The power of biotic ligand models : site-specific impact of metals on aquatic communities

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Chapter 2

Multimetal accumulation in crustaceans in surface water related to body size and water chemistry

ABSTRACT

Many relationships for bioaccumulation of metals have been derived in the past but verification in the field is often lacking. In this study we collected field data of bioaccumulation in caged *D. magna and G. roeseli* in twelve different contaminated brooks. Besides generating a comprehensive dataset on bioaccumulation for these species, we also aimed to check whether the bioavailability at the biotic ligand is useful to explain differences in observed bioaccumulation. Increasing bioaccumulation of Mn, Cd, Co and Ni was observed, which leveled off at higher concentrations. Whole-body concentrations of Ca, Na, Mg, K, Fe, Cu, Se and Zn were independent of exposure concentrations. Univariate and multivariate regressions were performed to examine relationships between accumulated metals and dissolved metal concentrations (Cw), fractional occupancy of the biotic ligand (f_{BL}) species weight (W) and other undefined species traits (Sp). Significant relations between body weight and bioaccumulation were found for Na, Fe, Mn, Cd, Co and Zn; smaller organisms accumulated larger amounts of these elements. Reduced body weight was accompanied by elevated concentrations of Co, Cu and Fe in *D. magna* and elevated concentrations of Mn in *G. roeseli,* indicating toxicity. Although significant relations were found between bioaccumulation and f_{BL} for Mn and Co, Cw was a better predictor for bioaccumulation.

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INTRODUCTION

Bioaccumulation of trace metals in organisms is a natural process that maintains metals at the required physiological level. It is a useful feature when external metal concentrations fluctuate, as is the case under natural field conditions. However, at higher environmental concentrations and for non-essential metals, bioaccumulation can reach critical levels, leading to adverse effect on growth, reproduction and survival. The ratio between exposure concentrations and whole-body concentrations is the simplest approach to quantify bioaccumulation. As a consequence of essential element regulation, the so-called bioconcentration factors from laboratory studies or bioaccumulation factors from field studies (both further referred to as BCF) are not intrinsic properties of a metal, as is demonstrated by the huge variation of reported BCF values (McGeer et al., 2003).

An important factor that explains some of the variability in accumulated metals is the exposure concentration. For trace elements such as Cd, Cu, Cr, Hg, Ni, Pb and Zn, BCF values decrease at higher metal concentrations (Goodyear et al., 1999; Hendriks et al., 2001). Also the chemical composition of surface water in terms of hardness, pH and dissolved organic carbon affects bioaccumulation. These parameters have a large impact on the chemical speciation and bioavailability of metals. It is generally thought that free metal ions are most bioavailable because free metal ions and are transported across cell membranes via protein carriers or membrane channels. In the past decades, biotic ligand models (BLMs) were developed that relate metal toxicity to metals bound to biological receptors. BLMs account for the competition between bioavailable metal species and major cations Ca, Na and Mg for binding to these receptors (De Schamphelaere et al., 2004c; Di Toro et al., 2001; Niyogi et al., 2004). The BLMs have shown to be useful tools for effect assessment because they relate effects to metal binding to the gill or gill-like organs, which is the primary site of action on the external side of the organism. Following this reasoning one would hypothesize that the fractional occupancy of the biotic ligand shows stronger relations with accumulated concentrations than with dissolved metals.

Another factor that contributes to variability in accumulated metals is size (i.e. body weight or length) of the organism; that is, metal concentrations dramatically increase in small individuals. (Hédouin et al., 2006; Hendriks et al., 2001; Newman et al., 1991; Rainbow et al., 1986). Whole-body concentrations roughly consist of two fractions: (1) metals absorbed to the internal body and (2) metals adsorbed to the body surface. The surface area to volume ratio is relatively high for small organisms. Whereas metals adsorbed to the external body surface may be a negligible fraction in larger animals, they take an increasingly larger proportion of total body burden in small animals.

Due to chemical similarity some metals may share uptake pathways, for instance Zn and Cd, and interactions between metals are to be expected. A meta-analysis showed that in the majority of studies effects of metal mixtures is not simply additive (Vijver et al., 2011). Synergistic as well as antagonistic effects are found, and depend on the metal-combination and the type of end point; i.e., accumulation, growth, reproduction or mortality studied. A clear general pattern of interaction effects was not observed.

Because mixture patterns are ambiguous in many laboratory studies, we focused our research on bioaccumulation of metals in caged crustaceans in diffusely contaminated natural surface waters. Fish and bivalves have been tested *in situ* for bioaccumulation, but field-data for smaller invertebrates are scarce (Burton et al., 2005; Schaller et al., 2011). Standardized laboratory tests often underestimate the risks of metals, in terms of accumulated amounts (Vink et al., 2005). The ecological relevance and robustness of field-data is high, because of the realistic exposure conditions, the high variation in chemical composition and other environmental conditions among sites (Crane et al., 2007).

The European Water Framework Directive (Point 1.2.6. of Annex V) states that laboratory data on persistence and bioaccumulation always should be compared with evidence from field studies. Because field-data on bioaccumulation are scarce, only available for a limited number of metals and hardly available for freshwater crustaceans our aim was to fill this gap by exposing caged organisms to natural surface waters, contaminated with a cocktail of metals and metalloids. The advantage of caged organisms is that the origin, life stage and exposure time of the organisms are controlled, while exposure conditions reflect realistic conditions well.

The aims of our study were as follows: (1) to derive model parameters for the relation between exposure concentration and whole-body concentrations and BCF for common freshwater crustaceans for a broad group of metals and metalloids under field conditions; (2) to test whether fractional occupancy of the biotic ligand is a better predictor for bioaccumulation than dissolved concentrations; and (3) to assess inter- and intraspecific size-dependent bioaccumulation.

METHODS

General set-up. We exposed the filter feeder *Daphnia magna* and the shredder *Gammarus roeseli in situ* to ambient field concentrations in twelve diffusely contaminated small rivers in semi natural areas in The Netherlands, and measured the accumulation of twelve common, essential and non-essential elements. We chose *D. magna* because it is the most common invertebrate laboratory test organism in toxicity experiments. It is a filter feeder, and individuals of our clone reached a size of approximately 5 mm under optimal laboratory conditions. In Dutch surface waters, *D. magna* is not abundant though the related *Daphnia pulex* is quite common. We selected *G. roeseli* because of its ecological relevance; it is widespread and abundant in Dutch rivers and plays a vital role in the food chain. It is an omnivorous species that shreds leaves and debris, eats smaller invertebrates but also constitutes an important food source for fish and birds. Male adult *G. roeseli* can reach a size of approximately 3 cm; females are smaller.

Accumulation of Ca, Na, Mg, K, Fe, Mn, Cd, Co, Cu, Ni, Se and Zn was measured. These twelve elements are natural constituents of surface waters but may occur at elevated concentrations due to anthropogenic activities (traffic, agriculture, industrial and domestic waste water, mining). Except Cd, these elements are essential for basic cellular functions such as maintenance of osmotic pressure. The presence of Ca, Na, Mg, K are known to affect bioavailability of Co, Cd, Cu, Mn, Ni, Zn. Whereas most of these metals mostly occur as cations under ambient conditions, Se is a metalloid that occurs as an oxyanion, predominantly SeO_4^{2-}. The elements Fe and Mn were included because of their role in cellular respiration (oxygen binding and redox conditions).

Site selection. Twelve upstream small rivers on sandy soils were selected in the Dommel catchment area in The Netherlands (Figure 2.1). The Dommel rises in the north-east of Belgium, and runs north through the southeast of the Netherlands until it joins the Meuse. The upstream part of the Dommel is heavily affected by ongoing zinc smelting activities dating back to 1904, industrial and municipal waste-water inflow and leaching and run-off from agricultural land (Petelet-Giraud et al., 2009). A natural process contributing to elevated concentrations of Ni and Co is pyrite oxidation enhanced by excessive nitrate from agricultural practice (Bernard et al., 2008). The area is shown to exceed water type-specific environmental quality criteria upto a factor 100, based on generic quality criteria.

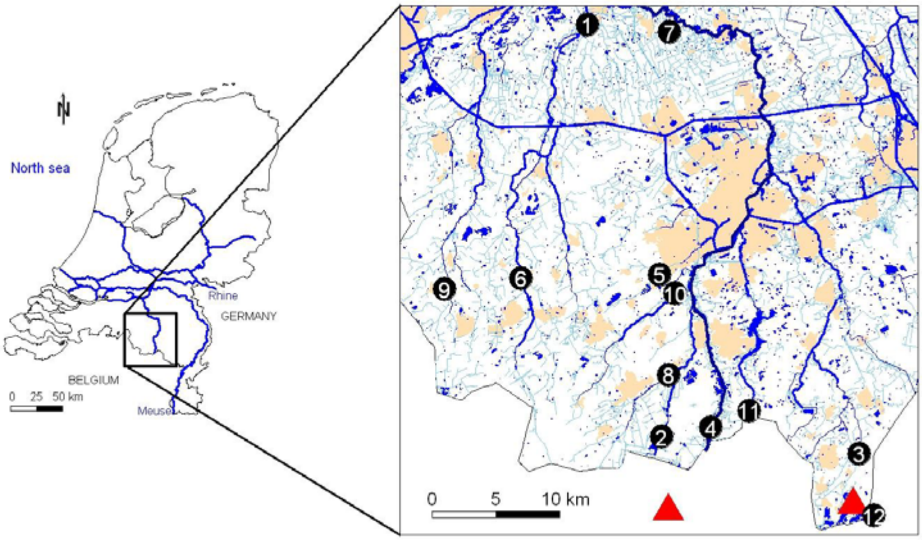


Figure 2.1 Map of The Netherlands (left) with Dommel tributary test region (square). The detailed map (right) shows rivers, brooks, lakes, fens and canals. Triangles indicate location of zinc manufacturers. Orange color represents urbanisations. Trial sites: 1. BEE (Beerze), 2. BEL (Beekloop), 3. BOL (Boschloop), 4. DOM (Dommel), 5. GEN (Gender), 6. GRB (Groote Beerze), 7. GRW(Groote Waterloop), 8. KSP (Keersop), 9. REU (Reusel), 10. RUN (Run), 11.TOR (Tongelreep) , 12.TUB (Tungelroyse Beek).

