Chapter 3

Field validation of biotic ligand models. The impact of multimetal exposure on *Daphnia magna*

ABSTRACT

The validity of single metal biotic ligand models to estimate effects of metal mixtures in natural surface waters was determined in two different experimental set-ups. Daphnids were exposed *in situ* for three weeks in twelve different rivers, as well as in the laboratory to water samples taken from the same sites. These waters have elevated concentrations of Cu, Ni, Zn, Cd, Mn and Co and other inorganic contaminants related to zinc-melting industry. A toxic unit approach was used to estimate mixture effects. Reduced survival was observed in the field experiment only, whereas reduced reproduction was observed in both experiments. These effects could be explained by Co and Ni in the laboratory experiment and by Cd and Zn in the *in situ* experiment. Evidence for causality was obtained by elevated (measured) whole body concentrations, and (calculated) occupancy of the biotic ligand. Sensitivity parameters ($f_{BL,50}$) of Co , Ni, Cd and Zn were calibrated to optimize the BLMs for application in mixed exposure scenarios. Field-exposed *Daphnia magna* was approximately a factor 20 more sensitive to Co and Ni and a factor 30 more sensitivity to Cd and Zn compared to the single metal laboratory experiments.

INTRODUCTION

Biotic ligand models (BLMs) were recently adopted for environmental regulation and management of metals in surface water (SCHER, 2009b). The concept of the BLM is that toxic effects of metals are related to the amount of free metal ions bound to an organism, i.e., a biological surface; for instance the fish gill (Pagenkopf, 1983). Thus, rather than the aqueous metal concentration, the surface concentration on biota determines the toxicological effect. Concentrations of the metal at a specific biological surface can be calculated with a BLM, as a function of free metal activities and activities of competing cations, such as H^+, Ca^{2+}, Mg^{2+} and Na^+. Free ion activities, on their turn, are heavily affected by water characteristics such as pH and the presence of ligands such as dissolved organic carbon, chloride, carbonate and sulphate.

The possibility to account for local water chemistry makes the BLM a suitable tool for higher tier risk assessment. Application of BLMs may result in a more accurate selection of sites that are at risk and effective application of risk reduction measures. In many cases, BLMs predicted lower risks for Cu, Ni and Zn than the generic approach based on total dissolved metal concentrations (Verschoor et al., 2011). Because of the economic and environmental interests involved, the reliability of BLMs is an important issue.

BLMs have been developed for single species exposed to single metals under optimized laboratory growth conditions. Studies to the validity and relevance of the model under realistic multi-stress, multimetal exposure conditions in a field situation are scarce. Most advanced are BLMs for Cu, Ni and Zn, which are available for different species under acute and chronic conditions in water. For these metals, we found 11 original research paper on the reliability of BLMs in water with a literature search in Scopes (5 February 2013) using the keywords “biotic ligand model” OR “bioavailability model” AND “*in situ*” or “field” or “natural” AND “validation” or “predictive capacity” or “prediction capacity”. Most studies concerned crustacean, of which 7 focused on Cu, 3 on Ni, and 1 on Zn. Most BLMs predicted toxic effects within a factor of 2 accuracy, although in some cases adjustment of parameters in the BLM or in the speciation model was necessary to obtain this performance. A closer look at these studies revealed that field validation is a flexible definition. It appeared that none of these papers described a BLM validation under realistic field, that is *in situ*, multimetal, exposure. Experiments are all performed under controlled exposure conditions in the

laboratory, either with filtered (0.45 µm) natural surface water or with reconstituted media, spiked with a single metal, sometimes containing DOC that is isolated from natural surface water samples, or with organisms sampled from natural surface waters.

Whereas the current BLMs are metal-specific and based on single metal exposures, realistic exposure in the natural environment is characterized by co-occurrence of metals. The toxic effect of metals mixtures is regarded as the sum of individual metal toxicities (additivity), but also may be enhanced (synergism) or reduced (antagonisms). In many cases, only a slight deviation of additivity was observed (Vijver et al., 2011). The effect of metal mixtures is difficult to predict and appears to be dependent on the species involved, the composition of the mixture, the exposure concentrations, the toxicological endpoint, and test duration (Posthuma et al., 2008). BLMs offer potential for a multimetal approach, because they are able to account for competition between metals for binding on biological surfaces. This approach however, is by definition only able to explain an antagonistic effect.

Several approaches have been described to couple mixture toxicity with BLM. 1) A toxic unit approach (additivity), based on metals bound to the biotic ligand, explained the combined effect of Cu and Cd on duckweed *Lemna paucicostata* (Hatano et al., 2008). With a similar method, relations were found for the chronic critical accumulation ratio and indices for the benthic macroinvertebrate community in streams (Schmidt et al., 2010a) , 2) Metals in a mixture were treated as competitive ions in combination with the competitive action of Ca, to estimate to combined toxicity of up to 6 metals in (Playle, 2004). Similar to this method , a mixture factor f_{mix} was introduced to explain the effect of Cd and Pb on the bacteria *Vibrio fischeri* (Jho et al., 2011), and 3) Instead of the occupancy of the biotic ligand by metals, the fraction of the biotic ligand remaining available for calcium binding was used to explain toxicity of Zn, Cu and Cd to rainbow trout *Oncorhynchus mykiss* (Kamo et al., 2008). None of these methods is able to account for increased sensitivity, due to exposure to multiple stressors.

From an ecological viewpoint, *in situ* tests are highly relevant, because they include exposure to a complex mixture of potential toxicants, ambient food quality, and dynamic instead of static conditions. However, *in situ* tests are only scarcely employed because the outcomes are highly variable (Baas et al., 2009) and statistical significance requires a high number of replicates. Large differences

between field and laboratory experiments are common and are often caused by differences in temperature, oxygen, and the availability of food. For example, the effect of cadmium on growth of *Daphnia magna* increased at elevated temperatures, whereas high food levels reduced the toxic effect of cadmium (Heugens et al., 2006). Similar effects were observed for the uptake of several metals (Cu, Ni, Zn, Pb, Cr, Cd), which was a factor 10-100 higher in natural surface water samples where no additional food was provided (Vink et al., 2005). This implies that BLMs are derived from experiments that may underestimate the sensitivity of organisms in a field situation.

The aim of this research was to determine the difference between a laboratory and an *in situ* experiment on *Daphnia magna* population growth and to determine the effect of complex mixtures on the sensitivity of *Daphnia magna* for Cu, Ni, Zn, Mn, Co and Cd toxicity. These metals were selected because they are typical contaminants in water affected by mining and metal industries. *D. magna* represents an important group of organisms in the aquatic food chain and is a common test organism (i.e. OECD 201, OECD 211). Toxicity data for *D. magna* are present for almost any substance, which we used to verify the contribution of other contaminants in our experiment. They are easy to breed in the laboratory and chronic experiments can be performed within a feasible timeframe of 3 weeks, which limits the chance of unexpected or extreme events interfering with the test. Moreover, intraspecies variability is low, because the reproduction is by parthogenesis, and life-stage of the test organisms can be controlled.

