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Nickel speciation and ecotoxicity in European natural surface waters: development, refinement and validation of bioavailability models

Final Report
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THE STRUCTURE OF THIS REPORT

This research report is structured into three parts:

1) An **EXTENDED SUMMARY**, which describes in brief the most important scientific insights that are described in detail in the core of the report. References to relevant sections, tables of figures are given when appropriate. The reader can find more information on the cited topic at these locations in the core of the report
2) The **CORE REPORT**, which contains introduction, materials and methods, model development and validation, and references
3) A large number of **ANNEXES**, dedicated to ease the future use of the data and models presented in the core report. Since the majority of the Annexes are very large tables, they are only available in spreadsheet format (Microsoft Excel®). These annexes can be obtained from the authors upon request. A few annexes are, however, added in the text at the end of this report.

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EXTENDED SUMMARRY

Introduction and aims

The accurate prediction of Ni ecotoxicity in natural surface water with bioavailability models such as the biotic ligand model (BLM) depends on how well these models can predict both the speciation of Ni (i.e. Ni^{2+} concentration), the toxicity of Ni^{2+} ions to an organism, and the effects of water chemistry parameters thereupon, such as dissolved organic carbon (DOC), pH, and water hardness. In a previous study we have developed BLMs based on ecotoxicity experiments in synthetic test waters (Deleebeeck et al., 2005). These models needed to be validated for their performance in natural surface waters.

However, although the accurate prediction of nickel (Ni) speciation in natural surface water is the first essential step towards the success of aquatic Ni bioavailability models, current speciation models such as WHAM V (Tipping, 1994) and WHAM VI (1998) are not well calibrated to Ni speciation in natural surface waters.

Therefore, we have measured free ionic Ni^{2+} concentrations in six natural surface waters with a wide range of water chemistry and at Ni concentrations relevant for toxicity to the organism currently known as the most sensitive to Ni, i.e *C. dubia* (Keithly et al., 2004). To ensure a large precision of free ionic Ni measurements at low Ni concentrations, a Donnan-membrane technique was applied, coupled with radiochemical determination of ^{63}Ni.

The aim of the study was to use the speciation data for testing and/or calibrating speciation models for further use in the surface water validation of the earlier developed bioavailability models (Deleebeeck et al., 2005). Additionally, by performing toxicity tests in the same natural waters with *C. dubia*, we wanted to test if bioavailability models developed for *D. magna* could accurately predict Ni toxicity to *C. dubia* or if separate models would be needed.

Although the toxicological focus of the study was on bioavailability to invertebrates, the study ends with a chapter about the refinement and field validation of Ni bioavailability models for fish and algae. Thus, the overall aim of the study was to calibrate existing

speciation models to Ni speciation in natural surface waters and to use these data to validate and/or refine bioavailability models for aquatic organisms from three trophic levels, i.e. algae, invertebrates (daphnids), and fish. IT was anticipated that this could allow to improve the accuracy and to reduce the uncertainty of the incorporation of speciation and bioavailability concepts into risk assessment of Ni and Ni compounds in the freshwater compartment.

Surface waters investigated in the present study (for C. dubia testing)

Surface waters were sampled from Ankeveen (NL), Bihain (B), Brisy (B), Eppe (F), Markermeer (NL) and Regge (NL) with a wide range of chemistries, such as pH 6.2 to 8.3, water hardness of 15 to 218 mg $CaCO_3$/L and a DOC content of 3.1 to 23.6 mg/L. A more detailed description and composition of these surface waters is given in Annex 1. Those surface waters were used throughout for speciation and toxicity testing.

Ni speciation – method

Ni speciation in spiked surface waters was determined by a highly sensitive Donnan-membrane technique to separate free Ni^{2+} ions from other dissolved Ni species, coupled to a radiochemical determination of ^{63}Ni. As such, Ni^{2+} concentrations as low as 0.1 µg/L could be detected. This method was used for all speciation measurements. A detailed description of the method is given in section 2.2.

Ni speciation – complexation kinetics

Measurements of Ni speciation were performed at 10 µg Ni/L in Ankeveen and Markermeer waters after 2 hours, 2 days and 7 days after spiking. No differences in free ionic Ni^{2+} concentrations were observed between the three equilibration times, suggesting that Ni speciation is at equilibrium after as little as 2 hours of reaction time (see section 3.1.1, Table 3.1). This was taken into account during the rest of the experiments to ensure equilibrium speciation in speciation and ecotoxicity experiments.

> *Significance for EU risk assessment. Since reaction times of Ni with ecotoxicity test solutions are usually not longer than 2 hours, it is very likely that for most literature ecotoxicity data Ni equilibrium conditions apply. It*

means that equilibrium speciation models can be used as a basis for an accurate normalization of toxicity data to other water chemistries. Applying equilibrium models to toxicity data with a shorter equilibration time is a worst case approach.

Ni speciation – equilibrium speciation in six surface waters at different Ni

Measurements of Ni speciation were performed in the six surface waters at dissolved natural background Ni (2.6 to 4.4 µg Ni/L) and at spiked Ni concentration representing the range of *C. dubia* chronic 10d-EC10 and 48h-EC50 levels in these waters (3.3 to 148 µg Ni/L). At background Ni, between 4% and 45% of the Ni was present as Ni^{2+}, depending on the water chemistry. In each individual water sample, increased dissolved Ni spikes yielded lower fractions of Ni^{2+}. Those varied between 14% and 60%. This increase is largely due to increased binding of Ni to DOC. A more detailed overview is available in section 3.1.2, Table 3.2)

Significance for EU risk assessment: The binding of Ni to DOC becomes increasingly important at lower Ni concentrations. Hence, DOC will be a very crucial factor for normalizing toxicity data, especially for sensitive organisms.

Ni speciation – effects of pH and hardness

A decreased pH (increased H^+) and increased water hardness resulted in an increase of the fraction of Ni^{2+} (see section 3.1.2., Table 3.3). This is mainly due to competition between Ni^{2+}, Ca^{2+}, Mg^{2+}, and H^+ ions for binding sites on the DOC.

Significance for risk assessment: These effects can be accounted for when a calibrated speciation model is used as the basis for all bioavailability models.

Ni speciation – Calibration of WHAM VI

All individual free Ni^{2+} measurements in the six waters at different dissolved Ni and in two waters with amended pH or hardness (n=33) were used to calibrate the WHAM VI speciation model. As in other studies (Bryan et al., 2002; Cheng et al., 2005) we assumed the natural

DOM to consist of a fraction of active fulvic acid (%AFA) and an inert fraction for ion binding. We found that both the binding strength of Ni to fulvic acid, i.e. log K_{MA}(Ni), and the %AFA needed to be calibrated, to achieve accurate speciation predictions over the whole range of investigated Ni concentrations. The best fit was obtained with log K_{MA}(Ni)=1.75 and %AFA = 40%. More details about WHAM VI and the fitting procedure can be found in section 3.1.3.). These best-fit assumptions were then used throughout the study whenever speciation calculations needed to be performed.

> *Significance for risk assessment: Speciation calculations in natural waters, needed for bioavailability normalizations should use these "best-fit" assumptions with regard to natural DOC.*

Ni speciation – Importance of other competing ions (trace metals)

Next to Ca^{2+} and Mg^{2+}, other cations also compete with Ni for binding sites on the DOC. The most important ones, based on both binding strength and concentrations in the environment are Fe^{3+}, Al^{3+}, Cu^{2+} and Zn^{2+}. An increase of these concentrations results in less Ni binding to DOC, more Ni^{2+} in solution and hence a higher bioavailability. Fe^{3+} activity is predicted by assuming equilibrium with colloidal $Fe(OH)_3$ and is only a function of pH; Al^{3+}, which presents a more complex situation, can be taken into account as described in section 3.1.3.1. Not taking into account the presence of Al^{3+} can result in an underestimation of 34% of Ni^{2+} at chronic EC10 levels of *C. dubia* (see section 3.2.2.). Cu and Zn can be taken into account by adding dissolved Cu or Zn to the input for the speciation calculations. Not accounting for Cu and Zn resulted in about 20% underestimation of Ni^{2+} at chronic EC50 levels of *C. dubia* (see section 4.3.5.3)

> *Significance for risk assessment: These competitive interactions should preferably be taken into account when carrying out bioavailability normalizations to regional or local water chemistry. They will result in lower normalized Ni NOECs, since less Ni will be predicted to bind to DOC when these ions are taken into account.*

Ni speciation – effects of background DOC and food additions in ecotoxicity tests

We found that 'background DOC' in deionized water does not contribute significantly to Ni binding (section 4.3.2.1). The influence of food additions, which potentially results in addition of dissolved ligands able to bind Ni, was investigated. Both algal food and the often-used YTC slurry (yeast-trout chow-cerophyl) were considered. We did not find any significant Ni-binding to ligands originating from algal food additions (section 4.3.2.1), but did find a significant contribution of YTC-ligands to Ni binding (section 4.3.2.2). Hence, the addition of YTC food introduces a greater deal of uncertainty to speciation calculations of Ni than addition of algal food.

> *Significance for risk assessment: All this knowledge is very important with regard to assumptions that often need to be made with regard to the DOC content of test solutions used in ecotoxicity tests, of which the resulting NOEC data are eventually to be normalized. How these results need to be translated into the most suitable assumptions is summarized in* section 4.3.2.3 *and also in Annex 16.*

Ni toxicity to C. dubia – as dissolved Ni

Ni toxicity to *C. dubia* varied substantially among the six water samples investigated, with 48h-LC50s between 34.6 and 183 µg/L (5-fold), 10d-EC50s between 4.9 and 68.4 µg/L (14-fold) , 10d-EC10s between 1.3 and 44.2 µg/L (34-fold) (see section 3.2.1.). The difference between toxicity values is larger for more sensitive endpoints (lower Ni-concentrations), highlighting the importance of carrying out bioavailability normalizations in regulatory exercises. Significant linear relations between acute and chronic toxicity and DOC were observed, stressing the importance of DOC as a modifier of Ni bioavailability (see Figure 3.2).

Ni toxicity to C. dubia – as Ni^{2+} and effects of water chemistry thereupon

In order to further explain differences among waters a second step was to calculate Ni-speciation and then to determine how the toxicity of the Ni^{2+} ion to *C. dubia* varies with modifying factors such as pH, Ca and Mg. We found that acute toxicity, expressed as Ni^{2+},

was not very much dependent on pH, Ca, or Mg; and that chronic toxicity of Ni^{2+} increased with increasing pH, Ca, and Mg. (See section 3.2.3, Figure 3.3). However, the interpretation of the importance of these data was difficult because of the significant correlation between pH, Ca, and Mg in the natural waters tested. The effects of pH, Ca, and Mg needed to be separated from one another, and this was done in the modelling section of the study, where it was aimed to develop, refine and validate acute and chronic Ni toxicity models to *C. dubia* and *D. magna* (see section 4).

Development, refinement and validation of Ni bioavailability models for daphnids

Data generated in a previous study (Deleebeeck et al., 2005) and in the current study were used to develop and refine toxicity bioavailability models. First, acute and chronic *D. magna* models described in Deleebeeck et al. (2005) were re-evaluated with WHAM VI, because WHAM VI yields slightly different speciation calculations than the previously used WHAM V (see section 4.1). Then, these models were validated against the *C. dubia* data obtained in the present study and also to a number of other existing Ni toxicity studies on the effect of hardness on acute and chronic Ni toxicity to *C. dubia* (Keithly et al., 2004) and *D. magna* (Chapman et al., 1980), on the effect of pH on acute Ni toxicity to *C. dubia* (Parametrix, 2004; Shubauer-Berigan et al., 1993), and on the combined effects of pH, hardness, alkalinity and natural DOM on chronic Ni toxicity to *C. dubia* (Wirtz et al., 2004). Similarities and differences between species and between different datasets were accounted for in the model development.

The following conclusions are the result from all these modelling analyses, taking into account our own studies and the above-mentioned studies published elsewhere. The conclusions start with some general observations made, i.e. (i) to (iv), continues with a description of how the final bioavailability models are constructed from those observations for acute and chronic Ni toxicity to *C. dubia* and *D. magna*, i.e. (v) to (ix), and ends with a recommendation on implementing this knowledge into risk assessment (x). Each conclusion is followed by a reference to a relevant section, Figure or Table that illustrates this conclusion.

(i) The pH effect on Ni^{2+} ion toxicity is more important in chronic than in acute exposures; toxicity of the free Ni^{2+} ion is generally increased at higher pH

(See Figure 3.3 for *C. dubia*, compare Figure 4.1 with Figure 4.9 for *D. magna*)

(ii) The pH effect on Ni^{2+} ion toxicity becomes increasingly important at pH levels > 8.0-8.2

(See Figure 4.1 for acute *D. magna*, Figure 4.4 for acute *C. dubia*, Figure 4.14 for chronic *C. dubia*)

(iii) The pH-effect on both acute and chronic Ni^{2+} ion toxicity cannot be modelled with a traditional single-site H^+ competition effect. Nevertheless, up to a pH of 8.2, an acute BLM-type model, which does not account for pH effects at all, is able to yield accurate acute toxicity predictions.

(See section 4.2.1 for acute *D. magna*, section 4.2.3.2 and 4.2.3.3. for acute *C. dubia*, 4.3.3.1 for chronic *D. magna*, and 4.3.4 and 4.3.5.3 for chronic *C. dubia*)

(iv) The protective effect of water hardness (Ca and Mg) can be modelled with traditional BLM-competition, because linear competitive effects are observed. The effects of Ca and Mg may be similar for both species.

(See Figure 4.1 for acute *D. magna*, Figure 4.3 for acute *C. dubia*, Figure 4.8 for chronic *D. magna*, Table 4.17 for chronic *C. dubia*)

(v) Alternative bioavailability models were developed, consisting of a traditional Ca, Mg competition effect, superimposed to a log-linear pH relation in the case of chronic Ni toxicity, characterized by a slope parameter, S_{pH}

(See equations 4.1 to 4.5 for acute Ni toxicity, equations 4.6 to 4.12 for chronic Ni toxicity)

(vi) The slope parameter varied considerably and significantly among species (*C. dubia* vs. *D. magna*), exposure times (acute vs. chronic, i.e. only pH effect considered for chronic), type of water (artificial vs. natural) and the pH range considered (<8.0-8.2 vs. > 8.0-8.2, see also conclusion ii).

(See section 4.3.6 for comparison *C. dubia* and *D. magna* and comparison of synthetic and natural waters for *D. magna*, see Figure 4.14 for comparison between different pH ranges for *C. dubia*)

(vii) Due to the latter, chronic toxicity data with *C. dubia* obtained at pH > 8.2 should only be used with great care when a Ni effects assessment needs to be conducted for waters with pH < 8.2. One possibility we recommend is a two-step normalization procedure, with the first step being a normalization to pH 8.2 with a model specifically developed for waters with pH > 8.2 (high pH-slope model) and the second step a further normalization to lower pH with the model developed for pH < 8.2 (low slope model). A similar approach could be followed when normalizations need to be carried out from pH < 8.2 to pH > 8.2.

(See Figure 4.14 for the different pH slopes in different pH ranges for *C. dubia*, i.e. a high slope at pH >8.2 and a lower slope at pH < 8.2)

(viii) When, below pH 8.2, species-specific pH-slopes based on natural waters data are used, very accurate predictions of chronic toxicity are obtained, typically resulting in a prediction error of less than factor 2.

(See Figure 4.13 for *C. dubia* and Figure 4.22 for *D. magna*)

(ix) Also, when a 'merged' average slope is used, based on natural waters test data only, very good predictive capacity of the models is observed for both *D. magna* and *C. dubia*.

(See section 4.3.6 and Figures 4.21 and 4.22)

(x) Significance for risk assessment: *We recommend that, for carrying out normalizations for risk assessment purposes, species-specific invertebrate models should be used whenever they are available for a given invertebrate species. For other invertebrate species, we recommend that the normalizations are carried out with both the C. dubia and the D. magna pH slopes. The knowledge of the overall impact of using different slopes on the final PNEC will allow taking into account uncertainty due to interspecies differences of bioavailability models. If using different slopes does not result in major differences in the final PNEC estimate, it may be more practical to only use the 'average' slope.*

Development, refinement and validation of Ni bioavailability models for fish

Based on the data reported in Deleebeeck et al. (2005), we developed a refined bioavailability model to predict chronic Ni toxicity to juvenile rainbow trout, *O. mykiss*. The model developed was identical in structure to the chronic models developed for *D. magna* and *C. dubia*. It also consists of a log-linear pH effects combined with linear protective effects of Ca and Mg. Interestingly the protective effects of Ca and Mg could be described by very similar values of the constants that describe competition between Ni and these ions and the pH slope was in the same range as the pH slopes the same constants pH slope (compare Table 5.2 with Table 4.14). The model was able to accurately predict toxicity in four out of five natural waters; 17 and 21d-LCx values were generally predicted by a less than 2-fold error (Figure 5.3). Toxicity was underestimated by about 2.9-fold in a soft acidic surface water (pH 5.6, hardness of 14 mg $CaCO_3$/L) (Figure 5.3), which suggest that the model should be used carefully under such conditions.

Development, refinement and validation of Ni bioavailability models for algae

Based on the data reported in Deleebeeck et al. (2005), we developed a refined bioavailability model to predict chronic Ni toxicity to growth rate of the green micro algae *P. subcapitata*. We found that above pH 6.4, a biotic ligand model could describe the protective effects of Ca^{2+}, Mg^{2+} and H^{+} ions (see Section 5.3.1. and Figure 5.4). Interestingly, the protective effect of Mg could be described with a very similar value of the 'Mg-competition' constant used for fish and daphnids (compare Table 5.4 with Table 5.2 and Table 4.14), while the protective effect of Ca on Ni toxicity to this alga was much less important (See Figure 5.4).

Significance for risk assessment: These observations with algae clearly illustrate the important role that Mg plays in modifying Ni toxicity to organisms for three trophic levels. The fact that the importance of this Mg 'competition' is quantitatively similar for daphnids, fish, and algae, may reflect the chemical similarity of ionic Mg^{2+} and Ni^{2+} (e.g., similar ionic radii) which may result in binding to similar sites on the organism surface as well as shared uptake pathways into an organism (e.g., Mg-transport channels). The large difference between the effect of Ca and Mg on Ni toxicity to algae also highlights that these ions should be considered separately in normalization of toxicity data to local or regional water chemistry, instead of being merged into water hardness.

Although there were some uncertainties related to differences in 'inherent' sensitivity of *P. subcapitata* across different test series (see section 5.3.3 and Table 5.6), the developed bioavailability model can reasonably accurately predict chronic effect concentrations (EC50's and EC10's) of Ni in natural waters when these inherent sensitivity differences are taken into account (generally by an error of less than a factor of 2, see Figure 5.7). In some cases, the model exhibited some tendency to underestimate toxicity at pH levels below 6.4, although the largest underestimations were only observed below pH 6.0.

Overall conclusion and significance for the risk assessment

The developed chronic Ni toxicity models for daphnids, fish and algae exhibit sufficiently high predictive capacities to yield a marked reduction of uncertainty associated with differences in chronic Ni bioavailability among different test waters. This is due to the fact that they can predict both Ni^{2+} concentrations as a function of dissolved Ni and water chemistry (mainly

DOC, pH, Ca, Mg), as well as the toxicity of the Ni^{2+} ion as a function of water chemistry (mainly pH, Ca, Mg). The use of the models presented in the present study for normalizing Ni toxicity data will therefore decrease the overall uncertainty of the risk assessment, provided that the variability of bioavailability modifying parameters across different EU regions and water bodies is acknowledged.

1. Introduction and aims of the study

The accurate prediction of Ni ecotoxicity in natural surface water with bioavailability models such as the biotic ligand model (BLM) depends on how well these models can predict both the speciation of Ni (i.e. Ni^{2+} concentration), the toxicity of Ni^{2+} ions to an organism, and the effects of water chemistry parameters thereupon, such as dissolved organic carbon (DOC), pH, and water hardness. In a previous study we have developed BLMs based on ecotoxicity experiments in synthetic test waters (Deleebeeck et al., 2005). These models needed to be validated for their performance in natural surface waters.

However, although the accurate prediction of nickel (Ni) speciation in natural surface water is the first essential step towards the success of aquatic Ni bioavailability models, there are little useful Ni speciation data available with natural waters. As a result of this, speciation models such as WHAM V (Tipping, 1994) and WHAM VI (Tipping, 1998) are not very well calibrated to taking into account the effect of dissolved organic matter (DOM) on Ni speciation in natural surface waters.

The description of Ni complexation to organic matter (i.e. humic substances) in WHAM V is only based on two data points for a soil fulvic acid and two for a soil humic acid (Tipping, 1993; Tipping and Hurley, 1992). Moreover, nickel concentrations applied were in the order of 10 µM (~600 µg Ni/L). Hence, the Ni binding-parameters in WHAM V may not be relevant or accurate for aquatic natural organic matter and/or for lower Ni concentrations, which are of larger relevance for the EU risk assessment. In other words, WHAM V has not been tested for its ability to accurately predict Ni speciation in natural surface waters. For the development of WHAM VI, one additional Ni speciation study was taken into account, i.e. a study of Ni complexation to groundwater humic substances (Higgo et al., 1993), which may also not be relevant for natural surface waters. Recent studies with Cu and Zn have demonstrated that both WHAM V and WHAM VI have to be calibrated to measured speciation data in spiked natural surface waters in order to obtain accurate speciation predictions (Bryan et al., 2002; Cheng et al., 2005). Hence, it was expected that this might also be the case for Ni.

Since the development of WHAM VI in 1998, additional Ni speciation studies have become available. As far as we are aware, however, only two studies can potentially be used for

calibration of speciation models in freshwater samples for Ni. Mandal et al. (2002) and Sekaly et al. (2003) measured Ni speciation in a number of Ni contaminated freshwaters from the Sudbury area using the Competitive Ligand Exchange Method (CLEM). Unfortunately, this method measures 'labile' Ni rather than truly free ionic Ni. Other Ni speciation studies are not considered directly applicable for use in the risk assessment. This is because they report Ni speciation in the presence of soil-derived humic (Guthrie et al., 2003) or fulvic acid (Mandal et al., 2000; Celo et al., 2001) or even citrate (Celo et al., 2001), which are unlikely to resemble true aquatic DOM. Malcolm and MacCarthy (1986) for example have clearly demonstrated that soil derived humic acid may bear little structural resemblance to true aquatic humic substances.

Therefore, it was deemed necessary to generate a new dataset of Ni speciation in natural surface waters with a wide range of water chemistry and at Ni concentrations relevant for toxicity to the organism currently known as the most sensitive to Ni, i.e *C. dubia* (Keithly et al., 2004). The aim of the study was to use the speciation data for testing and/or calibrating speciation models for further use in the surface water validation of the earlier developed bioavailability models (Deleebeeck et al., 2005). To ensure a large precision of free ionic Ni measurements at low Ni concentrations, a Donnan-membrane technique was applied, coupled with radiochemical determination of ^{63}Ni.

Additionally, by performing toxicity tests in the same natural waters with *C. dubia*, we wanted to test if bioavailability models developed for *D. magna* could accurately predict Ni toxicity to *C. dubia* or if separate models would be needed. The idea was that this assessment could be performed to a large degree of precision, as it would be based on measured and not on modeled speciation.

Thus, the overall aim of this study was to calibrate existing speciation models to Ni speciation in natural surface waters and to use these data to validate and/or refine bioavailability models for aquatic organisms. This would allow to improve the accuracy and to reduce the uncertainty of the incorporation of speciation and bioavailability concepts in risk assessment.

2. Materials and methods

2.1 Sampling and characterization of natural surface waters

Natural water samples were collected at 6 different sites, covering the range of water chemistry relevant for the EU. Table 2.1 provides some more detailed info about the locations of the sampling sites. A more complete description of the sampling sites is provided in Annex 1. This Annex 1 also includes the water chemistry that was measured immediately upon arrival in the laboratory.

Table 2.1 Sampling site information

Site ID	Name	Category	Village	Country
Ankeveen	Ankeveensche plassen	ditch [a]	Nederhorst den Berg	NL
Bihain	Ruisseau de St. Martin	stream	Bihain	B
Brisy	Ourthe Orientale	river	Brisy	B
Eppe	L'eau d'Eppe	stream	Eppe Sauvage	F
Markermeer	Markermeer	lake	Marken	NL
Regge	Beneden Regge	river	Ommen	NL

[a] Connected to large lake system in a natural reserve

Thirty liter of all surface waters was membrane filtered in the field (0.45 μm) and collected in metal-free poly-ethylene containers. The samples were immediately transported to the laboratory where they were stored at 4°C in the dark until analysis. pH, dissolved organic carbon (DOC) and inorganic carbon (IC) were measured upon arrival at UGENT on a TOC-analyzer (TOC-5000, Shimadzu, Duisburg, Germany). Sub-samples of five liters were then transported to KUL where they were initially (before all experimentation) characterized towards the following parameters: Ca, Mg, Na, K, Fe, Al, Mn, Ni, Cu, Zn, Pb, and Cd (ICP-OES, Perkin Elmer 3300 DV), Cl, NO_3, and SO_4 (Ion Chromatography, Dionex QIC analyzer, IONPAC AS4A).

It is noted that pH and IC were also measured during the speciation and ecotoxicity measurements. It is also noted that major cations and trace elements were also measured in the donor solutions during the speciation measurements, simultaneously with the

measurement of radio-active Ni in donor and acceptor solution (see section 2.2 for more detail). These *in-experiment* measurements were used instead of the initial measurements as inputs for all model calibrations, developments, and validations.

2.2 Measurement of Ni speciation

2.2.1. Kinetics of Ni complexation

Two surface waters (Ankeveen and Markermeer) were spiked with stable Ni to 10 µg Ni/L and the radioactive ^{63}Ni isotope. After 2 hours, 2 days and 7 days of spiking, speciation of Ni was determined with Donnan dialysis. The equilibration time of the Donnan dialysis system was also determined. A full description of the Donnan dialysis method is given below (see section 2.2.3).

2.2.2. Determination of Ni speciation in six surface waters and in two waters with adjusted pH and hardness

The free Ni^{2+} fraction was measured in all water samples at 3 Ni concentrations: the background concentration and concentrations in the range of 10d-EC_{10} and 48h-LC_{50} values of *C. dubia*, which were determined prior to these measurements according to the methods described in section 2.3, and which are different for each test water (see further, Table 3.7) Solutions were spiked with ^{63}Ni only for the speciation at background and were equilibrated for 16 hours prior to Donnan dialysis. An aliquot of Ni stock solution was subsequently added to the same donor solution to increase the concentration of stable Ni to about the 10d-EC10 level for *C. dubia* and the solution was equilibrated for 10 minutes prior to Donnan analysis. Finally, Ni was again added to the same donor solution up to about the 48h-LC50 for *C. dubia* and the speciation determined by dialysis. In order to determine the effect of pH and hardness, Ni speciation was also measured at four adjusted pH (6.2 to 8.1) and hardness (16 to 396 mg $CaCO_3$/L) levels for 2 surface waters (Ankeveen and Bihain). For Ankeveen, the pH level was decreased by adding HCl (from a 0.5 M stock solution) until the desired value was reached. The pH of Bihain was increased by adding NaOH (from a 1M stock solution). Increasing water hardness was realized by adding an appropriate aliquot of a Ca/Mg stock solution, i.e. 0.4M $Ca(NO_3)_2$ + 0.2M $MgCl_2$.

2.2.3. Donnan dialysis

The principle of Donnan Dialysis as speciation technique is the selective dialysis of very small quantities of the free metal ion from a large volume of donor solution to a small volume of acceptor solution. Free metal ions freely diffuse through the microporous membrane that carries a negative charge while anions and metal complexes with larger molecular weight are excluded based on charge or size. A full description of the method and its verification with model solutions is given elsewhere (Salam and Helmke, 1998; Helmke *et al.*, 1993; Helmke *et al.*, 1999).

Donnan equilibrations were carried out on 100 ml-samples of the surface waters, using a custom-machined exchange cell made from Teflon and Kel-f plastics (Figure 2.1). The cell holds a strong-acid cation-exchange membrane (Nafion-117, E.I. Dupont de Nemours) which separates the sample solution (donor) from an initially pure solution of $Sr(NO_3)_2$ (acceptor). Nafion-117 is a copolymer of tetrafluoroethylene and sulfonyl fluoride vinyl ethers. During equilibration, the sample solution was continuously circulated past the bottom of the membrane by a Teflon pump at a rate of 200 mL/minute. The acceptor solution (200 µL) rests on the top surface of the membrane and is hold by an annular ring in the top part of the cell. We used 2 Donnan cells that were placed in parallel to the pump. The data reported below refer to the average of the 2 values obtained from each of the acceptor solutions.

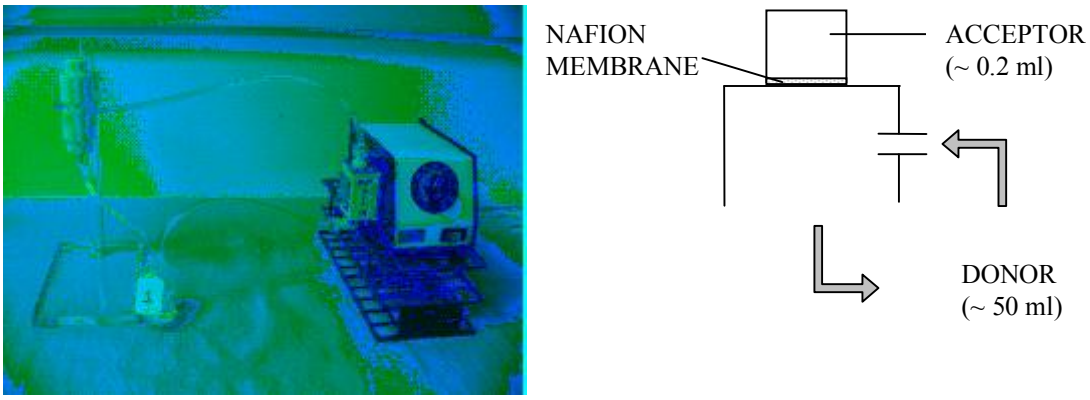


Figure 2.1 Scheme of the Donnan dialysis system (see text for detailed explanation)

In preparation for Donnan equilibration, the membranes were stored in a mixture of 10% methanol, 10% HNO_3 and 80% Milli-Q water to replace adsorbed cations with H^+. Prior to the membranes being used, they are rinsed and soaked in Milli-Q water, in $Ca(NO_3)_2/MgCl_2$ solutions (± 2.5 mM) and finally in a sub-sample of the spiked donor solution. The membranes were preloaded with Ca/Mg to avoid concentration decreases during the equilibration of the system, because of the low ionic strength of the samples.

Initially, the pH of the freshwaters was often found to increase by >0.5 pH units during Donnan dialysis due to the CO_2 degassing of the solutions. This shift is unwanted since metal speciation is largely pH dependent. Therefore solutions were pH buffered in all final experiments. The buffer MOPS (3N-Morpholinopropanesulfonic acid, pK_a 7.2) was added (3.6 mM) to the surface water samples (100 mL). The pH of the buffer was adjusted to the same pH as the water sample with NaOH (from a 1 M stock solution) prior to adding it to the solution. An experiment was set-up using water Markermeer (spiked with stable Ni to 10 µg/L) to assess the effect of adding buffer. The fraction of free Ni^{2+} in the original, non-buffered solution was 25.5% in which the pH changed from pH=8.15 initially to only pH=8.30 finally. The fraction free Ni^{2+} in the MOPS buffered solution was 24.2% while pH was maintained at pH=8.15. This experiment confirmed that MOPS buffer does not complex Ni.

The donor solution was spiked with a small volume of radio-isotope ^{63}Ni to determine the free fraction of Ni^{2+}. The acceptor solution (200 µL) is a $Sr(NO_3)_2$ solution whose ionic strength is identical to that of the sample solution as estimated by a complete analysis of the sample solution by ICP-OES and calculation.

Due to the low ionic strength of the surface waters, an equilibration time of 4 hours was necessary to attain Donnan equilibrium with the acceptor solution. Hereafter aliquots (150 µl) of donor and acceptor solution were taken and mixed with 850 µL water and 4 mL scintillation cocktail and sample radioactivity (counts per minute, cpm) was determined by a beta-counter (Parckard 1600CA). The fraction free Ni^{2+} in the sample was calculated with the following equation:

$$fraction \quad Ni^{2+} = \frac{cpm(acceptor)}{cpm(donor)} \qquad \text{(Eq. 2.1)}$$

From the donor solution, sub-samples of 5 mL were taken before and after the Donnan dialysis and analyzed by ICP-OES. The full composition of the solution after dialysis was used as input for WHAM and is reported in Annex 2.

2.2.3. Speciation modeling

The Ni speciation measurements were used to calibrate WHAM VI for taking accurately into account the effect of Ni-DOC complexation on the free ionic Ni^{2+} concentration. High molecular weight humic substances and are known to comprise a significant proportion (50 - 90%) of natural DOM (Thurman, 1985), with the remaining proportion being smaller organic molecules. Of these humic substances fulvic acids (FA) typically account for the majority of the DOC (~80%) with humic acids (HA) accounting for the remaining 20% (Thurman, 1985). The stability constant of Ni binding to fulvic acid (log $K_{MA(Ni)}$) and the percentage of 'active fulvic acid' (%AFA) were optimized to get the best fit with the experimental results. Details of the optimization method are given below.

2.3 Toxicity testing of Ni in natural waters

2.3.1. General

Acute (48h) and chronic (10d) toxicity experiments were carried out with *Ceriodaphnia dubia* (Crustacea:Cladocera). The test protocol was designed to comply as much as possible with US EPA (2002) and OECD (1984, 1998) guidelines. The most important deviation from the original US EPA test method 1002.0 was that no YTC slurry was provided as food source and that instead only a green algal mix was given in chronic toxicity experiments. This was to avoid the addition of potentially strong Ni-binding ligands to the test solutions, along with the YTC and thus to avoid complications in data analysis. Binding of Ni to exudates of the algal food and the daphnids was also determined using the Donnan-membrane technique.

2.3.2. Spiking of solutions

The test concentrations (nominal nickel concentrations) were selected based on the results of preliminary static-acute range-finding tests and information obtained from the literature. Test concentrations were spaced by a factor of 1.8 (nominal). For each acute and each chronic test, 5 or 6 nickel concentrations and a non-nickel spiked water control were tested. Test solutions were equilibrated for 24 hours before test initiation. Virtually no change in Ni speciation was observed between 2 hours and 7 days of equilibration (see 3.1.1), indicating that 24 hours is a suitable equilibration period. Test solutions for renewal of chronic tests were stored at 4°C in the dark during the whole testing period and were left to stand at 25°C one day prior to the renewal.

2.3.3. Test organisms

Ceriodaphnia dubia was obtained from a monoclonal in-house culture, which is routinely maintained on carbon-filtered city tap water (Gent, Belgium), conditioned by continuous passage over a biological filter. Six weeks (> three generations) before all experiments were started, organisms were acclimated to moderately hard reconstituted water (US EPA, 1993) with added Se and Vitamin B12 (Clesceri et al., 1998) and Zn (Muyssen and Janssen, 2002) to optimize culture health (Table 2.2). Dissolved Ni was below the detection limit of the GF-AAS, i.e. < 2 µg/L). Cultures were fed *ad libitum* with an algal mix of *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* in a 3:1 ratio on cell basis and were maintained on a light cycle of 12 hours light and 12 hours dark. Weekly, juveniles (150-350 µm) were sieved from the culture and were used to start up a new culture aquarium. No males were present in the culture for the whole period of conducting toxicity experiments.

Table 2.2 Composition[a] of moderately hard water used for culturing *Ceriodaphnia dubia*.

Major ions	Conc. (mg/L)	Supplements	Conc. (µg/L)
$NaHCO_3$	96	$NaSeO_4$ [b]	3
$CaSO_4 \cdot 2H_2O$	60	Vitamin B12 [b]	3
$MgSO_4$	60	Zn (as $ZnCl_2$) [c]	1
KCl	4		

[a] Based on US EPA's (1993) moderately hard water; this medium has a pH between 7.4 to 7.8, dissolved Ni background is <2.0 µg/L (detection limit)
[b] Recommended by APHA (1998)
[c] Recommended by Muyssen and Janssen (2002) to avoid Zn deficiency

All toxicity experiments were terminated within 14 weeks from the start of the acclimation to this culture water. Toxicity experiments were conducted simultaneously with all six waters, to ensure that all variability observed is due to differences in the test solutions.

2.3.4. Acute toxicity tests

Acute toxicity tests were initiated with 2 to 8 hour old juveniles. Tests were conducted in a temperature-controlled room at 25º±1ºC under a light cycle of 16 hours light and 8 hours dark. No food was provided to the organisms. Test containers were 30 mL polyethylene cups containing 20 mL of test solution. Ten *Ceriodaphnia dubia* individuals were impartially assigned to each test vessel and three replicates were tested per treatment. Mortality was determined after 24 and 48 hours. At test initiation and test termination pH and IC were measured in each test and samples were taken for total (only at test initiation) and dissolved Ni concentration (0.45 µm filtered). Ni concentrations were determined using GF-AAS (see 2.3.5). Dissolved oxygen was always >80% of air saturation.

2.3.5. Chronic toxicity tests

Chronic toxicity tests were initiated with 16 to 24 hour old juveniles. Tests were conducted in a temperature-controlled room at 25º±1ºC under a light cycle of 16 hours light and 8 hours dark. Test containers were 30 mL polyethylene cups containing 20 mL of test solution. One *Ceriodaphnia dubia* individual was impartially assigned to each test vessel and ten replicates were tested per treatment. Mortality and reproduction (number of juveniles) were determined every 24 hours. The test solution renewal and feeding scheme is summarized in Table 2.3. Feeding was by an algal mix of *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* in a 3:1 ratio on cell basis. No extra food was added on the 3rd day of exposure, because food remaining from the two previous feedings was still abundant and because overloading of food has previously been observed to act adversely on our *Ceriodaphnia* clone. Tests lasted 10 days, which was sufficient to ensure three broods to be completed in control organisms.

Table 2.3 Scheme of test medium renewal and feeding in chronic *Ceriodaphnia dubia* tests.

day	Day	Renewal	10^6cells/day	µg dry wt/day
0	Thu		4	52

1	Fri	X	6	70
2	Sat		6	70
3	Sun		0	0
4	Mon	X	6	70
5	Tue		6	70
6	Wed	X	6	70
7	Thu		6	70
8	Fri	X	6	70
9	Sat		6	70

pH and IC were measured in fresh test solutions prior to test initiation and test solution renewal and also in 'old' test solutions with every renewal. Samples for for total (only at test initiation) and dissolved Ni concentration (0.45 µm filtered) were taken at the same time. Ni concentrations were determined using GF-AAS (see section 2.3.5). Dissolved oxygen was always >70% of air saturation.