Site selection was based on evaluation of monitoring data over 2007 to 2009, calculated risks for Cu, Ni and Zn (Verschoor et al., 2011), geographical considerations, and visual inspection of candidate sites. The following selection criteria were applied: (1) selected sites cover a range of low to high metal exposure, (2) the pH of the sites are within the tolerance range of *D. magna, (3)* only natural streams are selected, and (4) as far from urbanization as possible. Selected rivers were Beekloop, Beerze, Boschloop, Dommel, Gender, Groote Beerze, Groote Waterloop, Keersop, Reusel, Run Tongelreep and Tungelroyse Beek.

***Daphnia magna* field experiment.** The *D. magna* clone K4 was cultured in our own laboratory at 20 ºC in M4-medium, according to Organisation for Economic Co-operation and Development 211 protocol (OECD, 2008). Four cages were installed on each site consisting of an inner and an outer cage. The inner cage was made of acrylic glass (methylmethacrylate), was 20 cm high and 9 cm in diameter and had a filter area of approximately 200 cm^2, covered with Monodur PA 500N (500 μm mesh) gauze. In a lab test it was confirmed that neonates were unable to escape from the cage with 500-μm mesh. The outer cage was applied for protective reasons and was made of steel with 1-mm steel mesh and was 21 cm high with 11 cm in diameter. Cages were installed approximately 10 cm below the water level. Pictures of the test with Daphnia magna are shown on page 45.

On June 9 and June 29, 2010, respectively 20 and 10 *D. magna* (<2 d old) were put in the cages. After three weeks of exposure, daphnids were collected from the field and brought to the lab. Adult and juvenile daphnids were separated and transferred to M4 medium for overnight depuration at 15ºC. Adults and juveniles were stored in separate Eppendorf tubes, freeze-dried, and kept at -18ºC.

***Gammarus roeseli* field experiment.** Adult *G. roeseli* were sampled 2 June, 2010, from the Tongelreep and transferred to the lab, where they were kept in breeding chambers in glass aquaria with 50% Tongelreep water and 50% M4 medium, under aeration at 20°C and 12-h light/dark regime (see http://www.youtube.com/watch?v=ICtcNRK3YTw). Gammarids were fed ad libitum with dried leaves (*Populus tremula)*, taken from the site, and juvenile *D. magna* from our lab-culture was supplied as additional food source once a week. Each week, half of the growth medium was replaced with fresh M4 medium and juveniles were separated from the parents. New batches of *G. roeseli* were started with juveniles. A field experiment was set-up with juveniles born between June 21 and 27, 2010. Individual juvenile gammarids were transferred to 100 mL polyethylene vessels with 250 µm nylon (Monodur PA 250N) mesh in the screw cap. The exchange surface was 3.5 cm in diameter. Eight vessels, containing 50 to 100 mg dried leaves and one gammarus per vessel, were installed on June 29, on the same sites as the *D. magna* trials. After three weeks, gammarids were transferred to the lab in their (original) vessels, taken out the next day and stored in separate Eppendorf tubes, freeze-dried and kept at -18ºC until further analysis. Pictures of the test with *Gammarus roeseli* are shown on page 46.

Water chemistry. At the start and the end of the experiment, the following parameters have been determined on site: pH, water temperature, O_2-saturation, conductivity. Water samples were taken for DOC and metal analysis in the laboratory. After filtration (0.22 µm) and acidification Na, Ca, Mg, Mn, Fe, K, Cd, Co, Cu, Ni, Se and Zn were analyzed by high-resolution inductively coupled plasma-mass spectrometry (ICP-MS). These measurements were supplemented with surveillance monitoring data from the waterboard authority from the same period as the field exposure took place. Average concentrations in water and variation coefficients (=standard deviation/mean*100) over the test period were calculated, based on a total of four to five measurements.

Bioaccumulation. Harvested biomass was divided or pooled to obtain samples of approximately 500 µg dry weight. Metal accumulation was determined by acid destruction of at least 100 µg freeze-dried biomass per vial with 2 mL HNO_3 (Ultrapure) at 120ºC. Blank controls (empty vials as well as daphnids grown in M4 medium) were also subjected to the destruction procedure. The residue was dissolved in 3 mL 9% (v/v) HNO_3. Standard samples of dogfish liver were analyzed as a control. After every 10^{th} sample two analytical blanks were measured. Detection limits in dissolved destruate were 80 µg Ca/L, 0.40 µg Cd/L, 0.30 µg Co/L, 0.6 µg Cu/L, 8 µg Fe/L, 40 µg K/L, 30 µg Mg/L, 0.9 µg Mn/L, 60 µg Na/L, 0.5 µg Ni/L, 1 µg Se/L and 1 µg Zn/L.

Analysis of these small amounts of biomass is sensitive for contamination during sampling and sample destruction. Outliers were characterized by extremely high Zn-concentrations (>1100 µg/g dry wt) when these values are not confirmed by duplicate samples. Outliers were removed from the dataset prior to statistical analysis of the data. It concerned six out of 24 samples of *G. roeseli* and three out of 52 samples of *D. magna* adults.

Modeling. The software WHAM7 was used to compute speciation for all Cd, Co, Cu, Mn, Ni, Zn; the major competing cations Ca, Na, Mg, K; and the major anorganic ligands Cl, SO_4, OH and CO_3. Calculated free ion activities are provided in Supplementary Data Table S2.1. The fractional occupation of the biotic ligand (f_{BL}) was computed by:

$$f_{BL} = \frac{K_{Me-BL} \times \alpha_{Me}}{1 + K_{Me-BL} \times \alpha_{Me} + \sum (K_{i-BL} \times \alpha_i)} \qquad \textbf{(Eq. 2.1)}$$

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). Affinity constants were obtained from literature (see Table 2.1). For Co, no data were available for binding to crustacea; therefore we used $\log K_{BL}$ obtained for the fish *Oncorhynchus mykiss*.

It is assumed that affinity constants for metal binding to the biotic ligand can be applied across different species. In case where both acute and chronic data were available, we selected chronic data.

Table 2.1 Affinity constants ($logK_{BL}$) for binding of metals and macro-ions to the biotic ligand and for binding to fulvic acids ($logK_{FA}$).

	Me=Cd	Me=Co	Me=Cu	Me=Mn	Me=Ni	Me=Zn
	D. pulex	*O. mykiss*	*D. magna*	*C. dubia*	*D. magna*	*D. magna*
reference	1	2	3	4	5	6
$logK_{BL}$						
-Me	7.0	5.1	8.02	2.23	4.0	5.31
-MeOH			8.02			
-MeCO3			7.44			
-H	6.1	6.2	6.67			5.77
-Ca	4.1	4.7		3.07	3.10	3.22
-Na		3.2	2.91			1.90
-Mg	3.7			3.08	2.47	2.69
$logK_{FA}$	1.51	1.35	2.17	1.76	1.43	1.68

1) (Clifford et al., 2010) ; 2) (Richards et al., 1998) ; 3) (De Schamphelaere et al., 2004c) ; 4) (Peters et al., 2011a) ; 5) (Deleebeeck et al., 2008a) ; 6) (Heijerick et al., 2005b).

Bioaccumulation was described by a simple empirical model (Goodyear et al., 1999; Hendriks et al., 2001):

$$Cb = a \times Cw^{b} \qquad \textbf{(Eq. 2.2)}$$

or expressed as a bioconcentration factor:

$$BCF = a \times Cw^{b'} \qquad \textbf{(Eq. 2.3)}$$

in which b'=b - 1. In this model the accumulated metal (Cb) is proportional to the metal concentration in water (Cw) raised to the power b. If b = 0, whole-body concentrations are constant, reflecting regulation by organisms. If b = 1, internal body concentrations are proportionally related to external concentrations, reflecting the absence or failing of a regulatory system. Usually, b is smaller than 1, which implies that bioconcentration factors (BCF=Cb/Cw) decrease at higher metal concentrations. To test whether fractional occupation of the biotic ligand is a better descriptor of bioaccumulation, we replaced Cw in Equations 2.2 and 2.3 by f_{BL}.

The relationship between bioaccumulation and body size is described by an allometric equation:

$$Cb = a \times W^{\kappa} \qquad \textbf{(Eq. 2.4)}$$

In this model whole-body concentrations (W) is proportional to the metal concentration in water raised to the power κ. Parameters a and κ are fitting constants. The factor Cb is independent of species weight when κ = 0, and inversely related to species weight when κ = -1. Model parameters in equations 2.2, 2.3 and 2.4 were derived by linear regression of the log-transformed equations for *D. magna* and *G. roeseli* separately (intraspecies relations) and for *D. magna* and *G. roeseli* together (interspecies relations).

Models that describe whole-body concentrations as a combined function of exposure concentrations and size were derived by regression after log transformation of:

$$Cb = a \times Cw^{b} \times W^{\kappa} \qquad \textbf{(Eq. 2.5)}$$

Additionally, it was tested whether inclusion of species as a binary covariable resulted in a significant improvement of the regression. If species is a significant covariable, a correction factor λ for *Gammarus* compared to *Daphnia* follows from the regression

$$\log(Cb) = \log(a) + b \cdot \log(Cw) + \kappa \cdot \log(W) + \lambda \cdot Sp \qquad \textbf{(Eq. 2.6)}$$

where Sp represents the presence of a particular species. For *D. magna* Sp= 0 and for *G. roeseli* Sp=1, and λ is the correction factor for the effect of species on bioaccumulation.