Different metals may have different target sites, and as a consequence may affect different endpoints. Survival, accumulation, reproduction and growth were measured as crucial parameters that determine population growth of a species. Accumulation of metals was measured because it provides additional evidence for a causal relation between a particular metal in the water phase and the observed effect. Comparison of field and laboratory experiments enables to distinguish the effect of mixture toxicity from other factors such as temperature, food and oxygen. We calibrated single metal BLMs, extended with a mixture model (based on the assumption of concentration addition), to optimize the prediction of metal toxicity in complex samples.

METHODS

Site selection. Twelve upstream small rivers and brooks were selected in an area contaminated with a mixture of metals (the Dommel catchment in The Netherlands) to cover a range of low to high metal exposure. A map of the study area is shown in Figure 2.1 (page 23). The rivers flow direction is from south to north. Contamination of the Dommel catchment is related to ongoing metal production (Zn, Pb, Cd, Co, Ni, Cu, Ag and Au) dating back to 1890 on two sites, indicated on the map. Most trials sites are located in different upstream braches of the Dommel river. Only site 1 (Beerze) and 6 (Groote Beerze) are in the same river system, with a distance of approximately 30 km in between. The trial sites are located in a large sandy area with forest and heathland, alternated with small villages and small scale agriculture (predominantly grassland). The contribution of pesticides and contaminants from municipal wastewater treatment is therefore limited.

Water chemistry. The trial sites were located in the proximity of regular sampling site of the surveillance monitoring network of water board the Dommel. The water board measured dissolved metal concentrations and many other parameters once or twice per month. Additionally, we took water samples for dissolved organic carbon and metal analysis at the start and the end of the experiment, and measured pH, water temperature, O_2-saturation and conductivity at each site. Na, Ca, Mg, K, Cd, Co, Cu, Ni and Zn were analyzed by High Resolution ICP-MS after filtration and acidification with HNO_3. An overview of the water characteristics of the trials sites is given in Table 3.1. Correlation of water chemistry parameters was computed after log-transformation of concentrations. A graphical presentation of correlations (Figure 3.1) was computed based on hierarchical clustering of the parameters in the correlation matrix (Sarkar, 2008).

Monitoring data of Se, Tl, Li, Mo, B, which are directly related to zinc smelter emissions, was lacking. Their potential relevance to aquatic toxicity is reported in several studies (Adams Kszos et al., 2003; Diamantino et al., 2000; Hamilton, 2004; Pickard et al., 2001; Schoderboeck et al., 2011). Measurements of the period 2006-2009 were used to estimate typical concentrations for these elements on our sites, using chloride as a proxy. The following regression functions were derived: log[Se] = -5.35 + 1.46 log[Cl] (adj. r^2= 0.66); log[Tl] = -4.82 + 1.03 log[Cl] (adj. r^2= 0.51); log[Li] = -3.36 + 0.69 log[Cl] (adj. r^2= 0.81); log[B] = -2.74 + 0.85

log[Cl] (adj. r^2= 0.64); and log[Mo] = -3.75 + 0.79 log[Cl] (adj. r^2= 0.41). Concentrations in the regression functions are expressed in mg/L.

Table 3.1 Water quality data in June 2010. Gray values are concentrations below the detection limit (=0.5*Limit of detection), bold values are concentrations exceeding the EC50 for *D. magna*.

		BEE	BEL	BOL	DOM	GEN	GRB	GRW	KSP	REU	RUN	TOR	TUB
O_2-sat.	%	73	71	62	109	61	80	77	85	75	67	89	57
EC	µS/cm	424	455	437	1113	384	527	476	369	498	378	519	1821
pH		7.3	7.2	6.9	7.2	6.5	7.1	7.5	7.1	6.5	6.6	6.6	7
NH_4	mg N/L	0.16	0.2	0.18	1.4	0.82	1.7	0.093	0.13	9.2	0.23	0.055	0.33
Cl	mg/L	27	36	32	120	30	46	39	29	39	22	46	326
NO_3	mg N/L	1.2	1.1	0.025	3.3	0.65	0.6	0.025	3.1	0.11	1.3	2.3	0.48
SO_4	mg/L	72	55	49	110	84	84	45	65	130	110	61	386
HCO_3	mg/L	72	130	81	140	41	86	160	61	110	30	150	193
TPO_4	mg P/L	0.31	0.13	0.3	0.34	0.11	0.15	0.096	0.084	1.6	0.29	0.24	0.40
DOC	mg C/L	11	6.1	9.7	6.7	3.8	11	6.2	9.2	30	8.2	4.5	6
Na	mg/L	21	27	22	100	22	39	24	21	25	16	34	400
Mg	mg/L	6.6	7.3	5.8	6.4	8.5	6.2	7.6	6.7	12	9.8	8.1	16
K	mg/L	9.8	5.7	4.9	67	5.7	15	1.8	9.4	26	9.8	5.6	12
Ca	mg/L	38	50	35	36	29	39	62	36	40	35	56	99
Mn	mg/L	0.34	0.091	0.12	0.082	0.35	0.37	0.2	0.055	0.56	0.3	0.044	1.2
Fe	mg/L	0.84	0.25	1.9	0.2	1.5	0.34	0.32	0.14	16	0.49	0.11	0.051
Cd	µg/L	0.05	0.05	0.05	0.55	0.22	0.05	0.05	0.13	0.05	0.05	0.05	0.035
Co	µg/L	4.06	2.36	0.246	2.56	**30.9**	7.49	0.29	3.39	6.06	**58.7**	1.02	0.35
Cr	µg/L	0.25	0.54	0.25	0.25	0.25	0.25	0.25	0.52	0.78	0.25	0.25	2.5
Cu	µg/L	3.1	1.4	1.3	1.9	1.1	1.2	0.5	3.3	2.5	2.3	1.4	1.1
Ni	µg/L	16	6.9	3.4	11	63	16	1.1	14	13	91	5.9	3.2
Pb	µg/L	0.1	0.21	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.12
Zn	µg/L	6.6	11	14	79	45	12	2.5	30	37	22	2.5	17
∑TU	dissolved	0.58	0.34	0.15	0.89	**3.5**	0.82	0.07	0.63	0.87	**5.96**	0.14	0.19
∑TU	biotic ligand	0.04	0.04	0.05	0.22	0.32	0.06	0.02	0.10	0.10	0.17	0.01	0.08

***In situ* field experiment.** Twenty juvenile *Daphnia magna* were exposed in the field on June 9, 2010, in four cages per site, permeable for water through 0.5 mm filter. Daphnia culture, cages and analytical methods were described in detail by Verschoor et al. (2012). Day temperatures were 19.4 ± 2.5 ºC. After three weeks, cages were brought to the laboratory and the total population (juveniles and adults) was counted. Organisms were left overnight for depuration in standardized

M4 medium (OECD 2008), freeze-dried and stored at -18ºC until the whole-body concentration of metals were determined by HR-ICP-MS. Pictures of the field experiment are shown on page 45.

Laboratory experiment. A laboratory experiment started a week before the first field experiment, with water samples from nine of the twelve field trials. BEE and REU were added to the field selection after the start of laboratory experiment, and GRW was spoiled by a mistake during refreshment of the water. Ten replicate vials were filled with 50 mL unfiltered surface water and one juvenile *D. magna* each. A control with M4 medium was also included in ten-fold. *D. magna* was fed ad libitum with algal species *Selenastrum* every 3 days. Half of the water in the vials was refreshed twice a week. Survival of adults and reproduction was determined after 8, 12, 15, 17, 19 and 21 days. Juveniles were removed after counting. Further treatment and analysis was performed similar to treatment of the daphnids collected in the field. Pictures of the laboratory experiment are shown on page 45.

