM)	Cl (mM)	Alkalinity (mg L^{-1} $CaCO_3$)	DOC (mg L^{-1})	% active DOM	% FA
96-h exposures												
Baseline RW	13	7.10	0.17	0.054	0.14	0.010	0.038	0.13	34	0.34	65	100
0.5 mM Ca	13	7.06	0.61	0.053	0.13	0.010	0.038	0.83	36	0.31	65	100
1.2 mM Ca	13	7.15	1.2	0.054	0.18	0.035	0.038	1.5	45	0.47	65	100
3.0 mM Ca	13	7.20	3.1	0.071	0.23	0.067	0.038	2.3	48	0.55	65	100
1.2 mM Mg	13	7.10	0.15	1.4	0.17	0.035	0.038	1.6	40	0.48	65	100
3.0 mM Mg	13	7.19	0.17	3.3	0.23	0.067	0.038	2.7	42	0.58	65	100
pH 5	13	5.00	0.09	0.041	0.10	0.010	0.030	0.45	7.2	0.5*	65	100
pH 6	13	6.20	0.08	0.040	0.10	0.010	0.030	0.32	13	0.5*	65	100
pH 7	13	7.10	0.17	0.054	0.14	0.010	0.038	0.13	34	0.34	65	100
pH 8	13	8.10	0.09	0.046	0.24	0.010	0.030	0.10	38	0.5*	65	100
pH 8.5	13	8.50	0.10	0.043	0.38	0.010	0.030	0.16	70	0.5*	65	100
1 mg/L DOC	13	7.04	0.15	0.055	0.12	0.012	0.038	0.13	34	0.93	65	40
2 mg/L DOC	13	7.09	0.15	0.050	0.12	0.015	0.038	0.62	32	2.1	65	40
5 mg/L DOC	13	7.02	0.16	0.058	0.14	0.017	0.038	0.21	40	5.5	65	40
10 mg/L DOC	13	7.01	0.18	0.065	0.16	0.022	0.038	0.38	42	11	65	40
30-d exposures												
Baseline RW	13	7.10	0.16	0.052	0.13	0.010	0.030	0.14	32	0.42	65	100
0.5 mM Ca	13	7.14	0.54	0.055	0.14	0.010	0.038	0.74	36	0.46	65	100
1.2 mM Ca	13	7.15	1.4	0.053	0.18	0.035	0.038	1.72	45	0.49	65	100
3.0 mM Ca	13	7.29	3.1	0.072	0.23	0.067	0.038	2.4	48	0.58	65	100
1.2 mM Mg	13	7.10	0.17	1.3	0.18	0.035	0.038	1.6	40	0.48	65	100
3.0 mM Mg	13	7.18	0.19	3.1	0.26	0.067	0.038	2.6	42	0.55	65	100
pH 5	13	5.10	0.07	0.044	0.12	0.010	0.030	0.49	6.8	0.5*	65	100
pH 6	13	6.20	0.08	0.047	0.11	0.010	0.030	0.42	15	0.5*	65	100
pH 7	13	7.10	0.16	0.052	0.13	0.010	0.030	0.14	32	0.42	65	100
pH 8	13	7.90	0.07	0.040	0.23	0.010	0.030	0.14	47	0.5*	65	100
pH 8.5	13	8.60	0.06	0.044	0.37	0.010	0.030	0.15	54	0.5*	65	100
2 mg/L DOC	13	7.10	0.15	0.054	0.13	0.015	0.038	0.63	32	2.2	65	40
5 mg/L DOC	13	7.02	0.17	0.057	0.14	0.017	0.038	0.22	40	5.6	65	40
10 mg/L DOC	13	7.02	0.17	0.065	0.16	0.022	0.038	0.39	42	11	65	40

Note: Baseline RW and pH 7 are the same tests.
* Corrected value (measured value ~1.4 mg L^{-1} in Ng et al., 2010; see Supplementaryinformation Section 2 for more information).

samples are required for PARAFAC modeling (Stedmon and Bro, 2008). This analysis provided the percentages of humic acid (HA) and fulvic acid (FA) like components in the maple leaves DOM, which were then used for Cu speciation modeling in the waters of the DOC-set (see Section 2.5).

The fish tissue samples from the pH-set were weighed and digested in 3–5 vols of 1 N HNO_3 (trace-metal grade, Fisher Scientific, Toronto, ON, Canada) at 65 °C for 48 h, with shaking at 24 h. The supernatant was collected after centrifugation of the digest (5000 g for 15 min), then diluted for Cu and Na analyses by GFAAS and FAAS respectively (Ng et al., 2010).

2.5. Chemical speciation modeling

Chemical speciation, notably Cu^{2+} activity, was estimated at each tested water chemistry (Table 1) in order to derive the acute and chronic BLM constants (see Section 2.6). These speciation calculations were performed at the observed 96-h and 30-d LC50 values (Table 2) in the Visual MINTEQ software version 3.1 (https://vminteq.lwr.kth.se/). Modeling of Cu binding to DOM was performed using the NICA-Donnan model embedded in Visual MINTEQ and using default assumptions which have been shown to provide good agreement with measurements in natural waters (Buffle, 1988; Bryan et al., 2002; Tipping et al., 2003). Specifically, we assumed that DOM contained 50% carbon (DOC) and that 65% of the DOM was chemically active with regard to Cu binding (*i.e.* 1 mg L^{-1} active DOM = 1.3 mg L^{-1} DOC). Furthermore, when no information on DOM composition is available, it is classically recommended to consider it as 100% FA. The latter assumption was used for all the tests with background DOM levels. On the other hand, we used the results of the EEM for the tests with maple leaves DOM, which suggested that it was composed of 40% FA and 60% HA (Fig. 1b). EEM characterization of relative humic and fulvic proportions has been used successfully before to improve BLM predictions for acute Cu toxicity to invertebrates (Al-Reasi et al., 2012).

2.6. Acute and chronic BLM parameterization

The formation constants K_{CaBL}, K_{MgBL}, K_{HBL} for the new acute and chronic BLMs were derived by linear regression analysis as described in detail by De Schamphelaere and Janssen (2002) and briefly in this section. The BLM is an equilibrium-based model which assumes that metal toxicity is directly proportional to the fraction of biological targets (*i.e.* biotic ligand, BL) bound by the metal, noted f_{CuBL} and expressed as:

$$f_{CuBL} = [K_{CuBL}\cdot(Cu^{2+}) + K_{CuOHBL}\cdot K_{CuOH}\cdot(Cu^{2+})\cdot(OH^-)] / [1 + K_{CuBL}\cdot(Cu^{2+}) + K_{CuOHBL}\cdot K_{CuOH}\cdot(Cu^{2+})\cdot(OH^-) + K_{CaBL}\cdot(Ca^{2+}) + K_{MgBL}\cdot(Mg^{2+}) + K_{HBL}\cdot(H^+)] \quad (1)$$

where () are ion activities in the water, K_{CBL} is the formation constant between BL and cation C (C: Cu^{2+}, $CuOH^+$, Mg^{2+}, Ca^{2+}, or H^+) and K_{CuOH} is the formation constant of $CuOH^+$. Note that both Cu^{2+} and $CuOH^+$ bind to BL in this model, as the toxicity of $CuOH^+$ is generally thought to contribute to the overall toxicity in acute Cu BLMs (Allen and Hansen, 1996; De Schamphelaere and Janssen, 2002). From Eq. (1), the Cu^{2+} activity at 50% mortality ($(Cu^{2+}) = LC50_{Cu2+}$) can be derived from the following equation:

$$LC50_{Cu^{2+}} = \frac{f_{CuBL}^{50}}{(1 - f_{CuBL}^{50})\cdot K_{CuBL}} \cdot \frac{1 + K_{CaBL}\cdot(Ca^{2+}) + K_{MgBL}\cdot(Mg^{2+}) + K_{HBL}\cdot(H^+)}{1 + \frac{K_{CuOHBL}}{K_{CuBL}}\cdot K_{CuOH}\cdot(OH^-)} \quad (2)$$

Table 2
Measured acute and chronic Cu LC values, with 95% confidence limits in parentheses, in rainbow trout in different water chemistries, in comparison to the values predicted by the new acute and chronic BLMs, and the observed Acute-to-Chronic Ratios (ratio of observed 96-h LC50 to 30-d LC20 values).

Nominal conditions	96-h LC50		30-d LC50	30-d LC20		30-d LC10	ACR
	Measured	Predicted New BLM	Measured	Measured	Predicted New BLM	Measured	
Baseline RW	9.2 [5.7–13]	7.7	8.2 [7.3–9.1]	5.2 [4.3–6.0]	7.5	3.9 [3.0–4.9]	1.77
0.5 mM Ca	12 [9.7–14]	9.6	11 [9.1–12]	6.6 [5.0–8.3]	10	4.6 [2.5–6.7]	1.82
1.2 mM Ca	29 [9.9–48]	18	42 [37–47]	31 [25–37]	18	24 [17–32]	0.935
3.0 mM Ca	45 [29–61]	32	70 [35–100]	49 [29–69]	43	39 [24–53]	0.918
1.2 mM Mg	10 [8.7–11]	8.5	13 [12–14]	10 [8.9–12]	5.8	9.3 [7.3–11]	1.00
3.0 mM Mg	13 [12–14]	10	18 [15–22]	16 [12–19]	6.9	14 [9.0–18]	0.813
pH 5	19 [10.–29]	19	8.1 [4.6–11]	5.0 [1.7–7.3]	3.7	3.8 [1.0–6.0]	3.80
pH 6	5.9 [4.6–7.2]	6.0	7.4 [6.4–9.6]	5.5 [4.5–6.3]	3.0	4.7 [3.4–5.4]	1.07
pH 7	9.2 [5.7–13]	7.7	8.2 [7.3–9.1]	5.2 [4.3–6.0]	7.5	3.9 [3.0–4.9]	1.77
pH 8	8.5 [7.1–10.]	20	9.3 [6.6–11]	8.2 [1.9–9.1]	16	7.6 [1.0–8.7]	1.04
pH 8.5	6.7 [5.8–7.5]	33	22 [17–28]	18 [10.–22]	39	16 [7.9–20]	0.372
1 mg/L DOC	31 [27–35]	21	n.d.	n.d.	–	n.d.	–
2 mg/L DOC	43 [38–49]	47	43 [37–49]	33 [25–40]	37	27 [17–37]	1.30
5 mg/L DOC	140 [120–150]	111	120 [110–140]	99 [75–120]	87	87 [55–120]	1.41
10 mg/L DOC	230 [210–260]	220	200. [190–220]	170 [140–190]	170	150 [110–180]	1.35

n.d.: no data.
Note: Baseline RW and pH 7 are the same tests.