Based on equation 2.6, twelve candidate models were fitted to the data: 1) Cb ~ Constant, 2) Cb~Cw, 3) Cb~W, 4) Cb~Sp, 5) Cb~Cw×W, 6) Cb~Cw×Sp, 7) Cb~W×Sp, 8) Cb~Cw×W×Sp, 9) Cb~f_{BL}, 10) Cb~f_{BL}×W, 11) Cb~f_{BL}×Sp and 12) Cb~f_{BL}×W×Sp. The same models are tested for BCF. The best regression model for each metal was selected based on Akaike information criterion (AIC) (Akaike, 1981). In general, model selection keeps a balance between predictive power and desired model simplicity. Theoretically, the model with the lowest AIC is the best model. However, to prevent over fitting, a statistical penalty is given based on the number of parameters; i.e. a three-parameter model should be at least 2 AIC units lower than the simpler two-parameter model to be selected. Additionally, the p-value must be <0.05 to be selected as best model. Adjusted r^2 is not less suitable for model selection, because leverage and skewness of the data distribution can lead to increased adjusted r^2, which does not reflect a real model improvement. All statistical analysis were performed in R version 2.14.0 (R Foundation for Statistical Computing, http://cran.r-project.org/).

RESULTS

Water chemistry. The mean water chemistry of trial sites, aggregated over a two months test period, is given in Table 2.2. Concentrations are variable in time, with variation coefficients mostly between 10 to 60, but with peaks upto >100. Several of the selected sites exceed environmental quality criteria (EQS), composed of maximum admissible concentrations plus background concentration: 0.9 + 0 µg Cd/L (specific value for hardness between 100-200 mg $CaCO_3$/L), 1.36 + 0.2 µg Co/L, 1.1+ 0.4 µg Cu/L, 20 µg Ni/L, 24.6 + 0.04 µg Se/L and 15.6 + 1 µg Zn/L (http://www.rivm.nl/rvs/normen). Criteria for chloride and sulphate are 200 and 100 µg/L, respectively. The sites BEL, GRW and TOR are the cleanest sites, with none or only slight exceedances, whereas Run and GEN exceed criteria for Co, Cu, Ni and Zn. DOM and TUB also constitute high concentrations of Zn but add relatively high concentrations Na, Se, Cl and SO_4. Cu-EQS is exceeded on 10 out of 12 sites, however DOC might reduce the effects of Cu significantly, especially on sites with DOC> 5 mg/L.

Maximum risk ratios (concentration/EQS) are Zn 5.5 in DOM, Ni 4.5 in Run, Cu 2.5 in KSP, Co 18 in Run. Most risk ratios are a factor 2 to 3 higher when concentrations are related to annual average EQS. An exception is Se, which exceeds annual average EQS on eight sites, up to a factor 50 in DOM and TUB.

Survival of test organisms. The amount of organisms that could be collected after three weeks of field exposure differed between sites. The total destructed (collected) biomass is given in Table 2.3. In BOL and DOM only a small amount of adult daphnids could be harvested, no juveniles were present and *G. roeseli* did not survive. Also, in KSP and TUB reproduction of *D. magna* failed. Reproduction of *G. roeseli* was not expected due to its young age. Whole-body concentrations are also expressed in Table 2.3. Due to the complex mixtures of elevated trace metal concentrations, it is difficult to establish causal relationships between individual elements and observed effects on survival and reproduction. The dataset, however, offers the opportunity to assess relationships between external exposure and accumulation of a large number of elements in a natural complex environment in two different species and to compare them with background accumulation in a standard M4 growth medium.

Body concentrations. Field exposed daphnids accumulate larger amounts of all metals except Ca compared to the control daphnids grown in M4 medium in the lab. Although Ca concentrations in the field are higher than in M4 medium, daphnids keep Ca constant at approximately 8%, which is normal in *D. magna* (Hessen et al., 2000). Whole-body concentrations of Co, Cd, Fe and Ni are a factor 20 to 30 higher; Na and Mn are a factor 8 to10 higher; and Mg, K, Cu, Se and Zn are a factor 2 to 3 higher in field-exposed adult daphnids compared to the control. The elements Cd and Se were also detected in control daphnids, whereas they are not constituents of M4 medium. The elements Cd and Se could have been present as impurities in trace-element or vitamin stock-solutions or they could have entered via the algae fed to the daphnids.

Accumulation in *D. magna* could be compared with accumulation in *G. roeseli* on seven sites. *G. roeseli s*howed significantly higher Ca contents and significantly lower contents of Fe and Co. Remarkably lower whole-body concentrations of Na, Mn, Cd, and Ni were found in *G. roeseli* but in a paired *t* test these differences were not significant. More observations at more sites are required to confirm these findings.

Dissolved concentrations. The relationship between whole-body concentrations, corresponding BCF values and aqueous concentrations in *D. magna* and *G. roeseli* is shown in Figure 2.2. Three elements clearly come forward for which whole-body concentrations depend on dissolved concentrations in one or more of the tested combinations of species and chemical forms: Mn, Cd and Co. Whole-body concentrations of the other elements (Ca, Na, Mg, K, Fe, Cu, Ni, Se and Zn) show no significant relation with exposure, implying that the whole-body concentrations of these elements are constant. In *G. roeseli* relations were found only between whole-body concentrations and dissolved Cd.

Occupancy of the biotic ligand. The occupancy of the biotic ligand is affected by competition between free ions. According to equation 2.1, two parameters are decisive in the ultimate ligand binding of a metal; (1) the level of the affinity constant; and (2) the concentration of the metal — both compared to the affinity constants and concentrations of its competitors.

Linear regression between log(Cb) and log(f_{BL}) revealed significant relations for Mn (slope=0.78 $\pm$ 0.25, adjusted r^2 =0.35), and Co (slope=0.43 $\pm$ 0.10, adjusted r^2 = 0.51) in *D. magna* and no significant relations in *G. roeseli.*

Size. Significant interspecies relations were found between accumulated Na, Fe, Mn, Cd, Co and Zn and species weight (W), see Figure 2.3. Interspecies differences in bioaccumulation reflect allometric phenomena, caused by changes in surface to volume ratios. Intraspecies relations were found for Mn, Fe, Co and Cu. Intraspecies relations between Cb and W most likely reflect toxic effects of accumulated concentrations on growth instead of an allometric phenomenon, as organisms were at the same lifestage and size at the start of the experiment. Size variations of *D. magna* were related to accumulated concentrations of Fe (slope= -1.35, adjusted r^2 = 0.32), Co (slope= -1.47, adjusted r^2 = 0.21), and Cu (slope= -0.59, adjusted r^2 = 0.33), whereas *G. roeseli's* size showed a significant relation with accumulated Mn (slope= -0.57, adjusted r^2 = 0.54).

Multivariate bioaccumulation models. Significant multivariate models (*p* value<0.05) describing whole-body concentration as a function of exposure concentration, weight or species could be derived for all metals in this study. From a pairwise *t* test with Holmes correction, it appeared that accumulation models containing f_{BL} (models 9 to 12) were significantly different from models without f_{BL} (models 1 to 8). The difference in AIC of twelve candidate regression models compared to the most simple reference model (Cb=constant) is plotted in Figure 2.4.

Based on this comparison of AIC of candidate models, it appeared that whole-body concentrations of Ca, Na and Zn were better described by a univariate model with either species (Cb~Sp) or weight (Cb~W) as variables. Fe, Mn, Cd and Co whole-body concentrations were best described by a bivariate model: Cb~Cw×Sp. For Mg and Cu, none of the candidate models fulfilled this criterion; therefore the reference model is selected as best model. Exposure concentrations, species or weight did not contribute significantly to improved model performance for Mg, K, Ni, and Se. Models for Cb as function of Cw and W, as function of Cw, W and Sp as well as models with the highest statistical reliability are summarized in Table 2.4. Models for BCF are presented in the Supplementary Data, Table S2.2. In general, species was selected as a better descriptor for bioaccumulation than weight. Because weight is an important species trait, some overlap in the predictive power of weight and species is to be expected. Univariate models for bioaccumulation as function of weight are given in Figure 2.3. Multivariate models Cb~Cw×W performed less well than Cb~Cw×Sp, though still offered an acceptable *p* value and adjusted r^2 for Mn, Cd and Co (see Table 2.4).

Table 2.2 Characteristics of filtered water samples of selected trial sites. Mean and variation coefficient (%) over June and July 2010[a].