Biotic ligand binding. The fractional occupancy of the biotic ligand was calculated as follows (De Schamphelaere et al., 2002b):

$$f_{BL} = \frac{K_{Me-BL} \times \alpha_{Me}}{1 + K_{Me-BL} \times \alpha_{Me} + \sum (K_{i-BL} \times \alpha_i)} \quad \textbf{(Eq. 3.1)}$$

where $K_{me\text{-}BL}$ is the affinity of the metal for the biotic ligand, α is the free ion activity, Me is the metal and i are competitive ions Ca, Mg, Na and/or H (De Schamphelaere et al., 2002b). Since $CuOH^+$ and $CuCO_3$ are known to contribute to Cu toxicity, $K_{Me\text{-}BL} \times \alpha_{Me}$ in nominator and denominator is replaced by $K_{Cu\text{-}BL} \times \alpha_{Cu} + K_{CuOH\text{-}BL} \times \alpha_{CuOH} + K_{CuCO3\text{-}BL} \times \alpha_{CuCO3}$ to describe Cu-binding to biotic ligands, according to (De Schamphelaere et al., 2002a).

BLM parameters are listed in Table 3.2. The parameters K_{BL} are provided by the BLMs and are available for *Daphnia magna* for Cu, Ni, and Zn, but not for Cd, Mn, and Co. Because it is impossible to derive BLM constants for each species, read-across is a common approach: a BLM derived for one species, is used for other species with phylogenetic similarity (De Schamphelaere et al., 2006; De Schamphelaere et al., 2010; Schlekat et al., 2010). Hence, we used a Mn-BLM derived for *Ceriodaphnia dubia*, a Cd-BLM of *Daphnia pulex* and a Co-BLM of *Enchytraeus albidus* to estimate the biotic ligand binding in *D. magna.* The software WHAM7 (Tipping et al., 2011) was used to compute free ion activities for

all metals and competitive ions. The following solution components were included: Ca, Na, Mg, K, Mn, Cd, Co, Cu, Ni, Zn, Cl, HCO_3 and SO_4. Dissolved organic carbon was assumed to consist of 50% active fulvic acids (Dwane et al., 1998).

Table 3.2 Input parameters for biotic ligand model. $logK_{BL}$ is the affinity constant and $f_{50\%}$ is the intrinsic sensitivity expressed as fractional occupancy of the biotic ligand at 50% effect level.

			$logK_{BL}$					
Me	Species	$f_{50\%}$	Me	H	Ca	Na	Mg	Ref.
Cd	*Daphnia pulex*	0.33*	7.0	6.1	4.1		3.7	1
Co	*Enchytraeus albidus*	0.32	5.13	6.53	3.83		3.95	2
Cu	*Daphnia magna*	0.39	8.0	6.67		2.91		3
Mn	*Ceriodaphnia dubia*	0.01	2.23		3.07		3.08	4
Ni	*Daphnia magna*	0.205	4.0		3.10		2.47	5
Zn	*Daphnia magna*	0.127	5.31	5.77	3.22	1.9	2.69	6

1)(Clifford et al., 2010); 2) (Lock et al., 2006); 3) (De Schamphelaere et al., 2004c); 4)(Peters et al., 2011a); 5) Deleebeeck et al (2008); 6) (Heijerick et al., 2005b)

*Intrinsic sensitivity was not available for *Daphnia pulex* and is taken from (Niyogi et al., 2008) for *O. mykiss*.

Mixture toxicity. Additivity was assumed for the effect modeling of metal mixtures. The toxic pressure of each trial site in each test period was expressed as a toxic unit (TU), which is the ratio of dissolved metal concentrations over the effect concentration (EC_x). Because toxic units are dimensionless, the toxicity of a mixture iwas computed by addition of the toxic units of individual compounds, as follows:

$$TU_{mixture,dissolved} = \sum_i TU_i = \sum_i \frac{[Me]_i}{EC_{50,i}} \quad \textbf{(Eq 3.2)}$$

where: i are individual metals in the mixture, $[Me]_i$ is the dissolved metal concentration and EC50, i is the 50% effect concentration.

The toxic unit concept implies that each component in a mixture contributes to toxicity, even though the individual component concentrations in a mixture may be below their toxicity threshold. To estimate the relevance of different potential contaminants in the surface water, their total dissolved concentrations were compared with chronic EC50 for *D. magna*, adopted from EPA's ecotoxicity database (www.epa.gov/ecotox), supplemented for Ni (Deleebeeck et al., 2008b). Chronic EC50s of potentially relevant contaminants for *D. magna* are 1400 µg

As/L, 53000 µg B/L, 8900 µg Ba/L, 34 µg Be/L, 2.2 µg Cd/L, 12 µg Co/L, 600 µg Cr/L, 101 µg Cu/L, 6.7 µg Hg/L, 3300 µg Li/L, 5200 µg Mn/L, 930 µg Mo/L, 97 µg Ni/L, 100 µg Pb/L,430 µg Se/L, 393 µgTl/L and 272 µg Zn/L.

The classical TU-formula is based on dissolved metal concentrations and does not account for the bioavailable fraction of the metals. In order to incorporate bioavailability in the toxic unit approach, the toxic unit formula was adjusted as follows (Schmidt et al., 2010b):

$$TU_{mix-BL} = \sum_i TU_i = \sum_i \frac{f_{BL,i}}{f_{BL,50\%,i}} \quad \textbf{(Eq. 3.3)}$$

where f_{BL}= the fractional occupancy of the biotic ligand and $f_{BL50\%}$ = the fractional occupancy of the biotic ligand coinciding with 50% effect. The $fBL_{50\%}$ is considered an intrinsic sensitivity of species, and is therefore a unique property of each species and metal. The parameters $f_{BL,50\%}$ are listed in Table 3.2.

Calibration. The biotic ligand binding corresponding with 50% reduction of population growth ($f_{BL,50\%}$) was computed from dose (f_{BL}) – response (population growth) curves, and wass compared with $f_{BL,50\%}$ literature values of single metal experiments. This $f_{BL,50\%}$ was considered to reflect realistic field conditions, and interclonal and interspecies differences. Significant dose response relations between accumulation (AC_{50}) and population growth were used as an indication for causal effects.

If the concept of additivity is valid, the sum of single metal TU_{50} should equal 1. Lower values may indicate synergism, and higher values may indicate antagonism, but other explanations are also possible. In our experiments, additional toxicants as well as deficiencies of essential trace elements or vitamins may have reduced the sensitivity of D. magna for metals. Moreover, inter-clonal (for Ni) and even inter-species (for Co) differences in the $f_{BL,50\%}$ can cause deviations between observed and predicted effects. Adjustment of $f_{BL,50\%}$ is justified to account for these differences in sensitivities. Calibration was performed by solving:

$$\sum_i \alpha_i \times TU_{50,i} = 1 \quad \textbf{(Eq. 3.4)}$$

in which i is a metal (Co, Ni, Cd, Zn, Cu or Mn). Values for α_i resulting in the lowest residual sum of squares were selected if all metals were uncorrelated. The

sensitivities of *Daphnia magna* to metals in complex field samples were subsequently computed by:

$$f_{mix,50\%,i} = f_{BL,50\%} \times \alpha_i \quad \textbf{(Eq. 3.5)}$$

in which i is a metal (Co, Ni, Cd, Zn, Cu or Mn), $f_{50\%,\ mix,i}$ is the sensitivity of Daphnia magna for metal i when exposed to field samples, and $f_{BL,50\%,i}$ is the sensitivity of Daphnia magna in standard media at single metal exposure.