where f^{50}_{CuBL} is the fraction of the BL bound by Cu at 50% mortality. According to this mathematical expression, a linear relationship should be observed between $LC50_{Cu2+}$ and the varying competing cation (Ca^{2+}, Mg^{2+} or H^+) activities. The ratio between the slopes and intercepts of these linear relations provide a matrix of equations for the determination of K_{CaBL}, K_{MgBL} and K_{HBL} (see De Schamphelaere and Janssen, 2002 for details). Note that any toxicity level could be used for this method, but because the $LC50_{Cu2+}$ is determined with the lowest uncertainty, it was selected for these calculations. Visual MINTEQ was used to determine the different ion activities at the observed 96-h and 30-d LC50 values (*cf.* Section 2.5). The linear regressions of $LC50_{Cu2+}$ *vs.*(Ca^{2+}), $LC50_{Cu2+}$ *vs.* (Mg^{2+}) and $LC50_{Cu2+}$ *vs.* (H^+) were then performed in Excel®. Note that the steepness of the H^+ slope tends to increase at higher pH where the toxicity of $CuOH^+$ is no longer negligible compared to the toxicity of Cu^{2+}. Thus, the linear regression $LC50_{Cu2+}$ *vs.* (H^+) was performed between pH 5 and 7, where $CuOH^+$ abundance and therefore toxicity were considered negligible. Indeed, at 50% mortality, Visual MINTEQ predicted that $CuOH^+$ was still ~5 times less abundant than Cu^{2+} at pH = 7 (see Results Section, Fig. 5a). If we make the reasonable assumption that $K_{CuOHBL} \leq K_{CuBL}$ (*i.e.* the toxicity of $CuOH^+$ should not be greater than the toxicity of Cu^{2+}), $CuOH^+$ should then not have importantly contributed to the overall Cu toxicity between pH 5 and 7. The acute and chronic ratio K_{CuBL}/K_{CuOHBL} (and the term $f^{50}_{CuBL}/((1 - f^{50}_{CuBL})\cdot K_{CuBL})$ in Eq. (2)) were determined by nonlinear regression of Eq. (2) from pH 5 to 8.5 using Sigmaplot®, with the previously determined K_{HBL}, K_{MgBL} and K_{CaBL} values and with the constraint that $K_{CuOHBL}/K_{CuBL} \leq 1$ (*i.e.* $CuOH^+$ is less toxic than Cu^{2+}). The acute and chronic K_{CuBL} values were then determined by iteration to obtain the best linear fit between the observed logit percent mortality and f_{CuBL} calculated with Eq. (1) for the whole toxicity data-set, using the Solver analysis tool in Excel® (as described by De Schamphelaere and Janssen (2002)). Finally, for each acute and chronic data-set, the corresponding f^{50}_{CuBL} value corresponded to the geometric mean of each f^{50}_{CuBL} calculated with Eq. (1) at the different observed 96-h and 30-d LC50s. Similarly, for the chronic data-set only, the f^{20}_{CuBL} was obtained from the different observed 30-d LC20s.

Table 3
Parameters in the newly developed rainbow trout acute and chronic Cu BLMs, the Windward BLM Cu acute BLM (W-BLM) and the *Daphnia magna* acute Cu BLM (D-BLM).

Parameter	Newly derived acute BLM	Newly derived chronic BLM	W-BLM	D-BLM
	Software and DOM binding conditions			
Software	Visual MINTEQ 3.1	Visual MINTEQ 3.1	Windward 3.1.2.37	Visual MINTEQ 3.1
Cu-DOM binding model	NICA-Donnan	NICA-Donnan	WHAM V	NICA-Donnan
% active DOM	65	65	100	65
% FA (rest is HA)	40–100[a]	40–100[a]	90	100
	Stability constants of inorganic aqueous complexes			
Log K_{CuOH}	6.48	6.48	6.48	6.48
Log $K_{Cu(OH)2}$	11.78	11.78	11.78	11.78
Log K_{CuHCO3}	12.13	12.13	14.62	12.13
Log K_{CuCO3}	6.77	6.77	6.75	6.77
Log $K_{Cu(CO3)2}$	10.2	10.2	9.92	10.2
Log K_{CuCl}	0.4	0.4	0.4	0.4
Log K_{CuSO4}	2.36	2.36	2.36	2.36
	BLM parameters			
Log K_{CaBL}	3.5	4.0	3.6	3.5
Log K_{MgBL}	1.4	3.4	3.6	3.6
Log K_{HBL}	6.2	5.8	5.4	5.4
Log K_{NaBL}	3.0[b]	3.0[b]	3.0	3.2
Log K_{CuBL}	7.1	7.2	7.4	8.0
Log K_{CuOHBL}	7.1[c]	6.5[c]	6.2[c]	7.3[c]
Log $K_{CuCO3BL}$	–	–	–	7.0[d]
f^{50}_{CuBL}	0.119	0.0876	0.123	0.47
f^{20}_{CuBL}	–	0.0560	–	0.47[e]
ACR	–	–	3.22	–

[a] See Table 1 for detailed assignment of %FA to the different solutions.
[b] Not estimated: default value from the W-BLM.
[c] Constant refers to the reaction: CuOH-BL = $CuOH^+$ + BL.
[d] Constant refers to the reaction: $CuCO_3$-BL = $CuCO_3$ + BL.
[e] This f^{20}_{CuBL} value corresponds to the f^{50}_{CuBL} value in the *D. magna* Cu acute BLM, which was directly used here to determine LC20 in this study (i.e. no adjustment was made).

2.7. Acute and chronic toxicity predictions: this study and literature data

In this study, acute (96-h LC50) and chronic (30-d LC20) toxicity were predicted using the BLM code incorporated in Visual MINTEQ 3.1, where inputs were the measured water chemistry (given in Table 1) and the newly derived sets of BLM parameters (given in Table 3). Accordingly, we evaluated the abilities of the newly derived acute and chronic BLMs to predict Cu toxicity across a wide range of water chemistries, using toxicity data from the literature (*i.e.* data different from those used in derivation of the models). A total of 19 acute studies (90 data points) (Fogels and

Sprague, 1977; Chapman, 1978; Chapman and Stevens, 1978; Howarth and Sprague, 1978; Seim et al., 1984; Cusimano et al., 1986; Mudge et al., 1993; Marr et al., 1999; Welsh et al., 2000; Hansen et al., 2002; Naddy et al., 2002; Taylor et al., 2003; Besser et al., 2007; Welsh et al., 2008; Little et al., 2012; Vardy et al., 2013; Calfee et al., 2014; Wang et al., 2014; Naddy et al., 2015) and 6 chronic studies (14 data points) (McKim et al., 1978; Waiwood and Beamish, 1978b; Seim et al., 1984; Besser et al., 2007; Wang et al., 2014; OSU, 2016) were evaluated (Table SI.1 in Supporting information). Tests with rainbow trout (*Oncorhynchus mykiss* or previously named *Salmo gairdneri*) of any life stage/weight and using any hydraulic regime (flow-through, intermittent flow, static renewal or static) were included, and the effects of both life stage/weight and hydraulic regime on Cu toxicity were evaluated. The acute BLM was evaluated with 96-h LC50s in tests where fish were not fed while the chronic BLM was evaluated with LC20s at various longer exposure times (see Results) up to 78 d in tests where fish were fed. Missing water chemistry parameters in the different publications were mostly obtained from recommendations of the USEPA (2007) and from personal communications with the authors. The tests provided a broad range of water chemistries, given in detail in Table SI.1. Notably, for the 90 acute tests, minimum and maximum values were: T = 4.4–18 °C, Hardness = 9.2–371 mg L^{-1}, Alkalinity = 0–263 mg L^{-1} $CaCO_3$, pH = 4.7–9.0 and DOC = 0–3 mg L^{-1}. For the 14 chronic tests: T = 10.8–12.0 °C, Hardness = 30–360 mg L^{-1}, Alkalinity = 6.7–250 mg L^{-1} $CaCO_3$, pH = 6.0–8.2 and DOC = 0.4–1.6 mg L^{-1}. The default assumption of 65% active DOM as 100% FA was adopted for all the studies.

We also compared the performance of the new acute and chronic BLMs to the performance of the other available BLM approaches used in the USA and in the EU, for predictions of toxicity to rainbow trout using both our present data-sets and literature data. Firstly, we used the rainbow trout acute Cu BLM, that is available in the BLM software version 3.1.2.37 (hosted on the website of Windward Environmental http://www.windwardenv.com/biotic-ligand-model/, formerly hosted by HDR-Hydroqual), to predict acute LC50 values for our data and literature data. This Windward BLM (noted W-BLM in this paper) is based on an unpublished data-set (personal communication with Robert C. Santore and Robert Dwyer) and is currently the only publicly available rainbow trout Cu BLM. The BLM parameters and specific modeling conditions of the W-BLM are given in Table 3. Note that this model uses thermodynamic constants from the Windermere Humic Aqueous Model version V (WHAMV) and different assumptions for metal complexation with DOM (Table 3). Specifically, the W-BLM uses the humic ion-binding model incorporated in WHAMV (Tipping, 1994) and assumes that 100% of DOM is active and is 10% HA (90% FA). Another important difference is the value of the copper carbonate stability constant: W-BLM/WHAMV uses the K_{CuHCO3} of $10^{14.62}$ from Mattigod and Sposito (1979), while Visual MINTEQ uses the K_{CuHCO3} of $10^{12.13}$ recommended by the International Union of Pure and Applied Chemistry (IUPAC) (Powell et al., 2007). Secondly, in accordance to the USEPA approach, we used the W-BLM to calculate chronic LC20 values, by dividing the predicted LC50 values by an ACR of 3.22 for Cu from the USEPA (2007). As described by the latter report, this Cu ACR is applicable to freshwater and saltwater species and corresponds to the geometric mean of ACRs reported for six acutely sensitive aquatic species (ACR = 1.48–5.59, with survival, reproduction and biomass as chronic endpoints), including *O. mykiss* (ACR = 2.88, with biomass as chronic endpoint, from Seim et al. (1984)).

Thirdly, in accordance to the EU approach, we used the *Daphnia magna* acute Cu BLM, available in the Visual MINTEQ BLMs library, to predict chronic LC20 values for our data and literature data. This *Daphnia* BLM (noted D-BLM in this paper) was developed by De Schamphelaere and Janssen (2004a) and was shown to satisfactorily predict chronic Cu toxicity to fish, on a relatively limited number of literature studies with fathead minnows and rainbow trout (De Schamphelaere and Janssen, 2008; Delbeke et al., 2010). This D-BLM uses the same thermodynamic database (Visual MINTEQ v. 3.1 database) and DOM assumptions as our new BLMs (Table 3). A major singularity in the D-BLM model is the contribution of $CuCO_3$ as a species that can bind to BL and elicit toxicity (Table 3). This incorporation of a log $K_{CuCO3BL}$ in the D-BLM was aimed at providing a better fit of the observed toxicity data at high pH levels and has no mechanistic basis. Indeed, the bioavailability of copper-carbonate complexes in *Daphnia magna*, or in fish, remains to be demonstrated. Therefore, this BLM parameter has not been used in our newly derived acute and chronic BLMs (Table 3).