Along with the natural waters, an assay with control culture water was run (see Table 2.x, no Ni spikes). The same feeding and renewal regime was applied and at the renewal of the 4th day of exposure, when contact time between algal food and test solution had been maximal. The old solution was filtered through 0.45 µm and 500 mL was sent to KUL for determination of Ni binding behavior of dissolved ligands present in this 'old' test solution. Ni binding to this solution was determined at background Ni and at about 2 µg/L added Ni to assess the potential importance of these exudates on Ni speciation in chronic toxicity tests.

2.3.6. Ni analyses

Ni was measured using Graphite Furnace Atomic Absorption Spectrometry (GF-AAS, SpectrAA100, Varian, Mulgrave, Australia) after acidification of the samples (0.14N ultrapure HNO_3, Normatom grade, VWR, Leuven, Belgium). Calibration standards (Sigma-aldrich, Steinheim, Germany) and a reagent blank were analyzed with every ten samples. The detection limit for Ni was 2 µg/L. Two certified reference samples, TMDA-62 and TM-25.2 (National Water Research Institute, Burlington, ON, Canada) with certified Ni concentrations (mean ± 95% confidence interval) of 10.0 ± 1.7 µg/L and 97.7 ± 8.5 µg/L, respectively, were analyzed at the beginning and end of each series of Ni measurements. Measured values during the period of the ecotoxicity experiments were between 9.1 and 10.4 µg/L for the lowest reference concentration and 90.3 to 99.8 µg/L for the highest reference concentration.

2.3.7. Data treatment

Test data were analyzed using Statistica software (Statsoft, Tulsa, OK, USA). The Mann-Whitney U test was used to test for significant differences between the reproduction of the Ni treatments and the control. The no observable effect concentration (NOEC) and lowest observable effect concentration (LOEC) were calculated on the basis of reproduction (number of juveniles/initial female, p <0.05). In addition, effects concentrations at the 10th, 20th nd 50th percentiles (EC10, EC20 and EC50) were calculated by the logistic regression (De Schamphelaere and Janssen, 2004)*:*

$$y = \frac{k}{1 + \left(\frac{x}{\exp(a)} \right)^{\frac{\ln\left(\frac{1}{9}\right)}{b-a}}} \qquad \text{(Eq. 2.2)}$$

and

$$y = \frac{k}{1 + \left(\frac{x}{\exp(a)} \right)^{\frac{\ln\left(\frac{2}{8}\right)}{c-a}}} \qquad \text{(Eq. 2.3)}$$

where

y = reproduction (No. of juveniles/replicate)
k = fitted control reproduction at Ni = 0 µg/l
a = ln (EC50) = $\log_e$ (EC50)
b = ln (EC10) = $\log_e$ (ErC10)
c = ln (EC20) = $\log_e$ (EC20)
x = measured dissolved nickel concentration

EC50s, EC20s and EC10s and their confidence limits are estimated directly from the fitting procedure. Parameter estimation and calculation of the 95% confidence limits was carried out using the Levenberg-Marquardt method (Levenberg, 1944; Marquardt, 1963).

48h-LC50s were calculated using the Trimmed Spearman-Karber method (Hamilton et al., 1977). All reported effect concentrations are based on dissolved Ni concentrations at test initiation. In acute experiments, measured dissolved Ni at test termination was within 10% of the value measured at test initiation. In chronic tests, dissolved Ni concentrations at test renewals were about 15% lower at test solution renewal.

3. Results and discussion

3.1. Ni Speciation

3.1.1. Ni complexation kinetics with DOC

The test of the equilibration time of the Donnan dialysis system itself indicated that for waters with low ionic strength 4 hours were necessary to reach equilibrium. The standard procedure is 2 hours in Donnan dialysis, but we found increasing fractions free Ni^{2+} ion between 1 hour and 4 hours, beyond which the values stabilised. The 4h equilibration time was selected for all the waters and all further measurements.

To assess the complexation kinetics of Ni with natural DOC, speciation measurements were carried out with the Donnan dialysis technique on 2 spiked surface waters (Ankeveen and Markermeer, spiked with 10 µg Ni/L) after 3 different equilibration times (Table 3.1, see Annex 1 for chemistry of the original samples). There was a small difference in the free Ni^{2+} ion fraction in the Ankeveen water between 2 days and other equilibration times, but this was mostly due to a pH effect. The experiment with the Markermeer water showed the same result It is concluded that complexation is nearly complete after 2 hours of equilibration and that an equilibration time of 16 hours (Ni speciation, sections 3.1.2) or 1 day (ecotoxicity testing, section 2.3) was more than sufficient for all further experiments.

Table 3.1 Kinetics of complexation of Ni in two surface waters after three different equilibration times

			2 hours	2 days	7 days
Ankeveen	Replicate 1	fraction Ni^{2+}	0.069	0.048	0.069
		pH	7.18	7.35	7.19
	Replicate 2	fraction Ni^{2+}	0.067	0.049	0.068
		pH	7.18	7.32	7.18
Markermeer	Replicate 1	fraction Ni^{2+}	0.21	0.22	0.20
		pH	8.17	8.09	8.21

3.1.2. Ni speciation in six natural waters and in two waters with amended pH and hardness

The aim was to measure the Ni speciation in 6 waters at 3 different Ni concentrations and in 2 waters with adjusted pH and hardness values. The % free Ni^{2+} at background ranged 4% to 45% (Table 3.2). Increasing Ni concentration decreased the % free Ni^{2+}. This indicates that, if Ni^{2+} is the dominantly bioavailable Ni species, Ni complexation by DOC will become increasingly important at lower Ni concentrations.

Table 3 Effect of Ni concentration on free Ni^{2+} fraction (pH is constant, except for Eau d'Eppe). Value between parentheses is the standard deviation of the duplicate analysis.

	pH	DOC (mg/L)	Hardness[a] (mg $CaCO_3$/L)	Ni concentration (µg/l)	fraction of Ni^{2+} (%)
Ankeveen	7.36	23.60	131.6	4.2	6.9 (±0.0)
	7.36	23.60	131.6	39	14 (±0.3)
	7.36	23.60	131.6	148	27 (±1.4)
Bihain	6.17	6.36	15.0	2.8	45 (±2.7)
	6.17	6.36	15.0	4.7	45 (±0.0)
	6.17	6.36	15.0	15	55 (±2.2)
Brisy	7.23	3.06	41.1	2.6	31 (±1.0)
	7.23	3.06	41.1	3.3	39 (±2.9)
	7.23	3.06	41.1	21	60 (±2.5)
Eau d'Eppe	7.85	5.02	108.4	4.4	31 (±2.0)
	8.04	5.02	108.4	5.8	27 (±2.6)
	8.17	5.02	108.4	21	37 (±3.3)
Markermeer	8.26	7.61	218.1	3.8	15 (±0.7)
	8.26	7.61	218.1	5.8	21 (±1.0)
	8.26	7.61	218.1	53	40 (±0.1)
Regge	8.58	12.60	204.0	3.7	4 (±0.7)
	8.58	12.60	204.0	6.8	8 (±0.7)
	8.58	12.60	204.0	107	23 (±0.3)

[a] hardness of original sample, final hardness during Donnan-measurement is in Annex 2

The effect of pH and hardness on Ni speciation was tested on two surface waters (Ankeveen and Bihain) at background Ni (Tables 3.3 and 3.4). Decreasing pH (increased H^+) and increasing hardness (Ca^{2+} and Mg^{2+}) increased the fraction of free Ni^{2+}, obviously related to ion competition effects. Indeed, higher concentrations of Ca^{2+}, Mg^{2+} or H^+ (lower pH) result in a larger Ni fraction being out-competed by these ions at ion binding sites on the DOC, resulting in a larger fraction of Ni^{2+}.

Table 3.3 Effect of pH on free Ni fraction. Value between parentheses is the standard deviation of the duplicate analysis.

	pH	Ni concentration (µg/l)	fraction of Ni^{2+} (%)
Ankeveen	8.06	4.4	4 (±0.3)
	7.71	4.5	4 (±0.1)
	7.15	4.8	5.9 (±0.1)
	6.46	5.0	8.2 (±0.5)
Bihain	6.16	2.5	45.2
	6.90	2.5	24.1
	7.59	2.7	19.6
	7.96	2.7	10.3

Table 3.4 Effect of hardness on free Ni^{2+} fraction. Value between parentheses is the standard deviation of the duplicate analysis.

	hardness mg $CaCO_3$/L	Ni concentration (µg/l)	fraction of Ni^{2+} (%)
Ankeveen	112	5.2	6.5 (±0.2)
	207	5	7.9 (±0.2)
	308	5	9.6 (±1.2)
	396	4.9	12.5 (±2.3)
Bihain	16	4.4	43.9 (±2.7)
	94	6.4	70.7 (±2.2)
	450	7.8	88.8 (±2.3)

3.1.3. Calibration of speciation models

3.1.3.1. Introduction to WHAM VI and the software

WHAM (Windermere Humic Aqueous Model) VI is a speciation code that is based on the Humic Ion Binding Model VI. This is a discrete site/electrostatic model for equilibrium ion binding by humic substances. The model has 4 metal-specific parameters, log K_{MA}, log K_{MB}, ΔLK_1 and ΔLK_2. The log K_{MA} and log K_{MB} are median log binding strengths for a given metal to carboxyl sites and weaker-acid sites, respectively. ΔLK_1 defines the spreading of values around these median constants. ΔLK_2 is the empirical parameter that increases the binding strengths of selected multi-dentate sites to provide for a greater range of binding strengths. Tipping (1998) found a correlation between log K_{MA} and log K_{MB}. Moreover, a universal average value of ΔLK_1 was obtained. As a result, the speciation data are fitted by the adjustments of only 2 parameters, log K_{MA} and ΔLK_2.

It is now appropriate to describe how water chemistry data need to be inserted into WHAM VI for speciation calculations. For all cations and anions, the WHAM VI software can deal with inputs as 'total' concentration, 'dissolved' concentration, 'free ionic' concentration, or 'free ion activity'. With respect to ion binding to humic and fulvic acid, one can insert these as either 'particulate' (total minus dissolved) or as 'colloidal' (dissolved), but WHAM VI assumes identical binding properties for 'particulate' and 'colloidal' humic or fulvic acid. Hence, when measurements of cations, anions and organic matter are available for the same fraction (e.g., dissolved fraction <0.45 μm), it does not affect calculated ion speciation if the measurements are inserted as 'total' or as 'dissolved'. Since 'total' is the default in WHAM VI and since the measured difference between total and dissolved concentration is usually minor for major cations and anions, we have always inserted those at 'total'. All trace metals were inserted as 'dissolved' to emphasize that they were measured on 'dissolved' samples. To emphasize

that we are working on the 'dissolved' fraction of organic matter (<0.45 µm), we always inserted organic matter as 'colloidal' fulvic acid. This was applied throughout all the analysis described in this report.

There were three exceptions to this. The first one relates to the case where effect concentrations needed to be predicted using the developed bioavailability models. In this case, we needed to make use of the option of inserting Ni as 'free ionic activity', i.e. (Ni^{2+}). This is explained in more detail in section 4.

The two other exceptions were related to the input of Fe(III) and Al in waters from a natural origin (such as the ones investigated in the current study). In natural waters, both Fe^{3+} and Al^{3+} may occur as colloidal $Fe(OH)_3$ and $Al(OH)_3$ (Bryan et al., 2002; Cheng et al., 2005). Since these colloids easily pass 0.45 µm filters, this may result in measured values of 'dissolved' Fe and Al that are higher than the actual 'dissolved' Fe and Al that can interact with dissolved ligands and DOC. Hence, the actual Fe an Al that is to be inserted in WHAM VI cannot directly be derived from the measured concentrations. The following solution has been suggested and successfully applied in several studies (e.g., Bryan et al., 2002; Cheng et al., 2005).

First, colloidal $Fe(OH)_3$ is predicted to occur in almost every surface water and hence, the Fe^{3+} activity can be directly estimated from the solubility constant of colloidal $Fe(OH)_3$:

$$K_{sol,Fe(OH)3} = (Fe^{3+}) / (H^{+})^3 \qquad \text{(Eq. 3.2)}$$

In literature $\log_{10} K_{sol,Fe(OH)3}$ varies from 2 to 5, but in accordance with Tipping et al. (2003) we used a value of 2.5 (at 25°C) throughout the present study, and a reaction enthalpy $\Delta H_{Fe(OH)3}$ = - 102,000 J/mol (Tipping et al., 2003). The solubility constant at any temperature *t* (in °C) can be calculated using the Van 't Hoff equation:

$$K_{sol,Fe(OH)_3,(t)} = K_{sol,Fe(OH)_3,(25°C)} \cdot \exp\left\{\frac{\Delta H_{Fe(OH)_3}}{R} \cdot \left(\frac{1}{298} - \frac{1}{273+t}\right)\right\} \qquad \text{(Eq. 3.3)}$$

Where R = the universal gas constant = 8.314 J mol^{-1} K^{-1}. Equation 3.2 clearly indicates that, when colloidal $Fe(OH)_3$ is present in solution, it controls the activity of Fe^{3+}, and this activity can be calculated from the measured pH only:

$$(Fe^{3+}) = K_{sol,Fe(OH)3} \cdot 10^{-3 \cdot pH} \qquad \text{(Eq. 3.3)}$$

The situation for Al^{3+} is a little more complex, since it is not always predicted to be in equilibrium with $Al(OH)_3$. The optimal way of calculating Ni speciation in presence of Al is to allow Al^{3+} to precipitate as colloidal $Al(OH)_3$ whenever the solubility product $K_{sol,Al(OH)3}$ is exceeded. This can be tested as follows.

First, the speciation problem is run with the assumption that colloidal $Al(OH)_3$ is present in solution, by inserting Al^{3+} activity into WHAM VI, calculated as follows:

$$(Al^{3+}) = K_{s,Al(OH)3} \cdot (H^{+})^3 \qquad \text{(Eq. 3.4)}$$

or

$$(Al^{3+}) = K_{s,Al(OH)3} \cdot 10^{-3 \cdot pH} \qquad \text{(Eq. 3.5)}$$

Where $K_{sol,Al(OH)3}$ is the solubility product of colloidal $Al(OH)_3$, with log $K_{sol,Al(OH)3}$ = 8.5 at 25°C, with a reaction enthalpy $\Delta H_{Al(OH)3}$ = -107,000 J/mol (Tipping et al., 2002). WHAM VI will then calculate, under the chemistry conditions of the modelled solution, the 'dissolved' Al concentration - all Al without colloidal $Al(OH)_3$ - in equilibrium with this Al^{3+} activity. When this predicted Al concentration is lower than the measured Al concentration, the difference is assumed to be due to the presence of colloidal $Al(OH)_3$, and the original assumption is valid. In this case, the speciation calculations performed are retained. In the other case, the original assumption was wrong and speciation calculations need to be performed by inserting the measured Al into the

software as ‘dissolved’. This approach was consistently followed throughout this report and is referred to as the ‘optimal’ scenario with respect to Al. Whenever appropriate, the influence of modelling Al in different ways will be discussed.

As mentioned earlier, dissolved organic matter was considered ‘colloidal’ and was assumed to consist of a certain fraction of active fulvic acid (%AFA) and a fraction of inert for ion binding (in line with the approach in Bryan et al., 2002, Cheng et al., 2005). The choice of fitting ‘active fulvic acid’ instead of ‘active humic acid’ has originally (e.g., Dwane and Tipping, 1998) been inspired by the fact that fulvic acid is usually much more abundant than humic acid in natural surface waters, i.e. typically 80% FA vs. 20% HA (Thurman, 1985). DOC was always multiplied by 2 to obtain FA concentrations, since on a weight basis, about 50% of DOM consists of carbon (Thurman, 1985). Based on Cu modeling data (Bryan et al., 2002) we used 65% AFA as a starting point for the modeling, but the aim was to calibrate this parameter to the measured speciation. Experiments with Cu and Zn have demonstrated 60-65% AFA is a good first approximation for modeling metal speciation in natural surface waters (Bryan et al., 2002, Cheng et al., 2005).

3.1.3.2. Calibration of WHAM VI

Because the parameters in WHAM model VI for Ni binding to humic substances are, for nickel, only based on data for isolated soil fulvic, isolated soil humic acid and isolated groundwater fulvic acid, the model needed to be calibrated using experimental results with truly aqueous DOM at background concentrations of Ni and at Ni concentrations reflecting the Ni sensitivity of a sensitive organism (here: *C. dubia*). The experimental results described in section 3.1.2 and Tables 3.2 to 3.4 were used for this calibration.

In order to calibrate WHAM model VI to the speciation data of all natural waters (including those with adjusted Ni, hardness and pH), the parameters were optimized through minimizing the squared residuals, i.e.

$$\sum\left[\left[\log(\%Ni^{2+}_{observed}) - \log(\%Ni^{2+}_{predicted})\right]^2\right] \quad \text{(Eq. 3.6)}$$

The values were transformed to log values to optimize predictions of free metal ion fraction *within similar order of magnitude* across the entire range of observations. Some parameters were adjusted before, because literature indicated already more relevant values. For the inorganic metal complexation, the default stability constants of Ni-carbonate complexes were adapted to those of NIST (*National Institute of Standards and Technology*). The default stability constant for the organic complex of fulvic acid (FA) with Zn and ZnOH was adjusted, based on the results of Cheng *et al.* (2005). In Table 3.5 default and adjusted parameters are shown. These parameters were used when optimizing the effective fulvic acid concentration and the metal-specific parameters of the Ni complex with fulvic acid.

Table 3.5 Default and adjusted stability constants of Ni with inorganic and organic ligands

	Parameter	Default stability constant (pK) in WHAM VI	Adjusted stability constant (pK)	Reference
Inorganic complexation	$K = [NiCO_3]/[Ni^{2+}].[CO_3^{2-}]$	5.78	4.57	NIST[1]
	$K = [NiHCO_3^+]/[Ni^{2+}].[H^+].[CO_3^{2-}]$	13.41	12.42	NIST[1]
Organic complexation	$K_{MA(Zn)}$	1.6	1.8	Cheng *et al. (2005)*

[1] *National Institute of Standards and Technology*

Adapting the ΔLK_2 parameter hardly affected the result, so the default value of 1.57 was maintained. However, adjusting the effective fulvic acid concentration and the stability constant log $K_{MA(Ni)}$ improved the fittings of the model with the experimental results (Figure 3.1). In most studies of Ni speciation, a fraction of 60 to 65% is considered as the chemically reactive fraction of the natural DOM, behaving as fulvic acid (Bryan et al., 2002; Cheng et al., 2005). Using this effective fraction, a value of 1.65

for the stability constant log $K_{Ma(Ni)}$ of the Ni-fulvic acid complex showed lowest residuals. However, the slope of the predicted/observed line was smaller than 1.0, suggesting a bias in the predictions. When both parameters were varied, the best fit was obtained for a log $K_{Ma(Ni)}$ of 1.75 and the % active fulvic acid (%AFA) of 40%, with a slope near unity (Fig. 3.1). Tables 3.6 and 3.8 show the main characteristics of the waters and the associated observed and predicted free Ni^{2+} fractions. The main difference between the calibration at log $K_{Ma(Ni)}$ = 1.65 and at log $K_{Ma(Ni)}$ = 1.75 is that the latter better predicts the lower Ni^{2+} fractions (Figure 3.1).

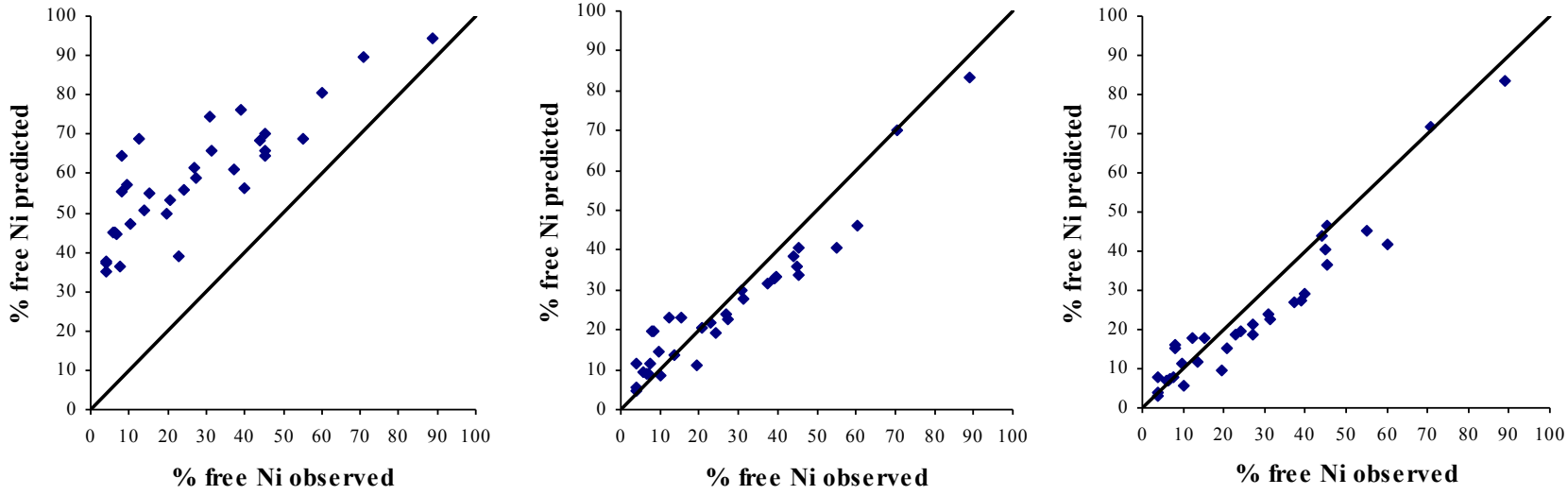
The 'optimized' parameters were used in all further speciation calculations, model developments and validations regarding the ecotoxicity datasets (see sections 3.2 and 4).

Table 3.6 Ni speciation in 6 surface waters at background and adjusted dissolved Ni concentrations; comparison between observed and predicted (WHAM model VI) free Ni fraction (%). Value between parentheses is the standard deviation of the duplicate analysis. Complete chemical composition in Annex 2. See also Figure 3.1 for observed vs. predicted free Ni^{2+} fractions

		pH	DOC mg/L	Ca conc (mM)	Mg conc (mM)	**Ni conc (µg/L)**	**% free Ni fraction**	
							observed	***predicted***
Ankeveen	background	7.36	23.60	0.87	0.29	**4.2**	**6.9 (±0.0)**	**7.2**
	'EC 10'	7.36	23.60	0.88	0.30	**38.9**	**13.8 (±0.3)**	**11.9**
	'LC50'	7.36	23.60	0.87	0.29	**147.3**	**27.2 (±1.4)**	**21.5**
Bihain	background	6.17	6.36	0.11	0.06	**2.8**	**45.3 (±2.7)**	**46.6**
	'EC 10'	6.17	6.36	0.11	0.06	**4.7**	**45.1 (±0.0)**	**40.6**
	'LC50'	6.17	6.36	0.11	0.06	**14.8**	**55.3 (±2.2)**	**45.1**
Brisy	background	7.23	3.06	0.23	0.15	**2.6**	**31.0 (±1.0)**	**24.1**
	'EC 10'	7.23	3.06	0.24	0.15	**3.3**	**39.0 (±2.9)**	**27.2**
	'LC50'	7.23	3.06	0.24	0.15	**20.7**	**60.2 (±2.5)**	**41.6**
Eppe	background	7.85	5.02	0.77	0.26	**4.4**	**31.4 (±2.0)**	**22.4**
	'EC 10'	8.04	5.02	0.76	0.26	**5.8**	**26.9 (±2.6)**	**18.9**
	'LC50'	8.17	5.02	0.77	0.26	**21.1**	**37.4 (±3.3)**	**26.9**
Markermeer	background	8.26	7.61	1.41	0.60	**3.8**	**15.3 (±0.7)**	**18.0**
	'EC 10'	8.26	7.61	1.35	0.61	**5.8**	**20.6 (±1.0)**	**15.3**
	'LC50'	8.26	7.61	1.34	0.60	**52.9**	**39.8 (±0.1)**	**29.0**
Regge	background	8.54	12.60	1.50	0.32	**3.7**	**4.0(±0.7)**	**7.9**
	'EC 10'	8.58	12.60	1.52	0.33	**6.8**	**7.7 (±0.7)**	**8.0**
	'LC50'	8.58	12.60	1.47	0.32	**106.8**	**23.0 (±0.3)**	**18.6**

Table 3.7 Ni speciation in 6 surface waters at background and adjusted pH and Ca concentrations; comparison between observed and predicted (WHAM model VI) free Ni fraction (%).Value between parentheses is the standard deviation of the duplicate analysis. Complete chemical composition in Annex 2.

		pH	DOC mg/L	Ca conc (mM)	Mg conc (mM)	**Ni conc (µg/L))**	**% free Ni fraction**	
							observed	***predicted***
Ankeveen	pH 1	8.06	23.60	0.85	0.30	**4.4**	**4.0 (±0.3)**	**2.9**
	pH 2	7.71	23.60	0.85	0.31	**4.5**	**4.0 (±0.1)**	**3.8**
	pH 3	7.15	23.60	0.86	0.31	**4.8**	**5.9 (±0.1)**	**6.8**
	pH 4	6.46	23.60	0.88	0.32	**5.0**	**8.2 (±0.5)**	**16.2**
	hardness 1	7.31	23.60	0.90	0.32	**5.2**	**6.5 (±0.2)**	**6.9**
	hardness 2	7.31	23.60	2.41	1.10	**5.1**	**7.9 (±0.2)**	**15.1**
	hardness 3	7.31	23.60	1.66	0.69	**5.0**	**9.6 (±1.2)**	**11.2**
	hardness 4	7.31	23.60	3.13	1.46	**4.9**	**12.5 (±2.3)**	**17.9**
Bihain	pH 1	6.16	6.36	0.12	0.06	**2.5**	**45.2**	**36.4**
	pH 2	6.90	6.36	0.13	0.07	**2.5**	**24.1**	**19.5**
	pH 3	7.59	6.36	0.16	0.08	**2.7**	**19.6**	**9.5**
	pH 4	7.96	6.36	0.19	0.10	**2.7**	**10.3**	**5.8**
	hardness 1	6.18	6.36	0.08	0.07	**4.4**	**43.9 (±2.7)**	**43.8**
	hardness 2	6.18	6.36	0.60	0.37	**6.4**	**70.7 (±2.2)**	**71.7**
	hardness 4	6.18	6.36	1.85	1.03	**7.5**	**88.8 (±2.3)**	**83.6**



Step 1 'typical' 65% FA (see text)
Default parameter log $K_{Ni\text{-}Fa}$ **1.4**
SSE (log fractions) = 10.4

Step 2 65% FA
Optimized log $K_{Ni\text{-}FA}$ **1.65**
SSE (log fractions) = 0.95

Step 3 Optimized % FA = **40%**
Optimized log $K_{Ni\text{-}FA}$ = **1.75**
SSE (log fractions) = 0.69

Figure 3.1 Comparison of % free Ni predicted and observed for three steps in the optimization.

3.2. Ecotoxicity of Ni to *Ceriodaphnia dubia*.

3.2.1. Ni toxicity as dissolved Ni in natural waters – effect concentrations

Selected chemical characteristics (DOC, hardness and pH) as well as 48h-LC50 and 10d-EC10, EC20 and EC50 are reported in Table 3.7. Full chemical characterization of the water samples during the ecotoxicity tests can be found in Annex 3, 10-day NOECs and LOECs, as well as uncertainty intervals about ECx values in Annex 4.

Table 3.7 Chemical composition[a] and 'dissolved' 48h-LC50 (survival) and 10d-ECx values (reproduction) of Ni to *C. dubia* (as µg Ni/L)

Site	DOC (mg/L)	H [b]	pH (acute)	pH (chronic)	48h-LC50	10d-EC50	10d-EC20	10d-EC10
Ankeveen	23.6	131.6	7.51	7.61	183	68.4	51.9	44.2
Bihain	6.36	15.0	6.34	6.56	35.2	23.1	12.8	9.0
Brisy	3.06	41.1	7.45	7.23	50.8	11.0	8.5	7.4
Eppe	5.02	108.4	7.95	7.86	34.6	4.9	(2.1) [c]	(1.3) [c]
Markermeer	7.6	218.1	8.04	8.01	88.7	12.1	(9.0) [c]	(7.6) [c]
Regge	12.6	204.0	8.00	8.18	161	20.1	11.0	7.8
Max-min factor[d]					5	14	25	34

[a] Full chemical characterization in annex 3
[b] H = calculated water hardness, as mg $CaCO_3$/L.
[c] EC extrapolated below lowest test concentration
[d] factor difference between lowest and highest L(E)Cx

Table 3.7 illustrates that Ni toxicity varied substantially among the different water samples, with 48h-LC50s between 34.6 and 183 µg/L (5-fold), 10d-EC50s between 4.9 and 68.4 µg/L (14-fold) , 10d-EC10s between 1.3 and 44..2 µg/L (34-fold). The difference between toxicity values is larger for more sensitive endpoints (lower Ni-concentrations). This illustrates the importance of taking into account site chemistry in regulatory exercises, especially since those differences are observed for one of the most sensitive organism for Ni.

A significant linear relation was found between DOC and acute ($p=0.015$, $r^2 = 0.81$) and chronic ($p=0.007$; $r^2 = 0.87$) Ni-toxicity (see Figure 3.2). DOC thus reduces acute and chronic Ni toxicity to *C. dubia*. Deleebeeck et al. (2005) also observed a significant linear effect of DOC on chronic Ni toxicity to *Daphnia magna*, whereas they found lesser effect of DOC on acute toxicity (lower regression slope). This suggests that differences in bioavailability may be more important at lower Ni concentrations and that this is largely due to the fact that a larger fraction of dissolved Ni is bound to DOC at lower Ni concentrations

(see section 3.1.2). This clearly illustrates that DOC is an important factor to consider in chronic bioavailability modelling.

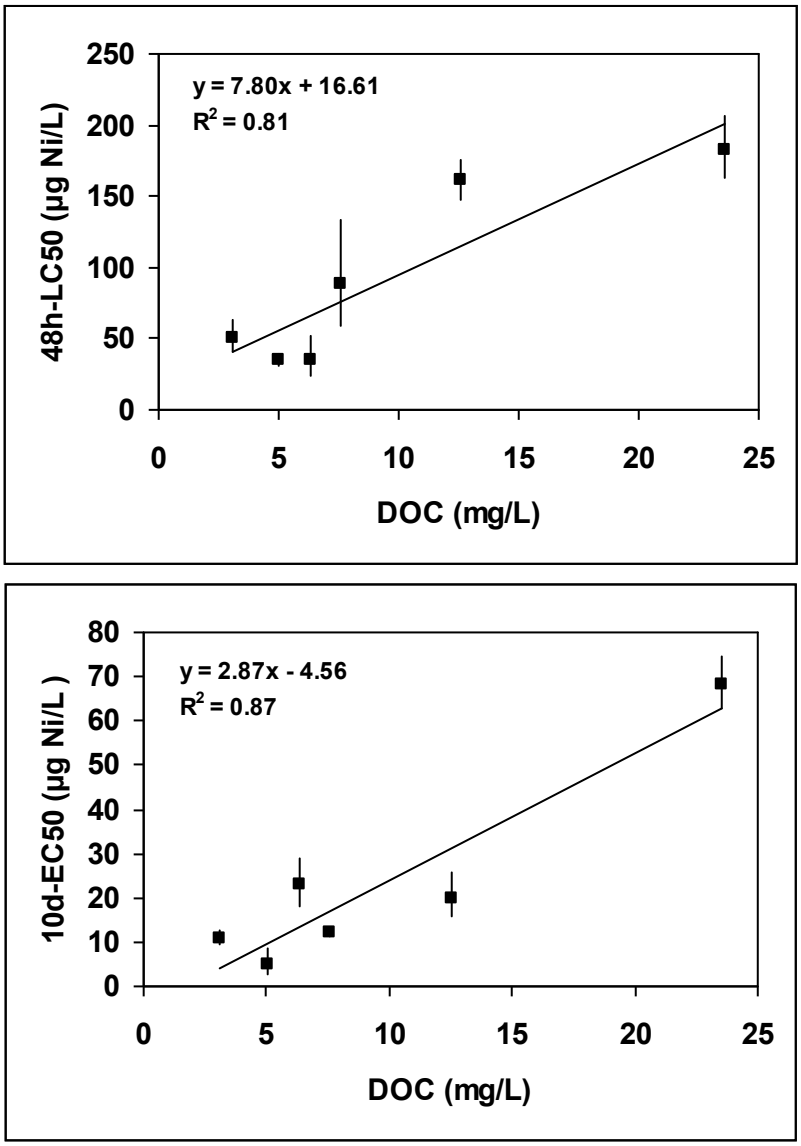


Figure 3.2 48h-LC50 and 10d-EC50 of Ni for *Ceriodaphnia. dubia* in spiked natural surface waters as a function of DOC.

A correlation on a dissolved Ni basis, as above, is only a first step in the understanding of Ni bioavailability. Here it only indicates that organic complexation of Ni is important enough to reduce Ni toxicity. In order to further explain differences among waters, and to also involve the effects of inorganic parameters (complexation and competition), a second step is to calculate Ni-speciation and then to determine how the toxicity of the Ni^{2+} ion varies with modifying factors such as pH, Ca and Mg. This is described in the following sections.

3.2.2. Ni toxicity as free Ni^{2+}

Speciation calculations were performed on all six test waters and all ECx values were expressed as Ni^{2+}-activity by using the ECx values as input to the WHAM 6 speciation model software. The input-file for these speciation calculations is given in Annex 3.

In order to investigate the importance of Al in Ni speciation at Ni concentrations which are relevant for toxicity to *C. dubia,* we modelled four possible Al scenarios (see also section 3.1.3.1):

(i) (Al^{3+}) was assumed to be in equilibrium with its colloidal $Al(OH)_3$ precipitate, i.e. $(Al^{3+}) = K_{s,Al(OH)3} \cdot (H^+)^3$ or $(Al^{3+}) = K_{s,Al(OH)3} \cdot 10^{-3 \cdot pH}$, with $K_{s,Al(OH)3}$ solubility product of colloidal $Al(OH)_3$, with log $K_{s,Al(OH)3}$ = 8.5 at 25°C, with a reaction enthalpy $\Delta H_{Al(OH)3}$ = -107 kJ/mol (Tipping et al., 2002). Al was inserted into the model as free Al^{3+} activity, since the WHAM 6 model software allows this option.

(ii) Measured dissolved Al was inserted as measured 'dissolved' Al.

(iii) Al was omitted from the model input (dissolved Al=0)

(iv) The 'optimal' way of modelling Al speciation, as explained in section 3.1.3.1

Under all scenarios, we used the optimal %AFA of 40% and a log K_{NiFA}=1.75 (see section 3.1.3.2). Annex 5 summarizes the calculated (Ni^{2+}) at all effect levels and under all scenarios for all surface waters tested.

In practice the calculated (Ni^{2+}) was very similar under scenario (i) and scenario (ii). An average ratio between the two scenarios of 1.02 was observed and a maximal difference of 10% was observed. The 'optimal' Ni^{2+} calculations (scenario iv) differed by up to 34% form the calculations from scenario (iii) (no Al assumed). Lower Ni^{2+}-activities are predicted when no Al is assumed, since Al competes with Ni for binding sites on the DOC. This illustrates the importance of considering Al competition for binding of Ni to DOC. Given the similarity of scenario (i) and (ii) calculations, it is more accurate to perform calculations under scenario (i) when no measured dissolved Al levels are available for a given water sample then to perform the calculations with Al = 0. The results of the 'optimal' speciation calculations are given in Table 3.8.

Table 3.8 48h-LC50 and 10d-ECx values of Ni to *C. dubia*, as Ni^{2+}-activity (mol/L). $Al(OH)_3$ was allowed to precipitate when its solubility product was exceeded. All other trace metals were also used as input.

Site	48h-LC50	10d-EC50	10d-EC20	10d-EC10	48h-pNi_{50} [a]	10d-pNi_{50} [a]
Ankeveen	5.08E-07	1.14E-07	7.62E-08	6.06E-08	6.29	6.94
Bihain	2.54E-07	1.37E-07	6.95E-08	4.70E-08	6.59	6.86
Brisy	3.95E-07	7.36E-08	5.56E-08	4.71E-08	6.40	7.13
Eppe	1.55E-07	1.35E-08	(4.90E-09) [c]	(2.81E-09) [c]	6.81	7.87
Markermeer	3.72E-07	3.05E-08	(2.10E-08) [c]	(1.69E-08) [c]	6.43	7.52
Regge	5.74E-07	3.30E-08	1.47E-08	9.36E-09	6.24	7.48
Max-min [b]	3.7	10	16	21		

[a] pNi_{50} = - log (EC50 or LC50 as Ni^{2+}-activity)
[b] factor difference between lowest and highest L(E)Cx
[c] EC extrapolated below lowest test concentration

The data in Table 3.8 illustrate that Ni-speciation does only explain little of the variability observed in dissolved LC50 and ECx values (Table 3.7), as the factor difference between minimum and maximum $LC50_{Ni2+}$ or ECx_{Ni2+} is only slightly lower. Indeed large differences (factor 3.7 to 21) are still observed between the different waters (Table 3.8), indicating that Ni^{2+} is not a much better predictor of Ni toxicity than $Ni_{dissolved}$ and that Ni-speciation alone does not explain all the variability observed on a dissolved basis. The fact that the variability is higher at lower effect levels (i.e., 10d-EC10) illustrates the importance of the development of a suitable bioavailability model.

3.2.3. Ni toxicity expressed as free Ni^{2+} as a function of water chemistry

This analysis will mainly focus on the 48h-LC50 and the 10d-EC50 levels. Ten day-EC50 levels bear less uncertainty than 10d-EC10 or 10d-EC20 levels (lower confidence intervals) and none of the EC50 values were 'extrapolated'.

We plotted 48h-$LC50_{Ni2+}$ and 10d-$EC50_{Ni2+}$ values against pH, hardness and DOC of the natural test waters (Figure 3.3) to determine how to further develop the bioavailability model and to compare the trends with those discussed previously for *D. magna* (Deleebeeck et al., 2005) and for *C. dubia* (Keithly et al., 2004).