To deal with the strong correlation between Co and Ni, and Cd and Zn, we assumed that the changes in sensitivity of D. magna were equal for Co and Ni, implying that $\alpha_{Co} = \alpha_{Ni}$, and $\alpha_{Cd=} \alpha_{Zn}$.

Statistical analyses. All statistical analyses were performed with R version 2.13 (www.cran-r.com). Significant differences between sites or between lab and field experiments were assessed with pairwise Wilcoxon test with *p* values adjusted according to Benjamini et al. (1995) to prevent false discoveries in multiple testing.

Analysis of variance was calculated to determine to what extent the type of experiment (field or lab) and the different sites were responsible for the variation in population growth and body weight.

The 50% effect parameters $f_{BL,50\%}$ (for biotic ligand binding), TU50 (biotic ligand binding for single metals and metal mixture) and AC50 (for accumulation) were computed using a three parameter log-logistic model; the lower limit was fixed to zero. Dose-response relations were considered significant when the *p*-value of fitted versus measured response parameters (both log-transformed) was<0.05.

RESULTS

Water chemistry and toxic pressure. The actual water composition and estimated toxic pressure of the selected sites in June 2010 are given in Table 3.1. Based on dissolved metal concentrations, the major contributors to toxic effects are Co (maximum concentration 5×EC50), Ni (0.5×EC50), Zn (0.3×EC50) and Cd (0.25×EC50). Other elements contribute less than 10% to the total toxic units. Maximum concentrations of non-measured elements were estimated with regression functions to be 21 µg Se/L, 5.9 µg Tl/L, 24 µg B/L, 249 µg Li/L and 17 µg Mo/L at TUB. Since their contribution to the overall toxicity is smaller than 0.1 TU, these elements were further ignored.

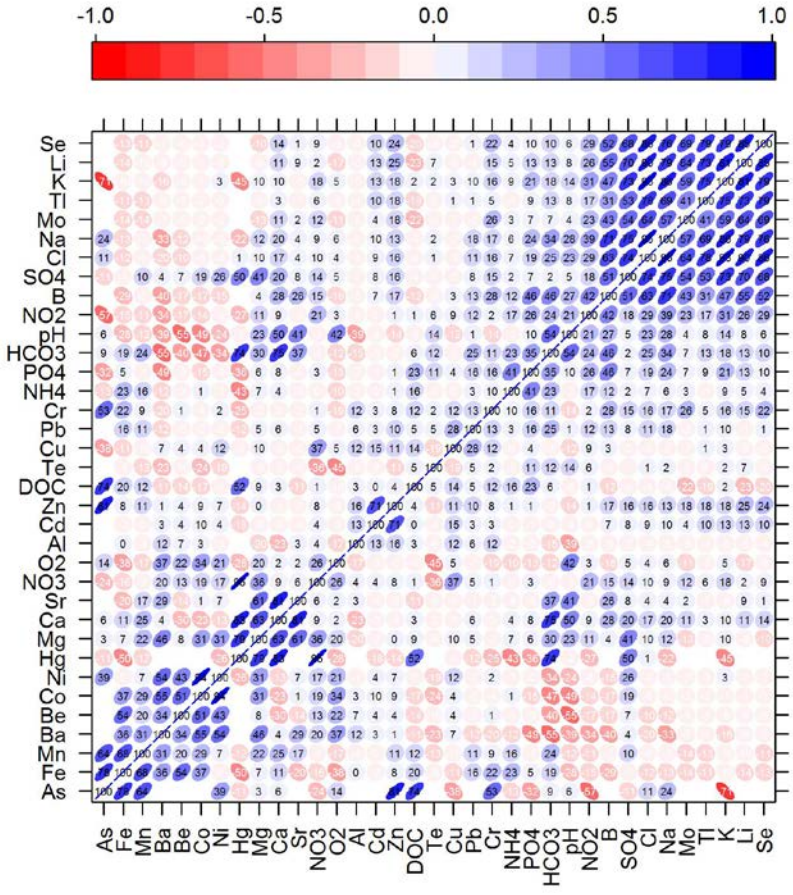

Figure 3.1 Correlation plot of water chemistry parameters, represented by ellipses. Parameters are clustered based on similarity of correlation coefficients. Plotted values are correlation coefficients x100. Positive correlations are colored blue with the angle of the ellipsoid to the right, negative correlations are red with the angles of the ellipse to the left. Strong correlations are indicated by narrow ellipses and darker blue or red colors. Uncorrelated parameters are colored white.

The co-occurrence of elements in natural surface waters of the Dommel catchment area is visualized in Figure 3.1. A cluster of correlated elements (Se, Li, K, Tl, Mo, Na and Cl) is directly related to emissions from zinc smelters. Another cluster of correlated elements contains Ni, Co, Be, Ba, Mn, Fe and As. The origin of this group is dictated by geological factors and is related to pyrite oxidation in areas with variable groundwater levels (Bernard et al., 2008). Cd and Zn are not correlated with these clusters because diffuse emissions are a major source of Cd and Zn emission. Strong correlations are observed for dissolved Co and Ni (*rho* =0.94), and for dissolved Cd and Zn (*rho* =0.71).

Survival. Survival of *D. magna* is shown in Figure 3.2. Survival of *D. magna* was lower in the field than in the laboratory, and variation in the field was high. Typical survival in the lab was 90 to 100%, with no significant differences between sites, whereas these survival rates are observed only occasionally in the field. Overall, three significantly different groups of sites (indicated by letters d, e and f) are distinguished in the field experiment: 1) Sites with a relatively high mean survival of 37% were BEE, BEL, TOR and GRW, indicated as control sites, 2) Sites with very low mean survival of 3% were BOL, DOM and TUB, and, 3) Sites with intermediate mean survival of 20% were GEN, GRB, REU, KSP and RUN.

At ∑TU=1, 50% effect is expected. This implies that significant reductions in the *D. magna* population may already occur at ∑TU<1. Based on EC50 values from literature, effects were expected to occur in GEN and RUN due to high Co and Ni concentrations (see Table 3.1) and possibly in DOM, GRB and REU. A significantly reduced survival is indeed observed at these sites, but only in the field experiment. However, three other sites (KSP, BOL en TUB) also show reduced or severely reduced survival, which cannot be explained by direct effects of metals at current concentrations.