		mg/L										µg/L					
Site	pH	DOC	Cl	SO_4	HCO_3	Ca	Na	Mg	K	Fe	Mn	Cd	Co	Cu	Ni	Se	Zn
BEE	7.4^{1}	12^{6}	34^{27}	75^{6}	84^{20}	40^{10}	26^{21}	6.4^{8}	11^{18}	0.47^{59}	0.3^{27}	0.04^{29}	3.3^{34}	3.4^{94}	15^{9}	0.022^{107}	8.5^{59}
BEL	7.3^{1}	5.8^{9}	38^{6}	50^{16}	140^{10}	51^{2}	28^{4}	6.9^{6}	5.7^{13}	0.21^{15}	0.08^{20}	0.06^{22}	2.1^{20}	1.6^{78}	6.6^{7}	0.15^{50}	14^{45}
BOL	7.7^{14}	9.2^{8}	26^{33}	46^{11}	49^{92}	36^{39}	23^{30}	5.5^{32}	4.7^{28}	1.6^{128}	0.06^{82}	0.10^{80}	0.25^{5}	3.1^{62}	2.9^{15}	0.21^{70}	41^{98}
DOM	7.2^{1}	6.5^{4}	190^{52}	175^{53}	140^{0}	35^{4}	130^{36}	6.2^{15}	100^{48}	0.11^{56}	0.05^{50}	0.70^{33}	2.1^{33}	2.6^{42}	11^{13}	2.9^{31}	91^{23}
GEN	6.6^{2}	3.2^{27}	30^{0}	83^{2}	48^{19}	32^{6}	23^{4}	8.0^{5}	6.2^{8}	0.66^{123}	0.26^{48}	0.20^{34}	18^{82}	1.9^{75}	52^{22}	0.048^{231}	43^{42}
GRB	7.2^{1}	10^{7}	49^{9}	85^{1}	90^{7}	43^{13}	43^{11}	6.3^{8}	17^{17}	0.3^{52}	0.28^{23}	0.06^{31}	6.0^{35}	2.1^{80}	15^{19}	0.06^{44}	19^{68}
GRW	8.2^{11}	6.0^{5}	39^{0}	47^{5}	150^{9}	58^{11}	23^{16}	6.7^{17}	3.0^{78}	0.25^{22}	0.13^{41}	0.04^{44}	0.34^{22}	1.3^{112}	1.4^{23}	0.06^{50}	5.4^{73}
KSP	7.2^{2}	9.4^{3}	27^{10}	63^{6}	74^{24}	39^{6}	20^{11}	6.2^{8}	9.8^{6}	0.12^{47}	0.05^{77}	0.09^{57}	1.9^{71}	3.8^{31}	11^{29}	0.22^{19}	25^{58}
REU	6.5	30	39	130	110	47^{11}	22^{11}	9.9^{15}	16^{47}	4.2^{185}	0.25^{87}	0.03^{44}	3.7^{57}	2.9^{68}	12^{38}	0.079^{43}	18^{75}
RUN	6.8^{3}	7.7^{9}	24^{9}	110^{0}	36^{22}	35^{8}	16^{5}	8.5^{17}	12^{25}	0.33^{49}	0.14^{86}	0.08^{59}	24^{127}	3.3^{31}	91^{37}	0.42^{30}	35^{81}
TOR	7.3^{13}	4.2^{12}	45^{5}	63^{3}	150^{0}	54^{19}	32^{25}	7^{21}	6.0^{25}	0.06^{62}	0.03^{50}	0.04^{28}	0.88^{22}	2.0^{60}	5.4^{12}	0.28^{34}	7.7^{87}
TUB	7.1^{3}	5.5^{14}	355^{12}	396^{3}	189^{3}	95^{9}	250	16^{20}	9.1	0.05^{39}	1.0^{81}	0.06^{67}	0.3^{55}	1.4^{84}	3.0^{35}	2.7^{NA}	32^{41}

[a] Superscript numbers express variation coefficient (%)

DOC=dissolved organic carbon; BEE=Beerze; BEL=Beekloop; BOL=Boschloop; DOM=Dommel; GEN=Gender; GRB=Groote Beerze; GRW=Groote Waterloop; KSP=Keersop; REU=Reusel; RUN=Run; TOR=Tongelreep; TUB=Tungelroyse Beek.

Table 2.3 Whole-body residues of macro-ions and trace elements in *D. magna and G. roeseli* after 3 weeks of field exposure[a].

		µg	mg/g dry wt						µg/g dry wt					
	n	Biomass	Ca	Na	Mg	K	Fe	Mn	Cd	Co	Cu	Ni	Se	Zn
Daphnia magna														
Control	6	400	84 ^{10}	6.8 ^{149}	1.1 ^{56}	5.2 ^{46}	0.7 ^{69}	0.12 ^{86}	0.19 ^{40}	0.85 ^{75}	31 ^{37}	2 ^{29}	0.6 ^{30}	98 ^{5}
BEE	9	552	60 ^{38}	89 ^{59}	2.7 ^{35}	11 ^{37}	13 ^{57}	0.78 ^{62}	1.3 ^{154}	18 ^{52}	76 ^{28}	130 ^{147}	1.4 ^{73}	230 ^{97}
BEL	4	875	80 ^{4}	50 ^{48}	2.6 ^{21}	9.1 ^{24}	18 ^{31}	0.26 ^{25}	1.3 ^{8}	12 ^{27}	110 ^{17}	210 ^{163}	2.3 ^{22}	250 ^{41}
BOL	1	569	67	37	2.1	8.8	2.1	0.053	1.0	0.78	42	29	0.53	140
DOM	1	144	67	92	2.5	13	12	0.14	20	4.1	130	3.9	5.8	260
GEN	4	404	77 ^{35}	38 ^{44}	2.1 ^{28}	8 ^{16}	44 ^{42}	0.57 ^{29}	2.8 ^{27}	65 ^{41}	110 ^{57}	49 ^{73}	2.7 ^{51}	190 ^{25}
GRB	6	535	69 ^{18}	34 ^{90}	2 ^{27}	7.3 ^{31}	41 ^{32}	0.47 ^{48}	0.74 ^{24}	19 ^{35}	92 ^{25}	10 ^{32}	2.8 ^{30}	240 ^{68}
GRW[c]	5	423	97 ^{23}	60 ^{82}	3.8 ^{27}	11 ^{46}	17 ^{29}	0.87 ^{28}	0.65 ^{34}	4.4 ^{39}	95 ^{84}	17 ^{134}	1.4 ^{37}	180 ^{17}
KSP[b]	5	372	75 ^{69}	19 ^{98}	1.3 ^{62}	5 ^{51}	19 ^{64}	0.44 ^{89}	1.8 ^{69}	22 ^{87}	110 ^{22}	56 ^{168}	2.1 ^{14}	160 ^{44}
REU	1	252	88	130	4.2	14	57	0.3	1.1	12	250	15	2.9	120
RUN	2	476	85 ^{7}	66 ^{76}	2.5 ^{31}	9.2 ^{23}	18 ^{14}	0.45 ^{97}	1.3 ^{38}	26 ^{33}	84 ^{17}	140 ^{102}	1.2 ^{34}	160 ^{2}
TOR	4	626	89 ^{14}	74 ^{55}	3.1 ^{28}	11 ^{30}	12 ^{24}	0.23 ^{8}	2.1 ^{21}	9.8 ^{17}	73 ^{40}	18 ^{92}	2.3 ^{42}	230 ^{16}
TUB	4	326	76 ^{22}	120 ^{56}	2.9 ^{29}	12 ^{29}	5.8 ^{30}	5.2 ^{28}	8.9 ^{79}	5.7 ^{14}	110 ^{14}	8.8 ^{123}	1.3 ^{72}	520 ^{26}
Mean			78 ^{14}	67 ^{52}	2.7 ^{30}	10 ^{26}	22 ^{77}	0.81 ^{172}	3.6 ^{157}	17 ^{103}	107 ^{47}	57 ^{116}	2.2 ^{60}	223 ^{47}
Gammarus roeseli														
BEE	4	243	100 ^{6}	20 ^{169}	2.2 ^{48}	10 ^{27}	0.81 ^{113}	0.19 ^{133}	0.36 ^{42}	2.4 ^{137}	120 ^{34}	26 ^{131}	1.9 ^{124}	140 ^{61}
GEN[b]	2	125	93	100	4.3	13	3.3	0.26	2.2	4.5	90	6.4	0.96	69
GRB	3	420	110 ^{10}	24 ^{85}	2.4 ^{6}	11 ^{16}	2.2 ^{102}	0.2 ^{75}	0.29 ^{14}	2.4 ^{81}	130 ^{20}	79 ^{168}	3 ^{103}	81 ^{19}
KSP	3	627	95 ^{22}	0.15 ^{29}	2 ^{13}	7.7 ^{18}	1.3 ^{62}	0.12 ^{88}	1.5 ^{15}	2.2 ^{43}	110 ^{7}	1.3 ^{29}	0.99 ^{87}	58 ^{5}
REU	2	537	85 ^{41}	27 ^{63}	3.1 ^{42}	9.3 ^{52}	1.4 ^{66}	0.084 ^{23}	0.31 ^{21}	2 ^{62}	83 ^{31}	1.5 ^{42}	0.3 ^{3}	53 ^{57}
RUN[c]	5	316	120 ^{1}	12 ^{129}	2.6 ^{27}	9.9 ^{25}	2.1 ^{32}	0.24 ^{42}	0.66 ^{13}	23 ^{37}	110 ^{13}	4.1 ^{131}	2.2 ^{23}	340 ^{72}
TUB	2	333	110	0.73	2.4	8.5	1.1	0.20	0.81	1.9	150	2.1	3.6	110
Mean	0		102 ^{12}	26 ^{130}	2.7 ^{29}	10 ^{17}	1.7 ^{49}	0.18 ^{34}	0.9 ^{82}	5.5 ^{142}	113 ^{20}	17 ^{166}	1.9 ^{64}	122 ^{83}

[a] Superscript numbers express variation coefficient (%); [b] One outlier removed from the dataset; [c] Two outliers removed from the dataset.

n=number of destruction replicates; Biomass=total destructed material; BEE=Beerze; BEL=Beekloop; BOL=Boschloop; DOM=Dommel; GEN=Gender; GRB=Groote Beerze; GRW=Groote Waterloop; KSP=Keersop; REU=Reusel; RUN=Run; TOR=Tongelreep; TUB=Tungelroyse Beek.