3. Results

3.1. Water chemistry

Fig. 1a shows the decaying maple leaf DOC concentration with time, reflecting bacterial decomposition of organic matter (*i.e.* oxidation into inorganic carbon). The emission–excitation spectra at the end of the ageing process (see Fig. 1.b for an example replicate) revealed the presence of two DOM components, for excitation wavelengths of 300–400 nm and at relatively long emission wavelengths, which are usually labelled as fulvic-like (410 nm) and humic-like (475 nm) components (DePalma et al., 2011; Al-Reasi et al., 2012). The two-component PARAFAC analyses gave relative abundances of 60 ± 4% HA and 40 ± 4% FA (mean ± SD, n = 3).

Water chemistry in the different tests is given in Table 1. In Ng et al. (2010), the toxicity tests at pH 5.0, 6.0, 8.0 and 8.5 were performed several years apart from the rest of the tests performed in this study, with a baseline RW that had slightly changed. The baseline RW for these pH tests contained approximately 50% less Ca and the reported DOC concentration was ~1.4 mg L^{-1}, somewhat higher than the concentration of ~0.5 mg L^{-1} of the other tests. For reasons presented in Supplementary Information Section 2, we now believe that the DOC value reported in Ng et al. (2010) was erroneous, and it was changed to 0.5 mg L^{-1} for the present modeling. In Supplementary Information Section 2 we also evaluate the impact of this change by presenting the modeling exercise without this DOC correction.

3.2. General observations on acute and chronic toxicity

No effect on growth was observed in the chronic tests (specific growth rate = 1.6 [1.4–1.9] % per day), so only mortality effects have been reported. In Table 2, acute toxicity is reported with the 96-h LC50 values (values of acute regulatory interest), while chronic toxicity is reported with the 30-d LC50 values (for comparison with the acute 96-h LC50 values), the 30-d LC20 values (values of chronic regulatory interest for the USEPA and the present study) and the 30-d LC10 values (values of chronic regulatory interest in the European Union).

As shown in Fig. 2 (raw data are given in Table SI.2 in Supporting Information), in approximately 70% of the tested conditions, the chronic 96-h, 10-d, 20-d and 30-d LC50 values were not significantly different from the acute 96-h LC50 values ($p > 0.05$, ANOVA and Tukey's test). Notably, the 96-h LC50 and 30-d LC50 values were not significantly different for all tested conditions, except at pH 8.5 and at 3 mM Mg. This similarity between acute and chronic toxicity is supported by the low calculated ACR (ratio of observed 96-h LC50 to 30-d LC20 values) of ~1.35 on average, which is more than 2-fold lower than the USEPA (2007) ACR value of 3.22.

Fig. 2 also shows that, for each test condition, the observed LC50 value was relatively stable over the exposure time in the chronic toxicity tests, except for a significant decrease (*i.e.* mortality increased) observed at pH 5 and, to a lesser extent, at pH 6, at 5 and 10 mg L^{-1} of DOC, and at 1.2 mM of Mg. For the pH-set (Fig. 2d), chronic 96-h LC50 values (food present) were available and could be compared to the acute 96-h LC50 values (food absent). Food protection seems to have occurred at pH 5, pH 6 (with a decrease over time) and at pH 8.5 (stable over time). The greatest food protection was observed at pH 8.5, where

the chronic LC50 value was more than 3-fold higher than the acute 96-h LC50 value.

3.3. Effects of water chemistry and BLM development

In the range of water chemistry tested, the observed 96-h LC50 values varied by a factor of 39 while the 30-d LC20 values varied by a factor of 27 (Table 2).

3.3.1. Effects of Ca and Mg

The increase of Ca concentration from 0.2 to 3 mM (15-fold) increased both the 96-h LC50 (5-fold) and the 30-d LC20 (9-fold) values (Table 2 and Fig. 3a). The ameliorative effect could be explained by the BLM framework, as linear relationships were observed between the calculated $LC50_{Cu2+}$ and Ca^{2+} activities ($p < 0.05$, F-test) (Fig. 4a). The derived acute and chronic log K_{CaBL} values were 3.5 and 4.0 respectively, very close to the values in the W-BLM and the D-BLM (3.6 and 3.5 respectively) (Table 3). The ameliorative effect of Mg was lower than that of Ca, as the increase of Mg concentration from 0.05 to 3 mM (60-fold) induced only a small protection, with a 1.4-fold increase in the 96-h LC50 values and a 3-fold increase in the 30-d LC20 values (Table 2 and Fig. 3b). In the chronic tests, the linear regression between 30-d $LC50_{Cu2+}$ and Mg^{2+} activities (Fig. 4b) provided a log K_{MgBL} value of 3.4, which was close to the acute W-BLM and D-BLM value of 3.6 (Table 3). However, in our own acute tests, the linear relationship between 96-h $LC50_{Cu2+}$ and Mg^{2+} activities was not significant ($p > 0.05$, *F*-test) (Fig. 4b), because of the very small change observed in the 96-h LC50 values with the Mg concentration (*cf.* Table 2 and Fig. 3b). If we use the linear regression parameters anyway, an acute log K_{MgBL} around 1.4 is obtained (Table 3), which is indeed not sufficiently high to provide a significant protection at the Mg^{2+} activities tested in this study.

3.3.2. Effects of DOM

Raising the DOC concentration from 0.3 to 10 mg L^{-1} (~30-fold) led to the highest level of protection observed in this study, with approximately a 30-fold increase in both the 96-h LC50 and the 30-d LC20 values (Table 2 and Fig. 3c). This ameliorative effect could be explained by the complexation of Cu by DOM, resulting in a decrease in the bioavailability of Cu. Indeed, the calculated LC_{50Cu2+} did not change significantly with the DOC concentration ($p > 0.05$, F-test) (Fig. 4c). This observation agrees with the BLM conceptual framework, stipulating that Cu^{2+} concentration is a better predictor of toxicity than total dissolved Cu, when the water ionic composition is held constant.

3.3.3. Effects of pH

As previously described in Ng et al. (2010), varying the pH showed little effects on acute and chronic toxicity in the midrange, but marked effects were observed at the extreme pHs (Table 2 and Fig. 3d). Protection against acute Cu toxicity was observed at pH 5 compared to the

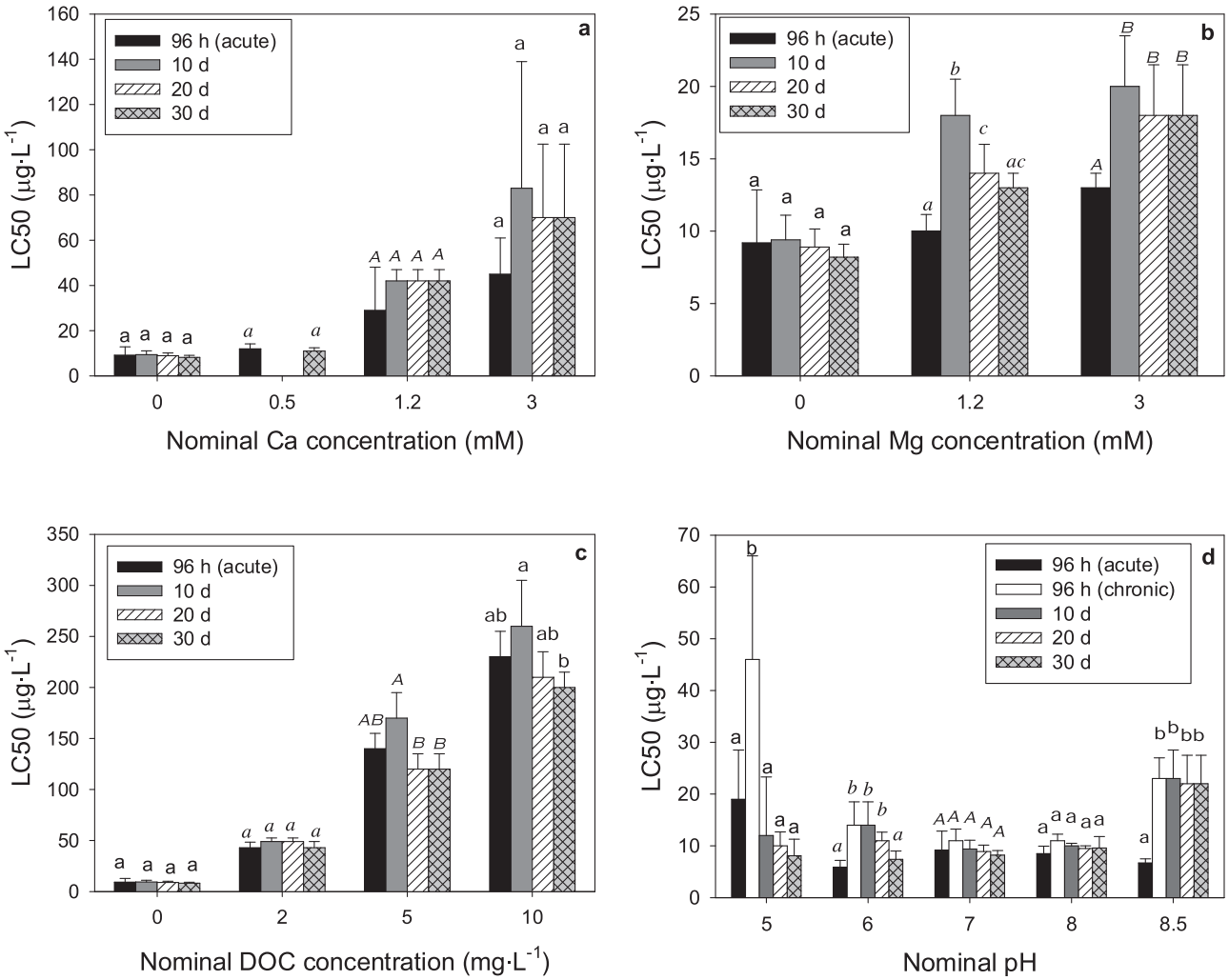

Fig. 2. LC50 values (as total dissolved Cu concentration) as a function of nominal dissolved concentrations of a) Ca, b) Mg, c) DOC and of d) pH, at 96 h (acute test) and 96 h, 10 d, 20 d and 30 d (chronic test). Error bars indicate 95% confidence limits. For a given parameter concentration, means not sharing the same letters are significantly different ($p < 0.05$, ANOVA with Tukey's test). Missing data: chronic 96-h LC50 values for the Ca-set, Mg-set and DOC-set and the 10-d and 20-d LC50 values at 0.5 mM Ca.