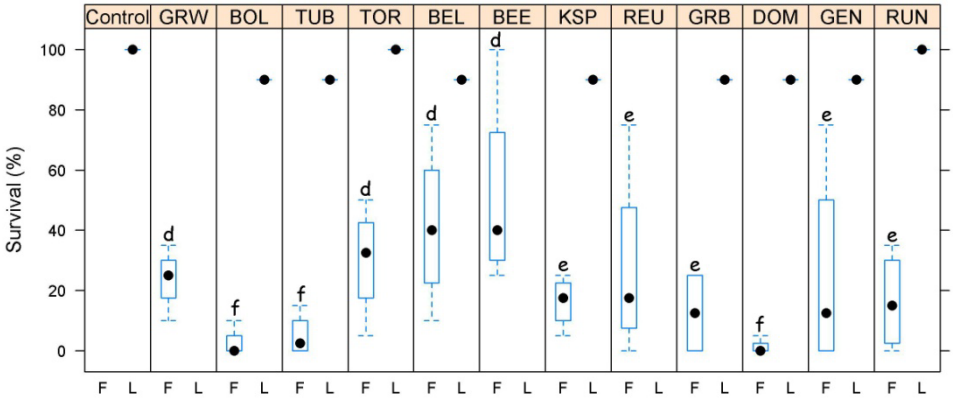

Figure 3.2 Survival of *Daphnia magna* after 3 weeks of exposure in twelve different surface water samples and a control under laboratory (L) and field (F) conditions. Sites are ranked with increasing predicted toxicity ($\sum TU_{dissolved}$). Similar letters indicate groups with similar effects (i.e.no significant difference).

Population growth. Population growth of *D. magna* is shown in Figure 3.3. The reproduction of *D. magna* was lower under field conditions than in a laboratory setting. The population growth obviously suffered from lower survival rates in the field. The correlation between these two endpoints is 0.85. Three groups of sites

(indicated by letters d, e and f) are distinguished with significantly different population growth.

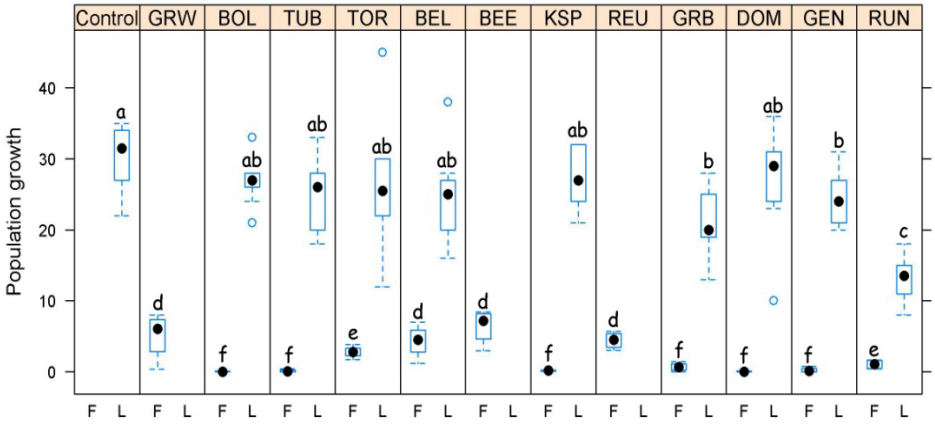

Figure 3.3 Population growth of Daphnia magna after 3 weeks of exposure in twelve different surface water samples and a control under laboratory (L) and field (F) conditions. Sites are ranked with increasing predicted toxicity ($\sum TU_{dissolved}$). Similar letters indicate groups with similar effects (i.e.no significant difference).

The ranking of sites based on their population growth was quite different between laboratory and field, and was inversely related (*rho* = -0.68). The four sites with highest population growth in the laboratory appeared to have the lowest population growth in the field and vice versa. An analysis of variance showed that type of experiment (field or lab) and trial site explained respectively 22 and 40% of the variance in population growth. The unexplained variation of 38% may be caused by experimental uncertainties, such as differences in food and oxygen levels and is quite common for field experiments.

Significantly reduced population growth was observed in the laboratory and field experiment in GEN, RUN, DOM and GRB, which was expected because $\sum TU_{dissolved}$ was approaching or exceeding 1 at these sites. In the field experiment, population growth reduction was also observed in BOL, TUB, KSP, which could not be explained by direct effects of the metal mixture.

Body weight. Body weight of adult daphnids after 3 weeks of exposure is shown in Figure 3.4. Body weight is considerably higher in the laboratory experiment than in the field experiments. Exceptions are RUN and BOL. The rank correlation between body weight in laboratory and field is slightly negative (*rho* = -0.37) as was also observed for population growth. These results confirm growth retardation in the field compared to the lab, and this may explain the reduced population

growth in the field. An analysis of variance showed that type of experiment (field or lab) and trial site explained respectively 29 and 33% of the variance in body weight.

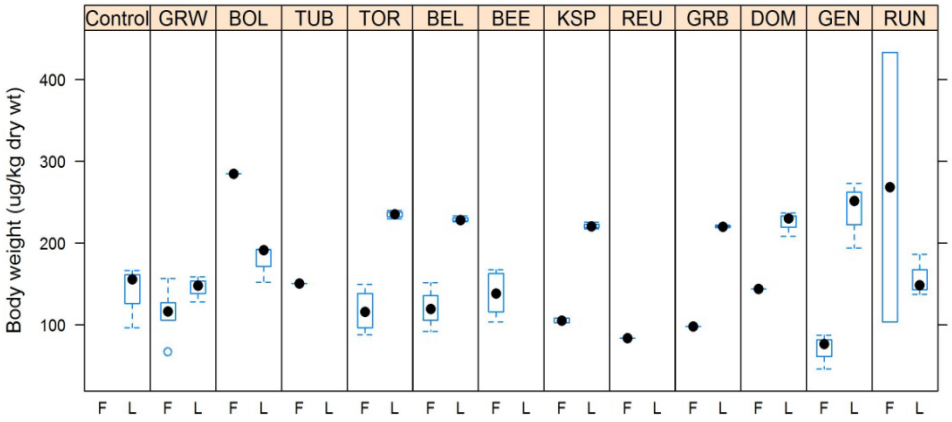

Figure 3.4 Body weight of *Daphnia magna* after 3 weeks of exposure in twelve different surface water samples and a control under laboratory (L) and field (F) conditions. Sites are ranked with increasing predicted toxicity ($\sum TU_{dissolved}$).

Accumulation. Whole body concentrations of Ca, Na, Mg, K and Cu were significantly (Wilcoxon $p<0.05$) higher, whereas Cd, Ni and Zn concentrations were significantly lower in field-exposed daphnids, compared to laboratory-exposed daphnids. No significant differences were found in body concentrations of Fe, Mn, Co and Se between field and laboratory-exposed daphnids. Accumulated whole body concentrations are listed in the Supplementary information.

A negative rank correlation between population growth and accumulation of Cd was found for the field experiments (*rho* = -0.47), whereas in the laboratory a positive rank correlation was observed with Cd (*rho* = 0.73) and Zn (*rho* = 0.65). Because Cd and Zn concentrations are below EC50, direct toxic effects of Zn or Cd on population growth were not expected. The negative effect of accumulated Cd on population growth in field experiments suggests an indirect effect of Cd. The positive correlation between population growth and accumulation of Zn in laboratory may be explained by inhibition of the toxicity of other, more toxic metals by competitive binding with Zn.