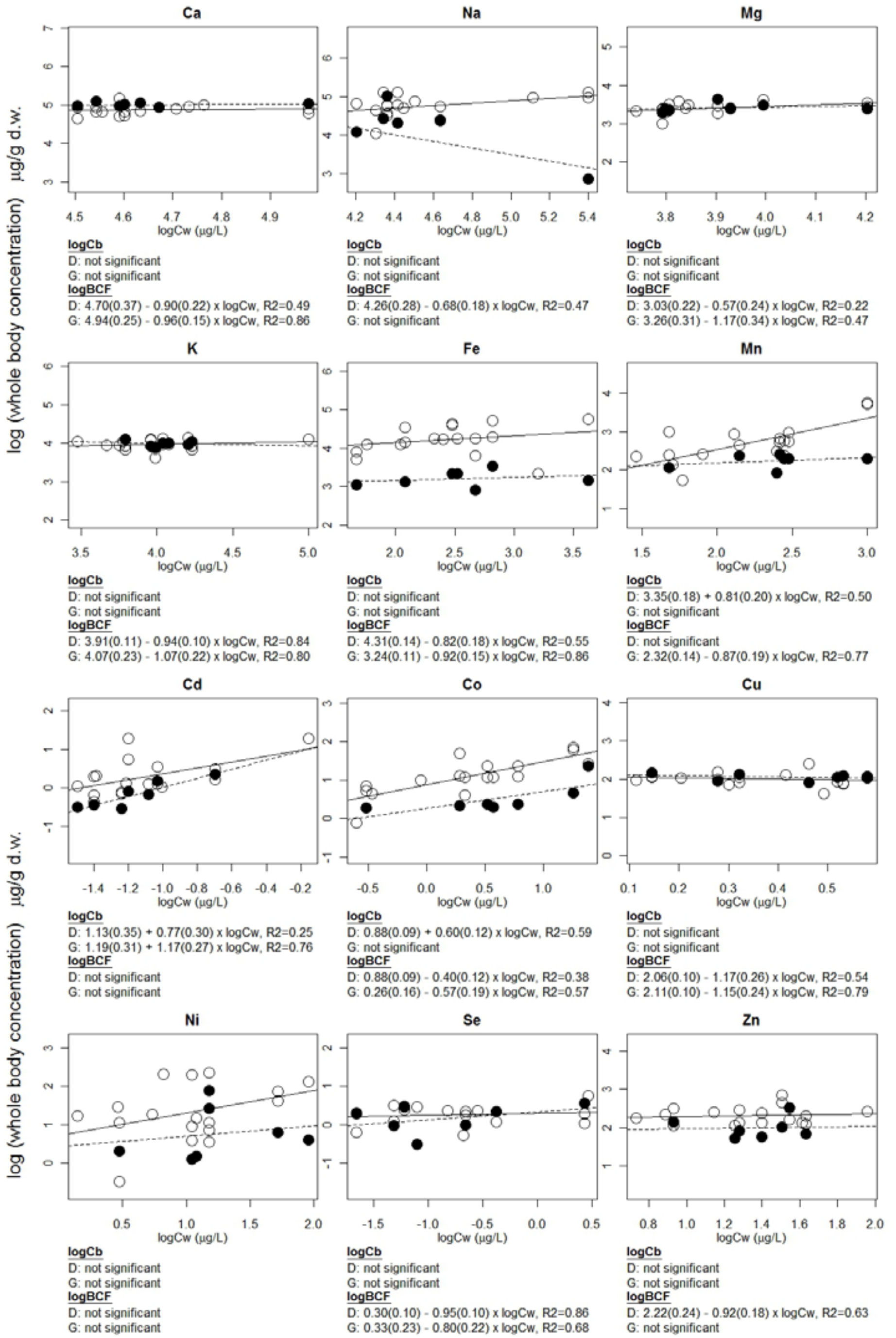


Figure 2.2 Whole-body concentrations of metals in D. magna(-○-) and G. roeseli (··●··) related to dissolved metal concentrations (Cw) in twelve natural surface waters. Regression functions of logCb~logCw and logBCF~logCw are printed below the figures, with standard error of the estimated coefficients between brackets.

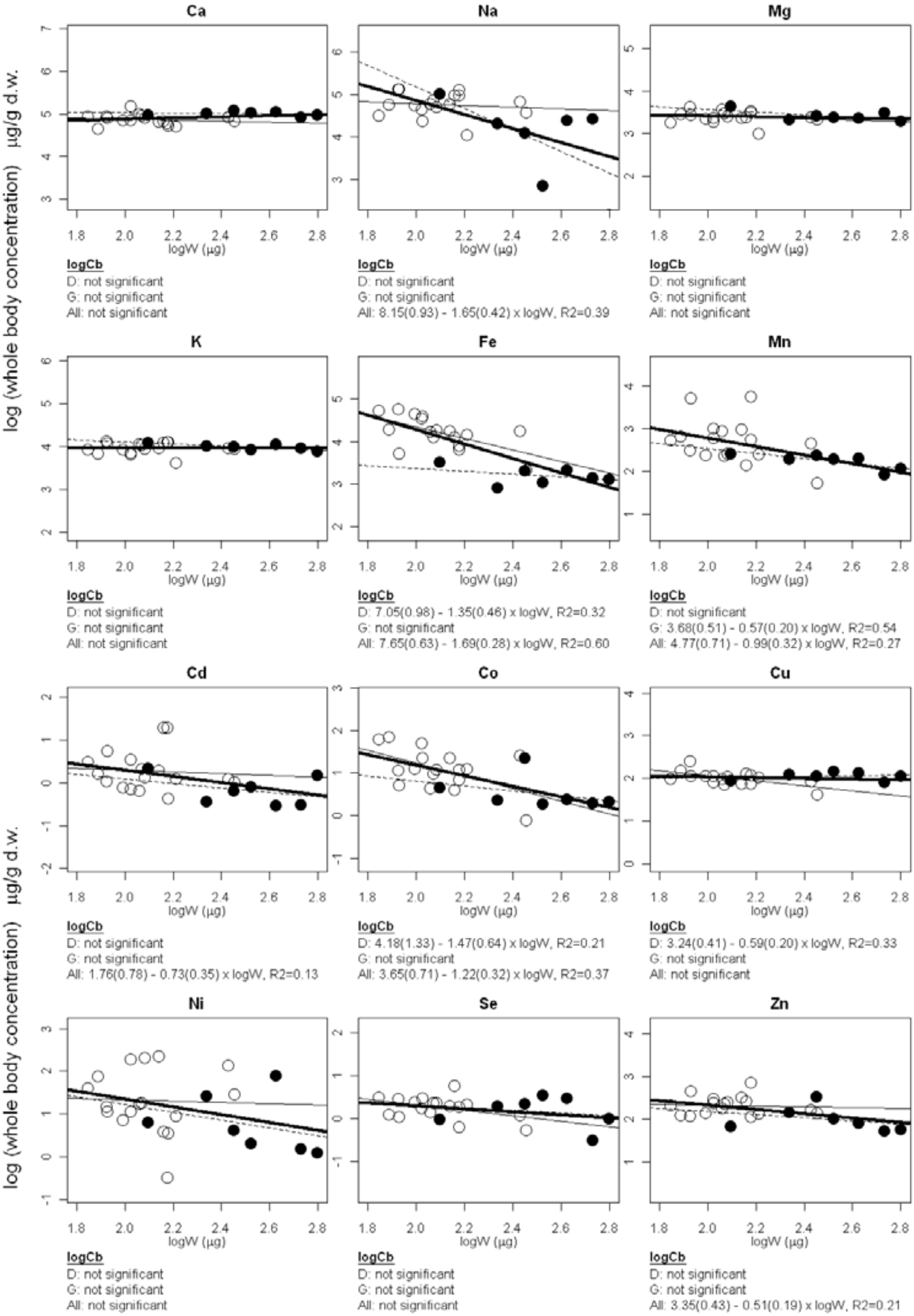


Figure 2.3 Allometric relations between body weight (W in µg) and whole-body metal concentrations (Cb in µg/g d.w.) in *Daphnia magna* (○) and *G. roeseli* (●) after three weeks exposure in natural surface waters. Separate regression lines are shown for *D. magna (—)* and *G. roeseli (····).* Bold line represents the regression over all data.

Table 2.4 Bivariate, trivariate and best models for Cb in µg/g/d.w.) as function of dissolved metal concentration (Cw in µg/L), species weight (W in µg/g d.w.) and other undefined species traits (Sp). Sp=0 for *D. magna*; Sp=1 for *G. roeseli*.

	Model: log Cb = a log Cw + κ log W	AIC	*p* value	Adj r^2
Ca	4.49(0.37) + 0.07(0.18) logCw + 0.14(0.10) logW	-97.0	0.365	0.01
Na	8.31(1.10) - 0.09(0.31) logCw - 1.66(0.43) logW	-25.8	0.004	0.36
Mg	3.27(0.28) + 0.35(0.19) logCw - 0.08(0.10) logW	-96.5	0.160	0.08
K	3.99(0.22) + 0.06(0.09) logCw - 0.04(0.09) logW	-98.5	0.765	-0.07
Fe	7.74(0.64) + 0.10(0.14) logCw - 1.7(0.29) logW	-45.3	<0.0001	0.59
Mn	4.95(0.62) + 0.50(0.17) logCw - 0.91(0.28) logW	-47.0	5.8e-4	0.46
Cd	2.34(0.68) + 0.81(0.26) logCw - 0.57(0.3) logW	-44.0	0.002	0.38
Co	3.31(0.55) + 0.42(0.1) logCw - 1.16(0.25) logW	-52.4	<0.0001	0.63
Cu	2.21(0.26) - 0.10(0.21) logCw - 0.07(0.12) logW	-88.5	0.664	-0.05
Ni	2.76(1.31) + 0.36(0.31) logCw - 0.9(0.56) logW	-12.4	0.164	0.08
Se	1.24(0.50) + 0.12(0.09) logCw - 0.41(0.22) logW	-56.2	0.112	0.12
Zn	3.28(0.49) + 0.06(0.18) logCw - 0.51(0.20) logW	-63.0	0.051	0.18
	Model: log Cb = a log Cw + κ log W + λ Sp			
Ca	4.89(0.38) + 0.09(0.16) logCw - 0.08(0.13) logW + 0.18(0.07) Sp	-101.1	0.066	0.19
Na	7.42(1.41) - 0.08(0.31) logCw - 1.21(0.61) logW - 0.36(0.35) Sp	-25.0	0.007	0.36
Mg	3.51(0.37) + 0.31(0.20) logCw - 0.18(0.14) logW + 0.08(0.08) Sp	-95.7	0.202	0.08
K	4.16(0.29) + 0.07(0.09) logCw - 0.12(0.14) logW + 0.07(0.08) Sp	-97.5	0.715	-0.08
Fe	6.16(0.70) + 0.14(0.12) logCw - 0.89(0.33) logW - 0.66(0.19) Sp	-54.1	<0.0001	0.73
Mn	4.02(0.80) + 0.61(0.17) logCw - 0.40(0.40) logW - 0.40(0.23) Sp	-48.3	6.2e-4	0.51
Cd	1.48(0.87) + 0.83(0.25) logCw - 0.13(0.41) logW - 0.36(0.24) Sp	-44.6	0.003	0.42
Co	2.16(0.72) + 0.50(0.10) logCw - 0.59(0.34) logW - 0.45(0.20) Sp	-55.9	<0.0001	0.69
Cu	2.65(0.34) - 0.02(0.20) logCw - 0.31(0.17) logW + 0.18(0.09) Sp	-90.5	0.232	0.07
Ni	1.24(1.84) + 0.48(0.33) logCw - 0.20(0.82) logW - 0.57(0.49) Sp	-12.0	0.181	0.09
Se	1.68(0.69) + 0.14(0.09) logCw - 0.63(0.32) logW + 0.17(0.18) Sp	-55.2	0.161	0.11
Zn	2.68(0.62) + 0.07(0.17) logCw - 0.22(0.27) logW - 0.24(0.16) Sp	-63.6	0.046	0.22
	Best model: log Cb =…….			
Ca	4.87 (0.03) + 0.14 (0.05) Sp	-104.8	<0.0001	0.23
Na	8.15 (0.93) – 1.65 (0.42) logW	-27.7	7.0e-4	0.39
Mg	3.42 (0.03)	-96.3	<0.0001	
K	3.98 (0.02)	-101.9	<0.0001	
Fe	6.10 (0.71) – 0.90 (0.34) logW – 0.64 (0.20) Sp	-54.5	<0.0001	0.72
Mn	3.23 (0.15) + 0.66 (0.16) logCw – 0.57 (0.15) Sp	-49.1	2.4e-4	0.51
Cd	1.22 (0.28) + 0.84 (0.24) logCw – 0.41 (0.16) Sp	-46.4	8.2e-4	0.44
Co	0.90 (0.08) + 0.55 (0.10) logCw – 0.71 (0.14) Sp	-54.4	<0.0001	0.66
Cu	2.02 (0.03)	-82.7	<0.0001	
Ni	1.15 (0.15)	-13.1	<0.0001	
Se	0.23 (0.06)	-61.0	<0.0001	
Zn	2.32 (0.06) -0.33 (0.11) Sp	-70.9	5.9e-3	0.27