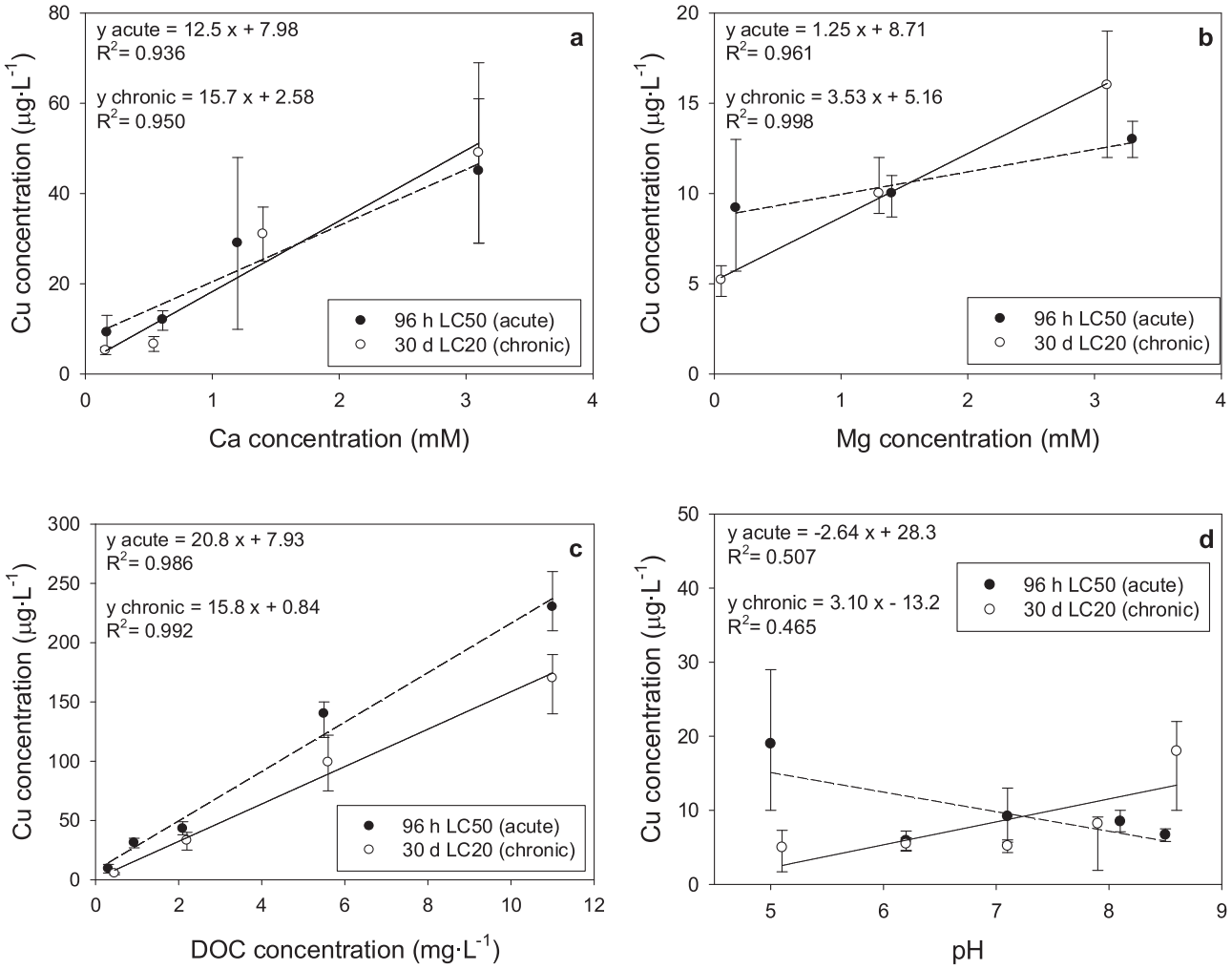

Fig. 3. 96-h LC50 and 30-d LC20 values (as total dissolved Cu concentration) as a function of total dissolved concentrations of a) Ca, b) Mg, c) DOC and of d) pH. Error bars indicate 95% confidence limits. Black dashed and solid lines are linear regression lines for the acute and chronic data respectively.

other tested pHs. A similar low-pH protection against chronic Cu toxicity seems to have been lost over time from 96-h to 30-d of exposure (*cf.* Fig. 2d). In the chronic exposure, only a significant protection at pH 8.5 was stable over time (*cf.* Fig. 2d).

As for the variation of DOC concentration, varying the pH had a strong effect on Cu speciation, specifically on Cu^{2+} and $CuOH^{+}$ activities which are the two chemical forms of interest here. Fig. 5 shows the speciation of the main Cu forms as a function of pH, as predicted by Visual MINTEQ (Fig. 5a) and the W-BLM (Fig. 5b). Note that the speciation is at 96-h LC50 values but would remain virtually the same at 30-d LC50 values. For both speciation softwares, Cu^{2+} activity decreases as the pH increases and $CuOH^{+}$ activity increases from pH 5–7 then decreases from pH 7 to 8.5. The free Cu^{2+} is more abundant from pH 5 to ~7.8, then $CuOH^{+}$ becomes more abundant. However, the activities of Cu^{2+} and $CuOH^{+}$ are respectively 3–11 times and 4–19 times higher according to Visual MINTEQ than according to the W-BLM. These differences come from the different assumptions on Cu-DOM complexation and from the different K_{CuHCO3} used by the models (Table 3).

From pH 5 to 7, linear relationships were observed between $LC50_{Cu2+}$ and H^{+} activities (as predicted by Visual MINTEQ) for both the acute and the chronic data (Fig. 4d) and the derived log K_{HBL} values were respectively 6.2 and 5.8, higher than the log K_{HBL} of 5.4 in the W-BLM and the D-BLM (Table 3). However, the comparison between the three models is complicated by the differences that they compute in the Cu speciation, more precisely by the differences in metal-DOM modeling assumptions and in the formation constants for carbonate complexes (Table 3). In our data-set, a significant contribution of $CuOH^{+}$ to the acute Cu toxicity could be hypothesized from the steeper slope observed at pH 8 and 8.5 (Fig. SI.1 in the Supporting Information), where $CuOH^{+}$ becomes more abundant than Cu^{2+}. The ratio K_{CuOHBL}/K_{CuBL} was estimated to be 1 for the acute test (*i.e.* the upper constraint set on this adjustable parameter) and 0.2 for the chronic test. These values mean that $CuOH^{+}$ and Cu^{2+} were equally toxic in the acute exposures and that Cu^{2+} was 5 times more toxic than $CuOH^{+}$ in the chronic exposures. Although they appear to be in strong contrast with the W-BLM which considers Cu^{2+} to be 15 times more toxic than $CuOH^{+}$ (Table 3), the differences in the speciation calculation complicate this comparison, as previously mentioned. Direct comparisons with the D-BLM are also hindered by the assumed contribution of $CuCO_3$ to the overall toxicity in this model.

3.3.4. *Estimations of log K_{CuBL} and critical f_{CuBL} values*

The linear relationships between the observed logit percent mortality and f_{CuBL} calculated with Eq. (1) are shown in Fig. 6a (whole acute data-set) and 6b (whole chronic data-set). The variability observed in these two graphs partially reflects the variability in the concentration-response curves from the acute and chronic toxicity tests. The best fits were obtained with a log K_{CuBL} of 7.1 (acute) and 7.2 (chronic), while the log K_{CuBL} (acute) of the W-BLM is 7.4 (Table 3). Using the K_{CuOHBL}/K_{CuBL} ratios estimated above, we assessed the log K_{CuOHBL} to be 7.1 (acute) and 6.5 (chronic), higher than the value of 6.2

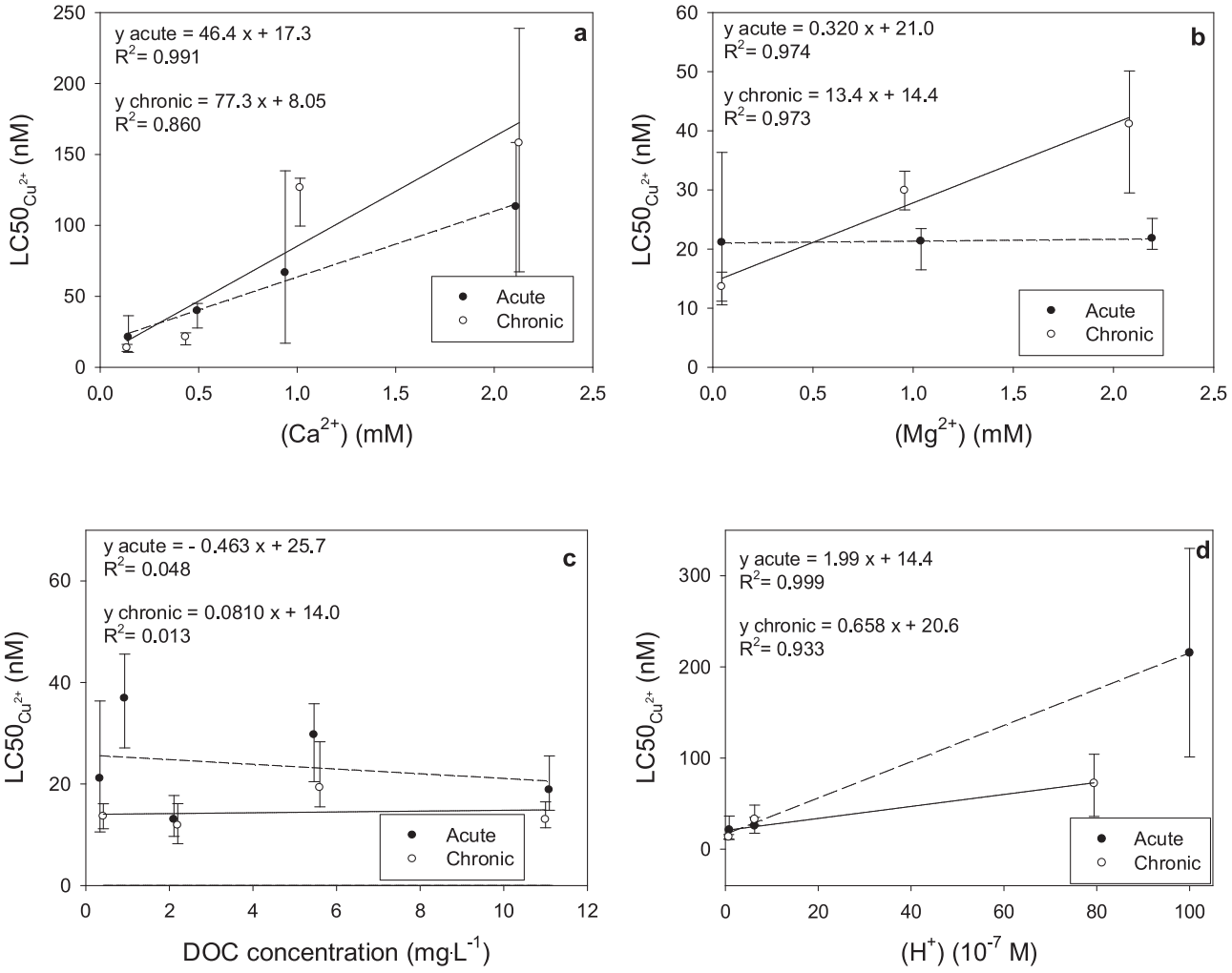

Fig. 4. 96-h and 30-d Cu^{2+} activities at 50% mortality as a function of a) Ca^{2+} activity, b) Mg^{2+} activity, c) DOC concentration and d) H^{+} activity from pH 5 to 7. Calculations were made with Visual MINTEQ (version 3.1) under conditions given in Tables 1 and 3 (newly derived BLMs columns). Error bars indicate 95% confidence intervals. Black dashed and solid lines are linear regression lines for the acute and chronic data respectively.

reported in the W-BLM.