Effect concentrations. AC50 and TU50 for population growth in laboratory and field experiments are summarized in Table 3.3. Significant dose response relations were found for Co and Ni bound to the biotic ligand in a laboratory setting

(Figure 3.5a). The causality of Co and Ni was confirmed by elevated whole body concentrations of Co (Figure 3.5b) and Ni in samples with a low population growth. Population growth was reduced by 50% at 188 µg Co/g dry wt and 773 µg Ni/g dry wt. The ∑TU50 for biotic ligand binding could be fitted when all 6 metals are included. The ∑TU50 of 19 is therefore meaningless. Only Co and Ni contribute significantly to the toxicity of the mixture, ∑TU50(Co+Ni) = 0.049. This implies either an antagonistic effect or increased sensitivity of *Daphnia magna* due to the presence of these other toxicants.

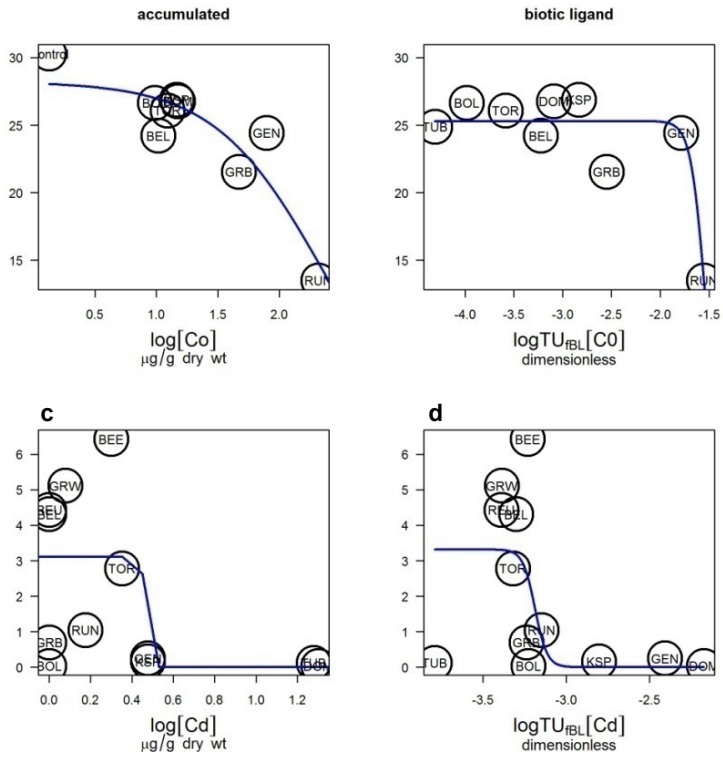

Figure 3.5 Dose-response relations of Co and Cd for *Daphnia magna* exposed to a cocktail of metals in natural surface waters. Figure a and b shows population growth in a laboratory experiment, Figure c and d are results of a field experiment. Exposure is expressed as whole body concentration (a and c) and fractional occupation of the biotic ligand (b and d). Labels express origin of the water sample or trial site (see Method section).

Other elements did not show significant dose-response relationships in the lab, implying that EC50 values were higher than the highest test concentrations. EC50 values are: Cd>1 µg/L, Cr>2.5 µg/L, Cu>3.3 µg/L, Pb>0.21 µg/L, Se>9.8 µg/L, Tl>2.81 µg/L and Zn>120 µg/L, which is in agreement with EC50 from literature.

The significance of the fractional occupancy of the biotic ligand with Cd (Figure 3.5d) and Zn is just slightly above p=0.05. More replicates are required to prove significant effects of Cd and Zn in *in situ* experiments. The adverse effect of Cd on population growth in the field gains evidence by the significant dose-response relations of Cd whole-body concentrations (Figure 3.5c).

Table 3.3 AC50 and TU50 and the lower and upper boundaries of the 95% confidence interval for the effect of metals on population growth of *D. magna*, estimated from a range of natural surface waters, tested in lab and field. AC50 and TU50 were computed by a three parameters log-logistic dose response equations. Significance *p*-value<0.001 *; <0.05 **, <0.1 *. RSE = Residual Standard Error.**

Reproduction ~ Whole-body concentrations (AC50 in µg/g dry wt)

	lab (overall RSE=4.71)[a]					field (overall RSE=2.42)[a]				
	AC50	**lower**	**upper**	**RSE**	***p***	**EC50**	**lower**	**upper**	**RSE**	***p***
Cd	>max	<min	>max	4.98		2.9	0.1	85	1.95	**
Co	231	188	284	2.09	***	4.5	<min	>max	2.21	
Cu	<min	<min	>max	4.76		<min	na	na	2.39	
Mn	<min	<min	261	4.76		218	110	432	2.08	*
Ni	803	773	833	2.49	**	>max	<min	>max	2.45	
Zn	>max	<min	>max	4.764		<min	na	na	2.34	

Reproduction ~ Biotic ligand binding (expressed as TU)

	lab (overall RSE=4.07)[a]					field[b]				
	TU50	**lower**	**upper**	**RSE**	***p***	**TU50**	**lower**	**upper**	**RSE**	***p***
Cd	<min	<min	>max	4.25		0.0007	0.0004	0.001	2.14	*
Co	0.029	0.023	0.036	1.80	***	0.009	<0.0001	4.9	2.50	
Cu	0.008	0.003	0.026	4.14		0.001	<min	>max	2.61	
Mn	0.32	<min	>max	4.25		0.06	na	na	2.61	
Ni	0.020	0.018	0.023	1.80	***	na	na	na	2.61	
Zn	0.031	<min	>max	4.50		0.03	0.008	1.1	2.14	*
sumTU[c]	19	<min	>max	4.75		0.07	0.018	0.24	2.14	

[a] Simultaneous fit of Cd, Co, Cu, Mn, Ni and Zn.

[b] Cd, Co, Cu, Mn, Ni and Zn were fitted separately because the simultaneous fit did not converge

[c] SumTU was fitted separate from individual elements

BLM calibration. Cd and Zn are the only metals that show a dose-response relation with population growth in the field experiment. The sensitivity of our *D. magna* population in the *in situ* experiment is approximately 30 times higher than indicated by the original single metal BLMs. Calibrated sensitivity parameters are $f_{mix,Cd}$ = 2.2e-5, and $f_{mix,Zn}$=3.9e-4.

Co and Ni are the only metals that explain differences in population growth in the laboratory experiment. A clear distinction between the contribution of Co and Ni to reduction of population growth is difficult because of the strong correlation between Co and Ni-concentrations in the aqueous phase (*rho*=0.94). Assuming that the suboptimal natural surface water composition affects the sensitivity of daphnids in a non-specific way, i.e. the increase of daphnia's sensitivity for these metals is increased equally ($\alpha_{Co}=\alpha_{Ni}$), optimized sensitivities are: $f_{mix,Ni}$ = 0.016 and $f_{mix,\ Co}$ = 0.01. The sensitivity of our *D. magna* population in complex field samples is approximately a factor 20 higher than indicated by the original single metal BLMs. Similar factors were reported by Deleebeeck (2008a), who derived values for $f_{mix,Ni}$ between 0.00874 and 0.0284 during validation of the BLMs with experimental data from other studies.