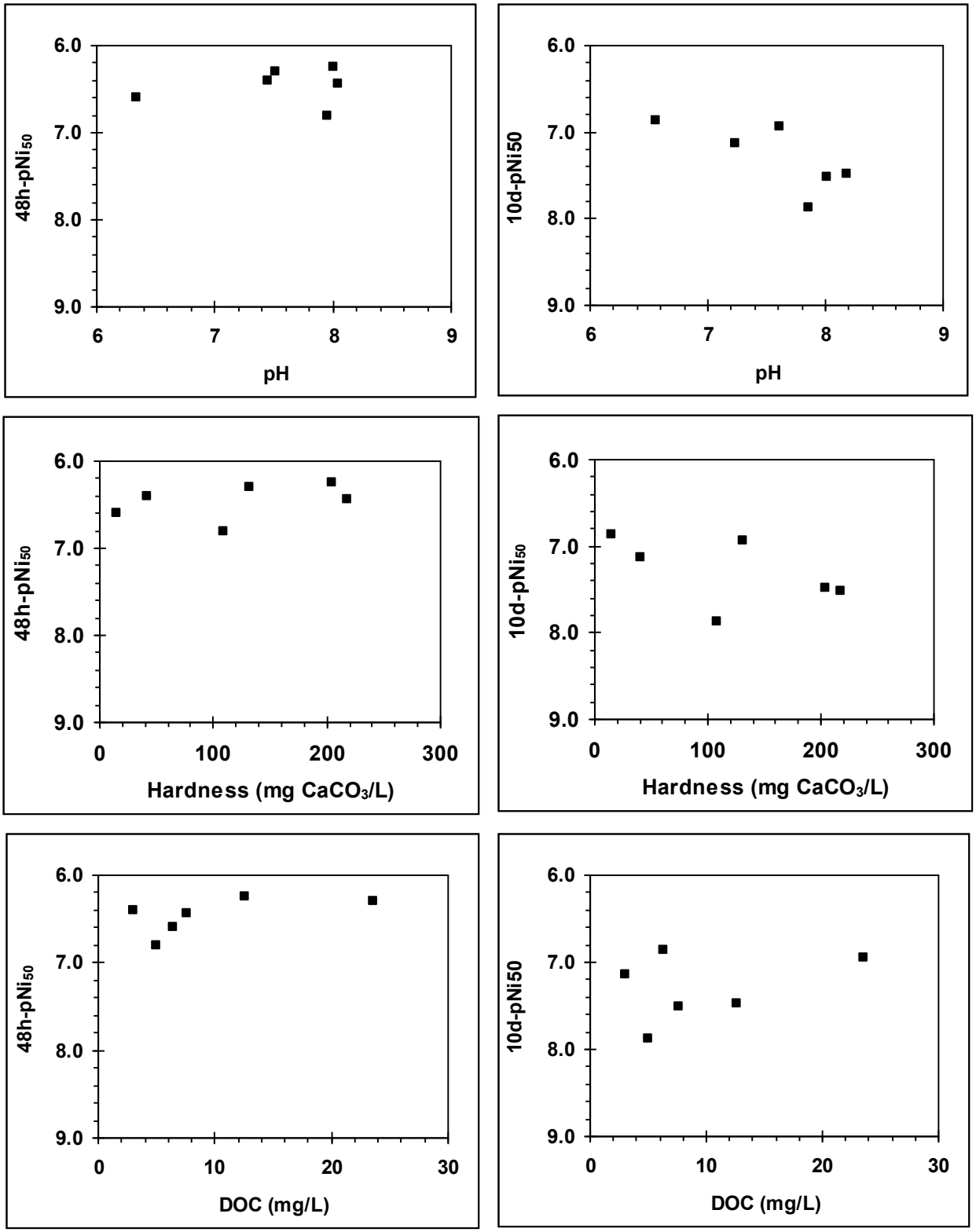


Figure 3.3 48h and 10d-pNi$_{50}$ of Ni to *C. dubia*, expressed as pNi = - log {Ni^{2+}-activity}, plotted against pH, water hardness and DOC of the natural test waters.

Figure 3.3 demonstrates that neither acute 48h-$LC50_{Ni2+}$ nor chronic 10d-$EC50_{Ni2+}$ exhibit a visible relation with DOC of the natural waters. This suggests that in this case DOC does not affect Ni toxicity beyond its effect on speciation.

Acute 48h-$LC50_{Ni2+}$ do also not exhibit a clearly visible trend with pH or hardness. The latter seems counter-intuitive since Keithly et al. (2004) demonstrated that 48h-LC50s of Ni to *C. dubia* increased with increasing hardness in reconstituted waters of pH 7.6 to 7.8 and since Deleebeeck et al. (2005) demonstrated that both Ca and Mg, which together constitute the hardness, protect *D. magna* against acute Ni toxicity. Acute toxicity of Ni^{2+} to *D. magna* was only affected by pH at pH>8 (Deleebeeck et al., 2005), and in *C. dubia* too pH has previously been shown to strongly affect acute Ni toxicity above pH 8 (Schubauer-Berigan et al., 1993). Thus, the fact that no pH effect was observed on acute Ni toxicity might at least party be due to the fact that no waters with pH exceeding pH 8 were investigated for acute Ni toxicity in the present study.

Ten day-$EC50_{Ni2+}$ seem to decrease with increasing pH and also with increasing hardness. The latter seems contradictory with the results obtained on the protective effects of Ca and Mg on chronic Ni toxicity to both *D. magna* (Deleebeeck et al., 2005) and with the increased chronic EC20s and EC50 of Ni to *C. dubia* at increased hardness (Keithly et al., 2004) in univariate experimental designs. The effect of pH, however, corroborates with the finding that chronic Ni toxicity was also reduced to *D. magna* (Deleebeeck et al., 2005).

However, it should be noted that pH and hardness are highly, positively correlated ($r = 0.90$) in our natural water samples. Since the effects of increased pH and increased hardness on Ni toxicity are typically counteractive, part of the pH and the hardness effect may have been masked by this 'natural' correlation. In the case of acute toxicity this may potentially explain why no effects of either pH or hardness are observed, i.e. both effects cancel out each other. In the case of chronic toxicity, it may suggest that the effect of pH is larger than the hardness effect.

To explore this further, pH and hardness effects should be separated from each other. This will be performed in the model development section, where different existing data-sets, including the one generated in the present study will be investigated into more detail.

4. Development, refinement and validation of aquatic Ni bioavailability models

4.1. Important initial considerations

In a previous report we have generated toxicity data with *Pseudokirchneriella subcapitata*, *Daphnia magna* (acute and chronic), and rainbow trout (chronic) tested in artificial waters without added DOC (Deleebeeck et al., 2005). Based on these data biotic ligand models (BLM) were developed and these models were then validated with spiked natural surface waters (Deleebeeck et al., 2005).

Both model development and validation were carried out using the biotic ligand model software, which uses the WHAM-Model V description of metal-DOC interactions. In the initial validation of the models, we assumed by default that Model V would accurately predict Ni speciation and we assumed that DOM consisted of 100% active fulvic acid.

Hence, it must be understood that all these validations made use of a speciation model which was not calibrated to measured Ni speciation in natural surface waters. Thus, in principle, these validations were meaningless, since one could not determine how good the metal toxicity predictions were and what the uncertainty around them was!

Therefore, any comparison concerning predictive capacities of models developed and validated with WHAM V (Deleebeeck et al., 2005) vs. WHAM VI (present study) is meaningless and will not discussed in this report. Also, all figures, tables, and discussion about predictive capacity of the former models in natural waters reported by Deleebeeck et al. (2005) must be neglected and replaced by the subsequent section of the present report!

Also, WHAM VI and WHAM V (as programmed into the BLM software) produce slightly different Ni^{2+} activities (<10%) in inorganic solutions not containing DOM, even when the same stability constants for inorganic metal complexes are used. This is due to a slightly different approach for calculating activity coefficients (Davies vs. Extended Debye-Hückel) and potentially also due to numerical differences (method to iteratively solve the large set of equations of which a speciation problem consist).

Since the calibrations of WHAM VI to Ni speciation in natural water are better and more realistic than WHAM V calibrations (data not shown), WHAM VI is the preferred

speciation model to integrate into the final bioavailability models. This implies that, in practice, although the discussed differences are relatively small, WHAM VI must also be used to calculate Ni^{2+}-activities in the artificial test waters of Deleebeeck et al. (2005) and that these calculations need to serve as a basis for the model development and validation in the present study. In other words, the original raw toxicity data (ECx values as dissolved Ni and reported water chemistry, Deleebeeck et al., 2005) must be re-evaluated using WHAM VI as must the BLM-parameters. In this report this will only be performed for acute and chronic *D. magna* toxicity data, because this project was specifically aimed at expanding the knowledge about Ni bioavailability to invertebrates (i.e. the daphnids *C. dubia* and *D. magna*) and since those organisms are the most sensitive anyway. Hence, more accurate bioavailability modeling is most crucial for these organisms in a risk assessment context.

In practice, all types of data-analyses reported by Deleebeeck et al. (2005) were repeated and the results of this are reported in the following sections. While these analyses were ongoing, we found important uncertainties around the pH values in the test solutions during the chronic *D. magna* experiments of the Bossuyt et al. (2001) dataset, of which the toxicity data were also validated in the Deleebeeck et al. (2005) study. Indeed, pH values during these tests have not been measured at the critical time points during the tests, i.e. in 'old' test solutions immediately before test water renewals, when solution pH may have increased substantially due to the presence of the algal food. This was not the case for their acute dataset (for which pH is reported in Annex A of the Bossuyt et al., 2001, report). Given the importance of pH in Ni bioavailability and toxicity, we do not wish to consider the chronic *D. magna* dataset of Bossuyt et al. (2001) in the validation (or further refinement) of our models, described hereunder. We also recommend not including the Bossuyt et al. (2001) data for the construction of an aquatic effects database for Ni for the risk assessment, because there are more reliable chronic data available with *D. magna* (e.g., the ones we do deal with hereunder).

The models developed and presented below will represent the most recent state-of-the-art on aquatic Ni-bioavailability, including additional evidence from peer-reviewed literature.

4.2. Development of an acute Ni toxicity model for aquatic invertebrates

4.2.1. Acute toxicity model for *D. magna*

The acute toxicity data from Deleebeeck et al. (2005) were re-analyzed using WHAM VI and BLM parameters were described according to the method of De Schamphelaere and Janssen (2002). The input table used for speciation calculations and selected output data are presented in annex 6 and Table 4.1, respectively.

Similar conclusions could be drawn as in Deleebeeck et al. (2005), see Table 4.1 for 48h-$LC50_{Ni2+}$ and Figure 4.1:

(i) Ca and Mg protected against the toxicity of Ni^{2+} up to a concentration of 3 mM, further concentrations of these cations again increased the toxicity.

(ii) Na (up to 14 mM) and K (up to 0.3 mM) did not affect toxicity of Ni^{2+}.

(iii) No pH effect was observed between pH 6.0 and 7.5, but the toxicity of Ni^{2+} was slightly higher at pH 8.1.

(iv) There was seemingly no effect of adding 0.75 g/L of MOPS as a pH buffer, as 48h-LC50s between pH 6 and 6.7 were virtually the same in MOPS-buffered and non-MOPS-buffered test solutions.

The fact that no pH effect is observed up to pH 7.5 seems to contrast with the proposed stability constant log K_{HBL} = 7.5 for binding of Ni to fish gills (Wu et al., 2001). It is noted, however, that the stability constants proposed by Wu et al. (2001) were based on a fitting exercise of the BLM on a dataset of measured Ni binding to fish gills at a range of different Ni and different Ca concentrations, whereas pH was not modified (Meyer et al., 1997). Wu et al. (2001) recognized that the set of model parameters they presented was not unique in terms of its predictive capacity. Hence, the apparent contrast between fish gill binding and acute Ni toxicity to *D. magna* is not definitive.

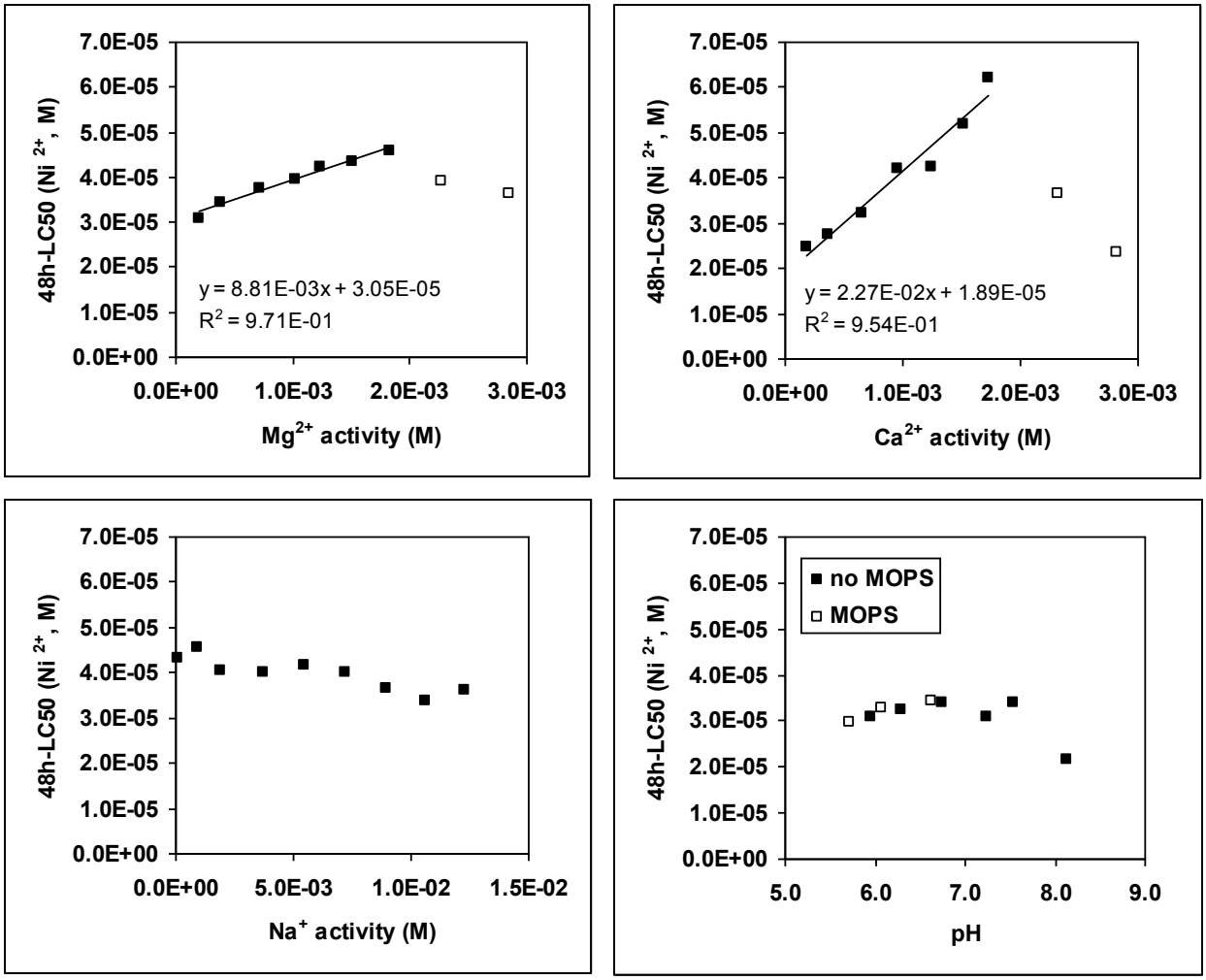


Figure 4.1 Observed 48h-LC50$_{Ni2+}$ (as Ni^{2+}-activity) for *D. magna* in artificial test waters from Deleebeeck et al. (2005). Data from Table 4.1. Data in the Ca and Mg graphs which are marked as open squares are not used for model development.

Table 4.1 Observed 48h-LC50$_{dissolved}$ and 48h-LC50$_{Ni2+}$ (Ni-activity) of Ni to *D. magna* in artificial test waters from Deleebeeck et al. (2005). See also Figure 4.1

Test water [a]	pH	Mg^{2+} [b] (M)	Ca^{2+} [b] (M)	$LC50_{diss}$ (mg/L)	$LC50_{Ni2+}$ [c] (M)
Mg 0.25 mM	6.62	2.03E-04	1.82E-04	2.26	3.08E-05
Mg 0.5 mM	6.58	3.88E-04	1.75E-04	2.61	3.46E-05
Mg 1.0 mM	6.60	7.13E-04	1.66E-04	2.96	3.75E-05
Mg 1.5 mM	6.48	1.02E-03	1.48E-04	3.24	3.97E-05
Mg 2.0 mM	6.59	1.23E-03	1.53E-04	3.54	4.22E-05
Mg 2.5 mM	6.79	1.51E-03	1.47E-04	3.77	4.37E-05
Mg 3.0 mM	6.82	1.83E-03	1.44E-04	4.06	4.57E-05
Mg 4.0 mM	6.85	2.27E-03	1.30E-04	3.63	3.93E-05*
Mg 5.0 mM	6.75	2.85E-03	1.22E-04	3.51	3.65E-05*
Ca 0.25 mM	6.50	1.97E-04	1.78E-04	1.82	2.48E-05
Ca 0.5 mM	6.63	1.85E-04	3.70E-04	2.08	2.76E-05
Ca 1.0 mM	6.71	1.71E-04	6.56E-04	2.53	3.22E-05
Ca 1.5 mM	6.77	1.59E-04	9.60E-04	3.41	4.19E-05
Ca 2.0 mM	6.89	1.52E-04	1.25E-03	3.56	4.24E-05
Ca 2.5 mM	6.89	1.59E-04	1.51E-03	4.49	5.20E-05
Ca 3.0 mM	6.86	1.50E-04	1.73E-03	5.50	6.23E-05
Ca 4.0 mM	6.90	1.41E-04	2.32E-03	3.40	3.68E-05*

Ca 5.0 mM	6.92	1.36E-04	2.81E-03	2.28	2.37E-05*
Na 0.078 mM	6.80	1.93E-04	1.76E-04	3.17	4.32E-05
Na 1.0 mM	6.80	1.86E-04	1.68E-04	3.49	4.58E-05
Na 2.0 mM	6.80	1.81E-04	1.65E-04	3.19	4.05E-05
Na 4.0 mM	6.80	1.75E-04	1.55E-04	3.32	4.00E-05
Na 6.0 mM	6.80	1.59E-04	1.42E-04	3.61	4.17E-05
Na 8.0 mM	6.80	1.54E-04	1.36E-04	3.61	4.02E-05
Na 10 mM	6.80	1.36E-04	1.46E-04	3.4	3.67E-05
Na 12 mM	6.80	1.35E-04	1.33E-04	3.23	3.38E-05
Na 14 mM	6.80	1.40E-04	1.29E-04	3.55	3.62E-05
K 0.078 mM	6.80	1.93E-04	1.76E-04	3.17	4.32E-05
K 0.3 mM	6.80	2.00E-04	1.97E-04	3.55	4.77E-05
pH 5.8 (1)	5.95	1.88E-04	1.26E-04	2.52	3.08E-05
pH 6.3 (1)	6.28	1.88E-04	1.26E-04	2.65	3.23E-05
pH 6.8 (1)	6.74	1.88E-04	1.27E-04	2.81	3.42E-05
pH 7.3 (1)	7.24	1.86E-04	1.26E-04	2.61	3.08E-05
pH 7.8 (1)	7.53	1.86E-04	1.24E-04	3.07	3.39E-05
pH 8.3 (1)	8.13	1.80E-04	1.17E-04	3.27	2.16E-05
pH 5.8 (2)	5.72	2.01E-04	1.34E-04	2.32	2.99E-05
pH 6.3 (2)	6.07	1.97E-04	1.24E-04	2.57	3.30E-05
pH 6.8 (2)	6.63	1.95E-04	1.27E-04	2.72	3.45E-05

[a] see Deleebeeck et al. (2005) for sample codes, in the pH test series: (1) refers to solutions without MOPS, (2) are solutions with 3.6 mM added MOPS.
[b] chemical activities of Ca, Mg
[c] *=not used for BLM development

Based on this, only stability constants for Ca and Mg binding to the *Daphnia magna* biotic ligand were calculated. They were calculated slightly higher than in Deleebeeck et al. (2005) due to slight differences in calculated Ni^{2+} activities, i.e. log K_{CaBL} = 3.10 and log K_{MgBL} = 2.47.

It is now appropriate to introduce the term $LC50^{*}_{Ni2+,i}$, which is the LC50 corrected for Ca and Mg competition in a given test solution *i*:

$$LC50^{*}_{Ni^{2+},i} = \frac{LC50_{Ni^{2+},i}}{1 + K_{CaBL} \cdot \left(Ca^{2+}\right)_i + K_{MgBL} \cdot \left(Mg^{2+}\right)_i} \qquad \text{(Eq. 4.1)}$$

In theory, the denominator could be extended with extra terms for other competing ions, but this was omitted here since Na^{+}, K^{+} or H^{+} exerted no significant competition effects over the investigated Na, K, or pH ranges. Therefore the asterisk * specifically refers to the correction for Ca and Mg competition effects and the $LC50^{*}_{Ni2+}$ represents the theoretical LC50 expressed as Ni^{2+} activity when Ca or Mg competition is negligible. The $LC50^{*}_{Ni2+,i}$ was calculated for all test solutions *i* (except at total Ca or Mg > 3 mM, total hardness > 325

mg $CaCO_3$/L) and the geometric mean of those values was used as the final model parameter for the acute *D. magna* BLM:

$$LC50^{*}_{Ni^{2+}} = \sqrt[n]{\prod_{i=1}^{n} LC50^{*}_{Ni^{2+},i}} \qquad \text{(Eq. 4.2)}$$

The absence of the subscript index '*i*' in the left-hand term of this equation indicate that this is a final bioavailability model parameter, optimized for a given set of toxicity data in *n* different test solutions. All *D. magna* acute Ni-BLM parameters are given in Table 4.2. Naturally, there is some uncertainty around this value, as indicated by the range of individual $LC50^{*}_{Ni2+,i}$ values for the individual test solutions (Table 4.2), but the impact of this uncertainty is reflected in the $LC50_{dissolved}$-based prediction errors (see Table 4.3, Figure 4.2). The magnitude of these prediction errors are indicative of how well the model is suited to predict toxicity, despite of the uncertainty associated with this model parameter.

Table 4.2 Parameters of the acute *D. magna* BLM

Model parameter	Value
log K_{NiBL}	4.00 [a]
log K_{CaBL}	3.10
log K_{MgBL}	2.47
$LC50^{*}_{Ni2+}$ (µM)	25.8 (16.2-36.5) [b]
f^{50}_{NiBL}	0.205 (0.139-0.267) [c]

[a] Assumed equal as in Wu et al. (2001), this assumption does not affect predictions of LC50's

[b] range (minimum and maximum) values for the separate exposure media between parentheses

[c] Calculated using equation 4.5

The model parameters in Table 4.1 can be used to predict $LC50_{Ni2+,i}$ for any test solution *i* with any given water chemistry (after rearranging Eq. 4.1):

$$LC50_{Ni^{2+},i} = LC50^{*}_{Ni^{2+}} \cdot \left\{1 + K_{CaBL} \cdot \left(Ca^{2+}\right)_i + K_{MgBL} \cdot \left(Mg^{2+}\right)_i\right\} \qquad \text{(Eq. 4.3)}$$

The prediction of $LC50_{Ni2+,i}$ can also be written in terms of a full BLM equation (De Schamphelaere and Janssen, 2002):

$$LC50_{Ni^{2+},i} = \frac{f_{NiBL}^{50}}{\left(1 - f_{NiBL}^{50}\right) \cdot K_{NiBL}} \cdot \left\{1 + K_{CaBL} \cdot \left(Ca^{2+}\right)_i + K_{MgBL} \cdot \left(Mg^{2+}\right)_i\right\} \quad \text{(Eq. 4.4)}$$

In this equation, f^{50}_{NiBL} is the fraction of the BL sites occupied by Ni at 50% effect. It is noted that, computationally, equations 4.3 and 4.4, yield identical predictions, since the values of f^{50}_{NiBL} and K_{NiBL} are linked through:

$$LC50^{*}_{Ni^{2+}} = \frac{f_{NiBL}^{50}}{\left(1 - f_{NiBL}^{50}\right) \cdot K_{NiBL}} \quad \text{(Eq. 4.5)}$$

The predicted $LC50_{Ni2+,i}$ for any test solution *i*, predicted with equation 4.3 are inserted into WHAM VI with the Ni-input set to 'activity'. The speciation model then calculates the dissolved Ni associated with this Ni^{2+}-activity, yielding the LC50 as dissolved Ni, i.e. $LC50_{dissolved,i}$. It is important that, before this step, the activities of Ca^{2+} and Mg^{2+} in each solution *i* need to be calculated, so that equation 4.3 can be used to predict the $LC50_{Ni2+,i}$ that needs to be inserted in WHAM VI.

The procedure outlined with equations 4.1 to 4.5 is the recommended procedure for the normalization of (a set of) existing toxicity data at (a set of) given water chemistries to any other given water chemistry.

In the following sections we will investigate the applicability of this acute *D. magna* BLM to several acute toxicity datasets with.

(i) *D. magna* in natural waters (Deleebeeck et al., 2005)
(ii) *D. magna* in well water with adjusted hardness (Chapman et al., 1980)
(iii) *C. dubia* in synthetic water at different hardness (Keithly et al., 2004)
(iv) *C. dubia* in synthetic water at different pH (Parametrix, 2004b)
(v) *C. dubia* in synthetic water at different pH (Schubauer-Bérigan et al., 1993)
(vi) *C. dubia* in natural waters (this study, see section 3.2)

4.2.2. Application of the acute toxicity model for *D. magna* to other datasets with *D. magna*

4.2.2.1. *D. magna* in natural waters (Deleebeeck et al., 2005)

These datasets report 48h-$LC50_{dissolved}$ for a range of spiked natural surface waters with the same laboratory clone of *D. magna* as the one used for the model development (Table 4.1). This dataset is reported in Deleebeeck et al. (2005) and contains a sub-set of test data reported previously by Bossuyt et al. (2001). The dataset covers a large range of DOC (1.8 to 25.8 mg/L), hardness (13 to 266 mg $CaCO_3$/L), Ca (3.0 to 73 mg/L), Mg (1.1 to 21 mg/L), pH (6.0 to 8.1), and alkalinity (0.4 to 161 mg $CaCO_3$/L) (Deleebeeck et al. (2005).

The input file for WHAM VI for this dataset is given in Annex 7. First, speciation calculations indicated that in the acute experiments of Deleebeeck et al. (2005) Al^{3+} was in equilibrium with colloidal $Al(OH)_3$ at the 48h-LC50s of Ni. Although no reliable Al measurements were available for the Bossuyt et al. (2001) subset, we also assumed Al^{3+} in equilibrium with $Al(OH)_3$, as this is a better option than not to include Al at all (see section 3.2). However, differences in calculated Ni^{2+} activity between Al^{3+} assumed in equilibrium with $Al(OH)_3$ and $Al_{dissolved}$ assumed 'zero' were less than 1% (data not shown). At the 48h-LC50 levels we only predicted between 5 and 23% of the dissolved Ni to be bound to DOC, indicating that the effect of DOC on acute Ni toxicity to *D. magna* is of limited importance.

Table 4.3 gives an overview of the calculated speciation of the solutions. The activities of Ca^{2+} and Mg^{2+} were used to predict 48h-$LC50_{Ni2+}$ using equation 4.3, and these values were inserted as input into WHAM VI to predict 48h-$LC50_{dissolved}$.

Table 4.3 Observed (obs) and predicted (pred) 48h-$LC50_{dissolved}$ and 48h-$LC50_{Ni2+}$ of Ni to *D. magna* in natural waters rom Deleebeeck et al. (2005) and Bossuyt et al. (2001).

Surface water [a]	pH	Mg^{2+} (M)	Ca^{2+} (M)	HCO_3^- (M)	$LC50_{obs}$ (µg/L)	$LC50_{pred}$ [b] (µg/L)	$LC50_{Ni2+,obs}$ (M)	$LC50_{Ni2+,pred}$ (M)
Bihain (1)	6.23	4.78E-05	6.78E-04	1.20E-05	2230	3807 *	2.78E-05	4.82E-05
Bihain (1)	6.21	4.78E-05	6.78E-04	1.16E-05	2110	3805 *	2.62E-05	4.82E-05
Ankeveen (1)	7.14	2.48E-04	8.78E-04	2.47E-04	5250	5798	5.01E-05	5.62E-05
Ankeveen (1)	7.14	2.48E-04	8.78E-04	2.47E-04	5440	5798	5.22E-05	5.62E-05
Markermeer (1)	7.92	5.50E-04	1.17E-03	1.99E-03	5490	8655	4.21E-05	6.78E-05
Markermeer (1)	7.96	5.50E-04	1.16E-03	1.99E-03	6130	8764	4.66E-05	6.78E-05
Mole (1)	7.58	2.46E-04	9.13E-04	1.62E-03	5010	5916	4.83E-05	5.73E-05
Mole (1)	7.62	2.46E-04	9.13E-04	1.63E-03	5130	5956	4.91E-05	5.73E-05
Clywydog (1)	5.94	5.06E-05	6.72E-05	8.64E-06	1040	1934 *	1.51E-05	2.84E-05
Clywydog (1)	5.96	5.06E-05	6.72E-05	8.94E-06	980	1934 *	1.42E-05	2.84E-05
Ankeveen (2)	6.79	1.76E-04	6.04E-04	2.25E-04	5720	5042	5.36E-05	4.68E-05
Bihain (2)	6.15	3.74E-05	7.75E-05	1.59E-04	860	2192 *	1.04E-05	2.86E-05
Brisy (2)	7.09	1.18E-04	1.06E-04	2.44E-04	2010	2279	2.64E-05	3.01E-05

Markermeer (2)	8.09	3.42E-04	7.58E-04	2.18E-03	4520	7910	2.94E-05	5.30E-05
Regge (2)	7.70	2.09E-04	9.35E-04	2.82E-03	6300	7752	4.65E-05	5.78E-05
Voyon (2)	8.02	2.13E-04	6.62E-04	2.21E-03	3840	5946	3.11E-05	4.89E-05

[a] (1) Data from Bossuyt et al. (2001); (2) data from Deleebeeck et al. (2005)

[b] 48h-$LC50_{pred}$ which overestimate the 48h-$LC50_{obs}$ by a factor of close to 2 or more than 2 are marked with a *

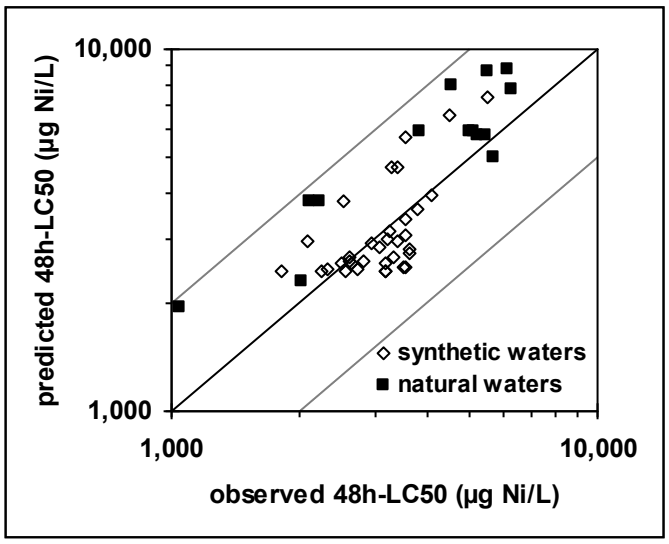


Figure 4.2 Predictive capacity of the acute *D. magna* model (Table 4.2) as shown by predicted vs. observed 48h-LC50. Data with synthetic waters from Deleebeeck et al. (2005, see also Table 4.1). Data with natural waters from Deleebeeck et al. (2005) and Bossuyt et al. (2001) (see also Table 4.3).

Figure 4.2 illustrates that the model is well-calibrated to the synthetic waters in the first place, but of course the true value of a model is that it should work in natural waters too. Table 4.3 and Figure 4.2 illustrate that 15 out of 16 of the 48h-$LC50_{dissolved}$ are predicted by an error of less than factor 2. Toxicity was underestimated (48h-LC50 overestimated) by a factor of more than 2 for Bihain (2) and by factors close to 2 for Bihain (1) and Clywydog (1) (Table 4.3). Compared with the other test waters, all these waters are characterized by very low Mg concentrations, i.e. between 1.1 and 1.5 mg/L, which is quite far below the lowest Mg concentration in test solutions used for model development, i.e. 6 mg Mg/L. Since acute Ni exposure is known to result in a loss of whole body Mg in *D. magna* (Pane et al., 2003) and because only the condition of such low Mg concentrations may have resulted in a net loss of body Mg, the daphnids may have been forced into a situation where they are extra sensitive to Ni stress. Additionally, the two test waters with the lowest hardness, Bihain (2) and Clywydog (1), were characterized by hardness levels of 14 and 13 mg $CaCO_3$/L respectively, which is well below the lowest hardness tested for model development, i.e. 50 mg $CaCO_3$/L and far below hardness levels which are considered 'normal' by common *D. magna* test protocols (e.g., Environment Canada indicates that below 25 mg $CaCO_3$/L, *D. magna* may be

unduely stressed). Thus, it is concluded that in 'normal' test waters, the acute *D. magna* model exhibits good predictive capacity for use in spiked natural surface waters.

4.2.2.2 *D. magna* in well waters with adjusted hardness (Chapman et et al., 1980)

An additional analysis was carried out on the experiments carried out with *D. magna* in well water with adjusted hardness levels (Chapman et al., 1980). They observed an increasing trend of 48h-LC50s (627 to 4,970 µg/L) with increasing hardness (50 to 200 mg $CaCO_3$/L) (Table 4.4). However, the interpretation is not very easy because other parameters (such as pH) co-varied with hardness, because not all experiments were conducted simultaneously, and because organisms were acclimated (for a non-reported duration) to the hardness before being tested.

We wanted to assess, given these uncertainties, to what extent our developed acute BLM could correctly reproduce the 48h-LC50s if only the sensitivity of the Chapman et al. (1980) *D. magna* strain was adjusted. As in other BLM applications (e.g., Santore et al., 2001; Santore et al., 2002) differences in sensitivity are dealt with by calibrating the f^{50}_{NiBL} (or the 48h-$LC50^{*}_{Ni2+}$) to the dataset, assuming other BLM-parameters (i.e. log K's) identical, by minimizing prediction errors. This is performed using equations 4.1 and 4.2.

The full composition of the test waters is given in Annex 8. Since the test water was derived from a natural source, Al and Fe(III) must have been present and they were taken into account as well, by assuming equilibrium with their colloidal hydroxy-precipitates. DOC in the well water used was assumed to be 1.3 mg/L (Santore et al., 2002) and to behave similar as surface water DOC (i.e., 40% active fulvic acid). The chemistry that was inserted into WHAM VI is given in Annex 8.

After speciation calculations we found 48h-$LC50^{*}_{Ni2+}$ = 10.5 µM, which is slightly lower than the value obtained for our *D. magna* clone, i.e. 25.6 µM. The former value was used for the prediction of $LC50_{dissolved,i}$ using equation 4.3 and WHAM VI (as explained above). Prediction errors were between factor 1.1 and 2.0 for all tests conducted (See Table 4.4).

Hence, the developed *D. magna* acute BLM (Table 4.2) does also reduce the uncertainty due to differences in water chemistry in another *D. magna* clone. Apparently, the importance of acclimating the organisms to the test water hardness does not affect Ni toxicity to such an extent that it causes prediction errors > factor 2.

Table 4.4 Chemical speciation of natural waters at 48h-LC50s of Ni to *D. magna* from Chapman et al. (1980)

Test water	pH	Mg^{2+} (M)	Ca^{2+} (M)	HCO_3^- (M)	$LC50_{obs}$ (µg/L)	$LC50_{pred}$ (µg/L)	$LC50_{Ni2+,obs}$ (M)	$LC50_{Ni2+,pred}$ (M)
Hardness50(1)	7.7	1.13E-04	2.72E-04	8.22E-04	1800	1264	2.07E-05	1.44E-05
Hardness50(2)	7.7	1.14E-04	2.72E-04	8.06E-04	627	1265	6.98E-06	1.44E-05
Hardness100(1)	7.9	1.98E-04	5.01E-04	1.50E-03	2360	1914	2.19E-05	1.77E-05
Hardness100(2)	8.2	1.98E-04	4.99E-04	1.51E-03	1920	2113	1.60E-05	1.77E-05
Hardness200(1)	8.3	3.27E-04	8.51E-04	2.84E-03	4970	3878	2.92E-05	2.27E-05

4.2.3. Application of the acute toxicity model for *D. magna* to datasets with *C. dubia* and development of the *C. dubia* acute Ni-BLM

4.2.3.1. Effect of hardness on acute Ni toxicity to *C. dubia* (Keithly et al., 2004)

We investigated if the effect of hardness on acute Ni toxicity to *Ceriodaphnia dubia* could be predicted with the acute *D. magna* BLM, characterized by log K_{CaBL} = 3.10 and log K_{MgBL} = 2.47 (Table 4.2). Keithly et al. (2004) found in synthetic waters that 48h-$LC50_{dissoolved}$ for *C. dubia* increased from 81 to 400 µg Ni/L between hardness levels of 50 to 253 mg $CaCO_3$/L. These values are considerably lower than the 48h-LC50 values observed for *D. magna* (Deleebeeck et al., 2005) in synthetic waters, i.e. between 1,820 and 5,500 µg/L. This is points to a higher acute sensitivity of *C. dubia* to Ni. This corroborates with the trend that smaller organisms tend to be more sensitive to metals than larger ones (Grosell et al., 2002; Bossuyt and Janssen, 2005; Muyssen et al., 2005). This also indicates that the *D. magna* BLM should be calibrated to account for this sensitivity difference, i.e. by calibrating f^{50}_{NiBL} or $LC50^{*}_{Ni2+}$, the 'sensitivity parameters' of the BLM. This was performed using equations 4.1 and 4.2. The results of this calibration and the predictions are given in Table 4.5. The full chemistry of their test solutions is reported in Annex 9.

Table 4.5 Chemical speciation of natural waters at 48h-LC50s of Ni to *C. dubia* at different hardness (Keithly et al., 2004). For observed vs. predicted 48h-LC50s see also Figure 4.3.

Hardness (mg/L)	pH	Mg^{2+} (M)	Ca^{2+} (M)	$LC50_{Ni2+,obs}$ (M)	$LC50_{obs}$ (µg/L)	$LC50_{pred}$ [a] (µg/L)	$LC50_{pred}$ [b] (µg/L)
50	7.66	7.50E-05	2.98E-04	2.07E-05	81	127	107
113	7.7	2.47E-04	5.21E-04	6.98E-06	148	172	171

161	7.61	3.33E-04	6.43E-04	2.19E-05	261	208	219
253	7.8	4.85E-04	9.64E-04	1.60E-05	400	275	310

[a] log K_{CaBL} = 3.10, log K_{MgBL} = 2.47, $LC50^{*}_{Ni2+}$ = 1.09 µM
[b] log K_{CaBL} = 3.3, log K_{MgBL} = 3.3, $LC50^{*}_{Ni2+}$ = 0.740 µM

An $LC50^{*}_{Ni2+}$ = 1.09 µM was found. Although all 48h-$LC50_{disolved}$ were predicted by an error between factor 1.2 and 1.6 (Table 4.5), there was a trend of underestimating the importance of the hardness effect (Figure 4.3), i.e. the predicted slope of the hardness effect was slightly lower than the observed slope. By increasing log K's to for example log K_{CaBL} = log K_{MgBL} = 3.3 and calibrating the 48h$LC50^{*}_{Ni2+}$ to 0.740 µM, the predicted slope was closer to the observed one (Figure 4.3). Other combinations of (even higher) adjusted log K_{CaBL} and log K_{MgBL} values were possible that resulted in similar fits, but it was chosen not to adjust the constants further, since this would mean modelling experimental noise. Indeed, the adjusted log K's resulted in predicted $LC50_{dissolved}$ which were all within the 95% confidence interval of the observed $LC50_{dissolved}$ (Figure 4.3). The separate effects of Ca and Mg could not be determined because the Ca:Mg ratio in the experiments was constant. Additional experimentation is needed to estimate the individual effects of Ca and Mg on acute Ni toxicity to *C. dubia*.