Finally, the fraction of Cu bound to BL estimated at 50% and 20% mortality are given in Table 3. The acute f^{50}_{CuBL} was estimated to be 0.118, very close to the fraction used in the W-BLM (0.123). For the chronic exposures, f^{20}_{CuBL} was the value of interest and it was estimated at 0.0560. Lethal accumulations LA50 and LA20 were estimated by considering the total density of BL on the gills to be 30 nmol g^{-1}, as reported in the W-BLM (*e.g.* LA50 = f^{50}_{CuBL} × 30 nmol g^{-1}). The resulting chronic LA20 was 0.0560 × 30 = 1.7 nmol g^{-1} and acute and chronic LA50 values were 0.119 × 30 = 3.6 nmol g^{-1} and 0.0876 × 30 = 2.6 nmol g^{-1}

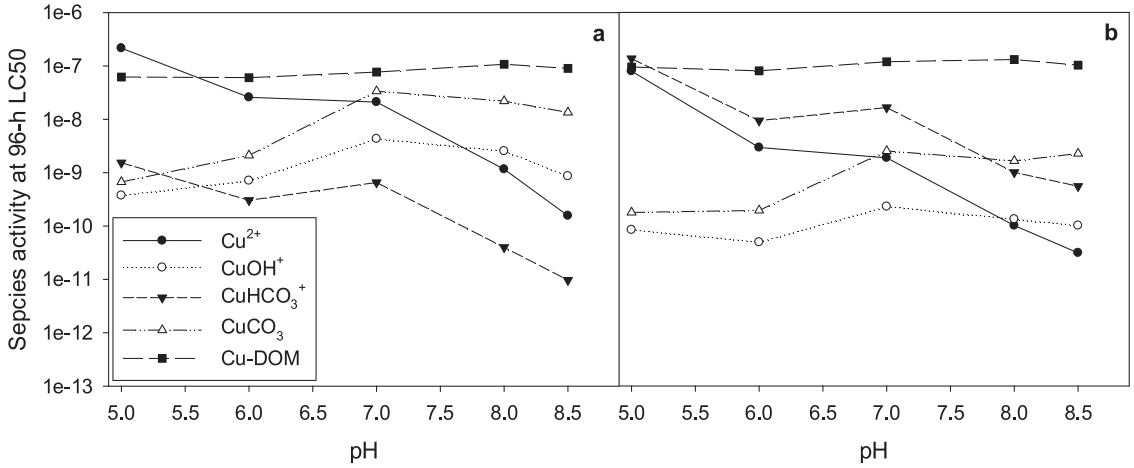

Fig. 5. Main Cu species activity at 96-h LC50 as a function of pH, as predicted by a) Visual MINTEQ (version 3.1) and b) the Windward BLM (version 3.1.2.37). Speciation calculations were performed under conditions given in Tables 1 and 3.

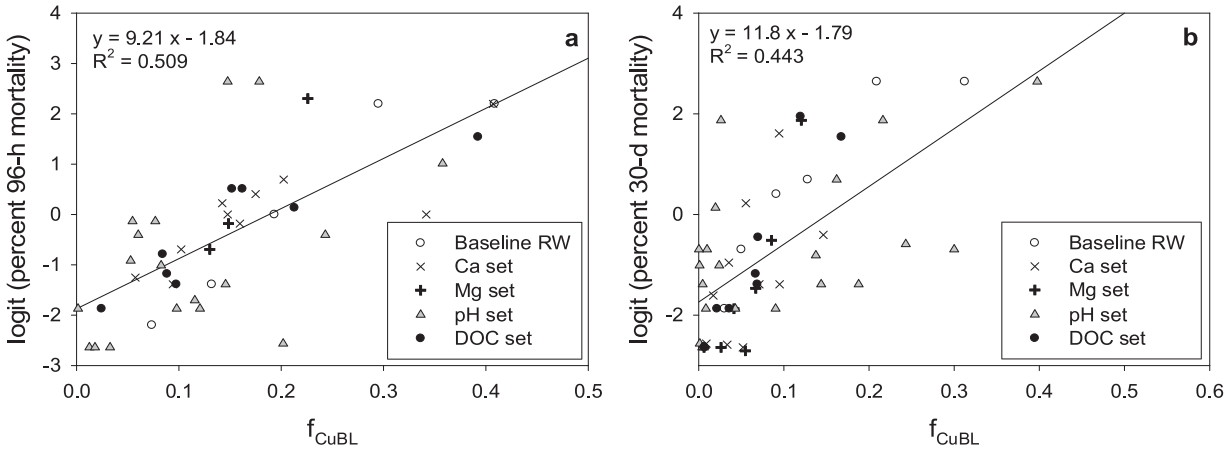

Fig. 6. Logit of the observed percent a) 96-h and b) 30-d fish mortality as a function of the calculated fraction of Cu bound to biotic ligand sites. The latter fraction was calculated with Eq. (1) using the newly derived acute and chronic BLM parameters (Table 3) at the different physico-chemical conditions in Table 1.

respectively. These latter LA50 values were in the same order of magnitude as the 24-h gill Cu accumulation values of ~8 nmol g^{-1} (wet weight) that were measured at 50% mortality between pH 6 and 8 by Ng et al. (2010) as reported in Table 2 of that paper.

3.4. Acute and chronic toxicity predictions

3.4.1. Model calibration–predictions of the present data-set

Table 2 and Fig. 7 present the comparison of 96-h LC50 and 30-d LC20 values measured in this study with those predicted by the newly derived BLMs. By convention, the BLM predictions are usually considered to be satisfactory when they are estimated within a 2-fold error from the observed toxicity. This was the case for 86% of the 96-h LC50 values predicted with the newly derived acute BLM (Table 2 and Fig. 7a). The exceptions were at pH 8 and 8.5 where the 96-h LC50 values were over-predicted (*i.e.* under-estimation of toxicity) by a factor of 2.3 and 5.0 respectively. The newly derived chronic BLM predicted similarly 85% of the 30-d LC20 values within a 2-fold error, with very slight deviations at pH 8.5 (2.1-fold over-estimation) and at 3 mM Mg (2.3-fold under-estimation) (Table 2 and Fig. 7b). The influence of pH in the chronic data-set was better captured by the newly derived chronic BLM, although a small residual effect was still observable, with an over-estimation of toxicity at low pH and an under-estimation of toxicity at higher pH.

3.4.2. Model validation–predictions of the literature data

Fig. 8 and Table SI.1 present the comparison of 96-h LC50 and LC20 values (variable exposure duration) measured in the selected independent studies and predicted with the newly derived BLM. The effects of rainbow trout weight, of flow regime and of chronic exposure duration on BLMs performance were assessed by evaluating the effect of each parameter on the ratio of predicted *vs.* observed LC values. These results are displayed in the Supporting Information (Figs. SI.2, SI.3, SI.4, SI.5). No trend could be detected between the performances of the new BLMs and fish weight (Figs. SI.2 and SI.3) or chronic exposure duration (Fig. SI.4) However, a significantly higher over-estimation of 96-h LC50 values was observed for tests performed with static renewal solutions (predicted LC50 *vs.* observed LC50 = 2.5 ± 1.1 (SD), n = 23, 4 studies) compared to flow-through tests (predicted LC50 *vs.* observed LC50 = 0.81 ± 0.59 (SD), n = 61, 14 studies) (Fig. SI.5). As discussed in Section 4.4, this effect was opposite to our expectations.

The 90 tests of the 19 acute studies provided 96-h LC50 values ranging from 2.8 to 516 µg L^{-1} (Table SI.1). The newly derived acute BLM captured 59% of this 184-fold variation within an error < 2-fold, while 21% of the 96-h LC50 values were more than 2-fold over-estimated (up to 4.4-fold) and 20% were more than 2-fold under-estimated (up to 6.7-fold) (Fig. 8a, Table SI.1). Mainly two studies were responsible for the 96-h LC50 over-estimation (Naddy et al., 2002) and under-estimation (Howarth and Sprague, 1978) and possible reasons are discussed in Section 4.4. Indeed, without these two studies, the

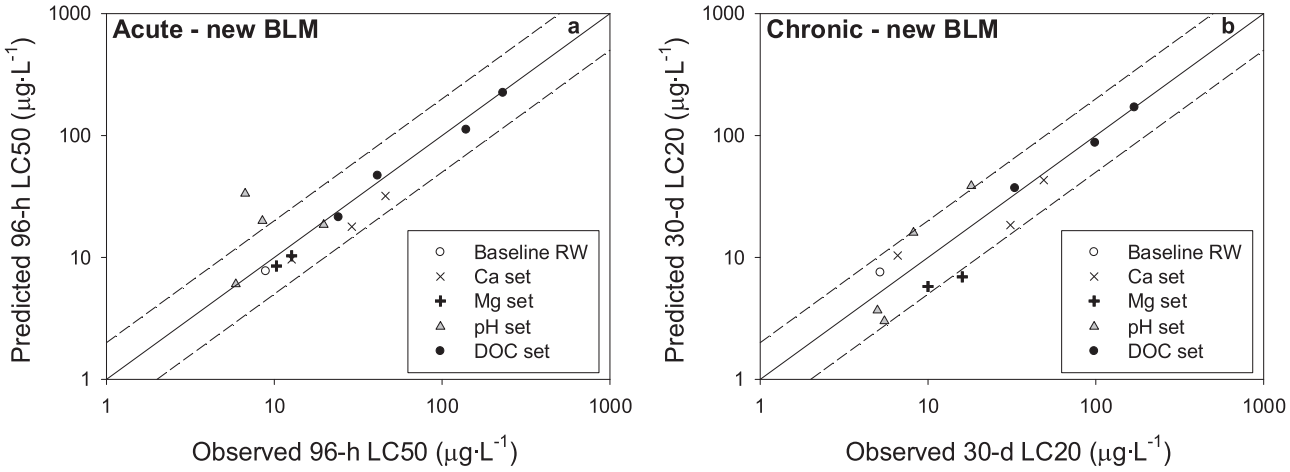

Fig. 7. New acute and chronic BLM calibration with the present data-set: Predicted *versus* observed a) 96-h LC50 values with the new acute BLM and b) 30-d LC20 values with the new chronic BLM. The calculations were made under the conditions and with the parameters given in Tables 1 and 3. Solid lines represent the 1:1 lines and dashed lines are the 2:1 and 1:2 lines.