DISCUSSION

Field experiments have a high ecological relevance because effects are studied under the most realistic circumstances, containing all interactions that affect biological responses. However, field experiments are not the most straightforward way to test the performance of BLMs, because many factors during the experiment are not in control or unknown. As a consequence, contaminants may only partly determine the studied responses. For example, the abundance of fish species in Ohio rivers was attributed to measured toxicants for only 7-10% (De Zwart et al., 2006), and similar findings for the river Rhine were reported (Hendriks, 1994). In our study we reduced the uncertainty of field effects by introduction of a controlled population *Daphnia magna* of the same exposure history, age, and sex, and excluded predation, adaptation and avoidance by keeping the daphnids in cages. Differences between sites are therefore mainly caused by differences in water chemistry.

Lab-to-field-comparison. In the field, lower survival, population growth and body weight of adults were observed, compared to the same waters tested in a laboratory experiment. Population growth in the field varied from <1 to 18% of population growth observed in the laboratory. Lower population growth was caused by mortality of the initial population, in combination with reduced reproduction rates of the surviving adults. In most of the trials it was possible to distinguish adults and juveniles at the end of the experiment. Mostly, one age group of juveniles was recognized, occasionally two age groups of juveniles were

found, apart from the adults. The observations indicate that the lower reproduction in the field is the result of mortality, smaller brood sizes and lower brood frequencies due to reduced growth rate and retarded maturation. These findings are confirmed by other *in situ* studies with caged daphnids (Baas et al., 2009; De Coen et al., 2006). Our experiment was more sensitive for mortality and reduced reproduction than the other studies, because we exposed neonates instead of adults. However, the origin and type of contaminants and the concentration ranges in our study are quite comparable with the study of De Coen et al (2006) who studied the impact of metallurgic effluents on caged *Daphnia magna.* After two weeks of exposure of the seven-day-old daphnids, the cumulative reproduction varied from 1 juvenile/surviving female closest to the discharge to 12 juveniles/per surviving female in control sites, which is just slightly above the reproduction in our study.

The main difference between laboratory and field experiments is the addition of algal food in the laboratory experiment, whereas *D. magna* exposed *in situ* grazes on naturally occurring algae. Food quantity and/or quality are most likely the limiting factors for reproduction on all sites. Differences in temperature between laboratory and field may be excluded since these were similar (19.4 $\pm$ 2.5 ºC versus 20 $\pm$ 2 ºC). Although the oxygen conditions of all the trials were generally considered good or sufficient, it cannot be excluded that (temporary) drops in oxygen concentrations occurred. It was observed, for example, that in BOL and KSP plant growth was exponentially increasing during the test in combination with falling water levels, and in TOR mechanical clearing of riversides upstream impacted the oxygen level temporarily.

Confirmation of causal effects was obtained by analysis of metal accumulation in *D. magna*. With this strategy we obtained evidence that Co and Ni caused direct effects on population growth of *D. magna* in the laboratory experiment because: 1) population growth shows a significant dose-response relationship with biotic ligand binding of Co and Ni, 2) reduced population growth coincides with elevated whole body concentrations of these elements and 3) dissolved concentrations were indeed near or above EC50 for Co and Ni. Moreover, the results indicate that in the field, Cd caused indirect effects on population growth of *D. magna* because: 1) an effect of Cd was not observed in the laboratory experiment, 2) accumulated whole body Cd concentrations were lower than in the laboratory experiment, and 3) dissolved Cd concentrations were lower than EC50.

Possible explanations for the fact that BLMs underestimated the effects of the metal mixture in natural surface water samples are: 1) the Co-BLM of *Enchytraeus albidus* may not be representative for *D. magna*; 2) the actual binding properties of natural organic matter differ from the properties of fulvic acids in the speciation model (Doig et al., 2006); 3) the presence of inorganic ligands such as phosphate and nitrate affected free metal-ion activities, which is not accounted for in the BLMs, or 4) the conditional affinity constants of metals to the biotic ligand are affected by the presence of multiple metals.

BLM calibration. BLMs of Co, Ni, Cd and Zn were calibrated to predict effects of metals on *Daphnia magna* in complex field samples. Two test systems were compared: *in situ* exposure and laboratory exposure to waters samples taken from the same sites. Because BLM modeling is a combination of chemical speciation modeling and biotic ligand binding and effect modeling, there are many calibration options. The parameters which were adjusted in published field validation studies are summarized in Table 3.4.

Species sensitivity is a powerful calibration parameter. This is related to many physiological and ecological traits, such as age, size, reproduction strategy, feeding type, breathing type etcetera (Rubach et al., 2010). The sensitivity for metals is a genetic property and may therefore vary between different clones of a species, which explains outcomes of different *Daphnia magna* experiments. Parameters related to the sensitivity of species are dependent on definition of the BLM. Sensitivity parameters in alternative BLMs are LA50, $f_{50\%}$, $EC_{50,0}$ and Q50. These parameters all refer to the binding of metals to the biotic ligand where 50% effect is observed.

Although intrinsic sensitivity parameters should be constant by definition, it is difficult to assess their true value. Experimental and model uncertainty is reflected by variation of the intrinsic sensitivity. The uncertainty is expressed by the calibration factor. When field validation of these parameters was performed in the same way as in the experiments used for BLM development (same research group, same species, same clones) the calibration factor was less than a factor 2 (De Schamphelaere et al., 2002a; De Schamphelaere et al., 2004a; Deleebeeck et al., 2008b). However, the calibration factor increased to 50 when data from other laboratory are used (Deleebeeck et al., 2008a; Villavicencio et al., 2005). In our study, the sensitivity of *D. magna* was approximately a factor 20 higher than indicated by the original BLMs. Sensitivities for Ni and Co in complex natural

surface water samples in the laboratory were $f_{mix,Ni}$ = 0.016 and $f_{mix,\ Co}$ = 0.01. Sensitivities for Cd and Zn under *in situ* conditions are even a factor 30 lower, but include the indirect effect of Cd and Zn on food quality and quantity.

Parameters related to speciation modeling in WHAM are %AFA (the fraction of active fulvic acids in DOC), nA (the number of binding sites on humic and fulvic acids) and pK_{MA} (the binding constant of a metal to fulvic acids). In studies that primarily focus on validation of chemical speciation, the heterogeneity of fulvic and humic acids (ΔLK2) is also calibrated (Van Laer et al., 2006), which is feasible when WHAM VI is used. The reactivity of the fulvic acid fraction varies considerably between sites; values between 17 and 85% were observed for copper in different natural water samples. A value of 50% was considered appropriate if a priori nothing is known about the reactivity of fulvic acids (Dwane et al., 1998). For Zn and Ni similar variations were observed (Cheng et al., 2005; Doig et al., 2007). Parameters related to refinement of the speciation modeling are adopted in later BLM publications, and also in the European Risk Assessment reports.

Summarizing the results, we found that the laboratory results were not always in line with the observations obtained in the field. *In situ* effects were in almost all cases larger than effects in laboratory studies, which implies that no-effect concentrations (derived from laboratory tests) may represent high bioavailability, but are not a-priori protective for effects of metals in natural waters. The sensitivity of *D. magna* in natural surface water samples containing a mixture of potentially toxic metals, suggests an antagonistic effect. Replacement of $f_{BL,50\%}$ by f_{mix} improved the performance of single metal BLMs.

Footnotes Table 3.4.