Summarizing, the effects of Ca and/or Mg on acute Ni toxicity to *C. dubia* and *D. magna* are fairly similar, although there are indications that the more sensitive *C. dubia* may experience a (slightly) larger protective effect from increased hardness. However, the difference is not important enough to result in large prediction errors. Indeed, prediction errors between factor 1.2 and 1.6 were in the same range as when the developed acute *D. magna* BLM was applied to another clone of the same species, i.e. 1.1 to 2.0 (see section 4.2.2).

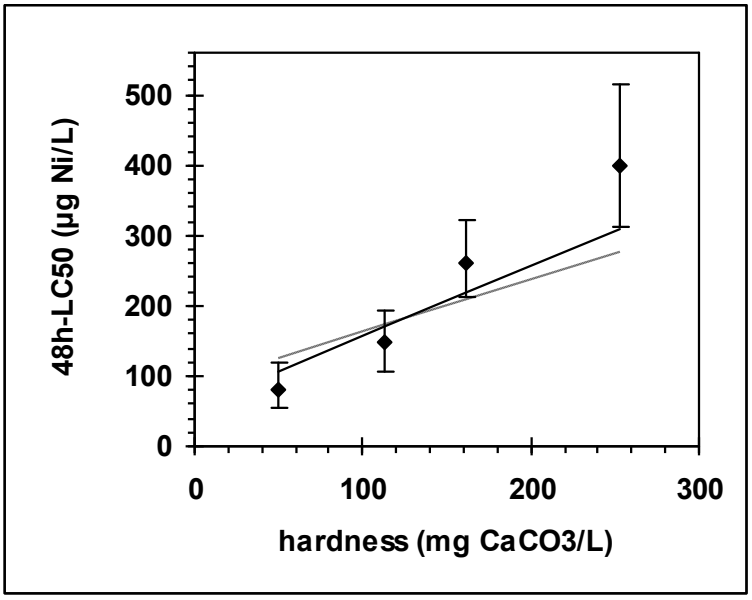


Figure 4.3 Acute 48h-LC50s of Ni to *Ceriodaphnia dubia* as a function of water hardness. Diamonds are observations reported in Keithly et al. (2004). The dashed line represents the BLM-predicted values, using log K_{CaBL} and log K_{MgBL} for *D. magna* (Table 4.2), and adjusted sensitivity. The full line represents the BLM-predicted values, using adjusted log K_{CaBL} = log K_{MgBL} = 3.3 and adjusted sensitivity.

4.2.3.2. Acute Ni toxicity to *C. dubia* in natural waters (this study)

As mentioned earlier, we wanted to separate the effects of pH from those of hardness, since they were positively correlated in the natural waters tested with *C. dubia* in the present study (see section 3.2). Therefore, using the data in Table 3.8, 48h-$LC50^*_{Ni2+,i}$ and 48h-$pNi^*_{50,i}$ were calculated using equation 4.1, with

$$48h\text{-}pNi^*_{50,i} = -\log\{48h\text{-}LC50^*_{Ni2+,i}\} \qquad \text{(Eq. 4.6)}$$

The uncertainty about the exact log K_{CaBL} and log K_{MgBL} for acute *C. dubia* Ni exposures was taken into account in this analysis by considering scenarios A and B: (A) log K_{CaBL} = 3.14, log K_{MgBL} = 2.47 (*D. magna* BLM-constants), (B) log K_{CaBL} = log K_{MgBL} = 3.3 (better fit to hardness relation observed by Keithly et al., 2004, see above).

Table 4.6 Chemical speciation of natural waters at 48h-LC50s of Ni to *C dubia* and LC50s corrected for Ca and Mg competition

				Scenario A	Scenario B
Site	**Ca^{2+} (M)**	**Mg^{2+} (M)**	**$LC50_{Ni2+,obs}$ (M)**	**$LC50^*_{Ni2+,obs,A}$ (M)**	**$LC50^*_{Ni2+,obs,B}$ (M)**
Ankeveen	6.64E-04	1.96E-04	5.08E-07	2.68E-07	1.87E-07
Bihain	9.13E-05	3.38E-05	2.54E-07	2.26E-07	2.03E-07
Brisy	2.05E-04	1.28E-04	3.95E-07	3.05E-07	2.37E-07
Eppe	6.08E-04	1.85E-04	1.55E-07	8.49E-08	5.98E-08

Markermeer	9.16E-04	3.92E-04	3.72E-07	1.64E-07	1.03E-07
Regge	1.08E-03	2.11E-04	5.74E-07	2.36E-07	1.60E-07
			geo-mean	*1.98E-07*	*1.44E-07*
Site	**pH**	**$pHCO_3^-$**	**$pNi_{50,obs}$**	**48h-$pNi^*_{50,obs,A}$**	**48h-$pNi^*_{50,obs,B}$**
Ankeveen	7.51	3.12	6.29	6.57	6.73
Bihain	6.34	4.32	6.59	6.65	6.69
Brisy	7.45	3.41	6.40	6.52	6.62
Eppe	7.95	2.79	6.81	7.07	7.22
Markermeer	8.04	2.76	6.43	6.79	6.99
Regge	8.00	2.57	6.24	6.63	6.80

The 48h-$LC50^*_{Ni2+,i}$ for all test solutions under scenario A were used to calculate the final model $LC50^*_{Ni2+}$, calibrated to the sensitivity of our *C. dubia* strain (the 'geometric mean' of the $LC50^*_{Ni2+,i}$ in Table 4.6). We found $LC50^*_{Ni2+,A}$ = 0.198 µM and $LC50^*_{Ni2+,B}$ = 0.144 µM, which is a factor 4 to 5 lower than the values obtained from the Keithly et al. (2004) dataset, i.e. 1.09 and 0.740 µM, respectively. Apparently, our *C. dubia* are clearly more sensitive than the strain used by Keithly et al. (2004). This may partly be explained by the fact that we have used 2 to 8 hour old juveniles, whereas Keithly et al. (2004) used <24 hour old juveniles. Hoang et al. (2004) demonstrated that larval fathead minnows were acutely more sensitive to Ni than 28d-old fathead minnows, thus corroborating with the idea that younger (and thus smaller) organisms may be more sensitive to acute Ni toxicity.

Now, under both scenarios, we again plotted pNi^*_{50} against pH to see what the residual effects of pH, separated from the hardness effect is (Figure 4.4). Although no effect of pH is observed between pH 6.3 and 7.5, one may suggest that at pH ~ 8 the toxicity seems slightly higher than at pH between 6.3 and 7.5. This becomes more obvious under scenario B, i.e. when Ca and Mg competition are modelled with potentially more appropriate constants for *C. dubia* This could be substantiated, under scenario B, by the observation that at pH ~ 8 $LC50^*_{Ni2+}$ is on average (of three values) 2-fold lower than at pH between 6.3 and 7.5 (compare values in Table 4.6), i.e. 0.10 vs. 0.21 µM. Interestingly, this closely resembles the observations made for the individual effect of pH on acute Ni toxicity to *D. magna*, which does also become apparent at pH around 8 and higher (Deleebeeck et al., 2005; see Table 4.1 and Figure 4.1). Also Schubauer-Berigan et al. (1993) observed a very strong pH effect above pH > 8, compared to pH ~ 7.5. All these data together suggest a trend of increased toxicity at higher pH values, starting at a pH around 8 for both *D. magna* and *C. dubia*.

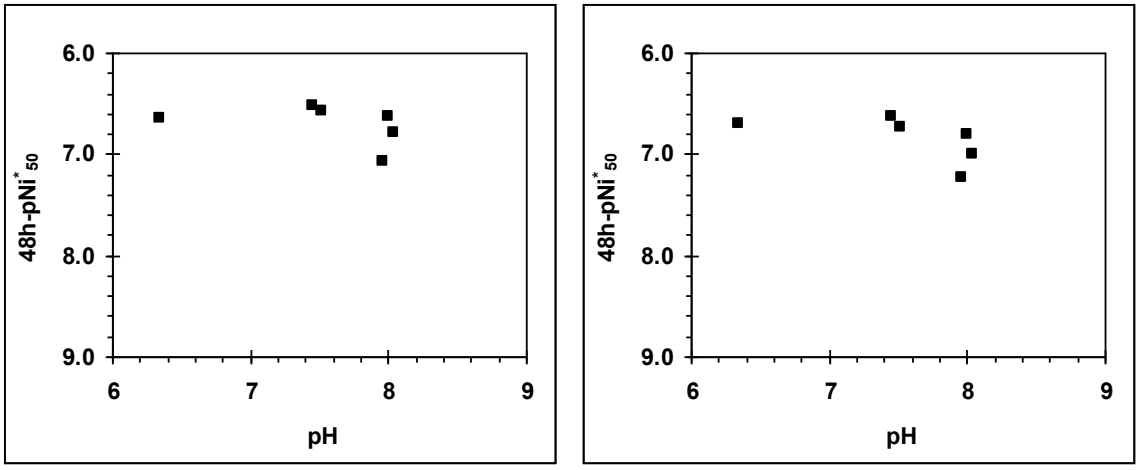


Figure 4.4 Acute Ni toxicity to *Ceriodaphnia dubia* in natural waters, corrected for Ca and Mg competition according to scenarios A (left) and B (right), plotted against the pH of the natural waters.

Using both scenarios, we now determined how well the acute Ni-BLM could reproduce the acute Ni toxicity to *C. dubia* in natural waters. Both models are summarized in Table 4.7. It is noted that the pH effect is currently not incorporated into this model, because the pH effect is quite limited, up to pH ~ 8 (or 8.2 for *D. magna*) and because the type of effect observed cannot be modelled by the usual BLM-like single-site H^+-competition.

Table 4.7 Model parameters of the acute *C.dubia* model

Model parameter	*D. magna*	*C. dubia* (A)	*C. dubia* (B)
log K_{NiBL}	4.0	4.0	4.0
log K_{CaBL}	3.10	3.10	3.3
log K_{MgBL}	2.47	2.47	3.3
$LC50^*_{Ni2+}$ (µM)	25.8[a]/10.6[b]	1.09[c]/0.198 [d]	0.740[c]/0.144 [d]
f^{50}_{NiBL} (as %)	20.5/9.58	1.08/0.198	

[a] For the clone used by Deleebeeck et al. (2005)
[b] For the clone used by Chapman et al. (1980)
[c] For the strain used by Keithly et al. (2004)
[d] For the strain used in the present study

The observed and predicted 48h-LC50s are reported in Table 4.8 and in Figure 4.5. Predictions errors were very similar for both scenarios, i.e. on average 1.3 (1.1 to 2.0) and 1.4 (1.1 to 2.0) for scenarios A and B, respectively. It is concluded that the acute *D. magna* Ni-BLM can be used to predict acute Ni toxicity to *C. dubia* in natural waters, but that, given the suspected pH effect at pH>8, it is not recommended to use the current model in solutions well over pH 8.

Table 4.8 Observed and predicted acute Ni toxicity to *C. dubia* using two scenarios

		Scenario A	Scenario B			
Site	**$LC50_{Ni2+,obs}$ (M)**	**$LC50_{Ni2+,pred,A}$ [a] (M)**	**$LC50^{*}_{Ni2+,pred,B}$ [a] (M)**	**$LC50_{diss,obs}$ (µg/L)**	**$LC50_{diss,pred,A}$ (µg/L)**	**$LC50_{diss,pred,B}$ (µg/L)**
Ankeveen	5.08E-07	3.75E-07	3.91E-07	183.0	148.2	152.6
Bihain	2.54E-07	2.23E-07	1.80E-07	35.2	31.5	26.1
Brisy	3.95E-07	2.57E-07	2.40E-07	50.8	35.3	33.3
Eppe	1.55E-07	3.61E-07	3.72E-07	34.6	68.7	70.3
Markermeer	3.72E-07	4.50E-07	5.20E-07	88.7	103.5	116.4
Regge	5.74E-07	4.81E-07	5.16E-07	161.0	140.3	148.1

[a] Inserted in WHAM VI as Ni^{2+} activity to predict LC50 as dissolved Ni

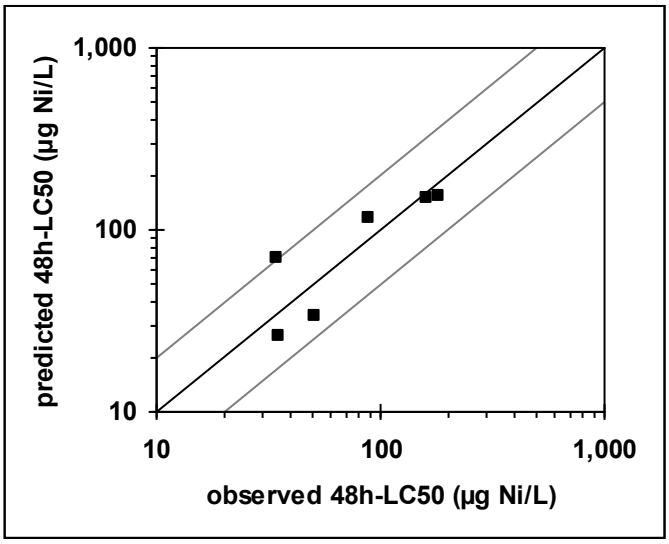


Figure 4.5 Predictive capacity of the acute *C. dubia* model (Table 4.7, scenario B) in natural waters as shown by predicted vs. observed 48h-LC50. Data are from the present study.

4.2.3.3. Comparison of pH effect with other datasets (Shubauer-Berigan et al.,1993; Parametrix, 2004)

Schubauer-Berigan et al. (1993) observed a marked decrease of the 48h-$LC50_{total}$ for *C. dubia* with increased pH. They report a 48h-$LC50_{total}$ of >200 µg Ni/L at pH 6.0 to 6.5, 140 µg/L at pH 7.0 to 7.3 and 13 µg/L at pH 8.5 to 8.7. This dataset confirms that Ni toxicity to *C. dubia* is only slightly affected between pH 6 and 7.5, but that acute Ni toxicity is markedly higher at pH levels well exceeding pH 8. Unfortunately, the authors reported LC50s based on total recoverable Ni instead of dissolved Ni, and the presence of YTC solids (6 mg/L) makes estimates of dissolved Ni and Ni speciation problematic. Also, the addition of food to acute toxicity tests with daphnids is no common practice anymore and may alter Ni toxicity and

also bioavailability relations. Indeed, when Mg influx via the water is affected by Ni (the suggested toxicity mechanism of Ni, Pane et al., 2003), this effect may potentially be counteracted by extra absorption of Mg via the food. When the toxic mechanism is affected by food, bioavailability relations may also be affected. This makes quantitative comparisons with other 'non-fed' datasets problematic.

In response to this, Parametrix (2004) recently finished a research project to investigate the separate pH effect on acute Ni toxicity to *C. dubia* in the absence of added food. They have observed a marked decrease of 48h-$LC50_{dissolved}$ between 266.3 and 23.6 µg Ni/L between pH 6 and 9 (Table 1 in Parametrix, 2004). Four test solutions (pH 6 to 9) were tested with addition of 3.6 mmol/L MOPS buffer, and two test solutions (pH 8 and 9) were also tested without addition of MOPS buffer. We have calculated the speciation of their test solutions using WHAM VI. Initial solution compositions were obtained from Parametrix by personal communication. Inorganic carbon was estimated from measured 'final' alkalinity (i.e. test solution after MOPS addition and/or pH adjustment, reported in Table 1 of Parametrix, 2004), taking into account that MOPS also contributes to the total alkalinity which measured with an acid titration method. Added Na (from NaOH used for pH adjustments) was estimated by charge balancing the solution. The full chemistry input data for speciation calculations is given in Annex 10. Table 4.10 gives the relevant speciation output.

Table 4.10 Chemical speciation of synthetic test solutions at the 48h-LC50 of Ni to *C dubia* and LC50s corrected for Ca and Mg competition (data from Parametrix, 2004)

					Scenario A	Scenario B
Test ID	**Ca^{2+} (M)**	**Mg^{2+} (M)**	**Na^{+} (M)**	**$LC50_{Ni2+,obs}$ (M)**	**$LC50^{*}_{Ni2+,obs,A}$ (M)**	**$LC50^{*}_{Ni2+,obs,B}$ (M)**
pH 6 - MOPS	2.99E-04	4.30E-04	5.70E-04	3.06E-06	2.04E-06	1.25E-06
pH 7 - MOPS	2.89E-04	4.16E-04	1.91E-03	2.20E-06	1.48E-06	9.16E-07
pH 8 - MOPS	2.41E-04	3.52E-04	4.41E-03	1.85E-06	1.32E-06	8.49E-07
pH 9 - MOPS	2.88E-04	3.38E-04	4.83E-03	4.30E-07	2.94E-07	1.91E-07
pH 8 - no MOPS	2.87E-04	4.16E-04	1.49E-03	9.49E-07	6.39E-07	3.95E-07
pH 9 - no MOPS	2.73E-04	4.03E-04	1.73E-03	1.22E-07	8.35E-08	5.20E-08
Site	**pH**			**$pNi_{50,obs}$**	**48h-$pNi^{*}_{50,obs,A}$**	**48h-$pNi^{*}_{50,obs,B}$**
pH 6 - MOPS	6.3			5.51	5.69	5.90
pH 7 - MOPS	7.1			5.66	5.83	6.04
pH 8 - MOPS	8.0			5.73	5.88	6.07
pH 9 - MOPS	8.9			6.37	6.53	6.72
pH 8 - no MOPS	8.1			6.02	6.19	6.40
pH 9 - no MOPS	8.9			6.91	7.08	7.28

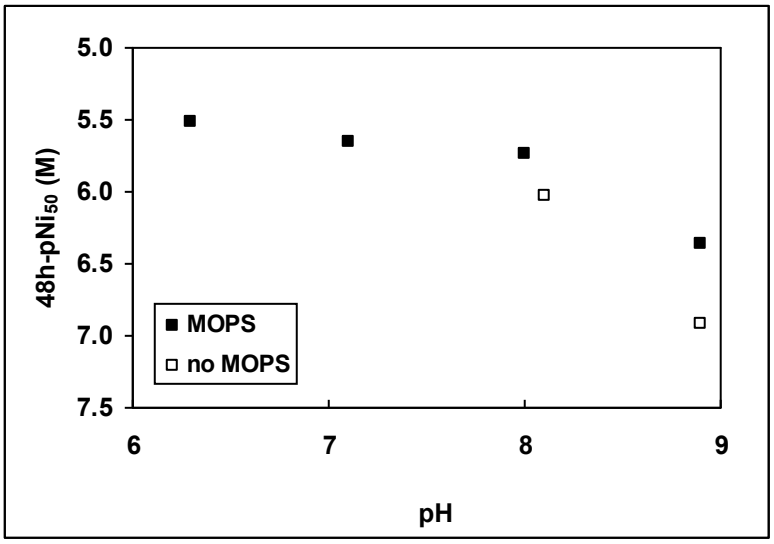


Figure 4.6 The effect of pH on acute 48h-pNi_{50} for *C. dubia*, with and without added MOPS; dataset from Parametrix (2004)

On the basis of the data in Table 4.10 and Figure 4.6, following observations can be made:

(i) In the test solutions with added MOPS, between pH 6 and pH 8.1, 48h-$LC50_{Ni2+}$ were hardly affected (less than 1.5-fold decrease and this corroborates with data observed for *D. magna* (see section 4.2.1.) and *C. dubia* in natural waters (present study, see section 4.3.3.2).

(ii) Between pH 8 and 9, 48h-$LC50_{Ni2+}$ were further reduced by 4.4 (MOPS-buffered) to 7.6-fold (not MOPS buffered). Again this confirms the marked effect of increasing pH well over pH 8 on acute Ni toxicity to daphnids.

(iii) 48h-$LC50_{Ni2+}$ were a factor of 2 (at pH 8) to 3.5 (at pH 9) higher in the MOPS buffered test media. These test solutions only differed in their MOPS and Na content (Na higher in MOPS-buffered test solutions).

Thus, considering the data with *C. dubia* in Table 4.10, either MOPS or Na ameliorates acute Ni toxicity, and this amelioration may be pH dependent. The fact that the protective effect of Na (or MOPS) is higher at higher pH and that the pH effect is smaller at higher Na (tests with MOPS) may suggest a competitive effect of both Na^+ and H^+ ions at the same Ni-BL site. Those observations were not made for *D. magna* testing, but it is noted that the effects of MOPS or Na were investigated at other pH levels, i.e. 6 to 6.8 and ~6.8, respectively. This may suggest that the pH effect, the Na effect, the MOPS effect and/or the interactive effects of these parameters may be species dependent.

Overall, however, the observed pH effects in both *C. dubia* and *D. magna* are not in line with H^+ competitive effects at a single BL-site. However, up to a pH of approximately 8 (or slightly higher), it appears that bioavailability effects can be quite accurately predicted by assuming only Ca and Mg competition and considering pH effects negligible. More research is clearly needed to fully explain the effect of bioavailability modifying factors at pH > 8, including the effects of pH, Ca, Mg, and Na.

4.3. Development of a chronic Ni toxicity model for aquatic invertebrates

4.3.1. Introducing remarks

This section will report on the further refinement and development of chronic Ni bioavailability models. Similar to section 4.2 we will first re-analyze the Deleebeeck et al. (2005) study to re-evaluate the individual effects of Ca, Mg and pH, using WHAM VI as the speciation model, also refining the chronic Ni-BLM. We will then validate/compare this chronic Ni-BLM with datasets of:

(i) Chronic Ni toxicity to *D. magna* in natural surface waters (Deleebeeck et al., 2005)

(ii) Chronic Ni toxicity to *D. magna* for different hardness levels (Chapman et al., 1980)

(iii) Chronic Ni toxicity to *C. dubia* at different hardness (Keithly et al., 2004)

(iv) Chronic Ni toxicity to *C. dubia* in natural surface waters.

(v) Chronic Ni toxicity to *C. dubia* at different pH/alkalinity/hardness (Wirtz et al., 2004)

One very important difference between acute and chronic toxicity tests with aquatic invertebrates is that in chronic toxicity tests food is added to the test vessels. Thus, in chronic toxicity tests additional Ni-binding (organic) ligands originating from this food (e.g., algal exudates) may be present and this may complicate the calculation of Ni speciation, especially in reconstituted waters, with no added organic matter. Therefore, it was considered interesting to conduct Ni speciation measurements in the presence of ligands from two different food sources:

(i) algal food (a 3:1 mix of *Pseudokirchneriella subcapitata* and *C. reinhardtii*) used in *D. magna* (Deleebeeck et al., 2005) and *C. dubia* experiments (this study)

(ii) YTC slurry (yeast-trout chow-cerophyl leaves) used in *C. dubia* experiments (Keithly et al., 2004; Wirtz et al., 2004).

The results of these experiments are described in the following section.

4.3.2. The effect of feeding on Ni speciation in test solutions

The same Donnan-membrane method for measuring Ni speciation was used as described in the materials and methods section for the natural waters (see section 2.2.3).

4.3.2.1. Algal food

The algal food used in our chronic *C. dubia* tests did not result in considerable modifications of Ni-speciation (e.g., via excretion of exudates or via excretion of digested algal material from daphnid guts) (Figure 4.7). At background Ni (0.5-0.7 µg/L) about 23-27% of the Ni was complexed to background 'unknown ligands' in the synthetic moderately hard water (USEPA, 1993), reconstituted on the basis of deionized water. No important difference in measured Ni^{2+} was observed when food and daphnids had been present in the solution. At 2 µg Ni/L only 11-13% was 'complexed' to unknown ligands, again with no important effect of added food and daphnids. The conclusions are that:

(i) ligands originating from (algal) food sources do not contribute significantly to Ni complexation during the experiments, and that

(ii) in synthetic water based on deionized water, 'unknown ligands' complex less than 10% of Ni at concentrations ≥ 2 µg/L.

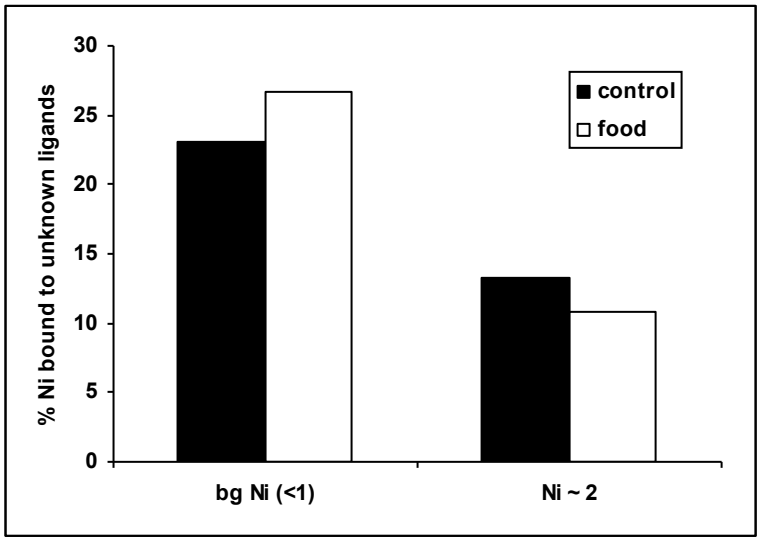


Figure 4.7 fraction of Ni bound to 'unknown ligands' in EPA moderately hard water (control) and EPA moderately hard water taken from 2 days old test solution with added food and C. dubia (food) at background Ni (<0.5-0.7 µg/L) and at about 2 µg Ni/L. 'Complexed' Ni was calculated as (predicted Ni^{2+} - observed Ni^{2+})/dissolved Ni2+; where predicted Ni^{2+} refers to WHAM6 simulations without DOC assumed; Here Ni^{2+} represent concentrations, not activities; input chemistry for speciation calculations is in Annex 11.

Since similar food concentrations were used in *D. magna* testing (only up to 2-fold higher; Deleebeeck et al., 2005), and since chronic effect concentrations for *D. magna* are much higher than 2 µg Ni/L, and given the fact that the fraction of free Ni^{2+} increases with increasing total or dissolved Ni (section 3.1.2), it is reasonable to assume that Ni binding to 'background' ligands in synthetic test waters with *D. magna* is insignificant too. Hence, for Ni speciation modeling in synthetic waters we assumed DOC = 0. It is stressed that this information can also be used to judge the potential importance of complexation of Ni to background ligands and ligands related to food addition in synthetic test waters used in chronic experiments with other species.

According to the US EPA protocol *P. subcapitata* is added daily to a new test chamber at a concentration of 2×10^5 cells/mL. With typical dry weights of *P. subcapitata* of 10-20 pg/cell, this corresponds to a food concentration of approximately 2 to 4 mg dry wt/L, whereas in our experiments, added algal food density amounted to 7 mg dry wt/L (see section 2.3.5). Hence, it is reasonable to assume that in ecotoxicity tests with *C. dubia* according to the US EPA protocol Ni binding to ligands associated with the addition of algal food is negligible. This will be taken into account in the subsequent speciation calculations.

4.3.2.2. YTC food

The fact that in our experiments Ni-binding to 'food associated' ligands was most likely unimportant, contrasts with the case of the chronic *C. dubia* experiments performed by Wirtz et al. (2004) and Keithly et al. (2004). They both used, in accordance with the US EPA test protocol (US EPA, 2002), a mixture of algal cells (*P. subcapitata*) and YTC in their chronic experiments with *C. dubia*.

We mimicked one of their test waters (Test No. 8 in Table 1 in Wirtz et al., 2004; pH ~ 7.9 to 8.1, hardness = 76 mg $CaCO_3$/L, alkalinity = 25 mg $CaCO_3$/L) and spiked dissolved Ni concentrations between 1.8 and 5.2 µg/L (close to *C. dubia* EC10=2.8 and NOEC=3.6

levels in this test water, Wirtz et al., 2004). Similarly as described before, we measured the % of Ni that was bound to 'background ligands' + 'ligands resulting from YTC addition'. Assuming that about 10% was again bound to background ligands in the deionized water (see 4.3.2.2), about 30-40% of Ni was estimated to be bound to 'YTC ligands'. This is an important contribution to overall Ni complexation in *C. dubia* test waters and it needs to be noted that this contribution may become even more important at higher pH levels, i.e. about > pH 8.

Upon addition of YTC, we measured 1.3 mg DOC/L, which is roughly 1 mg/L above the DOC level of our background deionized water. Keithly et al. (2004) measured, for similar YTC additions, a similar DOC addition of approximately 0.8 mg C/L (= DOC in chronic test – DOC in acute test). Assuming that the 'added' DOC behaved as natural surface water DOM (i.e., optimized %AFA of 40% and log $K_{Ni\text{-}FA}$ =1.75) we obtained good agreement between observed and predicted [Ni^{2+}]-concentrations. Hence, it may seem reasonable to assume that the DOC added due to YTC additions behaves as natural DOC.

However; it is uncertain if the Ni-DOC complexing would respond to pH (and hardness) changes in the same manner as natural DOM and if only organic ligands are at work. Indeed, with respect to the latter, it has been suggested that YTC slurry potentially contains sulfide, S^{2-} (Joseph Meyer, University of Wyoming, personal communication), which may also bind Ni, but which is likely to behave very differently than DOC in complexing Ni. How this uncertainty can be dealt with, must preferably be judged on a case-by-case basis (see further).

4.3.2.3. Implications for model development and risk assessment

For the development of chronic Ni toxicity models with aquatic invertebrates (below) and for future bioavailability normalizations of toxicity data from literature in the context of risk assessment, the following assumptions are recommended, in line with the results described above:

(i) 'Background' DOC present in reconstituted, artificial or synthetic waters prepared on the basis of deionized water does not contribute to Ni binding; hence the DOC-concentration of such waters should be set to 'zero'

(ii) Exudates originating from algal food added in toxicity tests do not significantly contribute to Ni binding; DOC originating from such food additions should be neglected too, as long as added food concentrations are not substantially higher than the ones used for our speciation measurements.

(iii) DOC originating from the addition of YTC to test solutions may potentially behave identical as natural surface water DOC; in this case, the amount of DOC assumed should be in relation to the concentration of YTC food added; it is also important, if DOC is measured in such tests, that the 'background' DOC is subtracted from the 'measured' DOC; it must be acknowledged however that the addition of YTC food introduces a greater deal of uncertainty to speciation calculations of Ni than addition of algal food.

These recommendations will be taken into account in the most appropriate way in the following sections on chronic Ni toxicity to aquatic invertebrates.

4.3.3. Development and validation of a chronic Ni-BLM for *D. magna*

4.3.3.1. Re-evaluation of the Deleebeeck et al. (2005) dataset

We re-analyzed the *D. magna* dataset with WHAM VI. All data were taken from Deleebeeck et al. (2005). The composition of all synthetic test waters, 21d-EC50 and EC10 values are given in Annex 13. We preferred to work with 21d-ECx values on the basis of net reproduction (R_0), since this was the most sensitive endpoint and also directly comparable to the identical reproduction endpoint of *C. dubia* used in the present study. Model development will be mainly based on EC50 values, as less uncertainty is associated with those than with EC10 or EC20 values, but validations will also be performed with EC10 and EC20 values where appropriate.

Besides working with WHAM VI instead of WHAM V, two other important differences need to be addressed with respect to the input data used for speciation calculations. First, in comparison with Deleebeeck et al. (2005), we were now able to use measured inorganic carbon (IC), which became available recently (measured during the tests of the pH test series). IC was measured weekly in new (just before organism introduction) and old test solutions (just after organism transfer to a new vessel) and was found to be very

similar throughout the experiment at each pH level. IC was slightly higher than expected based on nominal $NaHCO_3$ additions (i.e. 0.075 mM) between pH 6.4 and 7.4. It was lower then expected at pH 5.9 (Table 4.11). Those differences probably arose from the fact that these media were buffered with MOPS and then adjusted to the desired pH with NaOH. The added NaOH from concentrated stock solution may have contained traces of IC, which is plausible due to the capacity of alkaline solutions to absorb CO_2 from the atmosphere. Second, the presence of MOPS in some exposure solutions was accounted for in the speciation calculations, because it affects charge balance and ionic strength. This was not possible with the BLM software (Hydroqual, 2002) but can easily be incorporated into WHAM VI. A pK_a of 7.2 was used for the dehydrogenation of MOPS: (H^+) $(MOPS^-)$ $(H\text{-}MOPS)^{-1} = 10^{7.2}$.

Table 4.11 Measured IC-levels for the pH experiment in synthetic test waters for chronic *D. magna* tests (Deleebeeck et al., 2005).

pH[a]	MOPS added? (Y/N)	IC measured[a] (M)
pH series		
5.87	Y	5.26E-05
6.4	Y	1.23E-04
6.97	Y	2.72E-04
7.35	Y	4.81E-04
7.62	N	7.68E-04
8.22	N	3.50E-03
Ca and Mg series		
6.81[b]	Y	2.2E-04[c]

[a] average of all measurements
[b] average pH of all tests in the Ca and Mg series
[c] estimated based on linear regression between pH and log(IC) for tests with MOPS in the pH test series

The estimated IC-concentration (Deleebeeck et al., 2005) in the Ca and Mg tests is about 3 times higher than the expected one. However, this does not affect the calculated Ni^{2+}-activity at pH ~ 6.8. Indeed, using the estimated IC only results in a 1% lower Ni^{2+}-activity than using nominal IC.

Table 4.12 reports pH, Ca, Mg and Ni^{2+}-activities (and pNi) for all test waters at the 21d-EC50 of Ni. DOC was assumed = 0 because of the earlier discussed low complexing ability of DOC stemming from algal exudates under the conditions of chronic toxicity tests (see section 4.3.2.1). In Figure 4.8 the 21d-$EC50_{Ni2+}$ is plotted against Ca and Mg activity.

Table 4.12 21d-EC50s and corresponding chemistry of test waters (Ca, Mg, Ni as activity).

Test ID [a]	pH	Mg^{2+} (M)	Ca^{2+} (M)	Ni^{2+} (M)	pNi
Mg 0.25 mM	6.79	1.90E-04	1.39E-04	4.08E-07	6.39

Mg 0.5 mM	6.81	3.72E-04	1.40E-04	5.08E-07	6.29
Mg 1.0 mM	6.82	7.18E-04	1.35E-04	7.09E-07	6.15
Mg 1.5 mM	6.8	1.02E-03	1.33E-04	9.97E-07	6.00
Mg 2.0 mM	6.8	1.33E-03	1.27E-04	1.15E-06	5.94
Mg 3.0 mM	6.81	1.94E-03	1.24E-04	(1.19E-06)[b]	(5.93)
Ca 0.25 mM	6.85	1.81E-04	1.37E-04	3.06E-07	6.51
Ca 0.5 mM	6.81	1.78E-04	2.63E-04	4.46E-07	6.35
Ca 1.0 mM	6.81	1.72E-04	5.46E-04	5.23E-07	6.28
Ca 1.5 mM	6.79	1.68E-04	7.61E-04	5.32E-07	6.27
Ca 2.0 mM	6.79	1.64E-04	1.01E-03	8.46E-07	6.07
Ca 3.0 mM	6.8	1.51E-04	1.47E-03	(7.96E-07)[b]	(6.10)
pH 5.8	5.87	2.07E-04	1.81E-04	(7.10E-07)[c]	(6.15)
pH 6.4	6.4	2.07E-04	1.81E-04	8.05E-07	6.09
pH 7	6.97	2.08E-04	1.81E-04	6.45E-07	6.19
pH 7.6	7.35	2.10E-04	1.81E-04	5.75E-07	6.24
pH 7.6*	7.62	2.11E-04	1.77E-04	4.29E-07	6.37
pH 8.2*	8.22	2.08E-04	1.56E-04	3.50E-07	6.46

[a] Test codes identical to the ones used in Deleebeeck et al. (2005)

[b] not used for model development because 3 mM of Ca and Mg are less relevant for EU surface waters, i.e. they correspond to the higher percentiles of the Ca (85^{th}-95^{th} percentile) and Mg (>95^{th} percentile) distribution in EU surface waters (Heijerick et al., 2003) and are also not frequently tested in ecotoxicity testing. This is to make the final model as accurate as possible specifically for EU risk assessment purposes, i.e. for an as large proportion of the EU surface waters as possible.

[c] Not used for model development because discontinuity of pH effect below pH 6.4 (see text)

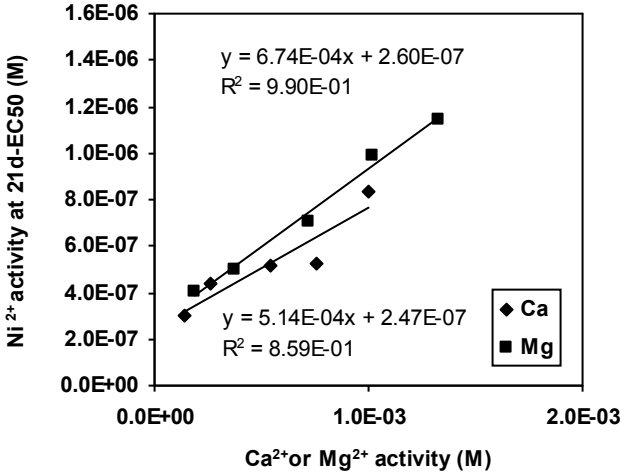


Figure 4.8 21d-EC50 as Ni^{2+}-activity for *D. magna* as a function of Ca^{2+} and Mg^{2+} activity; dataset from Deleebeeck et al. (2005); regression lines are used for derivation of stability constants for BLM (see text), only data used for model development are shown.

Again, as in Deleebeeck et al. (2005), a clear protective effect of Ca and Mg is observed (Figure 4.8). A Log K_{CaBL} = 3.53 and a log K_{CaBL} = 3.57 were derived and these were used throughout the rest of the analysis. Figure 4.9 gives the effect of pH on chronic Ni toxicity as 21d-$EC50_{Ni2+}$ vs. H^+ and as 21d-pNi^*_{50} vs. pH.