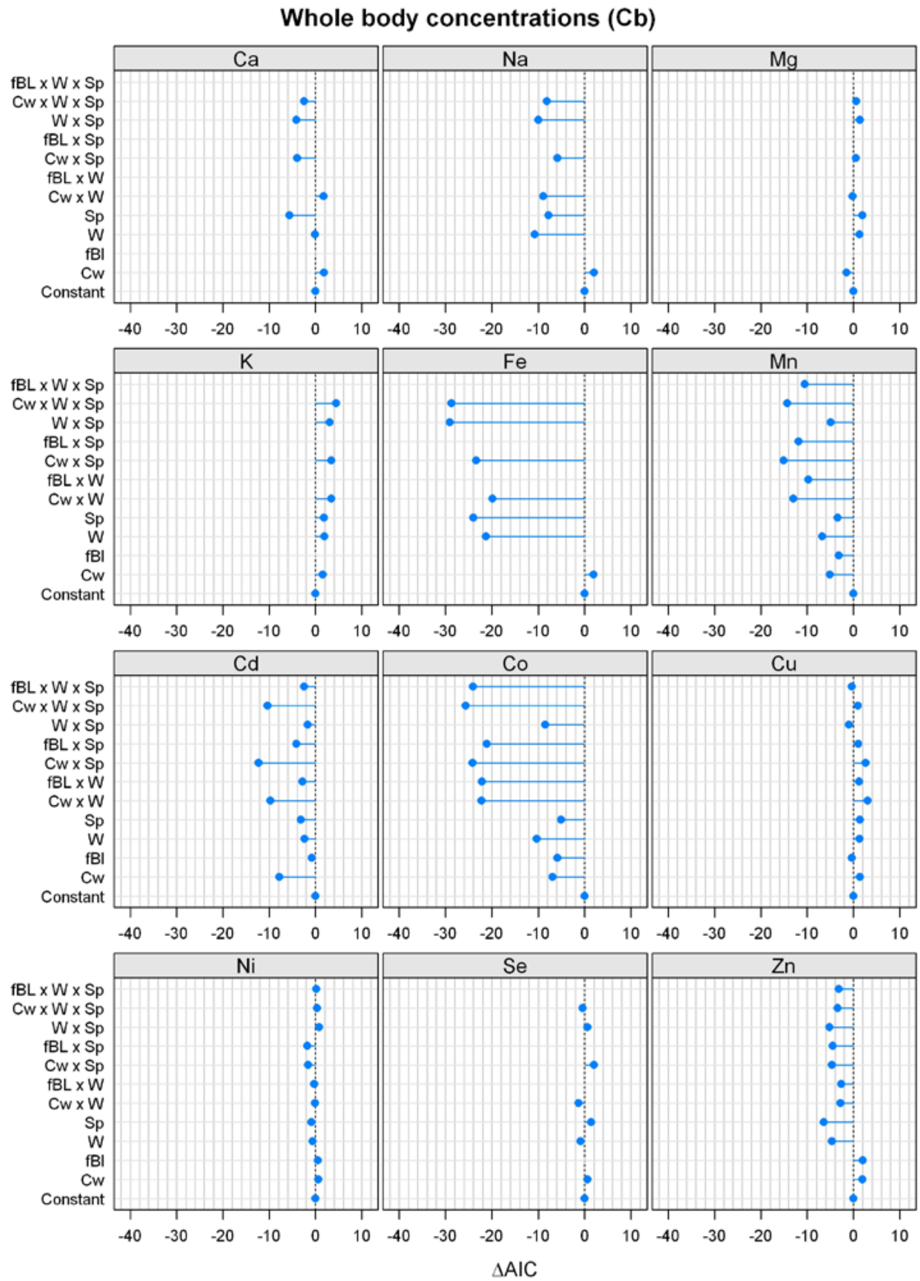


Figure 2.4 Comparison of candidate models for prediction of whole-body concentrations of metals in *D. magna* and *G. roeseli*. ΔAIC is the difference of Akaike Information Criterion between candidate models and the most simple reference model Cb~Constant. Candidate models with ΔAIC < -2 are significantly better than the reference model.

DISCUSSION

Regulation of whole-body metal concentrations. Strong regulatory systems to maintain optimal internal concentrations may partially explain the absence of significant relations between exposure concentrations and whole-body concentrations for Ca, Na, Mg, K, Fe, Cu, Se and Zn. In *G. roeseli*, Cd is the only metal with a significant relation between Cw and Cb. In *D. magna*, beside Cd, two essential elements (Mn and Co) show significant relations with exposure concentrations, suggesting that regulatory systems are not able to maintain concentrations at an optimum level. Both Mn and Co also showed significant relations with f_{BL}, and the performance of f_{BL} as model parameter was only slightly less than the performance of Cw. Ni accumulation for *D. magna* **(**Figure 2.2) also showed a trend (b= 0.59) with Cw, though non-significant due to the rather high variation in accumulated Ni-concentrations.

A certain level of bioaccumulation is essential for normal physiological functions; that is, 84 µg Cu/g dry wt and 70 µg Zn /g dry wt and 27 µg Fe /g dry wt were estimated to be the required amounts for optimal enzymatic functions in crustaceans (Rainbow et al., 1986; White et al., 1985). Above certain concentrations in surface water, also essential metals will be accumulated, which is reflected by the functions for Mn and Co. From this perspective it can be concluded that Ca, Na, Mg, K, Fe, Cu, Se and Zn concentrations at all the trial sites are within the physiological range, whereas Mn, Cd, Co and Ni concentrations exceed physiological thresholds, resulting in increasing whole-body concentrations.

Dissolved metal concentrations. Significant relations between exposure concentrations and BCFs of Cd, Cu, Ni and Zn were found in the literature for a variety of species (McGeer et al., 2003). The absence of significant relations between Cb and Cw implies that internal body concentrations are constant (b=0) and that BCF is inversely related to the exposure concentrations (b'=-1). Sometimes this distinction (either BCF is constant or Cb is constant) is not exclusive. Significant relations with exposure concentrations are found for Cb as well as the corresponding BCF for Zn, Cd, Cu and Ni (McGeer et al., 2003). This is caused by the fact that accumulation levels off at higher exposure concentrations. Lebrun (2011) found that whole-body Ni concentrations proportionally increased with exposure concentrations in water up to concentrations of 1000 µg Ni/L. At higher Ni concentrations BCF leveled off (Lebrun et al., 2011). We observed a leveling off of Mn (b=0.66) and Co (b=0.55) accumulation.

In some cases neither Cb nor BCF are related to exposure (i.e. Ni in *D. magna*), This seems contradictory because one of the options should be valid. For these cases the statistical power of our dataset is insufficient (caused by high variation coefficients of Ni-Cb) to conclude whether whole-body concentrations depend on aqueous concentration.

Absolute levels of BCF and whole-body concentrations are difficult to compare between studies because of the different expressions of concentrations in water (i.e. total concentrations (µg/L), dissolved concentrations (µg/L), standardized for suspended material (µg/kg), free metal ions (mol/L)) and in tissue (i.e. µg/kg wet weight, µg/g dry weight). Also differences in analytical methods may introduce large differences between studies. The type and mesh of the filter used for dissolved metal sample preparation (very common are 0.22 and 0.45 µm filters) affect the measured concentration of dissolved metals. Preparation of tissue samples and analysis differs in many ways: depuration time for gut clearance, washing (EDTA) to remove metals adsorbed to cuticles, and destruction methods may introduce large differences between studies. Therefore, comparison between literature data and results in the present study is focused on significance of allometric relations, and not to the exact values of whole-body concentrations and BCF.