Original BLMs:a. (HydroQual. 2002. *BLM Windows Interface, Ver 1.0.0.* Hydro-Qual, Mahwah, NJ, USA); b (De Schamphelaere et al., 2002b); c. (De Schamphelaere et al., 2002a) d.(The Biotic Ligand Model Interface (BLM), Version 2.2.3: Users Guide and Reference Manual. 2005. HydroQual, Mahwah, NJ, USA); e.(Schwartz et al., 2007); f. (Deleebeeck et al., 2008a)

References: 1 (Villavicencio et al., 2005); 2. De Schamphelaere, Vasconcelos et al. 2004); 3. (De Schamphelaere et al., 2002a); 4. (Bossuyt et al., 2004); 5. (De Schamphelaere et al., 2004c); 6. (Villavicencio et al., 2011); 7. (Schwartz et al., 2007); 8. (De Schamphelaere et al., 2005); 9.(Deleebeeck et al., 2008a); 10.(Deleebeeck et al., 2009b); 11.(Deleebeeck et al., 2007)

Table 3.4 BLM validation studies for Cu, Ni and Zn, and their calibration parameters reported in the literature. N is the number of natural surface water samples used for the calibration., sw = surface water.

Metal	Test duration	Species	N	Test medium	FIA measured?	Original BLM	Calibration parameters	Original value	Final value	Ref
Cu	Acute	*D. magna*	37	0.45 µm filtered sw	no	a	LA50	0.1	0.069	1
						b		3.4	11.7	
						c		8	14.1	
			6	reconstituted media with isolated DOC	no	a	%AFA.	50	68 (17-86)	2
			13	0.45 µm filtered sw	yes	b	$f_{50\%}$ $logK_{CuOH-BL}$ $logK_{CuCO3-BL}$ pK_{MHA}	0.39 7.45 - 1.75	0.47 7.32 7.01 1.9	3
		Several cladoceran	6	0.45 µm filtered sw	no	b	%AFA $f_{50\%}$	50 0.39	0.96-5.16 n.s.	4
	Chronic	*D. magna*	3	reconstituted media with isolated DOC	no	c	$f_{50\%}$ logK %AFA	0.47 6.68 50	0.393 6.67 41.4	5
		D.magna	20	0.45 µm filtered sw	no	d	LA50 nA humic acids nA fulvic acids	0.119 3.29--3 4.73e-3	0.153 1.65 e-3 2.37 e-3	6
		C. dubia	6	unfiltered sw	yes	a	%AFA	90	-	7
Zn	Chronic	*D. magna, P. subcapitata, O. mykiss*	6	0.45 µm filtered sw and reconstituted media with isolated DOC	no	a	%AFA	50	61 (23-81)	8
Ni	Acute	*D. magna*	5	0.45 µm filtered sw	no	9	$f_{50\%}$	0.205	0.095-0.205	9
		C. dubia	5						0.00469-0.0108	
	Chronic	*P. subcapitata*	5	0.45 µm filtered sw	no	10	$EC_{50,\ Ni,0}$	1.23	3.65	10
		O. mykiss P. promelas	5	0.45 µm filtered sw	no	11	Q_{50}	2.946	1.64-3.01	11

Supporting information

Table S3.1 Whole body concentrations (µg/g dry wt) of 12 elements in *Daphnia magna* after 3 weeks exposure under field (F) or laboratory (L) conditions.

Site	Exp.	n	Ca Mean ± sd	Na Mean ± sd	Mg Mean ± sd	K Mean± sd	Fe Mean ± sd	Cd Mean ± sd	Co Mean ± sd	Cu Mean ± sd	Mn Mean ± sd	Ni Mean ± sdi	Se Mean± sd	Zn Mean ± sd
Ctrl	L	3	69618 11174	41451 29938	2142 381	7896 1477	1438 983	2.5 0.2	1.3 0.4	59 1.5	38 15	312 276	1.0 0.2	163 99
BEE	F	5	65195 11104	59007 29821	2371 437	9435 2242	17963 5152	2.0 2.5	23 9.9	76 7.6	962 606	230 209	2.0 0.9	323 272
	L													
BEL	F	4	80353 3102	49966 23846	2584 546	9065 2186	18051 5552	1.3 0.1	12 3.2	108 18	258 63	40 40	2.3 0.5	250 103
	L	3	59582 2035	26760 21769	1751 494	6679 1150	25512 17898	5.3 0.3	10 1.3	31 12	234 52	492 124	1.5 0.6	214 35
BOL	F	1	67364	37208	2128	8788	2134	1.0	0.8	42	53	29	0.5	138
	L	3	63666 8867	16384 14431	1841 421	5969 1484	10161 11074	12 5.7	10 10	38 3.2	456 60	130 161	1.1 0.6	1083 303
DOM	F	1	67424	92397	2478	12602	12385	20	4.1	129	136	3.9	5.8	265
	L	3	57016 3324	19431 19580	1998 343	6494 876	10604 2372	15 1.2	15 3.0	40 3.0	345 66	101 68	1.8 0.7	431 142
GEN	F	3	88159 18588	32071 13753	1868 358	8426 1212	51912 10334	3.1 0.2	63 33	96 70	538 190	41 39	3.1 1.2	208 23
	L	3	61296 7678	47909 17069	2473 440	7978 1270	18992 3250	7.5 0.9	79 13	40 17	1179 924	80 17	1.5 0.9	267 29
GRB	F	2	69463 11100	55340 32808	2232 443	8527 3604	44290 15762	0.8 0.3	12 4.5	114 4.4	238 119	7.4 2.0	2.4 1.4	137 15
	L	3	58289 2747	25224 23580	1810 491	6410 1051	43905 14781	5.2 0.2	47 4.5	30 2.3	1026 70	140 65	2.0 0.6	658
GRW	F	5	89124 19631	38549 46178	3207 1046	9252 4673	18723 4142	1.2 1.3	6.2 2.7	58 11.5	874 176	17 23	1.4 0.4	179 31
	L	3	63614 1000	13390 16088	1823 370	6653 1209	10216 1588	1.7 0.1	2.5 1.1	21 7.0	351 57	354 542	1.2 0.3	222 104
KSP	F	2	140576 12320	64482 29130	2656 486	9353 2986	27956 9728	3.0 0.8	40 15	120 8.8	850 227	131 91	1.5 0.3	240
	L	3	58573 1872	33920 18627	2022 449	7529 1013	17047 8492	6.6 0.7	15 0.2	41 4.9	203 25	189 159	1.7 0.5	268
REU	F	1	87547	130374	4234	13775	57279	1.1	12	252	304	15	2.9	116
	L	0												
RUN	F	2	84528 5554	66126 49992	2460 765	9234 2089	17769 2402	1.3 0.5	26 8.5	84 15	446 432	135 138	1.2 0.4	164 3.3
	L	3	76906 3710	30178 25260	2400 496	8336 1420	18083 4296	7.0 0.7	209 20	36 7.4	766 139	794 509	1.7 0.3	456 180
TOR	F	4	88560 12633	74025 40730	3054 840	11041 3326	12379 2986	2.1 0.4	10 1.6	61 22	231 18	18 17	2.3 1.0	227 36
	L	3	55550 650	22852 12202	1755 21	5965 208	26331 26888	7.1 4.5	12 7.6	30 7.7	383 43	196 207	1.8 0.5	570 0.7
TUB	F	2	101062 57407	156794 89585	4340 1211	16391 5051	14000 8772	11 11.5	14 11	312 275	4592 1499	6.3 8.5	13 16.0	736 30
	L	0												