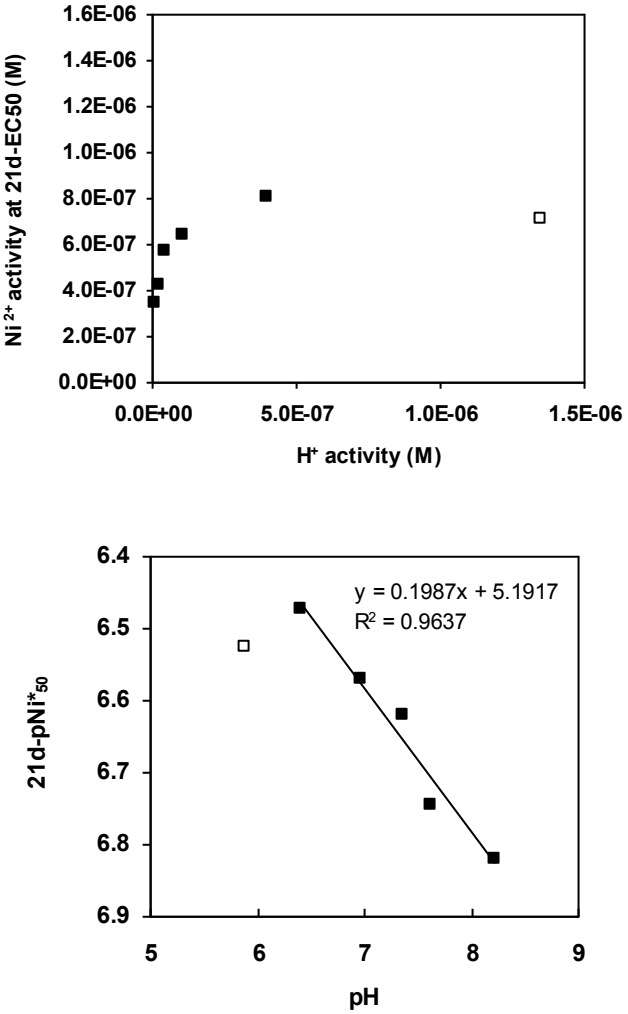


Figure 4.9 21d-EC50as as Ni^{2+}-activity and 21d-pNi^{*}_{50} as a function of H^{+} activity or pH; data from Deleebeeck et al. (2005), only filled squares are used for model development

It is clear that the pH effect, over the whole investigated pH range, does not seem to comply with the "single-site" BLM concept, which would require a linear relation between a the competing cation H^{+} and the $EC50_{Ni2+}$. The effect is obviously curvilinear with increasing slope at lower H^{+} activities (higher pH). Here too, a plateau is reached at pH levels < 6.4. Although it is possible to draw a straight line through the upper graph and derive a log K_{HBL}, in accordance with the BLM concept, it would not accurately reflect the true pH effect. The true pH effect here seems to follow more a log-linear relation, i.e. a straight line when pNi_{50} is plotted against pH. The continuity of this function is, however, invalidated at a pH < 6.4. At lower pH, *D. magna* is quite far from its optimal pH region (pH 7) and in nature it is usually not found at pH < 7. It is possible that a general stress response may have caused this non-

continuity of the pH effect. Additionally, pH levels below 6.4 are less relevant for typical EU surface waters (<5th percentile; Heijerick et al; 2003).

Several hypotheses may be put forward for this non-linear response, including differential chemistry of gill-microenvironment and bulk water, bioavailability of $Ni(OH)^+$ or $NiCO_3$ or $NiHCO_3^+$ complexes, the existence of more than one BL-site, and physiological pH effects. The data do currently not allow to test either of these hypotheses or to incorporate such effects into a mechanistic bioavailability model. Thus, the most straight-forward way of incorporating the pH effect at this time, which does not contrast with the observed pH effect, is to model it as a log-linear pH-effect, which acts independently from the BLM-type Ca and Mg competitive effects, i.e. superimposed on the traditional Ca and Mg competition. Such a log-linear pH effect, as an alternative to the BLM-type 'competition effect' has already been shown to accurately predict chronic Cu and Zn toxicity to green algae (De Schamphelaere et al., 2003; De Schamphelaere et al., 2005). The predictive model equation would in such a case become of the following form, for a test solution *i*, e.g. for the EC50 level:

$$EC50_{Ni2+,i} = 10^{-\left(S_{pH}\cdot pH_i + Q_{50}\right)} \cdot \left\{1 + K_{CaBL}\cdot\left(Ca^{2+}\right)_i + K_{MgBL}\cdot\left(Mg^{2+}\right)_i\right\} \qquad \text{(Eq. 4.6)}$$

Within this equation $EC50^*_{Ni2+,i}$ can be defined:

$$EC50^*_{Ni2+,i} = 10^{-\left(S_{pH}\cdot pH_i + Q_{50}\right)} = \frac{EC50_{Ni2+,i}}{\left\{1 + K_{CaBL}\cdot\left(Ca^{2+}\right)_i + K_{MgBL}\cdot\left(Mg^{2+}\right)_i\right\}} \qquad \text{(Eq. 4.7)}$$

This corresponds to the $EC50_{Ni2+}$ which is corrected for Ca and Mg competition, but which is dependent on the pH of the test solution. In equation 4.7 Q_{50} is the intercept of the (log-linear) pH function with the y-axis (Figure 4.9) after correction for Ca and Mg competition; *S* is the slope of the pH function.

It is interesting to note that this equation 4.7 can be interpreted as follows in terms of a typical BLM, by combining equations 4.5 and 4.7:

$$10^{-\left(S_{pH}\cdot pH_i+Q_{50}\right)}=\frac{f_{NiBL}^{50}}{\left(1-f_{NiBL}^{50}\right)\cdot K_{NiBL}} \quad \text{(Eq. 4.8)}$$

In words, if f^{50}_{NiBL} is independent of pH, it means that the log K_{NiBL} is dependent on pH, i.e. 'conditional' on pH. It would be interesting to investigate this assumption to provide more mechanistic background for model equation 4.6. Equations 4.6 and 4.7 can also be written in the following form:

$$pNi_{50,i}=Q_{50}+S_{pH}\cdot pH_i-\log\left\{1+K_{CaBL}\cdot\left(Ca^{2+}\right)_i+K_{MgBL}\cdot\left(Mg^{2+}\right)_i\right\} \quad \text{(Eq. 4.9)}$$

where

$$pNi^{*}_{50,i}=Q_{50}+S_{pH}\cdot pH_i=pNi_{50,i}+\log\left\{1+K_{CaBL}\cdot\left(Ca^{2+}\right)_i+K_{MgBL}\cdot\left(Mg^{2+}\right)_i\right\} \text{(Eq.4.10)}$$

For *D. magna* the derivation of S_{pH} is performed by linear regression analysis of $pNi^{*}_{50,i}$ vs. pH_i based on the results of the pH test series. This S_{pH} is then used to derive the $Q_{x,i}$ in each test solution *i*, according to a rearranged form of equation 4.10

$$Q_{x,i}=pNi^{*}_{x,i}-S_{pH}\cdot pH_i \quad \text{(Eq. 4.11)}$$

In this equation *x* denotes the effect level (e.g., 50%, 20%, 10%). For simplicity it is assumed that S_{pH} is independent of the effect level, similar to the non-dependency of BLM-stability constants of effect levels, and that differences between different effects levels are only due to differences in *Q*, which thus becomes is the 'sensitivity' parameter in this type of model, playing a similar role as the BL-Ni concentration (or fraction) in the usual BLM-models. The final model Q_x is calculated for each effect level as:

$$Q_x=\frac{\sum_{i=1}^{n}Q_{x,i}}{n} \quad \text{(Eq. 4.12)}$$

Table 4.13 gives the calculated pNi_x, the Ca, Mg activity and the $pNi^*_{x,i}$ for x=10, 20, and 50. The final chronic model-parameters, obtained using data in Tables 4.12 and 4.13 using equations 4.11 and 4.12 are summarized in Table 4.14.

Table 4.13 21d-ECx as pNi and pNi^* and corresponding chemistry of test waters (Ca, Mg, Ni as activity); data from Deleebeeck et al. (2005).

Test ID	pH	(Mg^{2+}) (M)	(Ca^{2+}) (M)	pNi_{50}	pNi_{20}	pNi_{10}	pNi^*_{50}	pNi^*_{20}	pNi^*_{10}
Mg 0.25 mM	6.79	1.90E-04	1.39E-04	6.39	6.57	6.68	6.73	6.91	7.02
Mg 0.5 mM	6.81	3.72E-04	1.40E-04	6.30	6.44	6.52	6.75	6.89	6.97
Mg 1.0 mM	6.82	7.18E-04	1.35E-04	6.15	6.24	6.29	6.77	6.85	6.90
Mg 1.5 mM	6.8	1.02E-03	1.33E-04	6.01	6.16	6.26	6.72	6.88	6.98
Mg 2.0 mM	6.8	1.33E-03	1.27E-04	5.94	6.01	6.06	6.75	6.82	6.86
Mg 3.0 mM	6.81	1.94E-03	1.24E-04	5.93	6.10	6.19	6.87	7.03	7.13
Ca 0.25 mM	6.85	1.81E-04	1.37E-04	6.52	6.79	6.95	6.85	7.12	7.28
Ca 0.5 mM	6.81	1.78E-04	2.63E-04	6.36	6.56	6.68	6.76	6.97	7.09
Ca 1.0 mM	6.81	1.72E-04	5.46E-04	6.29	6.48	6.60	6.83	7.02	7.14
Ca 1.5 mM	6.79	1.68E-04	7.61E-04	6.28	6.61	6.81	6.90	7.24	7.43
Ca 2.0 mM	6.79	1.64E-04	1.01E-03	6.08	6.28	6.40	6.78	6.98	7.10
Ca 3.0 mM	6.8	1.51E-04	1.47E-03	6.10	6.33	6.46	6.92	7.14	7.27
pH 5.8	5.87	2.07E-04	1.81E-04	6.15	6.39	6.53	6.53	6.77	6.91
pH 6.4	6.4	2.07E-04	1.81E-04	6.09	6.38	6.54	6.47	6.76	6.92
pH 7	6.97	2.08E-04	1.81E-04	6.19	6.31	6.38	6.57	6.68	6.75
pH 7.6	7.35	2.10E-04	1.81E-04	6.24	6.36	6.42	6.62	6.73	6.80
pH 7.6*	7.62	2.11E-04	1.77E-04	6.37	6.48	6.54	6.74	6.85	6.92
pH 8.2*	8.22	2.08E-04	1.56E-04	6.46	6.77	6.95	6.82	7.13	7.32

Note: Only underlined values are used for model development

Table 4.14 Model[a] parameters for chronic Ni bioavailability model for *D. magna*.

	D. magna - values
Log K_{CaBL}	3.53
Log K_{MgBL}	3.57
S_{pH} (slope of pH function)	0.1987
Q_{10}	5.646
Q_{20}	5.537
Q_{50}	5.352

Note: predictions of 21d-ECx_{Ni2+} with this model according to equation 4.6.

4.3.3.2 Validation with artificial and natural surface waters Deleebeeck et al. (2005)

This model was now validated with the artificial and natural surface waters. The input chemistry for WHAM VI of the natural waters is given in Annex 14. First, the activity of Ca^{2+} and Mg^{2+} were calculated and inserted into equations 4.6 to predict 21d-ECx_{Ni2+} for each test solution (except Bihain, as pH<6.4 and thus outside the model calibration). Those were then inserted into WHAM VI to obtain the 21d-$ECx_{dissolved}$. The predictive capacity of the models is plotted in Figure 4.10, for natural waters, as well as for the artificial test waters. The model

is able to predict most 21d-ECx values in artificial test solutions by an error of less than factor 2, indicating that the model is well calibrated to this dataset. This does not appear to be the case for the natural surface waters, where there is a trend of underestimating 21d-ECx values or overestimating the chronic Ni toxicity. Prediction errors were on average factor 2.0, 2.3 and 2.4 for the EC50, EC20 and EC10-levels respectively.

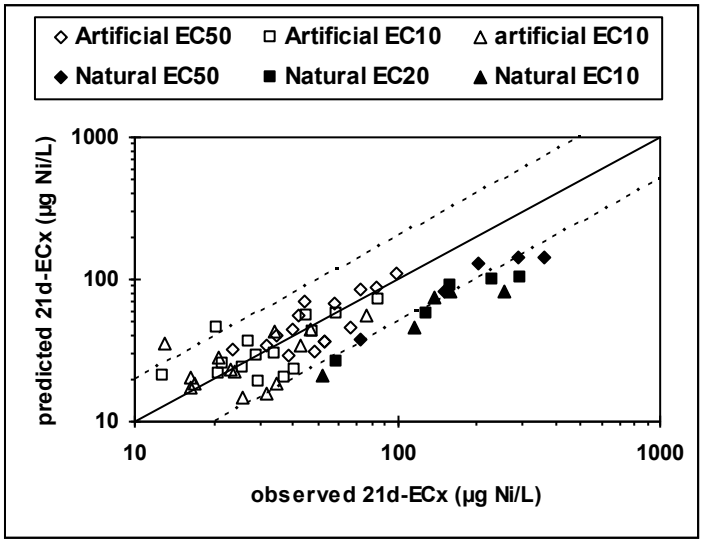


Figure 4.10 Predictive capacity of the chronic *D. magna* models (Table 4.14).

Possible hypotheses to explain why the chronic *D. magna* model is less accurate in natural waters include:

(i) At the time of ecotoxicity testing with natural waters, the chronic sensitivity of the daphnids to Ni might have been considerably lower (> the typically assumed 'normal' factor of 2) than at the time of testing in artificial waters (tested >1 year apart). However, the fitness of the daphnids was in both series of tests rather similar, as indicated by control reproduction (R_0) of on average 55 and 60 juveniles/adult in the artificial test waters and the natural test waters, respectively.

(ii) The model based on univariate testing does not capture potential interactive effects of different (co-varying) physico-chemical characteristics in natural waters. Interactive effects may include for example a more important competition of Ca and Mg at higher pH, a smaller pH effect at higher hardness. These are possibilities currently not accounted for in the model. Preferably, additional experimental data are needed to investigate these hypotheses. However, further on this possibility will be investigated.

(iii) The presence of DOC (humic substances) in natural waters (as opposed to artificial waters) ameliorates Ni toxicity beyond its effect on speciation. Glover et al. (2005) have shown that DOC may affect Na fluxes in *D. magna*. If the presence of DOC would also enhances Mg influx or reduces Mg efflux, an increased DOC level may potentially protect against the Mg-antagonist Ni, beyond the speciation effect of DOC.

4.3.3.3. Validation with well water with adjusted water hardness (Chapman et al., 1980)

These authors report increased 21d-chronic values for *D. magna* from 14.8 to 357 µg Ni/L, between a hardness of 50-200 mg $CaCO_3$/L. The data thus clearly confirm the earlier described protective effect of water hardness on chronic Ni toxicity. Since pH value in these experiments ranged between approximately 7.5 and 8, as opposed to pH 6.8 in Deleebeeck et al. (2005), it may be suggested that the protective effect of hardness is valid over a wide pH range, at least conceptually.

However, it is difficult or even impossible to analyze these data quantitatively for the following reasons:

(i) It is unclear whether the reported effect concentrations are on a dissolved or on a total recoverable Ni basis.
(ii) Only NOECs and LOECs are reported, which are, as opposed to ECx values, much more dependent on the choice of test concentrations and the within-treatment variability.
(iii) pH was different for the different hardness levels tested
(iv) Organisms are probably not tested simultaneously at each hardness level (inferred from different reported test temperatures)
(v) The diet was similar to YTC (blend of fish food and yeast) at a concentration of about 20 mg solids/L and this complicates speciation calculations.
(vi) Organisms have been acclimated to the test solutions (and thus to the hardness) before being tested, but it is not reported for how long

For these reasons it is not deemed appropriate to validate the chronic *D. magna* model for this dataset. This does, however, not mean that these data should not be considered for inclusion into an effects database for risk assessment. It should be acknowledged, however,

that the normalization of these toxicity data may be subject to more uncertainty than for example toxicity data from the Deleebeeck et al. (2005) dataset.

4.3.4. Development of a chronic Ni-BLM for *C. dubia*

First, the *D. magna* chronic model, described by equation 4.6, with parameters log K_{CaBL} = 3.53, log K_{MgBL} = 3.57, and S_{pH} = 0.1987 (Table 4.14) was validated for the *C. dubia* dataset generated in the present study with natural waters. Using the earlier calculated speciation in the test solutions (Table 3.8), and using these parameter values we optimized Q_{50}, Q_{20}, and Q_{10} values according to equations 4.6 to 4.11. This calibration is needed to account for the higher sensitivity of *C. dubia*. The results of this calibration are reported in Table 4.15. This model is referred to as *C. dubia* model 1.

Table 4.15 Model[a] parameters for different chronic Ni bioavailability models for *C.dubia* and *D. magna*

	D. magna	*C. dubia* Model 1 [a]	*C. dubia* Model 2 [b]
Log K_{CaBL}	3.53	3.53	3.53
Log K_{MgBL}	3.57	3.57	3.57
S_{pH} (slope of pH function)	0.1987	0.1987	0.8587
Q_{10}	5.646	6.462 [c]	1.581 [c]
Q_{20}	5.537	6.328 [c]	1.447 [c]
Q_{50}	5.352	6.320	1.321

[a] log K_{CaBL}, log K_{MgBL}, S_{pH} assumed identical as for *D. magna* (see Table 4.14)
[b] Species-specific S_{pH}
[c] Data from Eppe and Markermeer not used for calculation, because EC20 and EC10 were extrapolated values (see Table 3.8)

The Q_x values are indeed higher for *C. dubia* than for *D. magna*¸ confirming the higher sensitivity of *C. dubia*. This 'optimized' model 1 was now used to predict ECx_{Ni2+} values for the same dataset and, after insertion of $ECx_{Ni2+,predicted}$ into WHAM VI, also $ECx_{dissolved}$, in order to visualize how well this model was calibrated to the dataset (Figure 4.11). Observed and predicted EC50s are also reported in Table 4.16.

Figure 4.7 reveals that the *D. magna* model does reduce some of the observed variability. For example, there is a variability in observed 10d-$EC50_{dissolved}$ of factor 14, and the average prediction error about 10d-$EC50_{dissolved}$ is about factor 2.3 (range: 1.6-3.4). Two out of six 10d-$EC50_{dissolved}$ were predicted by an error of more than factor 2. Remarkably, the three EC50s at the three lowest pH levels were underestimated; the three EC50S at the highest

pH were all overestimated (Table 4.16). This suggests that the pH effect on chronic Ni toxicity to *C. dubia* might be more important than for *D. magna*. This is confirmed by the data presented in Figure 4.12, where 10d-pNi^*_{50} for *C. dubia* are plotted against pH.

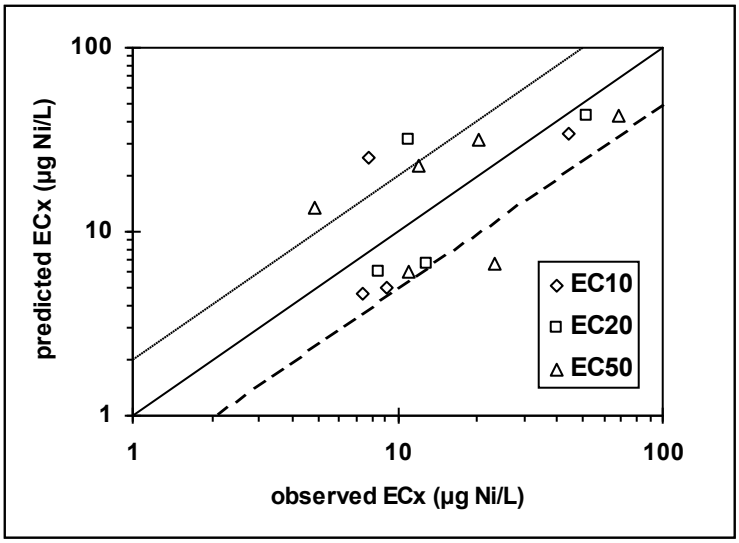


Figure 4.11 Observed and predicted chronic ECx of Ni to *C. dubia* in natural waters (data from present study, see Table 3.7); prediction carried out with *C. dubia* chronic model 1 (Table 4.15), which uses the same model parameters as the *D. magna* model (Table 4.15), but which is calibrated to the higher sensitivity of *C. dubia*

Table 4.16 Observed and predicted chronic 10d-$EC50_{dissolved}$ (as µg Ni/L) using different *C. dubia* chronic models

Site	DOC (mg/L)	H [a]	pH (chronic)	10d-EC50 (obs)	10d-EC50 (pred, model 1)[b]	10d-EC50 (pred, model 2)[b]
Ankeveen	23.6	131.6	7.61	68.4	43.1	41.5
Bihain	6.36	15.0	6.56	23.1	6.8	26.2
Brisy	3.06	41.1	7.23	11.0	6.1	9.7
Eppe	5.02	108.4	7.86	4.9	13.5	9.6
Markermeer	7.6	218.1	8.01	12.1	22.6	13.5
Regge	12.6	204.0	8.18	20.1	31.8	16.1

[a] H= calculated water hardness, as mg $CaCO_3$/L.
[b] parameters of model 1 and 2 are summarized in Table 4.15

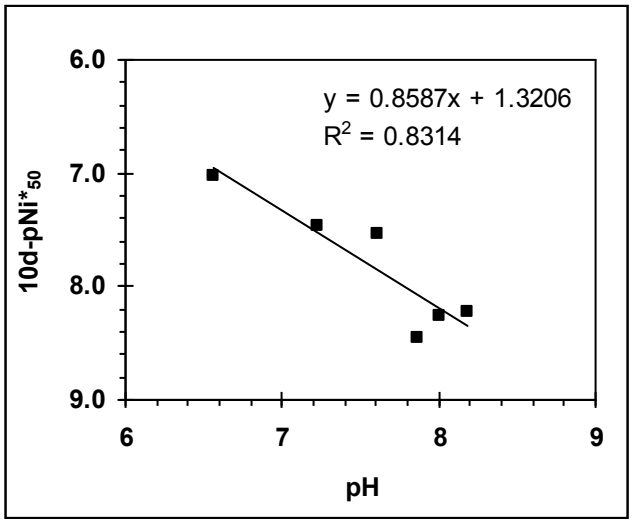


Figure 4.12 The effect of pH on chronic $EC50_{Ni2+}$ to *C. dubia* (this study) after correction for hardness effects, represented by $10d\text{-}pNi^{*}_{50}$

Ten day-pNi^{*}_{50} were calculated using equations 4.7 or 4.10, with log K_{CaBL} = 3.53, log K_{MgBL} = 3.57. The slope of the pH function for *C. dubia* between pH 6.6 and pH 8.2 appears to be S_{pH} = 0.8587 as opposed to 0.1987 for *D. magna* for a similar pH range, i.e. between pH 6.4 and 8.2. This explains why the *D. magna* model does not accurately predict Ni toxicity to *C. dubia* in natural waters. Indeed, when the species-specific *C. dubia* slope (model 2) was used for optimization of Q_x values for this *C. dubia* dataset, a much better calibration was obtained (Table 4.15, Figure 4.13).

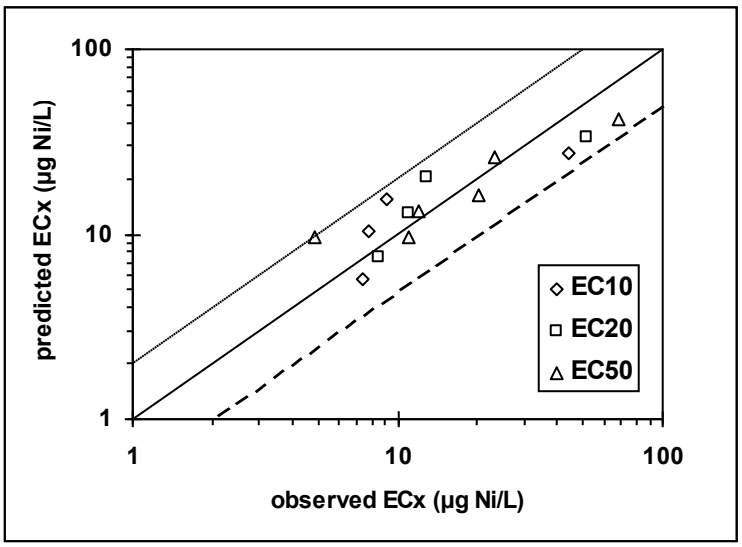


Figure 4.13 Observed and predicted chronic ECx of Ni to *C. dubia* in natural waters (data from present study, see Table 3.7); prediction carried out with *C. dubia* chronic model 2 (Table 4.15) .

The Q_x values belonging to this model (*C. dubia* chronic model 2) are reported in Table 4.15. The optimal calibration results in prediction errors lower than factor 2 for all test waters and effect levels, with an average prediction error for $EC50_{dissolved}$ of factor 1.4 (Table 4.16, Figure 4.13). This is a considerable amelioration compared to the factor 2.3 prediction error with *C. dubia* model 1.

This validation exercise suggests that different models might be needed for *C. dubia* and *D. magna* to obtain as accurate as possible predictions of chronic Ni toxicity for both organisms. The exact reasons for the different pH effect in both organisms cannot be inferred from the existing datasets.

In the following paragraphs we will validate this model with other datasets and investigate the effects of pH and hardness on chronic Ni toxicity to *C. dubia* into additional detail.

4.3.5. Validation of the chronic Ni-BLM for *C. dubia* with other existing datasets

4.3.5.1. General considerations

The following datasets, described hereunder, are considered in the analyses below:

(i) Keithly et al. (2004), who reports chronic Ni toxicity to *C. dubia* for 4 different hardness levels

(ii) Wirtz et al. (2004), who report chronic Ni toxicity to *C. dubia* in a number of synthetic test waters with varying pH, alkalinity and water hardness and in three filtered natural water samples.

Both datasets involve chronic toxicity tests in which a mixture of *P. subcapitata* and YTC was used as food source for *C. dubia*. It must be noted that this introduces considerable uncertainty into speciation calculations because ligands originating from YTC additions were shown to significantly complex Ni (see section 4.3.2). We will try to take this uncertainty into consideration in the most appropriate way

4.3.5.2. Effect of hardness effect on chronic Ni toxicity to C. dubia (Keithly et al., 2004)

Keithly et al. (2004) determined chronic 7d-LC20s and reproductive 7d-EC20s of Ni to *C. dubia* at 4 different hardness levels between 50 and 253 mg $CaCO_3$/L. The results of their tests are reported in Table 4.17. The chemistry of the test media is summarized in Annex 15.

Based on this dataset, it appears that increasing the water hardness between 50 and 253 mg CaCO3/L) slightly reduces chronic toxicity, based on survival LC20s (factor >3.1) and reproductive EC20s (>1.8). The effects are most likely even a little larger than this since at the lowest water hardness LC20 and EC20 are based on total (nominal) concentrations, which are typically higher than dissolved concentrations in chronic ecotoxicity tests with added food. Over a similar range of Ca or Mg (0.5 to 2 mmol/L), Deleebeeck et al. (2005) reported an increase of chronic reproductive EC50s in *D. magna* of approximately factor 2. Hence, the effect of hardness on chronic Ni toxicity is definitely not substantially more important in *D. magna* than in *C. dubia*.

Table 4.17 Chronic effect concentrations (and 95% confidence interval) of Ni (as dissolved Ni) to *C. dubia* as a function of water hardness (Keithly et al., 2004)

Hardness [a] ($mg\ CaCO_3$/L)	7d-$NOEC_s$ [b] (µg Ni/L)	7d-LC20 (µg Ni/L)	7d-$NOEC_r$ [c] (µg Ni/L)	7d-$EC20_r$ (µg Ni/L)
50 [d]	<3.8 [e]	<3.8 [e]	<3.8 [e]	<3.8 [e]
113	5.8	4.8	5.3	4.7
161	15.3	11.9	3.4	4.0
253	9.6	10.4	5.8	6.9

[a] Full chemistry is reported in Annex 15
[b] survival 7d-NOEC
[c] reproductive 7d-NOEC
[d] values are based on total nominal Ni concentration
[e] lowest Ni concentration caused >20% mortality and >20% reduction of reproduction

The nature of the data, i.e. only three 'unbounded' effect concentrations, do not allow the hardness effects on chronic Ni toxicity to *C. dubia* to be more quantitatively compared with *D. magna* to the same degree of certainty as for the effect on acute Ni toxicity (see section 4.2.3.1.). Another confounding factor is that the pH across the different hardness levels varied by 0.2 pH units, while it is observed that *C. dubia* exhibits a fairly large pH dependency with respect to chronic Ni toxicity (see section 4.3.4). Also, the presence of YTC and its complexing function would make such an assessment more uncertain.

We feel that the added uncertainty of all these separate elements does not justify a specific quantitative assessment or a specific calibration of log K_{CaBL} or log K_{MgBL} values for chronic Ni toxicity to *C. dubia*. The qualitative comparison with *D. magna* suggests that these values may be sufficiently similar not to cause substantial errors in toxicity predictions for *C. dubia*. In Annex 16, two possible approaches are recommended for taking into account the uncertainty about the Keithly et al. (2004) chronic dataset in a risk assessment context.

4.3.5.3. Validation of the chronic *C. dubia* model using another dataset (Wirtz et al., 2004)

Wirtz et al. (2004) determined reproductive toxicity of Ni to *C. dubia* in 14 synthetic waters and 3 filtered natural water samples from United States or Canada (Table 4.18). Along with those latter tests they also performed tests in unfiltered (raw) samples and in synthetic waters with DOC, obtained via reverse osmosis from these waters and added to the same concentration of these waters. Since there were generally no differences of Ni toxicity between unfiltered, filtered and RO-samples, we decided only to perform our data analysis on the data obtained with the filtered water samples.

Table 4.17 Chronic 7-day effect concentrations of Ni to *C. dubia* reproduction (data from Parametrix, 2004a); full chemistry is reported in Annex 17; data between parentheses not considered for data analysis (see footnotes)

Test ID[a]	Test No. in acute PMX-report[b]	Back-ground Ni (µg/L)	pH	Hardness (mg $CaCO_3$/L)	Alkalinity (mg $CaCO_3$/L)	DOC [c] (mg/L)	EC10[d] (µg/L)	NOEC[e] (µg/L)	EC50[e] (µg/L)
1	1	1.0	8.5	96	96	1.3	-	1.76	3.53
2	2	1.0	8.4	154	95	1.3	-	2.52	4.08
3	3	1.2	8.4	292	95	1.3	-	<2.98	5.18
4	4	1.2	8.7	194	194	1.3	-	<2.62	3.34
5	5	2.7	8.6	310	196	1.3	-	4.43	9.46
6*	6	6.3	8.4	586	197	1.3	-	(8.64)	(13.5)
7	7	0.9	8	42	26	1.3	4.94	3.60	10.3
8	8	0.9	8.1	76	25	1.3	2.80	3.60	17.0
9*	10	10.6	7.8	848	24	1.3	(24.1)	(22.1)[f]	(42.2)
10	11	1.2	8.6	182	183	1.3	2.87	3.66	4.51
11	12	1.4	8.3	192	96	1.3	-	<6.81	9.73
12 [g]	13	1.4	7.2	192	96	1.3	(7.58)	(6.89)	(24.5)
DJ lab (18)	-	1.2	8.4	246	86	1.3	NR	10.0	21.0
GR lab (22)	-	1.9	8.5	228	158	1.3	NR	3.28	7.01
DJ filt (16)	-	8.4	8.3	230	84	6.7	NR	19.6	52.6
GR filt (20)	-	2.9	8.6	236	166	7.5	NR	12.0	23.6
CP filt (14)	-	2.3	8.4	184	102	6.6	NR	27.9	46.4

[a] Refers to the test numbers (and/or codes) used in Table 1 and Table 2 of the chronic Wirtz et al. (2004) report; DJ = Desjardins Canal (Ottawa, ON, Canada), GR = Grand River (Ottawa, ON, Canada), CP = Cache la Poudre River (Fort Collins, CO, USA); 'filt' refers to spiked filtered natural water samples from these sources, 'lab' refers to lab match water samples, i.e. pH, alkalinity and water hardness identical to natural waters but without

added DOC; * denotes high background concentration of Ni in control background concentration combined with irrelevant hardness for EU waters, these data were not considered for data analysis.
[b] This test number refers to the test numbers used in Table 2 of the acute Parametrix report (Parametrix, 2004), note that test No. 10 from the acute report was not reported in the chronic report and was not considered for our data analysis.
[c] DOC in synthetic waters was assumed similar as in Keithly et al. (2004) since the same test procedures were used by PMX. DOC was assumed to consist of 0.5 mg/L background DOC, not contributing to Ni-binding, and 0.8 mg/L originating from YTC additions, which was assumed to behave as 40%AF.
[d] Only non-extrapolated EC10s are reported here (taken from Parametrix, 2004), assessment of 'extrapolated' made on basis of raw data received via personal communication with Dr. Bill Stubblefield (Parametrix, Albany, OR, USA), NR=not reported
[e] NOECs and EC50s were obtained via personal communication with Dr. Bill Stubblefield (Parametrix, Albany, OR, USA).
[f] reported NOEC = 55.1, but this is not possible when looking at the dose-response, we estimate it to be 22.1 instead
[g] test conducted at increased pCO2, reduced overall fitness of organisms, data not further considered.

Before initiating the data analysis, the toxicity data from Wirtz et al. (2004) were subjected to a critical evaluation. Tests No. 6 and No. 9 were not considered because high Ni background concentrations in controls, i.e. 6.3 and 10.6 µg/L, might have affected the concentration response relation and thus the 7d-EC50 estimate. The data from test No. 12 were not used because it is suspected that the addition of CO_2 to reduce the pH down to 7.2 may have reduced the overall fitness of the organisms resulting in an enhanced toxicity. The reduced fitness is obvious from the fact that control reproduction in this test water was considerably lower (about two-fold) than in all other test solutions (data received via personal communication with Dr. Bill Stubblefield, Parametrix, Albany, OR, USA).

After this data evaluation it is noted that the dataset covers a rather narrow pH range of 8.0 to 8.7, which does almost not overlap with the pH range for which the *D. magna* model and *C. dubia* model (model 2) were developed. It is also important to note upfront that the 80th, 90th and 95th percentiles of pH in EU surface waters are around 8.0, 8.1, and 8.2, respectively (Heijerick et al., 2003) and that consequently, most of the investigated test solutions represent conditions not relevant for the part of the EU surface waters represented in the monitoring databases used by Heijerick et al. (2003). In fact, only two test waters, i.e. No. 7 and 8, have a pH that fall within the 95th percentile of these EU surface waters. Therefore, less weight should be given to this dataset, and the conclusions drawn from it, then to the datasets with *C. dubia* generated in the present study and in the Keithly et al. (2004) study.

Nevertheless, from a point of view of better understanding bioavailability relationships, we wanted to determine how well the *C. dubia* chronic bioavailability model

(model 2, Table 4.15) could be calibrated to this dataset by only adjusting the sensitivity-parameter Q_{50} of the different *C. dubia* strains and by assuming log K_{CaBL}, log K_{MgBL} and S_{pH} the same as for *C. dubia* (model 2). In other words, can the *C. dubia* model, developed for a pH range between 6.6 and 8.2, be safely extrapolated to higher pH values?

First, Ni speciation at the 7d-$EC50_{dissolved}$ needed to be calculated with WHAM VI. Again, it is stressed that there is uncertainty with regard to the Ni binding by YTC-ligands. To address part of this uncertainty, speciation calculations were run under two scenarios. Scenario A with no binding at all assumed and scenario B assuming that the DOC from the YTC behaves identical to natural freshwater DOC. In scenario B 0.64 mg FA/L is added to the input of the speciation calculations. The conclusions from both scenarios will be compared. Since no measured Al concentrations were available, natural waters were modelled assuming either Al^{3+} in equilibrium with colloidal $Al(OH)_3$ or with Al=0, to assess the importance of Al on Ni speciation. Those two Al assumptions represent the minimum and maximum competitive effect of Al on Ni binding to DOC. The speciation calculations are reported in Table 4.19. WHAM VI input files for these calculations can be found in Annex 17.

Table 4.19 7d-$EC50_{Ni2+}$ and 7d- pNi^*_{50} for *C. dubia*; data from Wirtz et al. (2004); data between parentheses not considered for data analysis and interpretation

				Scenario A[b]		Scenario B [b]	
Test ID [a]	pH	(Mg^{2+}) (M)	(Ca^{2+}) (M)	$EC50_{Ni2+}$ (M)	pNi^*_{50}	$EC50_{Ni2+}$ (M)	pNi^*_{50}
1	8.5	2.65E-04	4.66E-04	2.21E-08	8.21	1.51E-08	8.37
2	8.4	3.63E-04	6.30E-04	2.64E-08	8.23	1.94E-08	8.36
3	8.4	6.50E-04	1.12E-03	3.13E-08	8.36	2.56E-08	8.45
4	8.7	4.31E-04	4.54E-04	1.11E-08	8.57	8.32E-09	8.70
5	8.6	6.71E-04	5.84E-04	3.44E-08	8.20	2.91E-08	8.27
6*	8.4	1.02E-03	1.28E-03	(5.63E-08)	(8.21)	(5.08E-08)	(8.25)
7	8.0	1.19E-04	3.36E-04	1.22E-07	7.32	8.50E-08	7.48
8	8.1	2.20E-04	4.89E-04	1.86E-07	7.27	1.46E-07	7.38
9*	7.8	1.35E-03	2.35E-03	(2.85E-07)	(7.69)	(2.70E-07)	(7.71)
10	8.6	4.33E-04	4.48E-04	1.65E-08	8.40	1.27E-08	8.51
11	8.3	4.03E-04	6.78E-04	6.50E-08	7.87	5.30E-08	7.96
12 [g]	7.2	4.09E-04	6.93E-04	(2.21E-07)	(7.34)	(1.97E-07)	(7.39)
DJ lab (18)	8.4	5.22E-04	8.09E-04	1.35E-07	7.62	1.17E-07	7.68
GR lab (22)	8.5	4.87E-04	6.60E-04	3.26E-08	8.19	2.66E-08	8.28
DJ filt (16)	8.3	4.45E-04	9.88E-04	2.21E-07	7.43	2.07E-07	7.46
GR filt (20)	8.6	5.42E-04	9.17E-04	4.90E-08	8.10	4.54E-08	8.13
CP filt (14)	8.4	3.55E-04	8.15E-04	1.77E-07	7.46	1.65E-07	7.49

[a] See footnote a of Table 4.17 for meaning of codes

[b] A = no DOC assumed from YTC addition, B=0.64 mg FA/L assumed from YTC addition; for natural waters reported data represent the scenario where Al^{3+} is assumed in equilibrium with colloidal $Al(OH)_3$

For the natural waters, the difference in calculated values of $EC50_{Ni2+}$ between minimum and maximum Al^{3+} activity is limited to less than 2%. We decided to work further with calculations obtained with Al^{3+}in equilibrium with colloidal $Al(OH)_3$ (see also section 3.2). However, when the presence of Cu and Zn (as competitors for Ni binding sites on DOC) is neglected, $EC50_{Ni2+}$ turn out to be calculated 17 to 26% lower than the values reported in Table 4.19 (data not shown). This indicates the potential importance of considering Cu and Zn in risk assessment of Ni, in order to improve the accuracy and protectiveness of this assessment. However, while the effect of Cu and Zn on Ni speciation can be taken into account using the speciation part (WHAM VI) of the bioavailability models, it is acknowledged that mixture toxicity effects of Cu and Zn on Ni toxicity can currently not be taken into account. We suggest that speciation and Ni toxicity predictions will be more accurate when Cu and Zn are taken into account in speciation modelling, as long as Cu and Zn have no important effect on single-metal Ni toxicity at the Cu and Zn concentrations under consideration. Taking into account the local or regional presence of Cu and Zn when normalizing Ni toxicity data to a given local or regional water chemistry, will result in lower normalized species-NOEC values, because less Ni will be predicted to bind at DOC in the presence of Cu and Zn, than in the absence. The same reasoning is valid for the competitive effects of Fe and Al discussed earlier.