Occupancy of the biotic ligand. Exposure to metals mixtures affects the bioaccumulation of individual metals. The relationship between exposure concentrations and whole-body concentrations might be masked by metal interactions, which are currently not very well understood. Norwood et al (2007) showed that Co, Cd and Ni bioaccumulation in *Hyelella azteca* was significantly inhibited with increasing number of metals in the mixture which included also As, Cr, Pb, Tl, Cu, Mn and Zn (Norwood et al., 2007). Bioaccumulation of Cu, Mn and Zn was not significantly affected by exposure to other metals. Synergistic and antagonistic effects were also found for simultaneous uptake of Cd, Co, Ni, Pb and Zn in *D. magna* and the zebrafish *Danio rerio*, which not only depended on the combination of metals but also on the exposure concentrations of the metals in the mixture (Komjarova et al., 2009a; b).

In the present study, we found that biotic ligand binding did not improve the significance of the regression models of Cb and BCF compared with Cw. In combination with Sp, Cw performed significantly better than f_{BL} for Cd (ΔAIC=8.10), Co (ΔAIC=3.12), and Mn (ΔAIC=3.28). Bioaccumulation of the other metals appeared to be independent of exposure concentrations (Cw or f_{BL}). The somewhat lower significance of the fractional occupancy of the biotic ligand for bioaccumulation prediction does not disqualify BLMs. Such models are suitable for effect assessment, often predicting effects within a factor 2 accuracy (De Schamphelaere et al., 2004c; Deleebeeck et al., 2008a; Heijerick et al., 2005b; Peters et al., 2011a). Bioaccumulation of metals is not always directly related to effects, because whole-body concentrations of essential metals are strongly regulated. Effects (i.e. reduced reproduction) are caused by energy costs involved in maintaining the body concentrations at a constant level. Therefore, effects rather than bioaccumulation are expected to be related to metal concentrations in the exposure medium or to the amount of metal bound to gill or gill-like organs. Moreover, whereas Cw is a measured value, f_{BL} is an estimated value, that contains uncertainties caused by the calculation of free ion activities and the use of conditional affinity constant for binding to the biotic ligand.

Biological variation. Increasing whole-body concentrations are found at lower exposure concentrations in *D. magna* than in *G. roeseli*. This might be caused by different boundaries of its regulatory system. Another explanation might be that *D. magna* feeds on naturally occurring algae, whereas G. roeseli feeds on leaves added into the cages. We assume that algal quality reflects water quality, whereas the quality of the leaves is independent of the water chemistry on the trial sites. Relations between water quality and whole-body concentrations are stronger in the filter feeder *D. magna* because whole-body concentrations are built up from waterborne as well as dietary exposure, whereas concentrations in the shredder *G. roeseli* originate from waterborne exposure only. The regression analysis shows that interspecies variation is important to take into account, and for some metals is the only significant parameter.

Since weight is an important species trait, overlap in the predictive power of weight and species as a covariable is to be expected. The coefficient for species as a covariable is a lumped parameter that contains all undefined species traits that result in different bioaccumulation. Beside size, other traits affect bioaccumulation, i.e. filtration rate, food consumption, uptake rate, elimination rate, storage capacity, detoxification. The present study shows that it is essential to account for intrinsic differences in bioaccumulation between species.

Size. Significant intraspecies relations between body weight and accumulated metals were found for Fe, Mn, Cu and Co in adult *D. magna* and for Mn in *G. roeseli*. Size variations of *D. magna* adults could be explained by accumulated concentrations of Fe, Co and Cu, whereas *G. roeseli's* size showed a significant relation with accumulated Mn. The relation between accumulated Co and weight of *D. magna* adults is not unexpected, as the surface waters in our study covers a broad concentration range from low values to values far above the environmental quality standard. The effects of Cu, Fe and Mn are more difficult to understand, because aqueous concentrations do not indicate a risk for these elements. Elevated whole-body concentrations of Cu, Fe and Mn, can be associated with the respiratory system. Copper is a constituent of haemocyanin, which is involved in oxygen binding. The level of haemocyanin in organisms is related to the oxygen tension in the environment. Elevated manganese concentrations were observed in the marine benthic crustacean *Nephrops norvegicus* proportional to duration of the hypoxic event and the exposure concentrations (Baden et al., 2003). Although in general the aeration in the streams in our study is good, temporary fluctuations or temporary hypoxia during the experiment can not be excluded.

Statistical power. The significance of effects in field tests is more difficult to prove than in laboratory tests, due to the inherently higher variability caused by variation of environmental conditions such as water temperature, aeration and availability and quality of food. Moreover, exposure concentrations are not controlled and vary in time. Variation in body weight can be caused by the effect of the exposure medium on development, growth and reproduction. Molting and release of offspring cause variation in body weight of *D. magna*. Upto 70% of Cd and Zn uptake in *D. magna* can be lost by molting, and upto 70% of the Se-uptake can be removed by release of offspring (Yu et al., 2002). In our experiment *G. roeseli* did not produce offspring. Variation in weight of *G. roeseli* can be caused by sex-differences and small age differences. At the start of the experiment, *G. roeseli* were 1-7 days old and sex differences were not visible at that time.

Less significant relations were found for *G.roeseli* than for adult *D. magna*, because data for these organisms are less abundant in our dataset. *G. roeseli* at eight out of twelve sites were obtained. The high variation and smaller number of trial sites resulted in a dataset with less statistical power for *G. roeseli*.

CONCLUSIONS

Differences in bioaccumulation between species were observed for most of the elements in this study, and often related to differences in weight. As far as effects are concerned, Co, Cu and Fe accumulation coincided with reduced growth of *D. magna* and Mn was related to reduced growth of *G. roeseli.* Smaller organisms accumulated higher amounts of metals per gram body weight compared to larger organisms for Na, Fe, Mn, Cd, Co and Zn, following an allometric model.

Field-exposed organisms accumulated increasing amounts of Mn, Cd, Co and Ni with increasing dissolved metal concentrations, but accumulation gradually stabilized at higher concentration levels. Significant relations were found between bioaccumulation of Mn and Co and both fractional occupancy of the biotic ligand and dissolved metal concentrations, where the latter showed to be more significant.

Supplementary information

Table S2.1 Calculated free ion activities on trial sites in mol/L (WHAM 7)

	H	Ca	Na	Mg	Cd	Co	Cu	CuCO3	CuOH	Mn	Ni	Zn
BEE	4.47E-08	1.15E-03	9.85E-04	1.09E-04	3.75E-10	2.06E-08	5.32E-08	3.01E-07	2.08E-08	2.30E-06	2.27E-07	7.58E-08
BEL	5.62E-08	1.48E-03	1.05E-03	1.17E-04	5.79E-10	1.13E-08	2.36E-08	1.71E-07	7.35E-09	7.02E-07	1.03E-07	1.25E-07
BOL	2.24E-08	1.20E-03	8.87E-04	1.03E-04	1.31E-09	1.75E-09	2.44E-08	1.69E-07	1.91E-08	5.68E-07	4.73E-08	4.87E-07
DOM	7.08E-08	8.39E-04	3.48E-03	8.15E-05	4.35E-09	1.26E-08	2.80E-08	1.55E-07	6.92E-09	4.67E-07	1.59E-07	7.61E-07
GEN	2.51E-07	9.39E-04	8.63E-04	1.41E-04	2.00E-09	1.67E-07	2.61E-08	1.05E-08	1.82E-09	2.94E-06	7.98E-07	3.7E-07
GRB	7.08E-08	1.21E-03	1.62E-03	1.05E-04	5.23E-10	3.86E-08	3.35E-08	1.22E-07	8.28E-09	2.21E-06	2.35E-07	1.67E-07
GRW	7.08E-09	1.66E-03	8.57E-04	1.13E-04	3.35E-10	8.24E-10	2.10E-08	1.41E-06	5.19E-08	8.93E-07	2.10E-08	4.83E-08
KSP	6.31E-08	1.13E-03	7.34E-04	1.09E-04	8.44E-10	1.36E-08	5.63E-08	1.91E-07	1.56E-08	3.59E-07	1.66E-07	2.05E-07
REU	3.16E-07	1.21E-03	8.28E-04	1.59E-04	2.72E-10	2.97E-08	4.08E-08	2.74E-08	2.26E-09	1.67E-06	2.05E-07	1.79E-07
RUN	1.78E-07	9.91E-04	6.16E-04	1.44E-04	8.27E-10	2.99E-07	4.87E-08	2.31E-08	4.79E-09	1.58E-06	1.44E-06	3.3E-07
TOR	5.62E-08	1.38E-03	1.03E-03	1.09E-04	3.57E-10	5.07E-09	2.13E-08	1.66E-07	6.63E-09	3.53E-07	7.92E-08	5.22E-08
TUB	7.99E-08	1.90E-03	8.61E-03	1.83E-04	3.43E-10	1.20E-09	2.34E-08	1.45E-07	5.13E-09	6.82E-06	4.44E-08	3.12E-07

Table S2.2 Bivariate, trivariate and best models for BCF as function of dissolved metal concentration (Cw in µg/L), species weight (W in µg/g d.w.) and other undefined species traits (Sp). Sp=0 for *D. magna*; Sp=1 for *G. roeseli*.