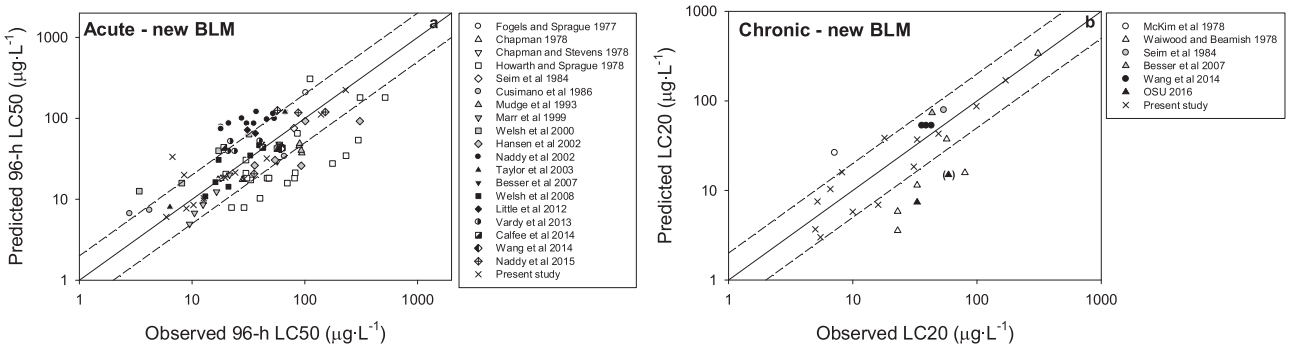

Fig. 8. New acute and chronic BLM validation with literature studies (present data-set included): Predicted *versus* observed a) 96-h LC50 values with the new acute BLM and b) LC20 values (10 d to 78 d) with the new chronic BLM. Note: For the OSU 2016 data point in brackets (), the observed LC20 value (59 µg L^{-1}) corresponds to a minimal estimate. The calculations were made under the conditions and with the parameters given in Tables SI.1 and 3. Solid lines represent the 1:1 lines and dashed lines are the 2:1 and 1:2 lines.

newly derived acute BLM captured 77% of the observed 110-fold variation in 96-h LC50 values within an error < 2-fold. For Howarth and Sprague (1978), where pH effects were investigated, the ratio of predicted *vs.* observed 96-h LC50 values increased by an average of ~3.4-fold from pH 5 to 9 (at all tested hardness), and up to 19-fold at a hardness of 360 mg L^{-1}. Cusimano et al. (1986) was the only other acute study which investigated pH effects on Cu toxicity to rainbow trout, at pH 4.7, 5.7 and 7 (Table SI.1). Although the 96-h LC50 values were relatively well predicted, the same increase with pH was observed in the ratio of predicted *vs.* observed LC50 values (by 4.6-fold from pH 4.7 to 7).

The 14 tests of the 6 chronic studies provided LC20 values ranging from 7–310 µg L^{-1} (Table SI.1). The newly derived chronic BLM captured 50% of this 44-fold variation within a 2-fold error, while 7% of the LC20 values were over-estimated (up to 3.7-fold) and 43% were under-estimated (up to 6.5-fold) (Fig. 8b, Table SI.1). The 45-d LC20 from McKim et al. (1978) was the only over-estimated value. For this study, most water chemistry parameters were not measured and recommendations from the USEPA (2007) were used (Table SI.1). Also, note that the LC20 value was not reported in this publication; instead we calculated it from the published concentration-response curve using TRAP. The 10-d LC20 values from Waiwood and Beamish (1978b) were generally under-estimated. In this study, it seems that the chronic BLM could not fully capture the observed pH effect on Cu toxicity. Indeed, as pH increased from 6 to ~7.8, the ratio of predicted vs. observed LC50 values increased by ~3.1-fold (from 2.2- to 4.5-fold from 30–100 mg L^{-1} hardness).

3.4.3. Evaluation of W-BLM and D-BLM with present and literature data sets

Fig. 9a and Table SI.1 show the predictive capacity of the W-BLM on our acute data-set and the acute literature studies. Overall, the W-BLM predicted 32% of the 96-h LC50 values within a 2-fold error, while 62% of them were more than 2-fold over-estimated (up to 16-fold) and 6% were more than 2-fold under-estimated (up to 28-fold). Residual pH effects were observed in the three studies where this parameter was widely varied. Indeed, for our pH-set, the W-BLM under-estimated 96-h LC50 by 3-fold at pH 5 and over-estimated 96-h LC50 by 13-fold at pH 8.5. For the data-set of Cusimano et al. (1986), the ratio of predicted *vs.* observed LC50 values increased by ~36-fold from pH 4.7 to 7. For the data-set of Howarth and Sprague (1978), this ratio increased on average by ~57-fold from pH 5 to 9 (at all tested hardnesses) and up to 200-fold at a hardness of 360 mg L^{-1}. In addition, the W-BLM, which considers an equal protection of Ca and Mg (Table 3), over-estimated Mg protection for our Mg-set and for the data-sets of Naddy et al. (2002) and Welsh et al. (2000).

Fig. 9b and Table SI.1 show the predictive capacity of the W-BLM with an ACR of 3.22 on our chronic data-set and the chronic literature studies. Note that for our data-set, the predicted 30-d LC20 values are similar to the W-BLM predicted 96-h LC50 values divided by 3.22, though not identical due to small differences in water chemistry between chronic and acute tests (Table 1). For most of our chronic data-set, this USEPA chronic modeling approach allowed for reasonable predictions of the 30-d LC20 values (*i.e.* within an error of 2-fold). In fact, only the 30-d LC20 at pH 5 was more than 2-fold under-estimated (by a factor of 2.5-fold). As for the new chronic BLM, the W-BLM with ACR = 3.22 over-estimated 45-d LC20 values from McKim et al. (1978) and generally under-estimated the 10-d LC20 values from Waiwood and Beamish (1978b).

Fig. 9c and Table SI.1 show the predictive capacity of the *Daphnia magna* acute Cu BLM (D-BLM) on our chronic data-set and the chronic literature studies. For most of our chronic data-set, this EU chronic modeling approach allowed for reasonable predictions of the 30-d LC20 values (*i.e.* within an error of 2-fold). This good performance is rather remarkable, notably as it was obtained without any adjustment of the D-BLM f^{50}_{CuBL} value of 0.47 (Table 3), a parameter that was originally calibrated for acute LC50 determination, not chronic LC20 determination, and in an invertebrate (*D. magna*), rather than in a fish. Only the 30-d LC20 at 1.2 mM Ca was more than 2-fold under-estimated (by a factor of 2.2-fold). As for the new chronic BLM and the W-BLM with ACR = 3.22, the D-BLM over-estimated 45-d LC20 values from McKim et al. (1978) and generally under-estimated the 10-d LC20 values from Waiwood and Beamish (1978b). For the latter study, the LC20 value at the highest pH and alkalinity was noticeably under-estimated (by a factor of 5.6-fold), which could be attributed to the contribution of $CuCO_3$ to the overall toxicity in the D-BLM.

4. Discussion

4.1. The acute nature of Cu toxicity in chronically exposed rainbow trout

Our study indicates that Cu chronic toxicity to rainbow trout was mainly of an acute nature, and that mortality was a more sensitive endpoint than growth. Indeed, fish growth was not significantly inhibited during chronic exposure. Furthermore, mortality occurred mostly within the first days of exposure at similar levels as in the acute exposure (except for the low pH exposures which are discussed in Section 4.3). All these conclusions are virtually identical to those reached by De Schamphelaere and Janssen (2004b) who performed a similar 30-d chronic exposure of juvenile rainbow trout to zinc in a range of water chemistries, resulting in the only previous chronic fish BLM. Accordingly, Naddy et al. (2007) reported similar toxicity between 7-day and 30-day early life stage tests on fathead minnow exposed to silver. In future, it will be of interest to evaluate whether the same is true for other fish species and other metals.

Previous studies on the effect of Cu on fish growth have led to contradictory observations. Several investigations have shown that Cu inhibits growth of fish, including that of rainbow trout (Lett et al., 1976;