The data in Table 4.19 also illustrate that under scenario A (no influence of YTC assumed), $EC50_{Ni2+}$ is between 5 and 46% higher than under scenario B (influence of YTC assumed) for the synthetic waters, but only 7% higher for the natural waters. This suggests that testing in natural waters, which contain natural DOC and which therefore better buffer the Ni^{2+}-activity than artificial waters, results in ecotoxicity data which are less uncertain with respect to Ni speciation. For both scenarios, we plotted the 7d-pNi^*_{50} against pH (as in previous modelling exercises, Figure 4.10).

The 7d-pNi^*_{50} values in the lab match waters are slightly higher than in natural waters, i.e. 0.1 to 0.2 pNi units, representing a factor difference between 1.3 and 1.6 on the basis of 7d-$EC50^*_{Ni2+}$. This can be considered within the normal range of testing variability (factor 2) and it cannot be concluded that *C. dubia* is more sensitive to free ionic Ni^{2+} in synthetic water than in natural waters, as was suggested for *D. magna*. However, the same trend as in *D. magna* is noted and further research into the individual effect of increased DOC on ionic Ni^{2+} toxicity is recommended.

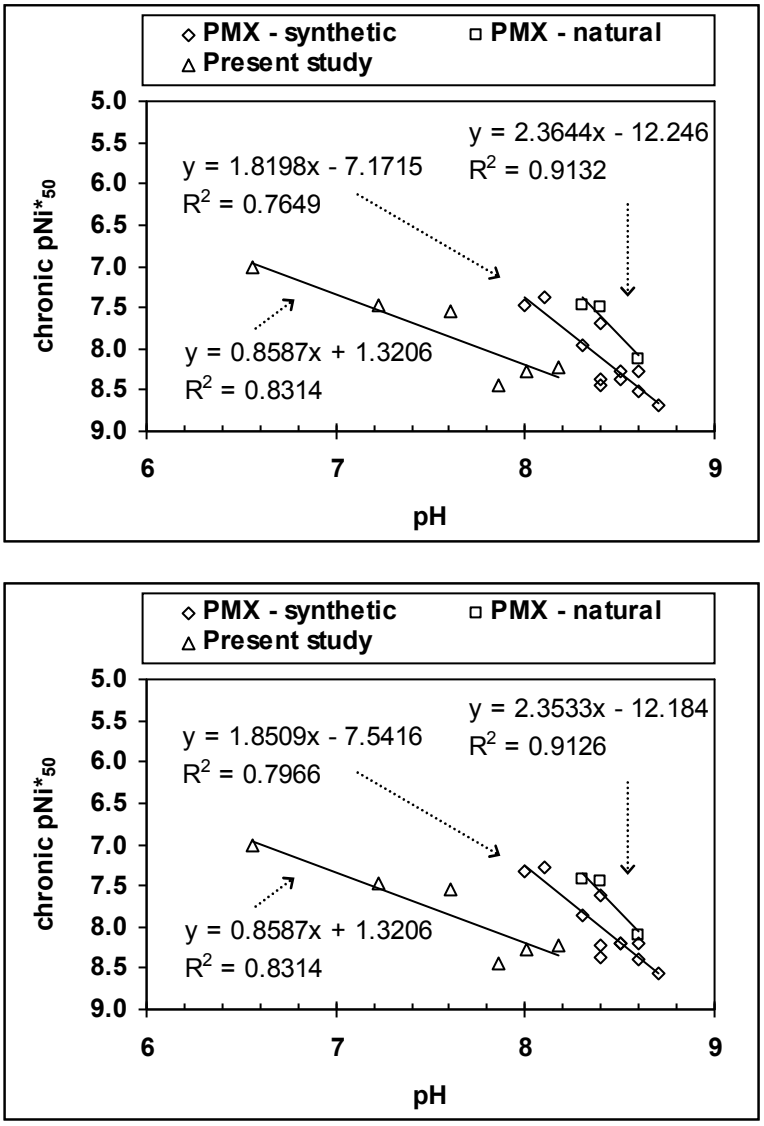


Figure 4.14 The effect of pH on chronic 7d-EC50$_{Ni2+}$ (data from Wirtz et al., 2004) to *C. dubia* after correction for hardness effects, represented by 7d-pNi$^{*}_{50}$, calculated for scenario A (upper panel) and B (lower panel) as explained in Table 4.19 and text. The 10d-pNi$^{*}_{50}$ for *C. dubia* from the present study is shown for comparison (see also figure 4.12).

Apparently, the slope of the pH function for this dataset is much larger, i.e. S_{pH} = 1.82 to 1.85 for synthetic waters and 2.35 to 2.36 for the natural water samples, than the slope obtained for our own *C. dubia* in natural waters based on the present study, i.e. S_{pH} = 0.8587 (see comparisons in Figure 4.14). This conclusion is not affected by the assumption with regard to the binding of Ni by YTC-ligands present in the test solutions. It is also noteworthy that the *C. dubia* strain used in the present study seems to be more sensitive to Ni^{2+} than the strain used by Wirtz et al. (2004). This is illustrated by the higher pNi*$_{50}$ (or lower EC50$_{Ni2+}$) observed in the present study at comparable pH values around 8.

Thus, it appears that the slope of the pH function is dependent on pH itself, and two distinct regions may be recognized: a slope close to 1 below pH 8.2 and a slope close to 2 above pH 8.2. This indicates that the earlier developed *C. dubia* model 2 will not work to accurately predict effect concentrations observed in the Wirtz et al. (2004) dataset. Different slopes of the pH function in different pH regions may suggest the presence of at least two biotic ligand sites, which may both be singly or doubly protonated (Borgmann et al., 2005). A slope of 1 suggests a singly protonated sites, and a slope of 2 suggests a doubly protonated site. Too few data are, however, available to develop a multi-site BLM. Moreover, when such type of model is to be developed, simultaneous experiments covering one large pH range (~ 6 to 9) need to be conducted.

The increased slope of the pH function with increasing pH confirms the trends observed in the acute toxicity datasets with both *D. magna* and *C. dubia* (see section 4.2) which suggest a marked increase of the toxicity of the Ni^{2+} ion at pH levels exceeding 8.2. We wanted to demonstrate how erroneous the *C. dubia* model 2 would be for predicting chronic toxicity in the Wirtz et al. (2004) dataset. Using the data in Table 4.19 and using equations 4.10 and 4.11 an optimal Q_{50} value of 0.812 for the whole dataset was found and this was used to predict 7d-$EC50_{dissolved}$ (Figure 4.15).

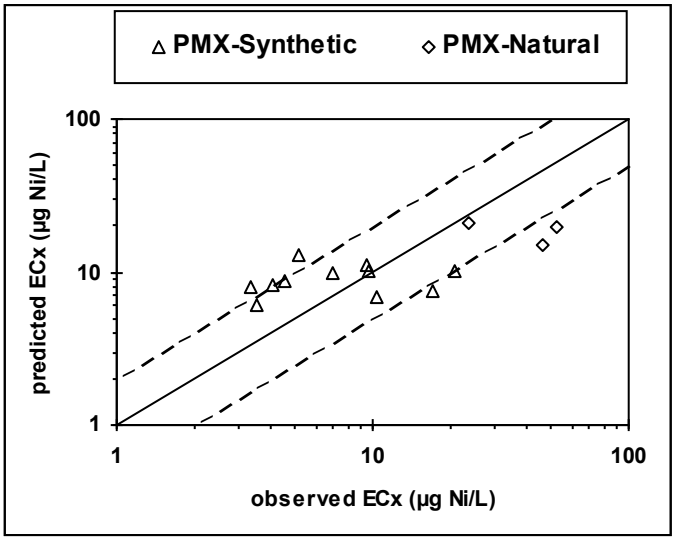


Figure 4.15 Predictive capacity of *C. dubia* model 2 (Table 4.15) for the *C. dubia* 7d-EC50 dataset of Wirtz et al. (2004), Q_{50} optimized to 0.812.

The data in Figure 4.15 indicate that all $EC50_{dissolved}$ are predicted by an error of less than factor 3, whereas the variability was originally a factor of 20. However, using the *C. dubia* model 2, developed for pH levels < 8.2 results in clearly biased predictions for the Wirtz et al. (2004) dataset, i.e. low EC50s are generally overestimated and high EC50s are generally underestimated.

Because of this important prediction bias, which is mainly due to a very different pH effect at pH > 8.2 vs. at pH <8.2, and because of the fact that pH 8.2 is the upper 95th percentile of EU surface waters, it is recommended not to use *C. dubia* ecotoxicity data for the EU risk assessment if they were obtained in test solutions with pH > 8.2.

The Wirtz et al. (2004) dataset, however, offers a unique possibility towards assessing the risk of surface waters with a pH > 8.2, because *C. dubia* is currently the species known to be most sensitive to Ni. It would be important, however, to also use the associated higher slope of the pH function in such a case.

4.3.6. An initial attempt to 'merge' the chronic *D. magna* and *C. dubia* models for predictions in natural waters

In sections 4.3.3 and 4.3.4 we found that the slope of the pH function, S_{pH} was quite different for *D. magna* (0.1987) and *C. dubia* (0.8587). These slopes were statistically significantly different *(*$p<0.001$, method used according to box 14.8 in Sokal and Rohlf, 1981). We also found that the predictive capacity of the chronic *D. magna* model, developed in artificial waters, was not very accurate for natural waters, i.e. overestimating Ni toxicity. We suggested that this might be due to interactive effects of Ca, Mg, and pH, not captured by the model. Since the pH-slope for *D. magna and C. dubia* were derived for artificial and natural waters, respectively, it is possible that this is the cause for the perceived large difference between S_{pH} of the two species.

We performed speciation calculations for the 21d-EC50 of *D. magna* in natural waters. The full chemistry for input into WHAM VI is given in Annex 14. Output of the speciation calculations is given in Table 4.20.

Table 4.20 21d-pNi_x and 21d-pNi^*_x for *D. magna* in natural waters; data from Deleebeeck et al. (2005)

Test water	pH	(Mg^{2+}) (M)	(Ca^{2+}) (M)	pNi_{50}	pNi_{20}	pNi_{10}	pNi^*_{50}	pNi^*_{20}	pNi^*_{10}
Ankeveen	6.79	1.72E-04	5.86E-04	5.64	5.76	5.83	6.20	6.32	6.39
Bihain	6.15	3.64E-05	7.52E-05	6.28	6.46	6.57	(6.42)[a]	(6.61)[a]	(6.71)[a]
Brisy	7.09	1.08E-04	9.63E-05	6.19	6.29	6.36	6.43	6.53	6.59
Markermeer	8.09	3.42E-04	7.56E-04	6.03	6.16	6.23	6.72	6.84	6.92
Regge	7.71	2.01E-04	8.98E-04	5.83	5.95	6.14	6.52	6.63	6.82
Voyon	8.02	2.04E-04	6.33E-04	6.04	6.12	6.17	6.64	6.71	6.77

[a] Not used for model development (see text)

Similarly as we did for our *C. dubia* dataset, we plotted the chronic pNi^*_{50} against the pH and found a slope of the pH function, S_{pH} = 0.3335 (Figure 4.16). This is two times closer to the *C. dubia* slope (i.e. 0.8589 than the *D. magna* slope derived based on artificial test waters (0.1987). Using the same statistical method (Sokal and Rohlf, 1981), we found that this new slope of 0.3335 is significantly different from the one derived in synthetic waters ($p=0.015$) and still different from the one estimated for our *C. dubia* in natural waters ($p<0.001$). The increased slope in natural waters seems to suggest that interactive effects of Ca, Mg, and pH in natural waters may indeed not be fully captured by univariate bioavailability experiments. Here too, the data point at pH 6.15 (Bihain) was not considered, because it represented a discontinuity in the pH function.

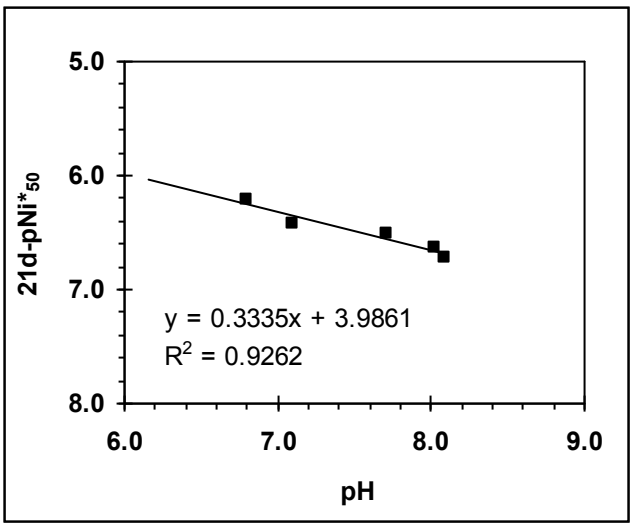


Figure 4.16 21d-pNi^*_{50} of *D. magna* in natural waters vs. pH; data of Deleebeeck et al. (2005).

Now, since the focus in risk assessment is on protecting natural waters, we will here try to calibrate the *D. magna* and *C. dubia* models to natural waters only, using a single 'merged' slope to determine the effect on the overall predictive capacity of the models. Arbitrarily, a 'merged' slope S_{pH} = 0.5961 was adopted (the mean of both species' slopes).

The final models, derived in a similar manner as described in all previous sections, are reported in Table 4.21. Predictive capacities of these models are given in figures 4.21 and 4.22.

Table 4.21 Model[a] parameters for 'unified' chronic Ni bioavailability models for *C. dubia* and *D. magna* and for the *D. magna* model solely based on natural waters

	D. magna (Natural)	*D. magna* (unified)	*C. dubia* (unified)
Log K_{CaBL}	3.53	3.53	3.53
Log K_{MgBL}	3.57	3.57	3.57
S_{pH} (slope of pH function)	0.3335	0.5961	0.5961
Q_{10}	4.183	2.203	3.524
Q_{20}	4.094	2.114	3.389
Q_{50}	3.986	2.006	3.310

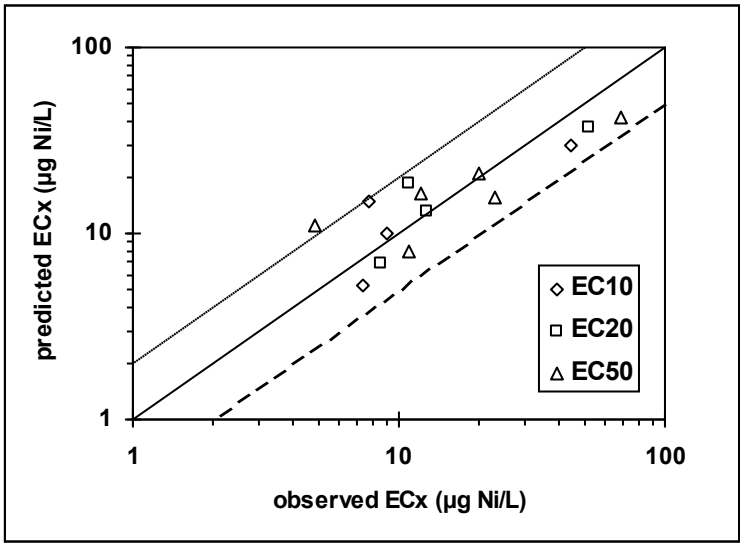


Figure 4.21 Predictive capacity of the 'merged' *C. dubia* chronic Ni toxicity model (Table 4.21) for natural waters

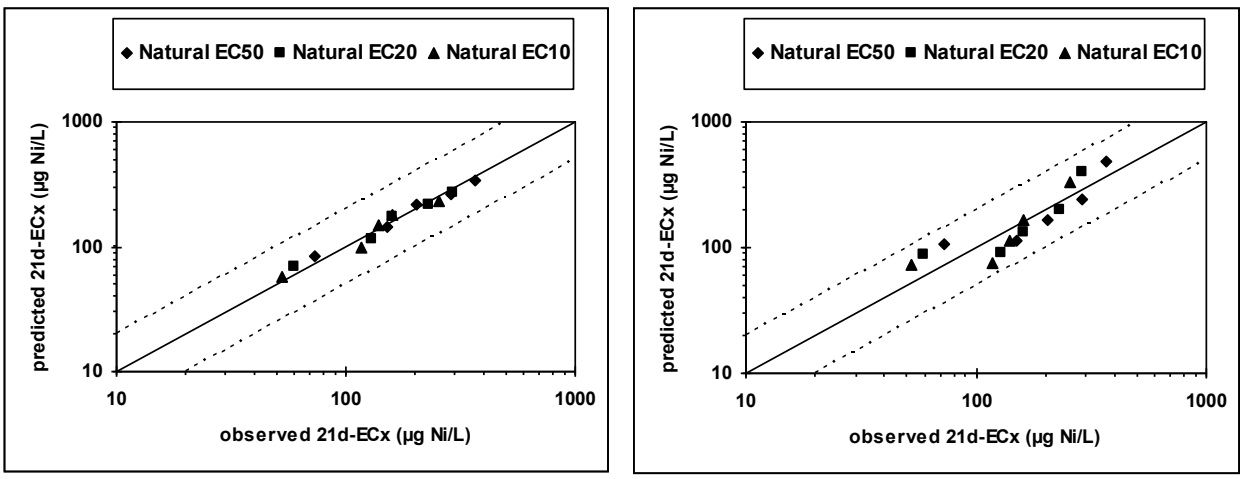


Figure 4.22 Predictive capacity of the *D. magna* chronic Ni toxicity models (Table 4.21) for natural waters; left: developed based on natural waters only; right: 'merged' model

The data in Figure 4.11 clearly demonstrate that:

(i) for *D. magna*, using the pH-slope, derived for natural waters, very accurate predictions are obtained; it must be recognized that this is a calibration rather than a validation.

(ii) For both organisms: using the average 'merged' pH-slope does not result in substantially important prediction errors; most effect concentrations are predicted by an error of less than factor 2.

The latter illustrates that using an 'average' model, does not result in substantially worse prediction errors compared to the species-specific models. We recommend that, for carrying out normalizations for risk assessment purposes, species-specific models should be used when they are available for a given species. For other invertebrate species, we recommend that the normalizations are carried out with both the *C. dubia* and the *D. magna* pH slopes. The knowledge of the overall impact of using different slopes on the final PNEC will allow to take into account uncertainty due to interspecies differences of bioavailability models. If using different slopes does not result in major differences in the final PNEC estimate, it may be more practical to only use the 'average' slope.

4.4. General conclusions for Ni toxicity modelling to aquatic invertebrates

The following overall conclusions are drawn with respect to the modelling of acute and chronic Ni toxicity to *D. magna* and *C. dubia*:

(xi) The pH effect on Ni toxicity is more important in chronic than in acute exposures; toxicity of the free Ni^{2+} ion is generally increased at higher pH

(See Figure 3.3 for *C. dubia*, compare Figure 4.1 with Figure 4.9 for *D. magna*)

(xii) The pH effect becomes increasingly important at pH levels > 8.0-8.2

(See Figure 4.1 for acute *D. magna*, Figure 4.4 for acute *C. dubia*, Figure 4.14 for chronic *C. dubia*)

(xiii) The pH-effect on both acute and chronic Ni toxicity cannot be modelled with a traditional single-site H^+ competition effect. Nevertheless, up to a pH of 8.2, an

acute BLM-type model, which does not account for pH effects at all, is able to yield accurate acute toxicity predictions.

(See section 4.2.1 for acute *D. magna*, section 4.2.3.2 and 4.2.3.3. for acute *C. dubia*, 4.3.3.1 for chronic *D. magna*, and 4.3.4 and 4.3.5.3 for chronic *C. dubia*)

(xiv) The protective effect of water hardness (Ca and Mg) can be modelled with traditional BLM-competition, because linear competitive effects are observed. The effects of Ca and Mg may be similar for both species.

(See Figure 4.1 for acute *D. magna*, Figure 4.3 for acute *C. dubia*, Figure 4.8 for chronic *D. magna*, Table 4.17 for chronic *C. dubia*)

(xv) Alternative bioavailability models were developed, consisting of a traditional Ca, Mg competition effect, superimposed to a log-linear pH relation in the case of chronic Ni toxicity, characterized by a slope parameter, S_{pH}

(See equations 4.1 to 4.5 for acute Ni toxicity, equations 4.6 to 4.12 for chronic Ni toxicity)

(xvi) The slope parameter varied considerably and significantly among species (*C. dubia* vs. *D. magna*), exposure times (acute vs. chronic, i.e. only pH effect considered for chronic), type of water (artificial vs. natural) and the pH range considered (<8.0-8.2 vs. > 8.0-8.2, see also conclusion ii).

(See section 4.3.6 for comparison *C. dubia* and *D. magna* and comparison of synthetic and natural waters for *D. magna*, see Figure 4.14 for comparison between different pH ranges for *C. dubia*)

(xvii) Due to the latter, chronic toxicity data with *C. dubia* obtained at pH > 8.2 should only be used with great care when a Ni effects assessment needs to be conducted for waters with pH < 8.2. One possibility we recommend is a two-step normalization procedure, with the first step being a normalization to pH 8.2 with a model specifically developed for waters with pH > 8.2 (high pH-slope model) and the second step a further normalization to lower pH with the model developed for pH < 8.2 (low slope model). A similar approach could be followed when normalizations need to be carried out from pH < 8.2 to pH > 8.2.

(See Figure 4.14 for the different pH slopes in different pH ranges for *C. dubia*, i.e. a high slope at pH >8.2 and a lower slope at pH < 8.2)

(xviii) When, below pH 8.2, species-specific pH-slopes based on natural waters data are used, very accurate predictions of chronic toxicity are obtained, typically resulting in a prediction error of less than factor 2.

(See Figure 4.13 for *C. dubia* and Figure 4.22 for *D. magna*)

(xix) Also, when a 'merged' average slope is used, based on natural waters test data only, very good predictive capacity of the models is observed for both *D. magna* and *C. dubia*.

(See section 4.3.6 and Figures 4.21 and 4.22)

(xx) We recommend that, for carrying out normalizations for risk assessment purposes, species-specific models should be used when they are available for a given invertebrate species. For other invertebrate species, we recommend that the normalizations are carried out with both the *C. dubia* and the *D. magna* pH slopes. The knowledge of the overall impact of using different slopes on the final PNEC will allow taking into account uncertainty due to interspecies differences of bioavailability models. If using different slopes does not result in major differences in the final PNEC estimate, it may be more practical to only use the 'average' slope.

5. Revision of toxicity modelling with fish and algae

The aim of this section was to refine and revalidate the Ni bioavailability models that were developed for fish and algae (Deleebeeck et al., 2005), using WHAM VI instead of the BLM software. The refinements were carried out according to similar fitting and modelling procedures as outlined in sections 3 and 4. Similar as in sections 3 and 4, the model developments and validations presented below replace all modelling chapters reported in Deleebeeck et al. (2005). However, all raw toxicity data reported in the latter study will be used for the current re-evaluation.

5.2. Development and validation of Ni bioavailability models for fish

All toxicity data with rainbow trout were extracted from Deleebeeck et al. (2005). Ni speciation in artificial waters was calculated at all investigated Ni concentrations in all test solutions, but also at the calculated 17d-LC50s and 21d-LC50s. The complete composition of all test waters and the input-files for the speciation calculations are given in annex 18. The analysis and the model development will be based on 17-day data, whereas the 21-data will be used as validation only. As opposed to Deleebeeck et al. (2005), who only used the full concentration response dataset to estimate BLM parameters, we have also performed a preliminary screening of the individual effects of Ca, Mg, and pH based on LC50s. The speciation of the test solutions at 17d-LC50s and 21d-LC50s is reported in Table 5.1.

When plotting 17d-pNi_{50} vs. pH a highly significant linear relation between 17d-pNi_{50} and pH was found over the whole pH range of 5.5 to 8.5, with the slope of the pH function of 0.324, which is within the range of the slopes found for chronic Ni toxicity to *D. magna* (slope = in artificial waters and 0.334 in natural waters) and *C. dubia* (slope = 0.859). This suggests that the pH function should preferably not be modelled by a single-site H^+ competition effect, since this requires a linear relation between the $LC50_{Ni2+}$ and the H^+ activity (De Schamphelaere and Janssen, 2002).

Table 5.1 Speciation calculations[a] at 17d- and 21d-LC50 for *O. mykiss* in synthetic test solutions; data from Deleebeeck et al. (2005); $LC50_{Ni2+}$ and pNi_{50} are reported

				17days		21days	
Test ID [b]	pH	(Mg^{2+}) (M)	(Ca^{2+}) (M)	$LC50_{Ni2+}$ (M)	pNi_{50}	$LC50_{Ni2+}$ (M)	pNi_{50}
pH 5.8	5.48	9.24E-05	8.69E-05	3.16E-05	4.500	3.05E-05	4.516
pH 6.4	6.76	9.19E-05	8.33E-05	1.31E-05	4.884	1.01E-05	4.994
pH 7.0	7.19	9.18E-05	8.70E-05	9.91E-06	5.004	8.72E-06	5.060
pH 7.6	7.67	9.23E-05	8.44E-05	7.43E-06	5.129	7.36E-06	5.133
pH 8.2	8.47	8.94E-05	7.97E-05	3.19E-06	5.497	2.73E-06	5.563
Mg basis	7.53	1.05E-04	8.09E-05	9.36E-06	5.029	8.57E-06	5.067
Mg 0.5	7.53	3.77E-04	7.67E-05	>1.03E-05	<4.987	1.24E-05	4.905
Mg 1	7.58	7.08E-04	8.64E-05	>1.82E-05	<4.740	>1.82E-05	<4.740
Mg 2	7.55	1.40E-03	6.75E-05	3.33E-05	4.478	4.29E-05	4.368
Mg 3	7.54	2.00E-03	6.13E-05	>1.77E-05	<4.752	>1.77E-05	<4.752
Ca basis	7.59	9.87E-05	8.82E-05	6.68E-06	5.175	4.85E-06	5.314
Ca basis 2	7.52	1.03E-04	9.07E-05	7.01E-06	5.155	ND	ND
Ca 0.5	7.63	9.67E-05	3.40E-04	8.53E-06	5.069	1.19E-05	4.926
Ca 1	7.62	9.08E-05	7.61E-04	2.38E-05	4.623	1.56E-05	4.806
Ca 3	7.52	8.25E-05	1.86E-03	2.19E-05	4.660	ND	ND

[a] See annex 18 for WHAM VI input

[b] Test ID's are the same as those used by Deleebeeck et al. (2005), consult this reference for detailed info

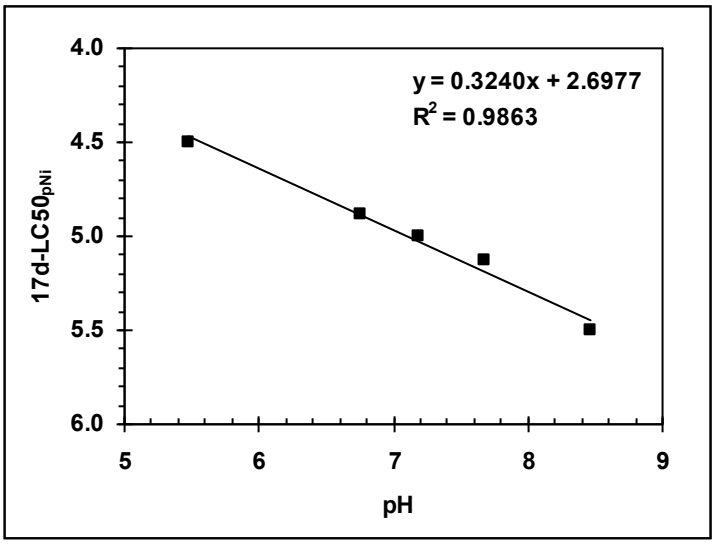


Figure 5.1 Toxicity of Ni^{2+} to *O. mykiss*, expressed as 17d-pNi_{50} as a function of pH

Therefore we developed a refined bioavailability model which is similarly constructed as the chronic models for *C.dubia* and *D. magna* (see section 4, equation 4.6), and which predicts the LC50, expressed as free Ni^{2+} activity, for any test solution *i*.

$$LC50_{Ni2+,i} = 10^{-\left(S_{pH}\cdot pH_i + Q_{50}\right)} \cdot \left\{1 + K_{CaBL}\cdot\left(Ca^{2+}\right)_i + K_{MgBL}\cdot\left(Mg^{2+}\right)_i\right\} \qquad \text{(Eq. 5.1)}$$

This model assumes that the slope of the pH function is independent of the Ca and Mg concentration and that the log K_{MgBL} and log K_{CaBL} are independent of the pH. The second term on the right hand of this equation, which describes competition by Ca and Mg, is justified by the fact that both Ca and Mg clearly reduce the toxicity of Ni^{2+}. This is demonstrated by increased values of the 17d-$LC50_{Ni2+}$ at concentrations of these cations higher than those in the basis medium (Table 5.1). As mentioned in Deleebeeck et al. (2005), the protective effects of Ca and Mg are not easy to compare because only one 17d-LC50 could be derived for an increased Mg concentration. However, based on the approximately 3.5-fold increase of the 17d-$LC50_{Ni2+}$ at 2 mM Mg (33.3 µM) compared to the one in the basis medium (9.36 µM) and the similar 3.5-fold increase 17d-$LC50_{Ni2+}$ at 1 and 3 mM Ca (23.8 and 21.9 µM) compared to the one in the basis medium (6.7 to 7.0 µM), it may be suggested that their protective effects are reasonably similar. The similarity of the LC50 at 1 and 3 mM of Ca suggest that there may be a limit (a 'plateau') to the protective effect of Ca.

The protective effect of Ca and Mg can be modelled on the basis of BLM-type, single-site competition, by means of deriving stability constants for these cations (See Eq. 5.1). Applying the method of De Schamphelaere and Janssen (2002) to the dataset of the Ca and Mg test series, while also accounting for the uncertainty associated with (i) the limited LC50 dataset for Mg and (ii) the apparent 'plateau' of the Ca protective effect, we found that initial estimates for both log K_{CaBL} and log K_{MgBL} between 3.1 and 3.8 were appropriate.

In the next step, these values were optimized using the full concentration response data, by fitting following 'normalized concentration'-response curve to survival (y %) observations at any investigated Ni concentration j in any test solution i.

$$y = \frac{100}{1 + \left(\frac{\exp\left(Q_{i,j}\right)}{\exp\left(Q_{50}\right)} \right)^{\beta}} \qquad \text{(Eq 5.2)}$$

Where

$$Q_{i,j} = -\log \frac{\left(Ni^{2+}\right)_{i,j}}{\left\{1 + K_{CaBL} \cdot \left(Ca^{2+}\right)_i + K_{MgBL} \cdot \left(Mg^{2+}\right)_i\right\}} - S_{pH} \cdot pH_i \qquad \text{(Eq. 5.3)}$$

is a measure of 'bioavailable Ni', being the Ni^{2+} activity normalized for pH, Ca, and Mg. It is the intercept of the linear relation between $EC50_{pNi}$, corrected for hardness effects, vs. pH. A lower Q means a higher level of bioavailable Ni. S_{pH} =0.3240, was taken from Figure 5.1, and K_{CaBL}, K_{MgBL}, Q_{50} and β were optimized. Based on the previous discussion, we started the optimization with assuming log K_{CaBL} = log K_{MgBL}. The best fit was obtained with log K_{CaBL} = log K_{MgBL} = 3.6, Q_{50} = 2946 (95% CI: 2.881-3.010), β = -4.477, with an r^2 of 0.74. The result of this fit is exemplified in Figure 5.2. A comparison is provided with a plot of survival vs. pNi. This comparison clearly illustrates that pNi is a much worse predictor of Ni toxicity then Q, which is a measure of the truly bioavailable Ni^{2+}. Assuming different values of log K_{CaBL} vs. log K_{MgBL} did not result in significant improvement of the fit, so our initial assumption seemed plausible, although additional research would be helpful to verify this in more detail.

Now 17d-$LC50_{Ni2+}$ can be predicted by equation 5.1, and by linking to WHAM VI, 17d-$LC50_{dissolved}$ van be predicted. Also, equation 5.2 can be rearranged to predict the 'bioavailable' Ni that would be expected to result in y% survival or x%=(100-y)% mortality.

$$Q_y = \frac{1}{\beta} \cdot \ln\left(\frac{100}{y} - 1\right) + Q_{50} \qquad \text{(Eq. 5.4)}$$

and

$$Q_x = \frac{1}{\beta} \cdot \ln\left(\frac{100}{100 - x} - 1\right) + Q_{50} \qquad \text{(Eq. 5.5)}$$

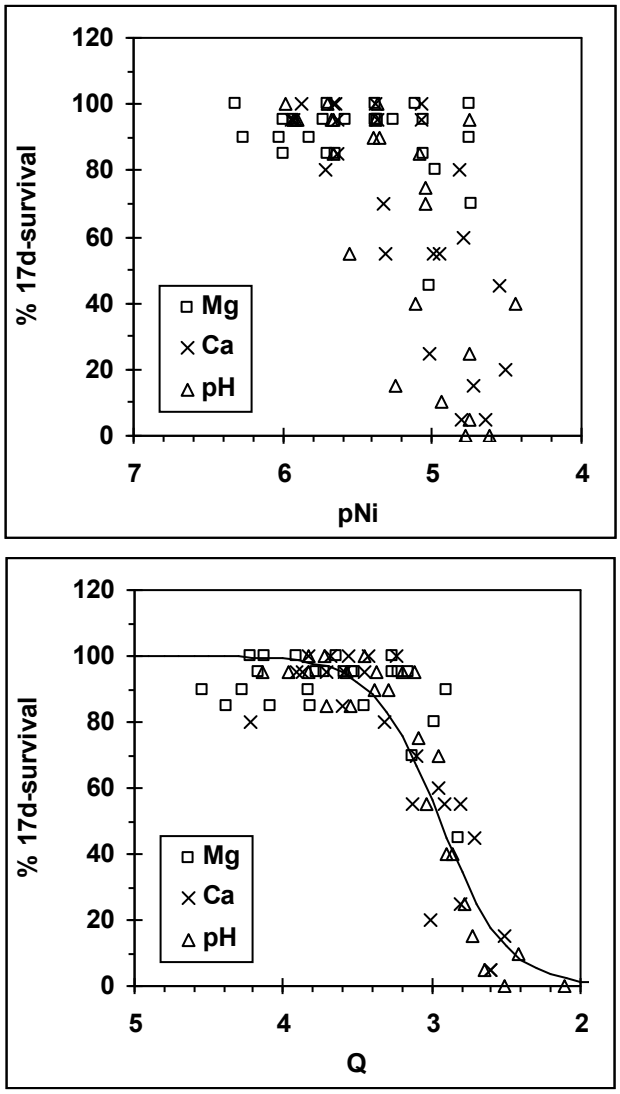


Figure 5.2. 17d-survival as a function of pNi and Q, a measure of bioavailable Ni, for all synthetic test waters and all investigated Ni concentrations.

Q_x can replace Q_{50} in equation 5.1 to predict the Ni^{2+}-activity expected at x% mortality (i.e., the LCx_{Ni2+}, as well as the dissolved Ni concentration associated with this, by using WHAM6:

$$LCx_{Ni2+,i} = 10^{-\left(S_{pH}\cdot pH_i + Q_x\right)} \cdot \left\{1 + K_{CaBL}\cdot\left(Ca^{2+}\right)_i + K_{MgBL}\cdot\left(Mg^{2+}\right)_i\right\} \qquad \text{(Eq. 5.6)}$$

These predicted concentrations can then be compared with the measured Ni concentrations associated with x% mortality, and this can be done for all Ni concentrations and test solutions.

Observed vs. predicted 17d-LC50s and 17d-LCx are plotted in Figure 5.3. The data in Figure 5.3 illustrate that most 17d-LC50s are predicted by an error of less than factor 2, not only for the synthetic test waters, but also for the natural test waters, the data of which have also been taken from Deleebeeck et al. (2005). Meaningful validations could only be performed for four test waters (i.e. Ankeveen, Bihain, Brisy, and Markermeer) because the 17d-LC50 in 'Eppe' was extrapolated and had very large confidence interval (filled triangle in Figure 5.3). The 17d-LC50s in Ankeveen, Brisy and Markermeer were predicted very accurately, i.e. by an error of 1.1-fold on average. The 17d-LC50 in Bihain was overestimated by 2.9-fold. Although this represents an important improvement compared to the model reported by Deleebeeck et al. (2005), where average prediction errors amounted to a factor of 6.4 for this surface water, it is still a much worse prediction than all other waters investigated. The latter may perhaps be explained by the fact that this test water had properties at the border of or even outside the chemistry ranges for which our model was developed. While in this water the pH of 5.6 was at the border, its water hardness of 14 mg $CaCO_3$/L (Ca = 0.09 mM, Mg = 0.05 mM, Table 2) was clearly lower than in all synthetic test waters, with a minimum hardness of 25 mg $CaCO_3$/L (Ca = Mg = 0.12 mM). The combination of those two rather 'extreme' conditions may have resulted in a higher-than-predicted sensitivity of the rainbow trout in this test water. In general, most 17d-LCx values could also be predicted by an error of less than a factor of 2. Again, three data points of the test in Bihain were inaccurately predicted. The same analysis was also repeated for the 21-day data, with similar observations (Figure 5.3), except that no 21-day data were available for Bihain water and the test at 3 mM Ca. The model constants for predicting 17-day and 21-day Ni toxicity to fish survival are summarized in Table 5.2.