		AIC	*p* value	Adj. r^2
	Model: log BCF = a log BCF+ κ log W			
Ca	4.49(0.37) - 0.93(0.18) logC_w + 0.14(0.1) logW	-97.0	1.4E-04	0.53
Na	8.31(1.1) - 1.09(0.31) logC_w - 1.66(0.43) logW	-25.8	2.4E-04	0.50
Mg	3.27(0.28) - 0.65(0.19) logC_w - 0.08(0.1) logW	-96.5	0.008	0.31
K	3.99(0.22) - 0.94(0.09) logC_w - 0.04(0.09) logW	-98.5	<0.0001	0.83
Fe	7.74(0.64) - 0.9(0.14) logC_w - 1.7(0.29) logW	-45.3	<0.0001	0.77
Mn	4.95(0.62) - 0.5(0.17) logC_w - 0.91(0.28) logW	-47.0	0.002	0.40
Cd	2.34(0.68) - 0.19(0.26) logC_w - 0.58(0.3) logW	-44.0	0.163	0.08
Co	3.31(0.55) - 0.58(0.1) logC_w - 1.16(0.25) logW	-52.4	<0.0001	0.67
Cu	2.21(0.26) - 1.1(0.21) logC_w - 0.07(0.12) logW	-88.5	<0.0001	0.58
Ni	2.76(1.31) - 0.64(0.31) logC_w - 0.9(0.56) logW	-12.4	0.058	0.16
Se	1.24(0.5) - 0.88(0.09) logC_w - 0.41(0.22) logW	-56.2	<0.0001	0.83
Zn	3.28(0.49) - 0.94(0.18) logC_w - 0.51(0.2) logW	-63.0	<0.0001	0.59
	Model: log BCF = a log C_w + κ log W + λ Sp			
Ca	4.89(0.38) - 0.91(0.16) logC_w - 0.08(0.13) logW + 0.18(0.07) Sp	-101.1	<0.0001	0.62
Na	7.42(1.41) - 1.08(0.31) logC_w - 1.21(0.61) logW - 0.36(0.35) Sp	-25.0	6.3E-04	0.50
Mg	3.51(0.37) - 0.69(0.2) logC_w - 0.18(0.14) logW + 0.08(0.08) Sp	-95.7	0.016	0.31
K	4.16(0.29) - 0.93(0.09) logC_w - 0.12(0.14) logW + 0.07(0.08) Sp	-97.5	<0.0001	0.83
Fe	6.16(0.7) - 0.86(0.12) logC_w - 0.89(0.33) logW - 0.66(0.19) Sp	-54.1	<0.0001	0.85
Mn	4.02(0.8) - 0.39(0.17) logC_w - 0.4(0.4) logW - 0.4(0.23) Sp	-48.3	0.002	0.45
Cd	1.48(0.87) - 0.17(0.25) logC_w - 0.13(0.41) logW - 0.36(0.24) Sp	-44.6	0.125	0.13
Co	2.16(0.72) - 0.5(0.1) logC_w - 0.59(0.34) logW - 0.45(0.2) Sp	-55.9	<0.0001	0.72
Cu	2.65(0.34) - 1.02(0.2) logC_w - 0.31(0.17) logW + 0.18(0.09) Sp	-90.5	<0.0001	0.63
Ni	1.24(1.84) - 0.52(0.33) logC_w - 0.2(0.82) logW - 0.57(0.49) Sp	-12.0	0.073	0.18
Se	1.68(0.69) - 0.86(0.09) logC_w - 0.63(0.32) logW + 0.17(0.18) Sp	-55.2	<0.0001	0.83
Zn	2.68(0.62) - 0.93(0.17) logC_w - 0.22(0.27) logW - 0.24(0.16) Sp	-63.6	<0.0001	0.62
	Best model: log BCF =…….			
Ca	4.72 (0.27) – 0.91 (0.16) logC_w + 0.14 (0.05) Sp	-102.6	<0.0001	0.63
Na	8.31 (1.10) – 1.09 (0.31) logC_w – 0.72 (0.19) logW	-25.8	2.4E-04	0.5
Mg	3.09 (0.17) – 0.65 (0.19 logC_w	-97.8	0.002	0.32
K	3.92 (0.09) – 0.94 (0.09) logC_w	-100.4	<0.0001	0.84
Fe	6.16 (0.70) – 0.86 (0.12) logC_w – 0.89 (0.33) logW – 0.66 (0.19) Sp	-54.1	<0.0001	0.85
Mn	3.23 (0.15) – 0.34 (0.16) logC_w – 0.57 (0.15) Sp	-49.1	6.9E-04	0.45
Cd	1.39 (0.09) – 0.40 (0.16) Sp	-48.0	0.018	0.19
Co	0.90 (0.08) – 0.45 (0.10 logC_w – 0.71 (0.14) Sp	-54.4	<0.0001	0.7
Cu	2.07 (0.08) – 1.14 (0.20) logC_w	-90.1	<0.0001	0.59
Ni	0.29 (0.18) – 0.76 (0.33) Sp	-13.1	0.030	0.16
Se	0.31 (0.09) – 0.90 (0.08) logC_w	-58.0	<0.0001	0.81
Zn	2.22 (0.24) – 0.93 (0.17) log(C_w) – 0.33 (0.11) Sp	-64.9	<0.0001	0.62

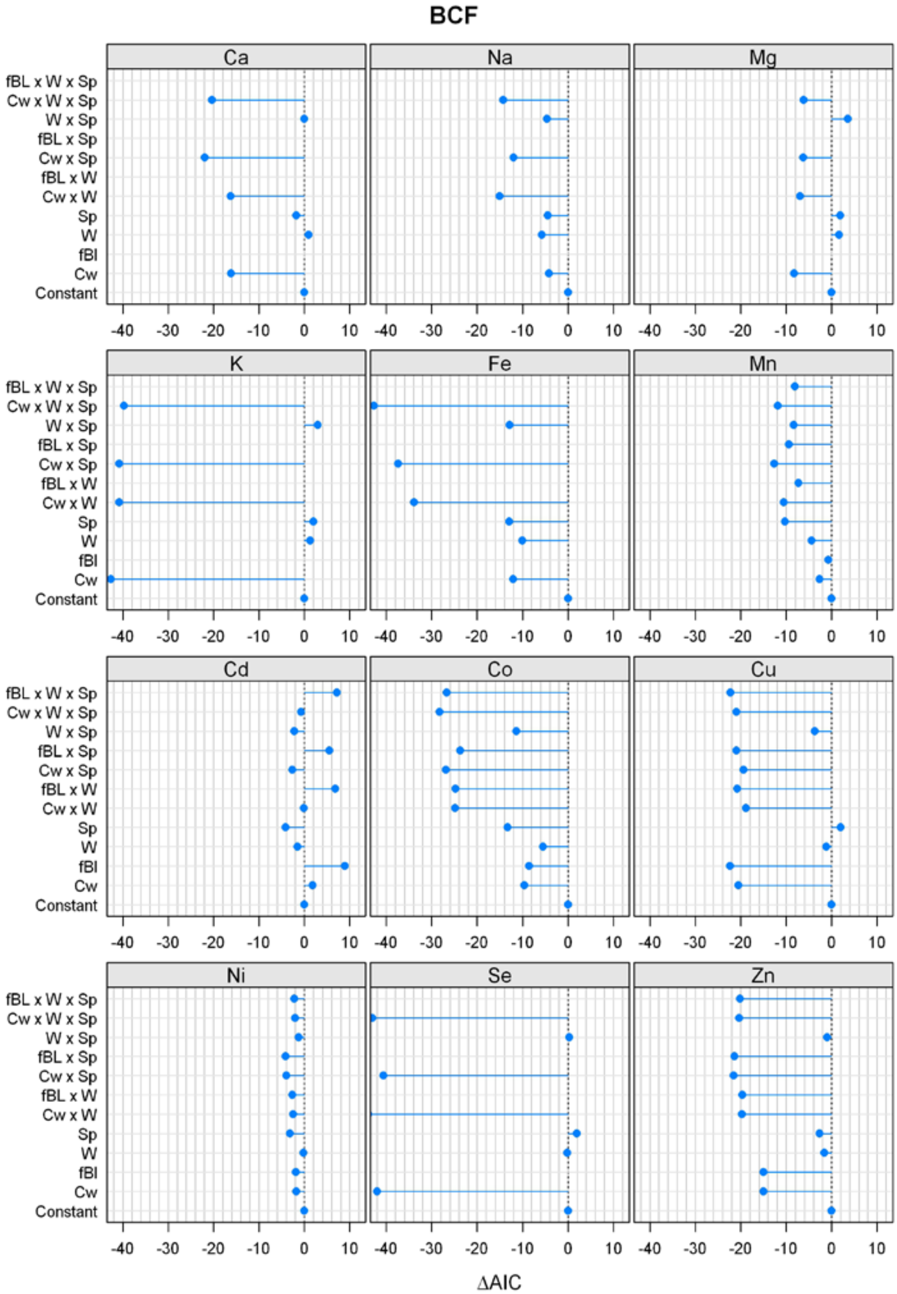
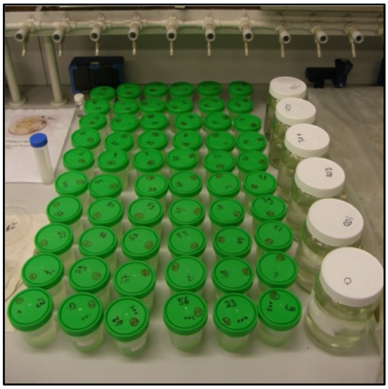


Figure S2.1 Comparison of candidate models for prediction of BCF of metals in *D. magna* and *G. roeseli*. ΔAIC is the difference of Akaike Information Criterion between candidate models and the most simple reference model C_b~Constant. Candidate models with ΔAIC <-2 are significantly better than the reference model.

1.

2.



3.

4.

1. Female *Daphnia magna* of 3 weeks old, carrying unfertilized female eggs in her brood pouch, together with a juvenile of 1-2 days old.
2. Laboratory set-up for *Daphnia* reproduction test.
3. Field set-up for *Daphnia* reproduction test.
4. Counting daphnids with Saskia.

Pictures 1-2 by the author at the Laboratory of Ecological Research
Picture 3 by Frans Lüers (Waterschap De Dommel)
Picture 4 by Frank Scheffer

1.

2.

3.

4.

1. Sampling and selection of gammarids with Sasha and Anouk
2. *Gammarus roeseli*
3. Beakers with leaves as food in the field test with *Gammarus roeseli*
4. Set-up of field test with *Gammarus roeseli*

Pictures 1, 2 and 4: by author
Picture 3 : http://en.wikipedia.org/wiki/Gammarus_roeseli