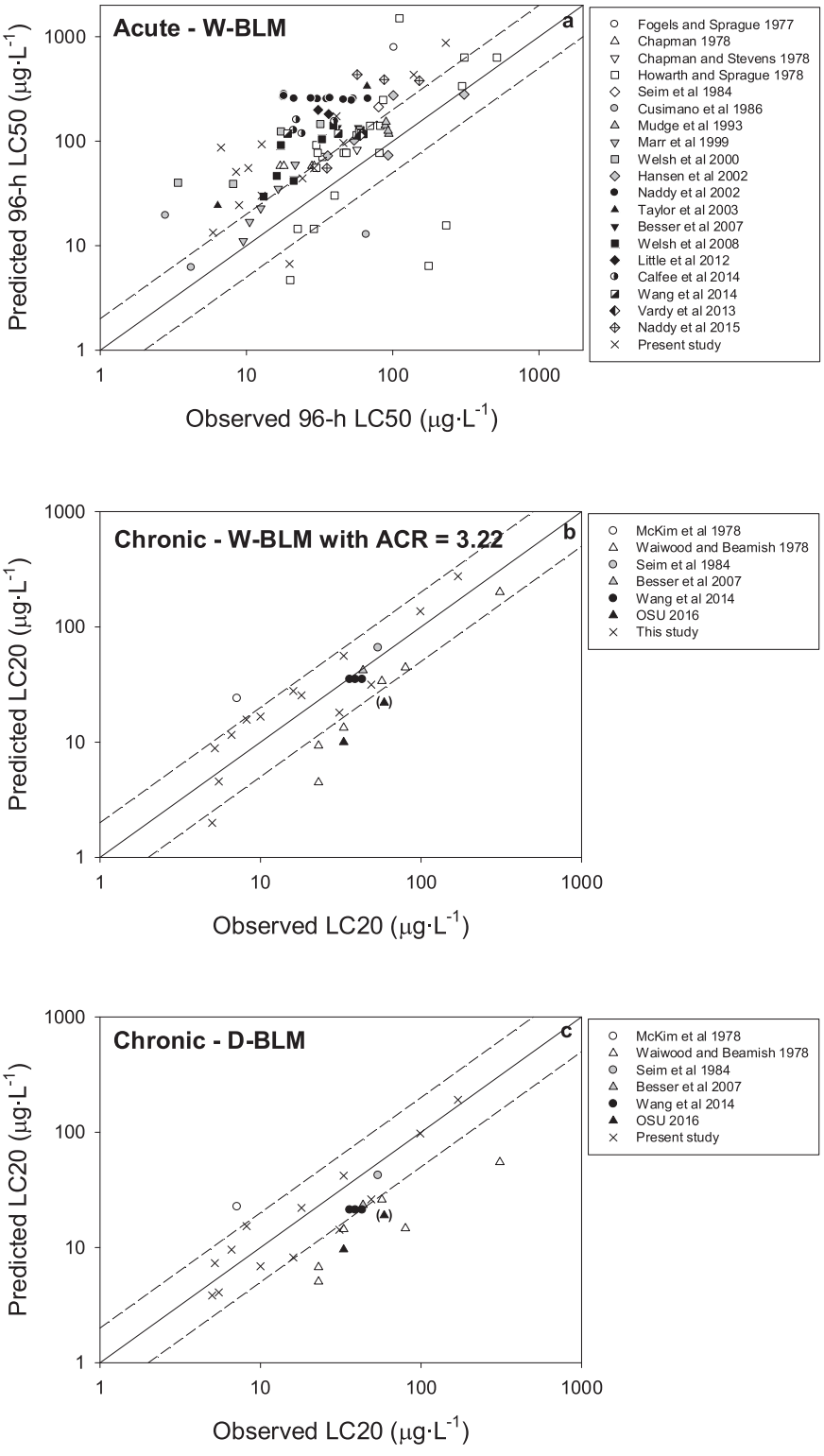

Fig. 9. The Windward BLM (W-BLM) and the *D. magna* acute Cu BLM (D-BLM) predictive capacities with the literature data-sets and the present data-sets: a) Predicted *versus* observed 96-h LC50 values with the W-BLM and predicted *versus* observed chronic LC20 values (10 d to 78 d) with b) the W-BLM with ACR = 3.22 and c) the D-BLM. Note: For the OSU 2016 data point in brackets (), the observed LC20 value (59 µg L^{-1}) corresponds to a minimal estimate. The calculations were made under the conditions and with the parameters given in Tables SI.1 and 3. Solid lines represent the 1:1 lines and dashed lines are the 2:1 and 1:2 lines.

Waiwood and Beamish, 1978a; Marr et al., 1996; De Boeck et al., 1997; Kamunde et al., 2005; Hashemi et al., 2008). This effect was generally associated with reduced appetite and/or decrease in efficiency of energy utilization associated with increased detoxification and maintenance costs (Woltering, 1984). In accordance with our observations, other studies have reported no decrease of growth in rainbow trout chronically exposed to Cu (Miller et al., 1993; Taylor et al., 2000; McGeer et al., 2000, 2002; Kamunde and MacPhail, 2008) and to Zn (De Schamphelaere and Janssen, 2004b). Differences in exposure duration, baseline fish sensitivity and metabolic activity, feeding quantity and frequency, as well as in food quality may explain these different observations on Cu effects on fish growth (Kamunde et al., 2005; Hashemi et al., 2008). With regards to diet quality, Kamunde et al. (2005) showed that growth inhibition of rainbow trout exposed to Cu was higher with low-Na food than with high-Na food. It is indeed now well established that Cu toxicity is associated with ionoregulatory disturbance. More precisely, Na plasma loss is usually considered to be the main cause of deleterious Cu effects (Laurén and McDonald, 1985;

Grosell et al., 2002; Grosell, 2012; Chowdhury et al., 2016). Proposed mechanisms for Cu-induced Na loss in freshwater fish are (i) a decrease in Na branchial influx, notably *via* inhibition of the basolateral Na/K-ATPase, and (ii) an increase in Na branchial diffusive efflux *via* an increase in the branchial epithelium permeability, which is believed to be induced by the displacement of Ca from the membrane tight junctions (Laurén and McDonald, 1985, 1987a,b; Grosell et al., 2002; Chowdhury et al., 2016).

In the present study, whole body Na concentration was indeed reduced in some of the acute Cu exposures (at 24 h and pH 6: $r^2 = 0.72$; at 96 h and pH 5: $r^2 = 0.72$; at 96 h and pH 7: $r^2 = 0.88$, in Fig. SI.1 in the Supporting Information of Ng et al., 2010). In the chronic Cu exposures, these data were collected only at 30 d and showed a constant Na level ($\sim 40\ \mu mol\ g^{-1}$ wet wt) in the whole fish body across the range of Cu concentration and pH tested (see Fig. SI.1 in the Supporting Information of Ng et al., 2010). Copper-induced disturbances of Na homeostasis have been shown mostly in acute studies (see Grosell, 2012 for a review) but a few chronic studies have also demonstrated these effects (McGeer et al., 2000, 2002). In these latter studies from McGeer and coworkers, an initial disruption of Na balance (within the first few days) was followed by a subsequent recovery to control levels, which was associated to an up-regulation of branchial Na/K-ATPase activity. This pattern of initial short "shock" phase followed by a gradual return to control conditions has been shown for other higher biological endpoints in chronic Cu toxicity studies in various fish: gill damage in the common carp (De Boeck et al., 2007; Hashemi et al., 2008), cessation of feeding in rainbow trout (Lett et al., 1976) and growth rate in rainbow trout (Lett et al., 1976; Waiwood and Beamish, 1978a; Dixon and Sprague, 1981). All these observations are in accordance with the damage-repair model described by McDonald and Wood (1993) for branchial acclimation to metals in freshwater fish. In our study, it is possible that the repair process rates were particularly fast so that growth was an insensitive endpoint. Yet, we cannot preclude possible impairments at other levels of biological organizations, such as olfactory impairment which has been shown to occur in fish exposed to very low waterborne concentrations of Cu (Green et al., 2010; Meyer and Adams, 2010).

4.2. Effects of DOM, Ca and Mg concentrations on Cu acute and chronic toxicity

Elevating DOM and Ca concentrations both reduced fish mortality in the acute and chronic exposures, in manners that were well captured by the BLM mathematical framework. The binding of aqueous metals to DOM has been shown to decrease metal bioavailability and hence toxicity to various aquatic organisms in both acute (Playle et al., 1993a; De Schamphelaere and Janssen, 2004a) and chronic (McGeer et al., 2002) exposures. In addition, a range of beneficial effects of DOM on ionoregulation in fish have been reported, and may also contribute an element of physiological protection (*e.g.* Wood et al., 2011; Crémazy et al., 2016). Increasing general water hardness (Ca and Mg concentrations) has also been shown to provide protection against metal toxicity to aquatic organisms in both acute (Howarth and Sprague, 1978) and chronic (Waiwood and Beamish, 1978a and b) exposures. Specific Ca protection against Cu toxicity has been relatively well studied and it is believed to be mainly due to the role of Ca in decreasing gill membrane permeability and hence in decreasing diffusive losses of plasma ions (Grosell, 2012), though it also provides some protection against the inhibition of Na influx (Chowdhury et al., 2016). Contrary to Ca, Mg protective mechanisms against Cu toxicity remain to be elucidated. In the present study, the two principal hardness ions, Ca and Mg, did not provide equal protections as assumed in the W-BLM. Indeed, compared to Ca, Mg protection was much lower in the chronic exposure and even negligible in the acute exposure. The relative protection of Ca and Mg against Cu toxicity seems to be species-specific, as De Schamphelaere and Janssen (2002) showed a similar protection by Ca and Mg in *Daphnia magna* while Ha et al. (2017) showed a much lower relative protection by Mg in *Daphnia galeata*. However for fish, Mg is usually found to be at best minimally protective against Cu toxicity. Indeed, as in the present study, no significant or small (relative to Ca) Mg protection against Cu toxicity has been observed in fathead minnows (Erickson et al., 1996), channel catfish (Perschbacher and Wurts, 1999), rainbow trout and chinook salmon (Welsh et al., 2000; Naddy et al., 2002). Hence, the newly derived acute BLM better predicted 96-h LC50 values for these latter rainbow trout studies compared to the W-BLM. The rationale for the log K_{MgBL} of 3.6 in the W-BLM is unclear since this model is based on an unpublished toxicity data-set. The reason why Mg protection would be higher in chronic than in acute Cu exposure is also unclear. It may be a nutritive effect of this essential cation.

4.3. Effect of pH on Cu acute and chronic toxicity

Very little effect of pH on acute and chronic toxicity was observed in our pH-set at pHs of 6.0-8.0, but marked effects occurred at pH extremes of 5.0 and 8.5, and these differed between acute and chronic exposures (Table 2). These pH effects were clearly the most challenging to model within the BLM construct. According to the BLM, pH effects can be of two different natures with opposite directions: proton competition effects (as shown in Fig. 4d and accounted for by K_{HBL}) and metal speciation effects (as shown in Fig. 5 and accounted for by Cu^{2+} activity calculations). Indeed, at low pH, the increase in H^+ competition (protection) may be counteracted by the increase in Cu^{2+} activity, while at high pH, the decrease in H^+ competition (loss of protection) may be counteracted by the decrease in Cu bioavailability by complexation of Cu^{2+} with carbonates and hydroxides. However, as the two examples in Fig. 5 illustrate, differences in aquatic geochemistry models greatly affect their outputs, notably the differences in stability constants of carbonato-complexes and in organic matter modeling. In our study, the effects of pH on Cu toxicity over the wide range of pH tested could not be fully captured by acute and chronic BLMs using either of these modeling frameworks. It is very likely that pH-dependent processes other than the ones classically assumed by the BLM framework, may be integrated within each toxicity data point. Therefore, the BLM constants, which are derived to obtain the best fit between observed and predicted toxicity, cannot be interpreted to exactly describe the geochemical processes underlying the observed relations.

At low pH, initial protection by food seems to have occurred in our study, as 96-h LC50 values in the chronic exposure were initially higher than their corresponding acute 96-h LC50 values. However, this additional protection at pH 5 and 6 decreased over the course of the chronic exposure, more rapidly at pH 5 than at pH 6 (Table S1.2; Fig. 2b). These findings may indicate that the protons directly contributed to the overall toxicity in the chronic exposure. Similar findings have been reported by Waiwood and Beamish (1978b) who showed that fish mortality occurred within the very first days of Cu exposure at pH ≥ 7.5, but was still occurring after 4 days of exposure at pH 6. This hypothesis of H^+ direct chronic toxicity is in agreement with the lower measured 24-h LA50 (for 30-d mortality) observed at pH 5 compared to the other pH tested in the chronic study (see Table 2 in Ng et al., 2010). Low pH has been shown to represent an additional stress on fish ionoregulation in chronic (and also acute) exposures, and feeding has been shown to provide some protection against this stress (Menendez, 1976; McDonald and Wood, 1981; Kwain et al., 1984; Wood, 1989; Reid, 1995; D'Cruz and Wood, 1998; D'Cruz et al., 1998). In our study, a long-term H^+ toxicity, lasting longer than food protection, could explain why the derived chronic K_{HBL} ($\log K_{HBL} = 5.8$) is 2.5-fold lower than the derived acute K_{HBL} ($\log K_{HBL} = 6.2$). Note that both values were higher than the W-BLM value, by 2.5- and 6.3-fold respectively. The log K_{HBL} of 5.4 in the W-BLM may come from the frequently reported biological surface pKa from 4 to 5.4 (Playle et al., 1993b). The higher log K_{HBL} values derived in our study may indicate the existence

of a physiological component in the mechanism of proton protection. Indeed, although proton protection against Cu toxicity has often been assumed to be of a competitive nature at the gill transporters (Di Toro et al., 2001; Paquin et al., 2002 Paquin et al., 2002), some studies have shown that H^+ protection may not be related simply to reduced metal uptake, but may also be physiologically based (Playle et al., 1992; Chowdhury and Blust, 2001; Grosell, 2012). This latter hypothesis was supported by the absence of a clear trend between pH and Cu bioaccumulation, as reported in Ng et al. (2010).