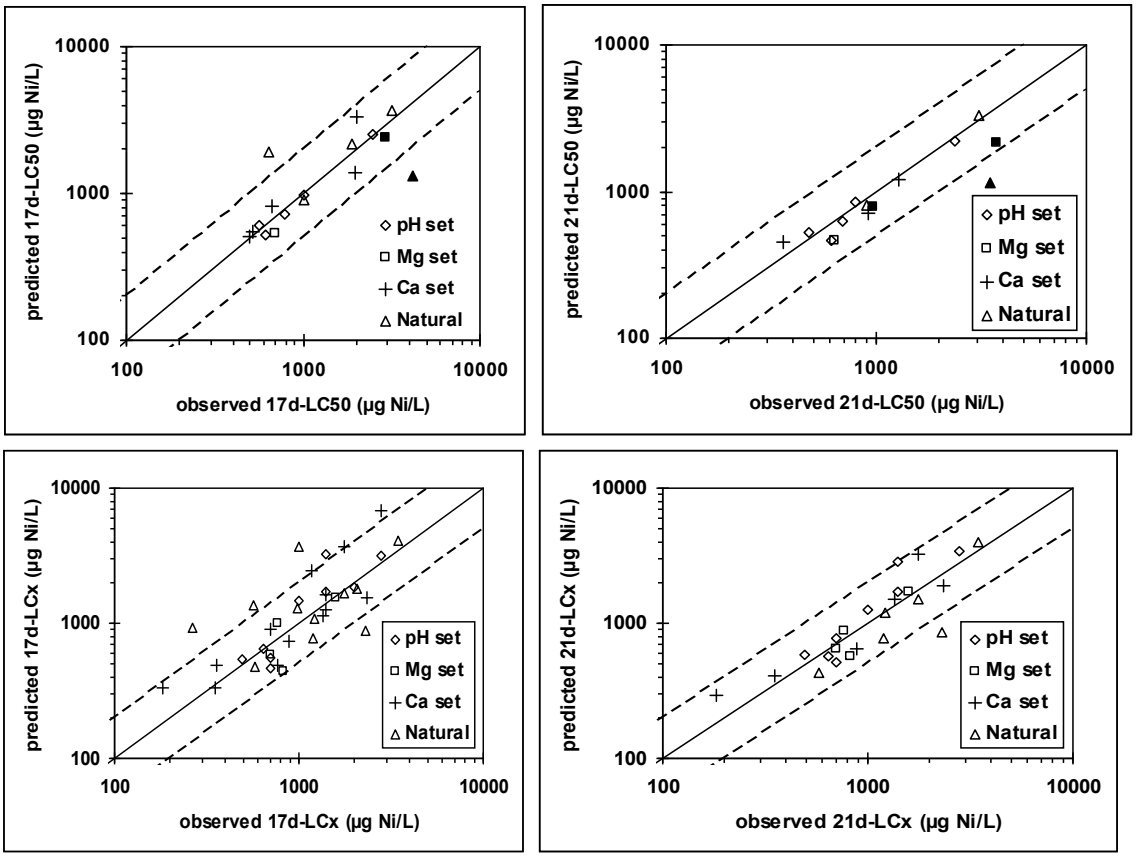


Figure 5.3 Predictive capacity of the model for LC50's (equation 5.1, top) and LCx's for x>10% and <100% (equation 5.6, down) after 17 (left) and 21 days of exposure (right). Filled symbols indicate 'extrapolated' LC50s and are less reliable to evaluate the predictive capacity of the models. The model constants for 17-day and 21-day Ni toxicity to fish survival are summarized in Table 5.2.

Table 5.2 Parameter values for the Ni bioavailability models for chronic rainbow trout survival to be used in equations 5.1, 5.5 and 5.6.

Parameter	Parameter value
log K_{Ca}	3.6
log K_{Mg}	3.6
S_{pH}	0.324
17d-Q_{50}	2.946
17d-β	-4.477
21d-Q_{50}	3.002
21d-β	-4.520

5.3. Development and validation of Ni bioavailability models for algae

5.3.1. Refinement of the Ni bioavailability model

We re-evaluated the 72-hour toxicity data with *Pseudokirchneriella subcapitata* reported in Deleebeeck et al. (2005) using WHAM 6 as the speciation model. First, All 72h-E_rC10 and E_rC50s obtained in synthetic test waters were taken from this study as well as the water chemistry associated with each test. The input-file for speciation calculations, with full chemistry of these solutions is given in annex 19. The most important components of the test media is given in Table 5.3. As in Deleebeeck et al. (2005), the model development described below will be based on 72h-E_rC50's; the model will then be validated for 72h-E_rC10s. The results of the speciation calculations, i.e. calculated activities of H^+, Ca^{2+}, Mg^{2+}, and Ni^{2+} are also given in Table 5.3.

Similar as in Deleebeeck et al. (2005), we observed that increased activities of H^+, Ca^{2+}, Mg^{2+} resulted in reduced toxicity of Ni^{2+}. While increased Ca^{2+} and Mg^{2+} resulted in linear increases of the 72h-E_rC50_{Ni2+}'s, an increase of H^+ only yielded a linear increase of the 72h-E_rC50_{Ni2+} up to an H^+ activity of 0.6 µM (or down to a pH of 6.5) (Figure 5.4). At a pH of 6.1 a similar 72h-E_rC50 was observed compared to pH 6.45.

In order to take into account the pH effect by means of a biotic ligand model, we therefore decided not to consider data points with a pH lower than 6.45 for model development. This also sets the lower boundary of this model's applicability to pH 6.45. Using the method described by De Schamphelaere and Janssen (2002) we derived parameter values for K_{HBL}, K_{CaBL}, and K_{MgBL} to be used in BLM-type Ni-toxicity equation which is used to predict the ECx_{Ni2+} for any test solution *i*, based on known activities of H^+, Ca^{2+}, and Mg^{2+}:

$$72h - E_rCx_{Ni2+,i} = 72h - E_rCx^*_{Ni2+} \cdot \left\{1 + K_{CaBL} \cdot \left(Ca^{2+}\right)_i + K_{MgBL} \cdot \left(Mg^{2+}\right)_i + K_{HBL} \cdot \left(H^+\right)_i\right\} \quad \text{(Eq. 5.7)}$$

Table 5.3 Test solution compositions[a], speciation [b] and 72h-E_rC50's and E_rC10's of Ni for *P. subcapitata* in synthetic test waters (data from Deleebeeck et al., 2005).

Test ID [c]	pH	Total Ca (M)	Total Mg (M)	Dissolved EC50 (µg/L)	Dissolved EC10 (µg/L)	Activity (H^+) (M)	Activity (Mg^{2+}) (M)	Activity (Ca^{2+}) (M)	EC50 Ni^{2+} activity (M)	EC10 Ni^{2+} activity (M)
Mg 0.12 mM *	7.48	7.73E-05	1.23E-04	124	25.3	3.31E-08	9.13E-05	5.73E-05	1.46E-06	2.99E-07
Mg 0.5 mM	7.51	7.49E-05	4.53E-04	255	75.2	3.09E-08	3.29E-04	5.42E-05	2.94E-06	8.66E-07
Mg 1.0 mM	7.5	7.73E-05	8.64E-04	321	108	3.16E-08	6.11E-04	5.45E-05	3.61E-06	1.21E-06
Mg 1.5 mM	7.52	7.73E-05	1.85E-03	399	124	3.02E-08	1.25E-03	5.21E-05	4.29E-06	1.33E-06
Mg 2.0 mM	7.53	7.73E-05	1.89E-03	596	162	2.95E-08	1.26E-03	5.16E-05	6.36E-06	1.72E-06
Mg 2.5 mM	7.54	7.73E-05	2.35E-03	742	252	2.88E-08	1.54E-03	5.06E-05	7.69E-06	2.62E-06
Mg 3.0 mM	7.53	7.49E-05	2.92E-03	812	213	2.95E-08	1.87E-03	4.79E-05	8.25E-06	2.17E-06
Mg 4.0 mM	7.54	7.49E-05	3.83E-03	821	284	2.88E-08	2.37E-03	4.62E-05	8.07E-06	2.79E-06
Mg 5.0 mM	7.52	7.49E-05	4.73E-03	1119	365	3.02E-08	2.84E-03	4.49E-05	1.07E-05	3.48E-06
Ca 0.12 mM *	7.4	8.48E-05	1.15E-04	93.7	30.3	3.98E-08	8.58E-05	6.31E-05	1.12E-06	3.61E-07
Ca 0.5 mM	7.38	3.49E-04	1.15E-04	108	36.6	4.17E-08	8.41E-05	2.54E-04	1.26E-06	4.28E-07
Ca 1.0 mM	7.4	6.99E-04	1.15E-04	114	37.3	3.98E-08	8.19E-05	4.97E-04	1.30E-06	4.25E-07
Ca 2.0 mM	7.39	1.45E-03	1.15E-04	136	51.9	4.07E-08	7.83E-05	9.85E-04	1.48E-06	5.64E-07
Ca 3.0 mM	7.39	2.17E-03	1.15E-04	122	31.5	4.07E-08	7.54E-05	1.42E-03	1.28E-06	3.30E-07
Ca 4.0 mM	7.41	2.87E-03	1.15E-04	144	42.1	3.89E-08	7.29E-05	1.82E-03	1.45E-06	4.26E-07
Ca 5.0 mM	7.44	3.59E-03	1.15E-04	141	40.5	3.63E-08	7.08E-05	2.21E-03	1.38E-06	3.97E-07
pH 6.0 [d]	6.01	8.48E-05	1.28E-04	125	47.5	9.77E-07	9.33E-05	6.17E-05	1.51E-06	5.74E-07
pH 6.4	6.45	8.73E-05	1.28E-04	145	51.9	3.55E-07	9.35E-05	6.36E-05	1.74E-06	6.22E-07
pH 7.2	7.29	8.73E-05	1.23E-04	91.8	37	5.13E-08	8.98E-05	6.36E-05	1.07E-06	4.33E-07
pH 7.6 *	7.65	8.98E-05	1.19E-04	83.1	44.3	2.24E-08	8.68E-05	6.53E-05	9.56E-07	5.09E-07
pH 8.0	7.92	8.73E-05	1.28E-04	81.5	35.9	1.20E-08	9.33E-05	6.34E-05	9.09E-07	4.00E-07
pH6-Mg 0.12 mM	6.23	1.15E-04	1.28E-04	172	57.6	5.89E-07	9.41E-05	8.44E-05	2.09E-06	6.99E-07
pH-6 Mg 1.5 mM	6.13	1.15E-04	1.48E-03	880	312	7.41E-07	1.00E-03	7.80E-05	9.88E-06	3.50E-06
pH-6 Mg 3.0 mM	6.08	1.05E-04	3.00E-03	883	145	8.32E-07	1.91E-03	6.67E-05	9.23E-06	1.52E-06
pH7-Mg 0.12 mM	7.2	1.12E-04	1.77E-04	108	26.5	6.31E-08	1.29E-04	8.16E-05	1.27E-06	3.12E-07
pH-7 Mg 1.5 mM	7.16	1.10E-04	1.36E-03	601	180	6.92E-08	9.23E-04	7.45E-05	6.54E-06	1.97E-06
pH-7 Mg 3.0 mM	7.15	1.12E-04	3.05E-03	914	292	7.08E-08	1.93E-03	7.07E-05	9.32E-06	2.98E-06
pH7.8-Mg 0.12 mM	7.95	1.07E-04	1.23E-04	98.3	31.5	1.12E-08	9.02E-05	7.82E-05	1.09E-06	3.52E-07
pH-7.8 Mg 1.5 mM	7.88	1.10E-04	1.48E-03	345	44.3	1.32E-08	1.00E-03	7.43E-05	3.61E-06	4.64E-07
pH-7.8 Mg 3.0 mM	7.85	1.07E-04	3.00E-03	395	91.2	1.41E-08	1.90E-03	6.77E-05	3.89E-06	8.97E-07

[a] See annex 19 for full WHAM VI input chemistry

[b] All speciation calculations were run with WHAM VI

[c] Test ID's are the same as those used by Deleebeeck et al. (2005), tests have been conducted in four separate test runs, i.e., the univariate Mg series, Ca series, and pH series, and the bivairate pH-Mg series series; consult this reference for detailed info; test media marked by an * have same pH, Ca, and Mg as standard OECD test water (OECD, 1984)

[d] data point not used for model development, implying model to be used carefully below pH 6.45 (see text)

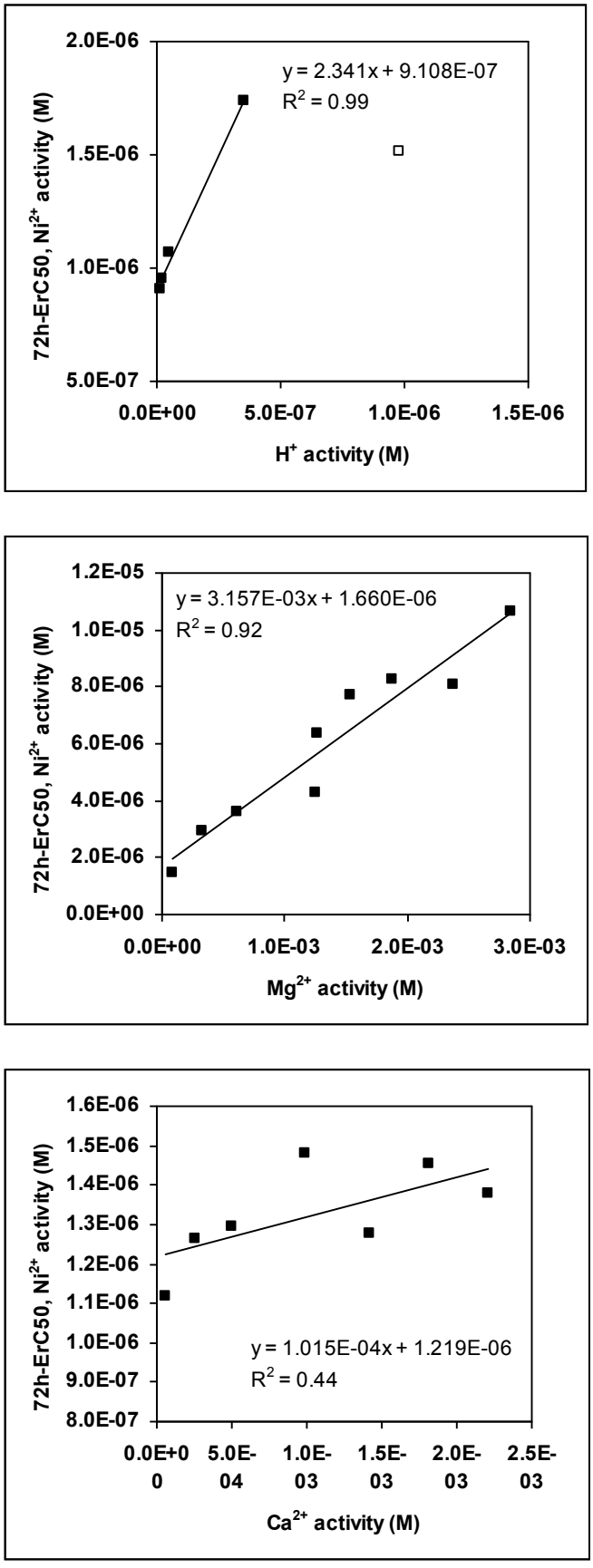


Figure 5.4 The effect of H^+, Mg^{2+}, and Ca^{2+} on the 72h-E_rC50_{Ni2+}. The open square data point in the upper panel is not used for modeling (see text).

Theoretically, the ECx^*_{Ni2+} is the ECx_{Ni2+} under the condition where competition by competing cations is negligible. It is thus a model constant which is, within the proposed BLM concept, assumed to represent the 'inherent sensitivity' of the algae, i.e. the sensitivity which is independent of water chemistry, and equals:

$$E_r Cx^*_{Ni2+} = \frac{f^x_{NiBL}}{\left(1 - f^x_{NiBL}\right) \cdot K_{NiBL}} \qquad \text{(Eq. 5.8)}$$

Where f^x_{NiBL} = the fraction of biotic ligand sites occupied by Ni at x% inhibition of algal growth rate, independent of water chemistry. Whatever value of K_{NiBL} is assumed, there is always a value of f^x_{NiBL} which is unambiguously associated with it, through equation 5.8. Although values can be assigned to both parameters, it is mathematically not strictly needed for toxicity predictions with equation 5.7. Accordingly, this was not carried out in the present study.

We derived values of log K_{HBL} = 6.5, log K_{CaBL} = 2.0 and log K_{MgBL} = 3.3. The large difference between stability constants for Ca and Mg reflects the much larger influence of Mg on Ni toxicity to *P. subcapitata* (Figure 5.5). For this reason it is important to explicitly differentiate between Ca and Mg in Ni effects estimations. The model parameter ECx^*_{Ni2+} was derived for x=10 and x=50, according to a similar approach as followed in Section 3:

$$72h - E_r Cx^*_{Ni2+} = \sqrt[n]{\prod_i^n \frac{72h - E_r Cx_{Ni2+,i}}{\left\{1 + K_{CaBL} \cdot \left(Ca^{2+}\right)_i + K_{MgBL} \cdot \left(Mg^{2+}\right)_i + K_{HBL} \cdot \left(H^+\right)_i\right\}}} \qquad \text{(Eq. 5.9)}$$

Briefly, the right-hand term was calculated for each exposure solution *i* investigated, using calculated activities of H^+, Ca^{2+}, Mg^{2+}, and $EC50_{Ni2+}$ and $EC10_{Ni2+}$ (annex x) and the parameter values for K_{HBL}, K_{CaBL}, and K_{MgBL} reported in Table 5.3. This term was then averaged (geometric mean) over the *n* test solutions investigated. Only the data from univariate test series of Ca, Mg and pH (except pH 6.0) were used for this estimation. Values of 1.12 µM and 0.365 µM of Ni^{2+} were found for the $EC50^*_{Ni2+}$ and the $EC10^*_{Ni2+}$, respectively. All model parameters are summarized in Table 5.4.

Table 5.4 Parameter values for the Ni BLM for *P. subcapitata* growth inhibition to be used in BLM-equations 5.7 and 5.9.[a]

Parameter	Parameter value
'Bioavailability parameters'	
log K_H	6.5
log K_{Ca}	2.0
log K_{Mg}	3.3
'Inherent sensitivity'	
$EC50^*_{Ni2+}$ (µM)	1.12 (3.57)[b]
$EC10^*_{Ni2+}$ (µM)	0.365 (0.549) [b]

[a] Equation 5.7 yields predicted values of EC50s and EC10 expressed as Ni^{2+}-activity; these activities need to be inserted into WHAM VI to predict EC10's and EC50's as dissolved Ni

[b] The lower value is the model based on tests from the Ca, Mg, and pH test series reported in Table 5.3 and was used to generate Figures 5.5 and 5.6; The higher value is the value calibrated to the lower 'inherent sensitivity' of the algae during tests with natural waters and was used to generate Figure 5.7 (see also text)

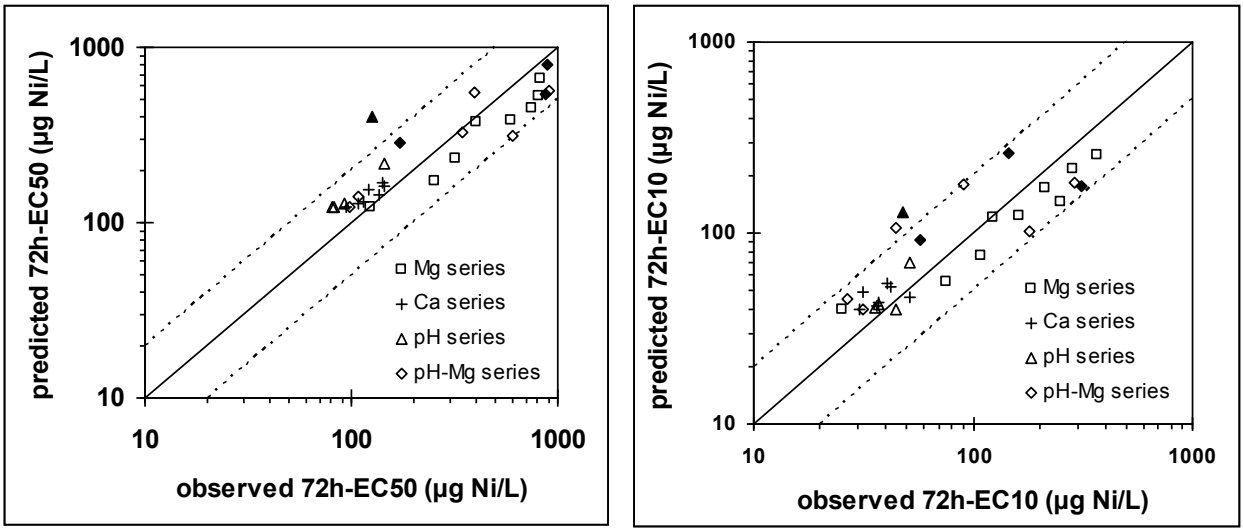


Figure 5.5 Predictive capacity of the Ni-BLM (Equation 5.7, parameter values in Table 5.4) for *P. subcapitata* in synthetic test waters from different univariate test series (data from Delebeeck et al., 2005) as shown by observed vs. predicted 72h-E_rC50's and 72h-E_rC10's. The calibration was performed on data from the Mg, Ca, and pH test series according to equation 5.7. The full line indicates perfect predictions; the dashed line indicates a 2-fold prediction error. Filled data points were obtained at pH < 6.4

Figure 5.5 demonstrates how well this model is calibrated to this dataset. When the data point obtained at pH < 6.4 was not considered (filled triangle), all EC50's and EC10's were predicted by an error of less than factor 2 with an average 1.3-fold prediction error for both EC50's and EC10's. The EC50 and EC10 for this data point at pH 6.0 were overestimated by 3.2 and 2.7-fold, respectively (filled triangle in Figure 5.5). This is logic since the model (equation 5.7) predicts a continuous increase of the ECx with decreasing pH, while the data suggest otherwise below pH 6.4 (see open square data point in Figure 5.4, upper panel).

5.3.2. Independent model validation with synthetic waters

A validation was also carried out with synthetic waters in which pH and Mg, which are the two most important factors affecting Ni toxicity to *P. subcapitata*, were varied in a bivariate manner (Deleebeeck et al., 2005). This validation was carried out to see whether the developed model could also cope with potential interactive effects of pH and Mg, which cannot be directly inferred from univariate testing. The model used is equation 5.7 with the parameter values in Table 5.4. The outcome of this validation is also presented in Figure 5.5. When the three data points obtained at pH = 6.0 were not considered (filled diamonds), All EC50's and all but one EC10 were predicted by an error of less than factor 2. The average prediction errors were 1.4-fold for the EC50's and 1.8-fold for the EC10's. Remarkably, All EC50's and EC10's obtained at pH 6.0 (i.e., outside the models calibration range, filled diamonds in Figure 5.5) and at Mg concentrations between 0.12 and 3.0 mM, were also predicted by an error of less than a factor of 2 (average = factor 1.6). Even the data point obtained at pH 6.0 and Mg = 0.12 mM was reasonably well predicted, although this is exactly the same synthetic test water (same composition) as the test water at pH 6.0 in the pH test series (open square data point in Figure 5.4). Hence, it might be possible that the ECx values from the latter test might have been an 'outlier' and that the model might perhaps roughly be valid even at pH < 6.4. This will be addressed below, where data from tests in natural waters will be validated.

5.3.3. Independent model validation with natural waters

A validation was also carried out with tests in natural waters reported by Deleebeeck et al. (2005). The latter study also included a data set generated earlier by Bossuyt et al. (2001). The main water chemistry and 72h-E_rC50 and 72h-E_rC10 values are reported in Table 5.5.

Although Deleebeeck et al. (2005) did not consider the part of the Bossuyt et al. (2001) dataset that was obtained in artificial waters (i.e., two tests in standard OECD test water), we wanted to include them in the present re-evaluation from a weight-of-evidence perspective. The model used is again equation 5.7 with the parameter values in Table 5.4. All calculations were performed with WHAM VI, using the DOC assumptions explained in detail in section 3.1.3.2, i.e. log $K_{MA(Ni)}$ = 1.75 and 40 % active fulvic acid. The outcome of this validation is presented in Figure 5.6.

Table 5.5 Test solution compositions[a], speciation [b] and 72h-E_rC50's and E_rC10's of Ni for *P. subcapitata* in natural waters and synthetic test waters tested along with these (data from Bossuyt et al., 2001; Deleebeeck et al., 2005, and from the present study).

Test water[c]	Data Source	pH [d]	DOC (mg/L)	Total Ca (M)	Total Mg (M)	Dissolved EC50 (µg/L)	Dissolved EC10 (µg/L)	Activity (H^+) (M)	Activity (Mg^{2+}) (M)	Activity (Ca^{2+}) (M)	EC50 Ni^{2+} activity (M)
Bihain	1	6.35	6.62	9.81E-04	1.80E-04	483	90	4.47E-07	1.22E-04	6.49E-04	4.84E-06
Bihain	1	6.35	6.62	9.81E-04	1.80E-04	508	88.2	4.47E-07	1.22E-04	6.49E-04	5.10E-06
Ankeveen	1	7.37	25.8	1.32E-03	4.57E-04	1236	314	4.27E-08	2.91E-04	8.16E-04	7.34E-06
Ankeveen	1	7.47	25.8	1.32E-03	4.57E-04	1043	219	3.39E-08	2.91E-04	8.14E-04	5.75E-06
Mole	1	7.99	5.14	1.40E-03	4.63E-04	584	154	1.02E-08	2.91E-04	8.56E-04	3.97E-06
Mole	1	8.01	5.14	1.40E-03	4.63E-04	750	73.7	9.77E-09	2.90E-04	8.56E-04	5.17E-06
OECD* [M]	1	7.70	0 [e]	1.20E-04	1.20E-04	339.0	67.1	2.00E-08	9.47E-05	9.44E-05	4.17E-06
OECD* [M]	1	7.70	0 [e]	1.20E-04	1.20E-04	399.8	44.6	2.00E-08	9.47E-05	9.44E-05	4.91E-06
Ankeveen	2	6.96	22.6	1.02E-03	2.91E-04	823	- [f]	1.10E-07	1.95E-04	6.66E-04	5.23E-06
Bihain	2	5.69	9.76	1.10E-04	4.00E-05	598	154	2.04E-06	3.32E-05	8.99E-05	6.80E-06
Brisy	2	6.89	2.54	2.53E-04	1.57E-04	376	75.5	1.29E-07	1.28E-04	2.04E-04	4.40E-06
Eppe	2	7.42	4.88	8.34E-04	2.50E-04	804	245	3.80E-08	1.85E-04	6.09E-04	7.30E-06
Markermeer	2	7.74	8.37	1.54E-03	6.40E-04	875	154	1.82E-08	3.92E-04	9.18E-04	5.95E-06
Regge	2	7.65	10.3	1.72E-03	3.25E-04	1072	251	2.24E-08	2.12E-04	1.10E-03	7.17E-06
OECD* [M]	3	7.42	0 [e]	1.20E-04	1.20E-04	362	63	3.80E-08	9.10E-05	9.08E-05	4.42E-06
$OECD^+$ [M]	3	7.42	0 [e]	2.00E-03	5.00E-04	669	99	3.80E-08	3.31E-04	1.32E-03	7.14E-06
Ankeveen	3	7.29	22.4	1.63E-03	4.10E-04	1627	425	5.13E-08	2.54E-04	9.79E-04	1.10E-05
Brisy	3	6.53	4.1	2.20E-04	1.57E-04	506	154	2.95E-07	1.24E-04	1.73E-04	5.82E-06
Regge	3	7.78	10.9	1.49E-03	3.22E-04	1186	301	1.66E-08	1.97E-04	8.92E-04	7.29E-06

[a] See annex 20 for full WHAM VI input chemistry; composition was calculated from measured concentrations in natural waters, taking into account additions of nutrients according to the OECD (1984) protocol, and pH adjustments with NaOH

[b] All speciation calculations were run with WHAM VI

[c] Test media marked by an * have same pH, Ca, and Mg as standard OECD test water (OECD, 1984); $OECD^+$ is standard OECD test water with Ca increased to 2 mM and Mg to 0.5 mM; tests marked with an [M] were used for model calibration to the specific inherent sensitivity of the algae in these test series (see text and also Table 5.4)

[e] (1) Bossuyt et al. (2001); (2) Deleebeeck et al. (2005); (3) Present study, newly generated data

[d] Average pH recorded during testing; for data from Bossuyt et al. (2001) average measured pH values were obtained via personal communication

[e] DOC in synthetic test media was assumed not to complex significant amounts of Ni (based on data reported in section 4.3.2.1)

[f] data point not considered because unreliable confidence intervals (see Deleebeeck et al. (2005) for more info

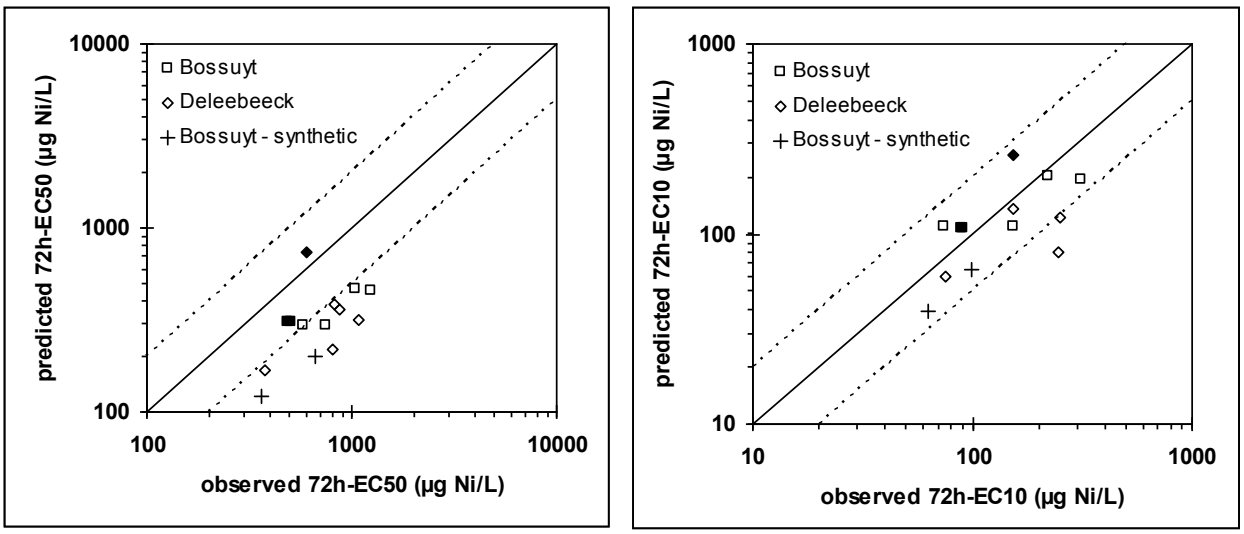


Figure 5.6 Independent evaluation of the predictive capacity of the Ni-BLM (Equation 5.7, parameter values in Table 5.4) for *P. subcapitata* in natural test waters (data from Bossuyt et al., 2001; Delebeeck et al., 2005) and two standard synthetic waters tested along with the natural waters in Bossuyt et al. (2001). Figures show observed vs. predicted 72h-E_rC50's and 72h-E_rC10's. The calibration was performed on data from the univariate Mg, Ca, and pH test series according to equation 5.7. The full line indicates perfect predictions; the dashed line indicates a 2-fold prediction error. Filled data points were obtained at pH < 6.4.

When the three data points obtained at pH < 6.45, i.e. pH 5.7 in Bihain water tested by Deleebeeck et al. (2001) and pH 6.35 in Bihain water tested by Bossuyt et al. (2001) were not considered (filled symbols in Figure 5.6), all EC50's were underestimated by more than 2-fold, with an average of 2.6. Although predictions of EC10's exhibited an average 1.6-fold difference with observed EC10's, most of them were also underestimated (i.e., below the 1:1 reference line, Figure 5.6).

Initially, this looked as if the model developed based on synthetic waters does not capture the effects of bioavailability modifying factors in the field and/or the interactive between these factors. However, in section 5.3.2 we showed that the interactive effects of Mg and pH were reasonably well predicted. Another potential explanation, as also put forward previsouly for chronic *D. magna* test results (section 4.3.3.2), might have been that the presence of humic substances in the natural test waters (as opposed to synthetic waters) might have ameliorated Ni toxicity beyond its effect on Ni-speciation. This would indeed lead to higher-then-observed predictions of ECx levels in the presence of natural DOC.

However, when the test data obtained in standard synthetic test water are considered ("+" in Figure 5.6), which were tested simultaneously with the natural waters by Bossuyt et al. (2001), it is observed that EC50's and EC10's are also largely underestimated, i.e. by 3.2-fold and 1.5-fold, respectively. Again, the underestimation was worse for the EC50 then for the EC10. This put forward the idea that perhaps the differences of the inherent sensitivity of *P. subcapitata* between different test series, might have been larger than anticipated. To investigate this, we calculated $EC50^*_{Ni2+}$ and $EC10^*_{Ni2+}$ separately for each test series using equation 5.9, since these are good measures of the 'inherent' sensitivity (see above). If possible, only the synthetic test waters were used to calculate these values; pH values lower than 6.45 were not considered. The results of these calculations are presented in Table 5.6.

Table 5.6 'Inherent sensitivity' [a] of *P. subcapitata* in different test runs

Data source	**Test series / waters [b]**	$EC50^*_{Ni2+}$ (µM)	$EC10^*_{Ni2+}$ (µM)
Deleebeeck et al. (2005)	Mg	1.52	0.448
Deleebeeck et al. (2005)	Ca	0.953	0.299
Deleebeeck et al. (2005)	pH (without pH<6.45)	0.794	0.314
Deleebeeck et al. (2005)	pH-Mg (without pH<6.45)	1.18	0.300
Bossuyt et al. (2001)	two OECD waters	3.60	0.543
This study	OECD and $OECD^+$ water	3.55	0.567
Deleebeeck et al. (2005)	Natural (without pH < 6.45)	3.46	0.685

[a] 'Inherent sensitivity' is reported as $EC50^*_{Ni2+}$ and $EC10^*_{Ni2+}$, and was calculated as explained in text by applying equation 5.9 to data from Tables 5.3 and 5.5

[b] See Tables 5.3 and 5.5 for more information on these test series

The calculation reveals important inter-test series differences of the inherent sensitivity. Indeed, when only artificial test waters are considered, $EC50^*_{Ni2+}$ values varied between 0.79 and 3.6 µM (factor 4.5), while $EC10^*_{Ni2+}$ values only varied between 0.30 and 0.54 (factor 1.5). Interestingly, the inherent sensitivity in the Bossuyt et al. (2001) dataset seemed lower than the inherent sensitivity in the synthetic waters dataset of Deleebeeck et al. (2005), although the differences are less for the EC10-level than for the EC50-level. Therefore, this may very well explain why the natural waters EC50 values of Bossuyt et al. (2001) were underestimated (Figure 5.6), while it was less obvious for their EC10 values. A similar conclusion is reached when the 'dissolved' EC50 and EC10 values in synthetic media with the same pH, Ca, and Mg, but performed in different test series, are compared. This can be done for the pH, Ca, and Mg test series of Deleebeeck et al. (2005) as well as for the Bossuyt et al. (2001) test series, which all contain a test at pH, Ca, and Mg of the OECD standard test water (media marked with a * in Tables 5.3 and 5.5). While EC50's in this

OECD medium were between 83.1 and 124 µg Ni/L in the univariate pH, Ca, and Mg test series, they were between 339 and 400 µg Ni/L in Bossuyt et al. (2001) (See Tables 5.3 and 5.5). Again, EC10's were less different, i.e. between 25.3 and 44.3 µg/L in the univariate pH, Ca, and Mg test series, and between 44.6 and 67.1 µg Ni/L in Bossuyt et al. (2001). These data confirm the conclusion that is reached on the basis of the 'inherent' sensitivity parameters $EC50^*_{Ni2+}$ and $EC10^*_{Ni2+}$.

A similar independent evaluation of the natural waters dataset of Deleebeeck et al. (2005) was not possible because no parallel (simultaneous) tests in synthetic waters were tested along with those. However, when the $EC50^*_{Ni2+}$ and $EC10^*_{Ni2+}$ values were calculated for these natural waters, we found values of 3.46 and 0.69, respectively, which are similar as in the Bossuyt et al. (2001) dataset, but also much larger than in the synthetic waters tested by Deleebeeck et al. (2005) (Table 5.6).

As a preliminary conclusion we therefore hypothesize that the erroneous predictions of nickel toxicity in natural waters was more likely due to inherent sensitivity differences between test series rather than due to a failure of the proposed model structure, i.e. the BLM (Equation 5.7, Table 5.4). However, since the evidence was largely based on the limited dataset of Bossuyt et al. (2001), i.e. one type of artificial water, and two natural surface waters with pH > 6.4, we decided to perform some additional testing.

We sampled three natural surface waters, which had also been tested at previous occasions, i.e. Ankeveen, Brisy, and Regge. Water chemistry was determined according to procedures mentioned in section 2.1. Samples were spiked with a range of different Ni concentrations and ecotoxicity tests with *P. subcapitata* were conducted as described in Deleebeeck et al. (2005). Simultaneously, the standard OECD medium was also investigated, as well as OECD medium with increased levels of Ca (from 0.12 mM to 2 mM) and Mg (from 0.12 mM to 0.5 mM) (marked as $OECD^+$ in Table 5.5).

The water chemistry of these waters, as well as the EC50 and EC10 levels of Ni are reported in Table 5.5. The EC50 and EC10 levels in OECD medium in this test series were 362 and 63 µg/L, respectively, which is similar to the values obtained for the Bossuyt et al. Data (2001), and again higher than the values obtained for this water by Deleebeeck et al. (2005). When the inherent sensitivity of the new test series is calculated on the basis of the

two synthetic test waters (OECD and OECD^{+}), we found values for EC50*$_{Ni2+}$ and EC10*$_{Ni2+}$ of 3.55 and 0.57 µM, respectively, which are both very similar to the values obtained for the Bossuyt et al. (2001) dataset and the natural waters dataset of Deleebeeck et al. (2005) (Table 5.6).

When all datasets are considered, they may be divided into two different subsets, depending on the 'inherent sensitivity' of the algae during testing. One group consists of all test data from Deleebeeck et al. (2005) obtained in synthetic waters; the other group consists of the datasets of Bossuyt et al. (2001), the natural waters dataset of Deleebeeck et al. (2005), and the dataset generated in the present study. It is currently unclear why such a large sensitivity difference exists between these two subsets, especially because all tests were carried out according to the same test protocol – all datasets were generated in our laboratory.

At this point it is appropriate to state that bioavailability models are by definition not able to predict 'inherent sensitivity' differences. Rather, they are designed to predict toxic effect levels for waters with different combinations of bioavailability modifying factors when the 'inherent sensitivity' is known. Therefore, to evaluate the true predictive capacity of the algal BLM developed here - which consists of the stability constants describing modifying effects of Mg, Ca, and pH – we will now perform predictions of EC50's and EC10's, with calibrated inherent sensitivities. The values used for EC50*$_{Ni2+}$ and EC10*$_{Ni2+}$ were 3.57 µM and 0.55 µM, respectively (Table 5.4). Those were obtained by applying equation 5.9 only to the toxicity data obtained in synthetic waters from Bossuyt et al. (2001) and the newly generated data in OECD and OECD^{+} test waters (Table 5.5). By calibrating the model to the sensitivity in these datasets on the basis of synthetic waters alone, a new independent validation of the natural waters data was possible. The results of this validation are presented in Figure 5.7.