At high pH, food seemed to have provided some durable protection: at pH 8.5, chronic LC50 values were consistently higher than the acute 96-h LC50 values. As a result, derived chronic K_{CuOHBL} ($\log K_{CuOHBL} = 6.5$) was 4-fold lower than the derived acute K_{CuOHBL} ($\log K_{CuOHBL} = 7.1$). Yet, as for K_{HBL}, both K_{CuOHBL} values were higher than the value in the W-BLM. For the acute BLM, despite bringing the $CuOH^+$ contribution equal to that of Cu^{2+} to the overall toxicity, toxicity was still being under-predicted at pH ≥ 8. To satisfactorily predict toxicity at high pH values within the construct of the BLM, the toxicity of the hydroxo-complex would have to be higher than that of the free ion (*i.e.* $\log K_{CuOHBL} > \log K_{CuBL}$), a rather unrealistic condition. However, this trend at high pH seems to be confirmed by the Howarth and Sprague (1978) data-set. Different 24-h LA50 values (for 96-h and 30-d mortality) were measured at pH 8.5 compared to the lower pH values tested (see Table 2 in Ng et al., 2010), potentially indicating the occurrence of some physiological modifications in the fish gills at high pH.

Previously, Ng et al. (2010) proposed that the inability of the BLM to fully capture the pH effect on Cu toxicity in the wide range of pH tested here may be due to the buffering of pH in the fish gill microenvironment. Indeed, this buffering would explain the relatively stable acute and chronic LC values observed in the pH range tested in our study (Table 2 and Fig. 3d) (in strong contrast with the W-BLM predictions) (Tao et al., 2001; Playle and Wood, 1989). This hypothesis of a different and much more stable pH at the immediate proximity of the gill surface would indeed complicate BLM predictions based on bulk water pH.

4.4. Performance of the new acute and chronic BLMs, and comparison with W-BLM and D-BLM

The newly derived BLMs did relatively well at predicting the majority of the literature data, despite the wide experimental conditions used in the different studies. For example, fish life stages varied from embryos to adults (with corresponding variations in weight of two orders of magnitude) without a noticeable effect on acute or chronic Cu toxicity to rainbow trout (Figs. SI.2, SI.3). Indeed, among the different studies surveyed, only a few reported an effect of fish life stage/weight on Cu toxicity to rainbow trout (Howarth and Sprague, 1978; Vardy et al., 2013), while more have shown little to no variation of fish sensitivity with this parameter (Chapman and Stevens, 1978; Besser et al., 2007; Little et al., 2012; Calfee et al., 2014; Wang et al., 2014). Similarly, chronic exposure duration ranged from 10 d (Waiwood and Beamish, 1978b) to 78 d (Seim et al., 1984) without a detectable effect on Cu toxicity to rainbow trout (Fig. SI.4). We selected this wide range of exposure durations based on our findings that not much additional mortality was occurring after 10 d in chronic exposures. In agreement, in Waiwood and Beamish (1978b), fish were in fact exposed for up to 30 d, but most mortality occurred within the first 5 days and thus only the 10-d LC20 values were reported. Finally, in Wang et al. (2014), 21-d and 52-d Cu exposures, started with 1-dph larval rainbow trout, led to LC20 values that differed by only 1.2-fold.

On the other hand, the flow regime seemed to noticeably influence Cu toxicity to rainbow trout, but in an unexpected manner (Fig. SI.5). As for the present study, most laboratory fish toxicity tests use flow-through systems (14 out of the 19 acute studies and 4 out of the 6 chronic studies, Table SI.1), where the toxicant is added to the test water only minutes prior to being introduced into the test chamber. Concerns have been raised that the rapid water turnover rates of flow-through systems do not allow sufficient time for the metal to equilibrate between its different forms in solutions. Indeed, Ma et al. (1999) and Kim et al. (1999) demonstrated that increasing equilibration time between Cu and water containing DOC (2.5–20 mg L^{-1}) led to *Ceriodaphnia dubia* being exposed to lower free Cu^{2+} concentrations and thus to lower toxicity (*i.e.* higher LC value). More recently, Welsh et al. (2008) showed that static renewal tests generated lower Cu toxicity to rainbow trout (higher observed LC50 values) than in flow-through tests, with waters containing low DOC concentrations (0.3–2 mg L^{-1}). However, these differences could be explained by an observed accumulation of DOC (originating from fish) in the static test chambers, suggesting that equilibration time was probably rapid at the Cu and DOC concentrations used in this study. In our flow-through study, where residence time was about 80 min, it is possible that thermodynamic equilibrium was not reached in the exposure. This would lead the newly derived BLMs to under-predict LC values for static renewal tests that allowed water pre-equilibration.

Two studies were largely responsible for the over-estimation (Naddy et al., 2002) and under-estimation (Howarth and Sprague, 1978) of 96-h LC50 values by the newly derived acute BLM. Two reasons may contribute to these discrepancies. First, uncertainties in water chemistry inputs may have led to erroneous LC50 predictions. For example, DOC concentrations were not measured in either study (default DOC values in Table SI.1 are based on USEPA (2007) recommendations). In fact, in Howarth and Sprague (1978), only the temperature, hardness and pH have been reported. Second, fish used in these two studies may have presented somewhat different sensitivities than the fish used in our study (*i.e.* a different f^{50}_{CuBL} would apply). Differences in sensitivities may notably originate from different acclimation protocols. For the chronic studies, the 10-d LC20 values from Waiwood and Beamish (1978b) constituted the major deviations from the newly derived chronic BLM predictions. Interestingly, this study had almost identical experimental conditions as in Howarth and Sprague (1978): juvenile rainbow trout around the similar age and size were likewise exposed in a series of dilutions of well waters from the University of Guelph (Canada), probably around similar dates. As for Howarth and Sprague (1978), only the temperature, hardness and the pH were measured during these tests, the rest of the chemistry being estimated from USEPA (2007) recommendations for the modeling purpose (Table SI.1).

In general, the newly derived acute BLM did better at predicting rainbow trout Cu 96-h LC50 values than the W-BLM. Indeed, the W-BLM generally over-estimated 96-h LC50 values, *i.e.* under-estimated toxicity, suggesting its less conservative nature for regulatory uses. Furthermore, the newly derived acute BLM was noticeably better at capturing pH and Mg effects, by assuming a higher H^+ protection, a higher $CuOH^+$ toxicity and a negligible Mg protection. A residual pH effect was however observed, as measured toxicity at pH ≥ 8 was higher than predicted. On the other hand, W-BLM with an ACR = 3.22 was actually better at predicting chronic toxicity than acute toxicity, although it was derived from an acute data-set. This higher performance was due to two reasons. First, the W-BLM parameters (log K values) were actually closer to the new chronic BLM parameters than to the new acute BLM parameters, so it could better capture water chemistry effects of the chronic data-set. Second, the W-BLM, although over-estimating acute LC50 values, yielded reasonable chronic LC20 values because of the use of a high ACR of 3.22 from USEPA (2007). A lower ACR value of ~1.4 was observed between the acute and chronic data-sets in our study. Similarly, for the same endpoint of survival, an ACR of ~1.9 was estimated in Wang et al. (2014), of ~1.5 in Seim et al. (1984), of ~1.3 in Besser et al. (2007) and of ~1.2 for Howarth and Sprague (1978) and Waiwood and Beamish (1978b). The overall performances of the three chronic BLMs tested (newly derived BLM, W-BLM and D-BLM) were relatively similar, although the W-BLM provided slightly better predictions. The relatively satisfactory performance of

the daphnia BLM (D-BLM) on fish data supports the hypothesis of similar Cu toxicity mechanisms among freshwater animals (*i.e.* ionoregulatory disturbances (Grosell et al., 2002)). All three models tended to under-estimate the LC20 values from Waiwood and Beamish (1978b). However, a particularly strong under-estimation from the D-BLM of the LC20 value at the highest pH and alkalinity questions the validity of adding $CuCO_3$-BL binding to the model. More chronic studies testing a wider range of physico-chemical conditions are needed to thoroughly evaluate the performance of the new chronic BLM, as well as the D-BLM and W-BLM approaches, to predicting chronic toxicity to rainbow trout.

5. Conclusions and recommendations

In conclusion, rainbow trout responded very similarly to acute and chronic Cu exposures, with toxicity differences mainly observed in the Mg-sets (Mg protection against chronic toxicity only) and at the extreme pHs 5 and 8.5 (with opposite pH effects on the chronic and acute toxicity). The experimental data-sets collected in this study offer critically needed quantitative evidence of the relationships between water chemistry parameters and acute and chronic Cu toxicity to fish. The new acute and chronic Cu BLMs developed from these data-sets offer a significant improvement for assessing Cu toxicity to fish in a wide range of water chemistry. Notably, the new acute BLM appears more robust than the W-BLM in the pH range of 5–8.5 and at varying Mg concentrations. Nevertheless, it should be noted that the current approaches for predicting chronic Cu toxicity to fish using acute BLMs (W-BLM with ACR for USEPA and D-BLM for EU) may yield reasonable predictions between pH 6 and 8, and in the case of the W-BLM approach when chronic Mg protection is relatively low [note that Ca:Mg ratios of 1–5 are typically found in natural freshwaters (Welsh et al., 2000)]. Yet, because of the similar acute and chronic toxicity, the USEPA ACR value of 3.22 may over-estimate the chronic toxicity of Cu to rainbow trout. For future work, we recommend that the mechanisms underlying the pH effects on Cu toxicity be further investigated. Indeed, our study suggests that there may be effects other than H^+ competition and $CuOH^+$ toxicity which contribute to the pH-dependant modification of Cu toxicity to rainbow trout. Additionally, we suggest that Mg effects on Cu toxicity to fish should be re-evaluated, as the considerable acute Mg protection incorporated into the W-BLM was not supported by our study, or by a number of literature studies. The differences in Mg protection and pH effects between acute and chronic exposures also need further mechanistic investigation. Finally, the framework of the acute and chronic BLMs will not be complete without an evaluation of possible Na effects, which could not be undertaken in the present study.

Acknowledgments

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Appendix A. Supplementary data

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