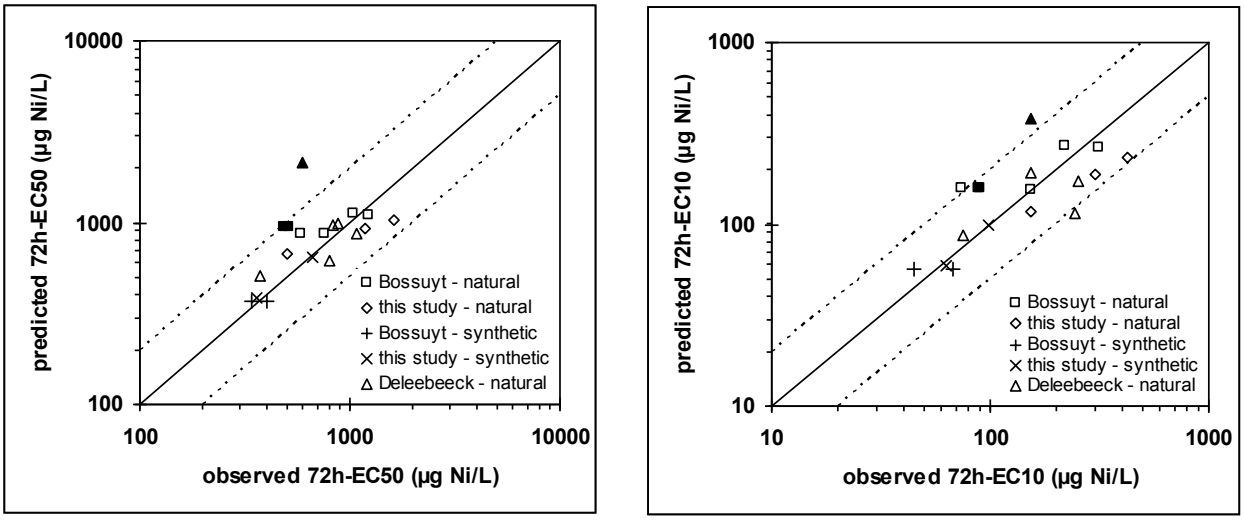


Figure 5.7 Independent evaluation of the predictive capacity of the Ni-BLM (Equation 5.7, parameter values in Table 5.4, adjusted 'inherent sensitivity', i.e. $EC50^*_{Ni2+}$ = 3.57 µM and $EC10^*_{Ni2+}$ = 0.55 µM) for *P. subcapitata* in natural test waters (data from Bossuyt et al., 2001; Deleebeeck et al., 2005 and also from the present study) and two standard synthetic waters tested along with the natural waters in Bossuyt et al. (2001) and the present study (see Table 5.5, see also text for more details). Figures show observed vs. predicted 72h-E_rC50's and 72h-E_rC10's. The full line indicates perfect predictions; the dashed line indicates a 2-fold prediction error. Filled data points were obtained at pH < 6.4.

When the data points obtained at pH < 6.45 are not considered, all EC50's and most EC10's are predicted by an error of less than 2-fold, with average prediction errors of 1.4-fold for EC50's and 1.5-fold for EC10's. As opposed to Figure 5.6, it is now noted that the predictions are not skewed to the 'underestimation' side of the 1:1 reference line, but that they are equally spread along both sides of this line.

Interestingly, The EC50 and EC10 in the Bihain test waters investigated by Bossuyt et al. (2001) were also predicted reasonably well, despite the fact that pH of these waters was below 6.45. However, pH in these test waters was only slightly lower, i.e. 6.35, again suggesting that the valid pH range of applying the algal BLM might be extended to lower pH's (see also section 5.3.2). However, the EC50 and EC10 in Bihain water tested at pH 5.7 by Deleebeeck et al. (2005) were still overestimated by 3.6 and 2.5-fold respectively, suggesting that there is clearly a limit to this extrapolation. Based on the reasonable model validations with data from Bihain water at pH 6.35 tested by Bossuyt et al. (2001) and from the tests carried out at pH 6.0 in the pH-Mg test series (Deleebeeck et al., 2005), we suggest that the model may be reasonably safely extended to a pH as low as 6.0, although the

uncertainty about model predictions between pH 6.0 and 6.4 is likely to be higher than at pH > 6.4

5.3.4. Conclusion on the Ni bioavailability model for algae

Although there are some uncertainties related to differences in 'inherent' sensitivity of the alga across different test series, the developed bioavailability model can reasonably accurately predict chronic effect concentrations of Ni in natural waters when these inherent sensitivity differences are taken into account. When data below pH 6.4 are not taken into account, average prediction errors based on tests in 12 samples were 1.4-fold for EC50's and 1.5-fold for EC10's. All ECx values were predicted by an error of less than factor 2.1. This represents an important reduction of uncertainty due to differences in bioavailability, since 'dissolved' EC50's and EC10's varied between 425 and 1630 µg Ni/L (factor 3.8) and between 73.3 and 376 µg Ni/L (factor 5.1).

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7. Annexes

Since the majority of the Annexes are very large tables, they are only available in spreadsheet format (Microsoft Excel®). These annexes can be obtained from the authors upon request. Following Annexes are also available in the text below: 1A, 1B, 1C, 2, 3, 4, 5, and 16.

ANNEX 1A – Description of sampling sites

Ankeveen

This body of water is a ditch, which connects to a large system of interconnected lakes called the Ankeveensche Plassen (NL). It is located in a lowland peat area in the Netherlands close to the village of Nederhorst-den-Berg. The surface water is characterized by an intermediate pH (usually around 7), a moderate hardness and a high DOC concentration.

Bihain

A small creek (named Ruisseau de St. Martin) located in the nature reserve named Hoge Venen, which is a highland peat rich area in Belgium (Walloon region). The water of this creek is characterized by low pH and low to intermediate DOC concentration. Usually hardness is low, but higher Ca levels may occasionally be observed during drought periods.

Brisy

This sampling site is part of the eastern branch of the river Ourthe, called the 'Ourthe Orientale'. It is located near the village of Brisy (Belgium, Wallon region) and is situated in a forested area. This site is characterized by low DOC concentration, low hardness and a pH close to 7.

Eppe

This is a small stream just outside the protected forest area called 'Le Val Joly'. The sample was taken close to where it joins the river 'Helpe Majeure', which is a tributary of the river Samber. The water is charactezired by low DOC, moderate hardness and a pH between 7 and 8.

Markermeer

This site is a large lake in the north of the Netherlands, cut off by a dam from Lake Ijssel and the 'Waddenzee' sea. It is characterized by high pH, high hardness and an intermediate DOC concentration.

Regge

This river in the north of the Netherlands is situated in the province of Overijssel and joins the river Vecht near the village of Ommen, where the sample was taken. The water is characterized by high pH and hardness and a moderate to high DOC concentration.

ANNEX 1B
Site chemistry (trace elements and macro-ions in µg or mg/L units)
note: final chemistries used for data analysis are in Annexes 2 and 3

	pH	DOC (mg/L)	IC (mM) UGENT	IC (mM) KUL	hardness mg CaCO3/L
Ankeveen	7.1	23.56	0.79	0.75	131.6
Bihain	6.1	6.36	0.13	0.13	15.0
Brisy	7.5	3.06	0.41	0.33	41.1
Eppe	7.7	5.02	1.76	1.50	108.4
Markermeer	8.1	7.61	1.94	1.86	218.1
Regge	7.9	12.57	2.95	2.60	204.0

Background of trace elements (µg/L)

	Ni	Cd	Cu	Pb	Zn	Fe	Al	Mn
Ankeveen	2.8	0.4	1.4	0.5	6.1	139.7	28.6	0.7
Bihain	2.0	1.7	2.0	1.1	21.0	65.0	23.0	BDL
Brisy	1.7	0.5	1.6	1.4	6.6	98.7	23.6	2.6
Eppe	1.2	0.4	0.4	0.3	2.1	109.5	20.2	5.0
Markermeer	1.4	0.2	1.0	1.2	3.2	5.3	2.7	BDL
Regge	3.2	0.2	1.0	0.4	5.6	52.0	BDL	0.5

BDL=below detection limit

	Macro-cations (mg/L)				Macro-anions (mg/L)		
	Ca	Mg	Na	K	Cl	SO4	NO3
Ankeveen	41.1	7.1	17.2	7.4	29.1	81.0	3.1
Bihain	4.4	1.0	4.1	0.8	8.8	6.4	3.6
Brisy	10.1	3.8	6.8	2.3	16.2	8.6	13.3
Eppe	33.4	6.1	9.0	4.5	15.5	18.5	6.9
Markermeer	61.8	15.6	78.3	9.4	95.5	110.2	3.1
Regge	68.8	7.9	43.2	12.0	44.9	56.3	7.6

Note: pH and IC are values measured upon arrival in the laboratory, measured pH and IC during speciation and toxicity experiments are used in all data analyses.

ANNEX 1C

Site chemistry (trace elements and macro-ions in molarity units, M)

note: final chemistries used for data analysis are in Annexes 2 and 3

	Background of trace elements							
	Ni	Cd	Cu	Pb	Zn	Fe	Al	Mn
Ankeveen	4.78E-08	3.13E-09	2.19E-08	2.65E-09	9.39E-08	2.50E-06	1.06E-06	1.24E-08
Bihain	3.39E-08	1.51E-08	3.15E-08	5.31E-09	3.21E-07	1.16E-06	8.52E-07	BDL
Brisy	2.89E-08	4.31E-09	2.55E-08	6.69E-09	1.01E-07	1.77E-06	8.76E-07	4.74E-08
Eppe	2.10E-08	3.47E-09	6.58E-09	1.48E-09	3.25E-08	1.96E-06	7.48E-07	9.04E-08
Markermeer	2.36E-08	1.95E-09	1.51E-08	5.69E-09	4.93E-08	9.58E-08	9.97E-08	BDL
Regge	5.54E-08	1.95E-09	1.56E-08	1.72E-09	8.62E-08	9.31E-07	BDL	9.87E-09

	Macro-cations				Macro-anions		
	Ca	Mg	Na	K	Cl	SO4	NO3
Ankeveen	1.02E-03	2.91E-04	7.49E-04	1.89E-04	8.22E-04	8.44E-04	5.00E-05
Bihain	1.10E-04	4.00E-05	1.80E-04	2.00E-05	2.48E-04	6.66E-05	5.76E-05
Brisy	2.53E-04	1.57E-04	2.96E-04	5.86E-05	4.57E-04	8.90E-05	2.15E-04
Eppe	8.34E-04	2.50E-04	3.91E-04	1.16E-04	4.38E-04	1.93E-04	1.11E-04
Markermeer	1.54E-03	6.40E-04	3.41E-03	2.40E-04	2.70E-03	1.15E-03	5.00E-05
Regge	1.72E-03	3.25E-04	1.88E-03	3.07E-04	1.27E-03	5.87E-04	1.23E-04

ANNEX 2

Input table for WHAM 6 speciation calculations for the speciation experiments (for WHAM 6 calibration)

Assumption: Al3+ in equilibrium with colloidal Al(OH)3 = scenario (i)

For scenario (ii), i.e. Al input as dissolved, replace with the data in the Al-dissolved column

Description	SPM (mg/L)	Temperature (K)	pCO2 (atm)	pH	Colloidal fulvic acid (g/L)	Na (M) TOTAL	Mg (M) TOTAL	Al (M) ACTIVITY	K (M) TOTAL	Ca (M) TOTAL	Mn (M) DISSOLVED	Fe(III) (M) ACTIVITY	Ni (M) DISSOLVED	Cu (M) DISSOLVED	Zn (M) DISSOLVED	Cd (M) DISSOLVED	Pb (M) DISSOLVED	Cl (M) TOTAL	NO3 (M) TOTAL	SO4 (M) TOTAL	CO3 (M) TOTAL
Ankeveen	0.00E+00	298.15	999	7.36	0.01888	2.31E-03	2.91E-04	1.06E-06	1.69E-04	8.72E-04	1.00E-09	2.63E-20	7.21E-08	7.98E-08	8.32E-08	1.00E-09	1.00E-09	8.21E-04	5.00E-05	8.44E-04	6.43E-04
Ank EC10	0.00E+00	298.15	999	7.36	0.01888	2.49E-03	2.95E-04	1.06E-06	1.91E-04	8.83E-04	1.00E-09	2.63E-20	6.63E-07	3.54E-08	5.82E-08	1.00E-09	1.00E-09	8.21E-04	5.00E-05	8.44E-04	6.43E-04
Ank LC50	0.00E+00	298.15	999	7.36	0.01888	2.46E-03	2.93E-04	1.06E-06	1.93E-04	8.73E-04	1.00E-09	2.63E-20	2.51E-06	2.91E-08	6.24E-08	1.00E-09	1.00E-09	8.21E-04	5.00E-05	8.44E-04	6.43E-04
Bihain	0.00E+00	298.15	999	6.17	5.09E-03	4.45E-04	5.74E-05	7.48E-07	3.06E-05	1.09E-04	1.00E-09	9.77E-17	4.72E-08	1.40E-07	1.96E-07	1.00E-09	1.00E-09	2.47E-04	5.76E-05	6.68E-05	4.70E-05
Bihain EC10	0.00E+00	298.15	999	6.17	5.09E-03	4.85E-04	5.74E-05	7.48E-07	3.64E-05	1.08E-04	1.00E-09	9.77E-17	8.03E-08	6.40E-08	1.47E-07	1.00E-09	1.00E-09	2.47E-04	5.76E-05	6.68E-05	4.70E-05
Bihain LC50	0.00E+00	298.15	999	6.17	5.09E-03	4.84E-04	5.91E-05	7.48E-07	3.75E-05	1.09E-04	1.00E-09	9.77E-17	2.53E-07	5.66E-08	1.35E-07	1.00E-09	1.00E-09	2.47E-04	5.76E-05	6.68E-05	4.70E-05
Brisy	0.00E+00	298.15	999	7.23	2.45E-03	1.95E-03	1.52E-04	1.85E-08	5.31E-05	2.30E-04	1.00E-09	6.46E-20	4.50E-08	2.04E-08	1.21E-08	1.00E-09	3.99E-09	4.56E-04	2.15E-04	8.91E-05	3.09E-04
Brisy EC10	0.00E+00	298.15	999	7.23	2.45E-03	2.07E-03	1.45E-04	1.85E-08	5.85E-05	2.36E-04	1.00E-09	6.46E-20	5.60E-08	2.31E-08	3.26E-08	1.00E-09	1.00E-09	4.56E-04	2.15E-04	8.91E-05	3.09E-04
Brisy LC50	0.00E+00	298.15	999	7.23	2.45E-03	2.13E-03	1.46E-04	1.85E-08	6.11E-05	2.38E-04	1.00E-09	6.46E-20	3.53E-07	1.72E-08	3.62E-08	1.00E-09	1.00E-09	4.56E-04	2.15E-04	8.91E-05	3.09E-04
Eau d'Eppe	0.00E+00	298.15	999	7.85	4.02E-03	3.02E-03	2.58E-04	9.97E-08	1.08E-04	7.69E-04	1.00E-09	8.91E-22	7.58E-08	3.72E-08	3.59E-09	1.00E-09	4.73E-09	4.37E-04	1.11E-04	1.93E-04	1.42E-03
Eppe EC10	0.00E+00	298.15	999	8.04	4.02E-03	3.07E-03	2.57E-04	9.97E-08	1.14E-04	7.62E-04	1.00E-09	2.40E-22	9.91E-08	1.73E-08	4.13E-09	1.00E-09	1.68E-09	4.37E-04	1.11E-04	1.93E-04	1.42E-03
Eppe LC50	0.00E+00	298.15	999	8.17	4.02E-03	3.14E-03	2.63E-04	9.97E-08	1.23E-04	7.70E-04	1.00E-09	9.77E-23	3.59E-07	2.22E-08	4.99E-09	1.00E-09	1.00E-09	4.37E-04	1.11E-04	1.93E-04	1.42E-03
Markermeer	0.00E+00	298.15	999	8.26	6.09E-03	6.12E-03	6.00E-04	8.76E-07	2.22E-04	1.41E-03	1.00E-09	5.25E-23	6.48E-08	6.58E-08	1.95E-08	1.00E-09	2.37E-09	2.69E-03	5.00E-05	1.15E-03	1.81E-03
Mark EC10	0.00E+00	298.15	999	8.26	6.09E-03	7.53E-03	6.05E-04	8.76E-07	2.32E-04	1.35E-03	1.00E-09	5.25E-23	9.92E-08	2.56E-08	7.82E-09	1.00E-09	3.82E-09	2.69E-03	5.00E-05	1.15E-03	1.81E-03
Mark LC50	0.00E+00	298.15	999	8.26	6.09E-03	7.52E-03	6.02E-04	8.76E-07	2.36E-04	1.34E-03	1.00E-09	5.25E-23	9.01E-07	3.25E-08	9.74E-09	1.00E-09	3.14E-09	2.69E-03	5.00E-05	1.15E-03	1.81E-03
Beneden Regge	0.00E+00	298.15	999	8.54	0.01008	6.96E-03	3.21E-04	8.52E-07	2.77E-04	1.50E-03	1.00E-09	7.59E-24	6.22E-08	5.03E-08	5.65E-08	1.00E-09	2.56E-09	1.26E-03	1.23E-04	5.87E-04	2.50E-03
Regge EC10	0.00E+00	298.15	999	8.58	0.01008	7.49E-03	3.28E-04	8.52E-07	2.87E-04	1.52E-03	1.00E-09	5.75E-24	1.16E-07	3.54E-08	5.14E-08	1.00E-09	2.50E-09	1.26E-03	1.23E-04	5.87E-04	2.50E-03
Regge LC50	0.00E+00	298.15	999	8.58	0.01008	7.35E-03	3.21E-04	8.52E-07	2.88E-04	1.47E-03	1.00E-09	5.75E-24	1.82E-06	2.80E-08	5.16E-08	1.00E-09	1.00E-09	1.26E-03	1.23E-04	5.87E-04	2.50E-03
Ankeveen pH 1	0.00E+00	298.15	999	8.06	0.01888	3.71E-03	3.01E-04	1.06E-06	1.84E-04	8.47E-04	1.00E-09	3.31E-22	7.53E-08	2.80E-08	2.50E-08	8.53E-09	5.12E-09	8.21E-04	5.00E-05	8.44E-04	6.43E-04
Ank pH 2	0.00E+00	298.15	999	7.71	0.01888	3.82E-03	3.05E-04	1.06E-06	1.91E-04	8.53E-04	1.00E-09	3.72E-21	7.67E-08	7.94E-09	1.95E-08	1.00E-09	4.55E-09	1.37E-03	5.00E-05	8.44E-04	6.43E-04
Ank pH 3	0.00E+00	298.15	999	7.15	0.01888	3.87E-03	3.12E-04	1.06E-06	2.00E-04	8.64E-04	1.00E-09	1.78E-19	8.10E-08	2.55E-10	6.24E-08	1.00E-09	2.93E-09	2.57E-03	5.00E-05	8.44E-04	6.43E-04
Ank pH 4	0.00E+00	298.15	999	6.46	0.01888	3.93E-03	3.18E-04	1.06E-06	2.06E-04	8.84E-04	1.00E-09	2.09E-17	8.60E-08	2.28E-09	9.70E-08	1.00E-09	5.03E-09	4.07E-03	5.00E-05	8.44E-04	6.43E-04
Ank Ca 1	0.00E+00	298.15	999	7.31	0.01888	2.41E-03	3.22E-04	1.06E-06	1.71E-04	9.02E-04	1.00E-09	5.89E-20	8.84E-08	2.60E-08	9.36E-08	1.00E-09	1.00E-09	8.21E-04	5.00E-05	8.44E-04	6.43E-04
Ank Ca 2	0.00E+00	298.15	999	7.31	0.01888	2.48E-03	1.10E-03	1.06E-06	1.89E-04	2.41E-03	2.51E-09	5.89E-20	8.64E-08	3.91E-08	1.13E-07	1.00E-09	1.00E-09	5.46E-03	5.00E-05	8.44E-04	6.43E-04
Ank Ca 3	0.00E+00	298.15	999	7.31	0.01888	2.44E-03	6.92E-04	1.06E-06	1.82E-04	1.66E-03	2.16E-09	5.89E-20	8.49E-08	3.31E-08	1.03E-07	1.00E-09	1.00E-09	3.11E-03	5.00E-05	8.44E-04	6.43E-04
Ank Ca 4	0.00E+00	298.15	999	7.31	0.01888	2.49E-03	1.46E-03	1.06E-06	2.02E-04	3.13E-03	3.93E-09	5.89E-20	8.39E-08	3.91E-08	1.14E-07	1.00E-09	1.00E-09	7.63E-03	5.00E-05	8.44E-04	6.43E-04
Bihain pH 1	0.00E+00	298.15	999	6.16	5.09E-03	4.57E-04	6.24E-05	7.48E-07	3.41E-05	1.21E-04	1.00E-09	1.66E-16	4.19E-08	2.12E-08	1.62E-07	1.00E-09	1.00E-09	2.47E-04	5.76E-05	6.68E-05	4.70E-05
Bih pH 2	0.00E+00	298.15	999	6.9	5.09E-03	1.50E-03	7.03E-05	7.48E-07	3.88E-05	1.33E-04	1.00E-09	1.00E-18	4.29E-08	1.26E-08	6.82E-08	1.00E-09	1.00E-09	2.47E-04	5.76E-05	6.68E-05	4.70E-05
Bih pH 3	0.00E+00	298.15	999	7.59	5.09E-03	2.79E-03	8.06E-05	7.48E-07	4.35E-05	1.57E-04	1.00E-09	8.51E-21	4.57E-08	1.79E-08	3.22E-08	1.00E-09	4.22E-09	2.47E-04	5.76E-05	6.68E-05	4.70E-05
Bih pH 4	0.00E+00	298.15	999	7.96	5.09E-03	3.67E-03	9.66E-05	7.48E-07	5.56E-05	1.91E-04	1.00E-09	6.61E-22	4.57E-08	1.04E-08	3.07E-08	1.00E-09	1.59E-09	2.47E-04	5.76E-05	6.68E-05	4.70E-05
Bih Ca 1	0.00E+00	298.15	999	6.18	5.09E-03	4.98E-04	6.53E-05	7.48E-07	3.64E-05	8.27E-05	4.23E-09	1.45E-16	7.56E-08	1.12E-07	2.68E-07	3.16E-09	1.00E-09	2.47E-04	5.76E-05	6.68E-05	4.70E-05
Bih Ca 2	0.00E+00	298.15	999	6.18	5.09E-03	5.24E-04	3.65E-04	7.48E-07	4.37E-05	5.95E-04	1.36E-08	1.45E-16	1.09E-07	7.88E-08	5.75E-07	1.26E-09	1.00E-09	1.90E-03	5.76E-05	6.68E-05	4.70E-05
Bih Ca 4	0.00E+00	298.15	999	6.18	5.09E-03	5.53E-04	1.03E-03	7.48E-07	6.27E-05	1.85E-03	1.89E-08	1.45E-16	1.27E-07	8.38E-08	8.17E-07	1.61E-09	4.02E-09	5.78E-03	5.76E-05	6.68E-05	4.70E-05

ANNEX 3

Input table for WHAM 6 speciation calculations at ECx levels of Ceriodaphnia dubia tests with Ni in natural waters.

Assumption: Al3+ in equilibrium with colloidal Al(OH)3 = scenario (i)

For scenario (ii), i.e. Al input as dissolved replace with the data in the Al-dissolved column

	Description	SPM (mg/L)	Temperature (K)	pCO2 (atm)	pH	Colloidal fulvic acid (g/L)	Na (M) TOTAL	Mg (M) TOTAL	Al (M) ACTIVITY	K (M) TOTAL	Ca (M) TOTAL	Mn (M) DISSOLVED	Fe(III) (M) ACTIVITY	Ni (M) DISSOLVED	Cu (M) DISSOLVED	Zn (M) DISSOLVED	Cd (M) DISSOLVED	Pb (M) DISSOLVED	Cl (M) TOTAL	NO3 (M) TOTAL	SO4 (M) TOTAL	CO3 (M) TOTAL	Al (M) DISSOLVED
Ankeveen	Acute LC50	0.00E+00	298	999	7.51	0.0189	7.49E-04	2.91E-04	9.33E-15	1.89E-04	1.02E-03	1.24E-08	9.33E-21	3.12E-06	2.19E-08	9.39E-08	3.13E-09	2.65E-09	8.22E-04	5.00E-05	8.44E-04	8.76E-04	1.06E-06
Bihain	Acute LC50	0.00E+00	298	999	6.34	5.09E-03	1.80E-04	4.00E-05	3.02E-11	2.00E-05	1.10E-04	0.00E+00	3.02E-17	6.00E-07	3.15E-08	3.21E-07	1.51E-08	5.31E-09	2.48E-04	5.76E-05	6.66E-05	9.96E-05	8.52E-07
Brisy	Acute LC50	0.00E+00	298	999	7.45	2.45E-03	2.96E-04	1.57E-04	1.41E-14	5.86E-05	2.53E-04	4.74E-08	1.41E-20	8.66E-07	2.55E-08	1.01E-07	4.31E-09	6.69E-09	4.57E-04	2.15E-04	8.90E-05	4.43E-04	8.76E-07
Eppe	Acute LC50	0.00E+00	298	999	7.95	4.02E-03	3.91E-04	2.50E-04	4.47E-16	1.16E-04	8.34E-04	9.04E-08	4.47E-22	5.90E-07	6.58E-09	3.25E-08	3.47E-09	1.48E-09	4.38E-04	1.11E-04	1.93E-04	1.80E-03	7.48E-07
Markermeer	Acute LC50	0.00E+00	298	999	8.04	6.09E-03	3.41E-03	6.40E-04	2.40E-16	2.40E-04	1.54E-03	0.00E+00	2.40E-22	1.51E-06	1.51E-08	4.93E-08	1.95E-09	5.69E-09	2.70E-03	5.00E-05	1.15E-03	2.05E-03	9.97E-08
Regge	Acute LC50	0.00E+00	298	999	8	0.0101	1.88E-03	3.25E-04	3.16E-16	3.07E-04	1.72E-03	9.87E-09	3.16E-22	2.74E-06	1.56E-08	8.62E-08	1.95E-09	1.72E-09	1.27E-03	1.23E-04	5.87E-04	3.13E-03	0.00E+00
Ankeveen	Chronic EC10	0.00E+00	298	999	7.61	0.0189	7.49E-04	2.91E-04	4.68E-15	1.89E-04	1.02E-03	1.24E-08	4.68E-21	7.54E-07	2.19E-08	9.39E-08	3.13E-09	2.65E-09	8.22E-04	5.00E-05	8.44E-04	9.91E-04	1.06E-06
Bihain	Chronic EC10	0.00E+00	298	999	6.56	5.09E-03	1.80E-04	4.00E-05	6.61E-12	2.00E-05	1.10E-04	0.00E+00	6.61E-18	1.54E-07	3.15E-08	3.21E-07	1.51E-08	5.31E-09	2.48E-04	5.76E-05	6.66E-05	2.11E-04	8.52E-07
Brisy	Chronic EC10	0.00E+00	298	999	7.23	2.45E-03	2.96E-04	1.57E-04	6.46E-14	5.86E-05	2.53E-04	4.74E-08	6.46E-20	1.26E-07	2.55E-08	1.01E-07	4.31E-09	6.69E-09	4.57E-04	2.15E-04	8.90E-05	5.14E-04	8.76E-07
Eppe	Chronic EC10	0.00E+00	298	999	7.86	4.02E-03	3.91E-04	2.50E-04	8.32E-16	1.16E-04	8.34E-04	9.04E-08	8.32E-22	2.21E-08	6.58E-09	3.25E-08	3.47E-09	1.48E-09	4.38E-04	1.11E-04	1.93E-04	1.83E-03	7.48E-07
Markermeer	Chronic EC10	0.00E+00	298	999	8.01	6.09E-03	3.41E-03	6.40E-04	2.95E-16	2.40E-04	1.54E-03	0.00E+00	2.95E-22	1.29E-07	1.51E-08	4.93E-08	1.95E-09	5.69E-09	2.70E-03	5.00E-05	1.15E-03	2.08E-03	9.97E-08
Regge	Chronic EC10	0.00E+00	298	999	8.18	0.0101	1.88E-03	3.25E-04	9.12E-17	3.07E-04	1.72E-03	9.87E-09	9.12E-23	1.32E-07	1.56E-08	8.62E-08	1.95E-09	1.72E-09	1.27E-03	1.23E-04	5.87E-04	3.07E-03	0.00E+00
Ankeveen	Chronic EC20	0.00E+00	298	999	7.61	0.0189	7.49E-04	2.91E-04	4.68E-15	1.89E-04	1.02E-03	1.24E-08	4.68E-21	8.85E-07	2.19E-08	9.39E-08	3.13E-09	2.65E-09	8.22E-04	5.00E-05	8.44E-04	9.91E-04	1.06E-06
Bihain	Chronic EC20	0.00E+00	298	999	6.56	5.09E-03	1.80E-04	4.00E-05	6.61E-12	2.00E-05	1.10E-04	0.00E+00	6.61E-18	2.18E-07	3.15E-08	3.21E-07	1.51E-08	5.31E-09	2.48E-04	5.76E-05	6.66E-05	2.11E-04	8.52E-07
Brisy	Chronic EC20	0.00E+00	298	999	7.23	2.45E-03	2.96E-04	1.57E-04	6.46E-14	5.86E-05	2.53E-04	4.74E-08	6.46E-20	1.46E-07	2.55E-08	1.01E-07	4.31E-09	6.69E-09	4.57E-04	2.15E-04	8.90E-05	5.14E-04	8.76E-07
Eppe	Chronic EC20	0.00E+00	298	999	7.86	4.02E-03	3.91E-04	2.50E-04	8.32E-16	1.16E-04	8.34E-04	9.04E-08	8.32E-22	3.59E-08	6.58E-09	3.25E-08	3.47E-09	1.48E-09	4.38E-04	1.11E-04	1.93E-04	1.83E-03	7.48E-07
Markermeer	Chronic EC20	0.00E+00	298	999	8.01	6.09E-03	3.41E-03	6.40E-04	2.95E-16	2.40E-04	1.54E-03	0.00E+00	2.95E-22	1.53E-07	1.51E-08	4.93E-08	1.95E-09	5.69E-09	2.70E-03	5.00E-05	1.15E-03	2.08E-03	9.97E-08
Regge	Chronic EC20	0.00E+00	298	999	8.18	0.0101	1.88E-03	3.25E-04	9.12E-17	3.07E-04	1.72E-03	9.87E-09	9.12E-23	1.87E-07	1.56E-08	8.62E-08	1.95E-09	1.72E-09	1.27E-03	1.23E-04	5.87E-04	3.07E-03	0.00E+00
Ankeveen	Chronic EC50	0.00E+00	298	999	7.61	0.0189	7.49E-04	2.91E-04	4.68E-15	1.89E-04	1.02E-03	1.24E-08	4.68E-21	1.17E-06	2.19E-08	9.39E-08	3.13E-09	2.65E-09	8.22E-04	5.00E-05	8.44E-04	9.91E-04	1.06E-06
Bihain	Chronic EC50	0.00E+00	298	999	6.56	5.09E-03	1.80E-04	4.00E-05	6.61E-12	2.00E-05	1.10E-04	0.00E+00	6.61E-18	3.93E-07	3.15E-08	3.21E-07	1.51E-08	5.31E-09	2.48E-04	5.76E-05	6.66E-05	2.11E-04	8.52E-07
Brisy	Chronic EC50	0.00E+00	298	999	7.23	2.45E-03	2.96E-04	1.57E-04	6.46E-14	5.86E-05	2.53E-04	4.74E-08	6.46E-20	1.87E-07	2.55E-08	1.01E-07	4.31E-09	6.69E-09	4.57E-04	2.15E-04	8.90E-05	5.14E-04	8.76E-07
Eppe	Chronic EC50	0.00E+00	298	999	7.86	4.02E-03	3.91E-04	2.50E-04	8.32E-16	1.16E-04	8.34E-04	9.04E-08	8.32E-22	8.27E-08	6.58E-09	3.25E-08	3.47E-09	1.48E-09	4.38E-04	1.11E-04	1.93E-04	1.83E-03	7.48E-07
Markermeer	Chronic EC50	0.00E+00	298	999	8.01	6.09E-03	3.41E-03	6.40E-04	2.95E-16	2.40E-04	1.54E-03	0.00E+00	2.95E-22	2.05E-07	1.51E-08	4.93E-08	1.95E-09	5.69E-09	2.70E-03	5.00E-05	1.15E-03	2.08E-03	9.97E-08
Regge	Chronic EC50	0.00E+00	298	999	8.18	0.0101	1.88E-03	3.25E-04	9.12E-17	3.07E-04	1.72E-03	9.87E-09	9.12E-23	3.42E-07	1.56E-08	8.62E-08	1.95E-09	1.72E-09	1.27E-03	1.23E-04	5.87E-04	3.07E-03	0.00E+00

ANNEX 4 Efffect concentrations of Ni to *Ceriodaphnia dubia*

10-day NOECs and LOECs of Ni to *Ceriodaphnia dubia* reproduction

Site	NOEC (µg Ni/L)	LOEC (µg Ni/L)
Ankeveen	38.6	62.4
Bihain	20.2	33.4
Brisy	21.5	34.9
Eppe	<3.7	-
Markermeer	<12.2	-
Regge	<8.3	-

48-hLC50s (mortality) and 10d-ECx (reproduction) with 95% confidence limits for *C. dubia (µg/L)*

Site	48h-LC50	low CL	high CL
Ankeveen	183	163	206
Bihain	35.2	24.2	51.3
Brisy	50.8	41.1	62.8
Eau d'Eppe	34.6	30.7	39.1
Markermeer	88.7	58.7	134.1
Regge	161	148	175

Site	10d-EC50	low CL	high CL	10d-EC20	low CL	high CL	10d-EC10	low CL	high CL
Ankeveen	68.4	62.9	74.4	51.9	45.3	59.6	44.2	36.6	53.5
Bihain	23.1	18.3	29.1	12.8	8.5	19.3	9.0	5.2	15.7
Brisy	11.0	9.7	12.5	8.5	7.2	10.2	7.4	5.9	9.2
Eppe	4.9	2.7	8.7	*2.1*	0.8	5.8	*1.3*	0.4	4.7
Markermeer	12.1	11.2	13.0	*9.0*	7.4	10.8	*7.6*	5.8	9.9
Regge	20.1	15.7	25.8	11.0	7.1	17.0	7.8	4.4	13.7

ANNEX 5

Calculated Ni2+-activity for different scenarios of Al input for C dubia tests

			Scenario (i)		Al-assumption Al(OH)3 Scenario (i)	Al total Scenario (ii)	Al = 0 Scenario (iii)	'Best' Scenario (iv)		
		Measured dissolved Al (M)	Predicted dissolved Al (M)	Al(OH)3 Precipitated? (Y/N)	Calculated (Ni2+) (M)	Calculated (Ni2+) (M)	Calculated (Ni2+) (M)	Calculated true' Ni2+ (M)	(iv)/(iii)	(ii)/(i)
Ankeveen	Acute LC50	1.06E-06	8.04E-07	Y	5.08E-07	5.24E-07	4.41E-07	5.08E-07	1.15	1.03
Bihain	Acute LC50	8.52E-07	9.55E-07	N	2.59E-07	2.54E-07	2.11E-07	2.54E-07	1.20	0.98
Brisy	Acute LC50	8.76E-07	2.88E-07	Y	3.95E-07	4.17E-07	3.63E-07	3.95E-07	1.09	1.05
Eppe	Acute LC50	7.48E-07	6.32E-07	Y	1.55E-07	1.55E-07	1.48E-07	1.55E-07	1.04	1.00
Markermeer	Acute LC50	9.97E-08	7.85E-07	N	3.76E-07	3.72E-07	3.69E-07	3.72E-07	1.01	0.99
Regge	Acute LC50	0.00E+00	7.38E-07	N	5.85E-07	5.74E-07	5.74E-07	5.74E-07		
Ankeveen	Chronic EC10	1.06E-06	9.13E-07	Y	6.06E-08	6.20E-08	4.86E-08	6.06E-08	1.25	1.02
Bihain	Chronic EC10	8.52E-07	7.97E-07	Y	4.70E-08	4.78E-08	3.50E-08	4.70E-08	1.34	1.02
Brisy	Chronic EC10	8.76E-07	3.02E-07	Y	4.71E-08	5.17E-08	3.66E-08	4.71E-08	1.29	1.10
Eppe	Chronic EC10	7.48E-07	5.58E-07	Y	2.81E-09	2.88E-09	2.39E-09	2.81E-09	1.17	1.02
Markermeer	Chronic EC10	9.97E-08	7.61E-07	N	1.75E-08	1.69E-08	1.62E-08	1.69E-08	1.04	0.96
Regge	Chronic EC10	0.00E+00	1.08E-06	N	1.01E-08	9.36E-09	9.36E-09	9.36E-09		
Ankeveen	Chronic EC20	1.06E-06	9.00E-07	Y	7.62E-08	7.80E-08	6.19E-08	7.62E-08	1.23	1.02
Bihain	Chronic EC20	8.52E-07	7.93E-07	Y	6.95E-08	7.07E-08	5.26E-08	6.95E-08	1.32	1.02
Brisy	Chronic EC20	8.76E-07	3.01E-07	Y	5.56E-08	6.08E-08	4.36E-08	5.56E-08	1.28	1.09
Eppe	Chronic EC20	7.48E-07	5.57E-07	Y	4.90E-09	5.01E-09	4.22E-09	4.90E-09	1.16	1.02
Markermeer	Chronic EC20	9.97E-08	7.60E-07	N	2.17E-08	2.10E-08	2.01E-08	2.10E-08	1.04	0.97
Regge	Chronic EC20	0.00E+00	1.08E-06	N	1.58E-08	1.47E-08	1.47E-08	1.47E-08		
Ankeveen	Chronic EC50	1.06E-06	8.75E-07	Y	1.14E-07	1.17E-07	9.43E-08	1.14E-07	1.21	1.03
Bihain	Chronic EC50	8.52E-07	7.81E-07	Y	1.37E-07	1.39E-07	1.07E-07	1.37E-07	1.28	1.02
Brisy	Chronic EC50	8.76E-07	2.98E-07	Y	7.36E-08	8.02E-08	5.85E-08	7.36E-08	1.26	1.09
Eppe	Chronic EC50	7.48E-07	5.54E-07	Y	1.35E-08	1.37E-08	1.19E-08	1.35E-08	1.13	1.02
Markermeer	Chronic EC50	9.97E-08	7.59E-07	N	3.14E-08	3.05E-08	2.94E-08	3.05E-08	1.04	0.97
Regge	Chronic EC50	0.00E+00	1.08E-06	N	3.50E-08	3.30E-08	3.30E-08	3.30E-08		

Note: scenario (iv) = Best=Allowing Al(OH)3 to precipitate when Solubility product of Al(OH)3 is exceeded
This is when the dissolved measured Al > predicted dissolved Al under scenario (i)

ANNEX 16 – Recommendations on how to deal with the uncertainty of the Keithly et al. (2004) dataset in a risk assessment context

When the chronic reproductive NOECs of Keithly et al. (2004) are to be used in risk assessment, an appropriate assessment of the Ni speciation might at a certain point be desired, despite the uncertainty associated with the presence of YTC-ligands in the test solutions. This assessment could be performed along two different lines.

First, one could assume that the complexation of Ni is entirely due to DOM coming from the YTC and that this DOM behaves similarly as natural DOM, i.e. it can also be modeled by WHAM VI, according to the calibration discussed earlier (AFA=40%, pK_{NiFA}=1.75). Hereby it is also implicitly assumed that the effects of pH and hardness on Ni binding to YTC-ligands are also well predicted by WHAM VI, since the assessment of 40%AFA was made for one single pH and hardness combination in the present study. In this case speciation calculations would need to be run with a DOC input of 0.8 mg/L (= 1.3 mg/L measured after YTC addition - 0.5 mg/L background DOC in dilution water), or with an assumed FA concentration of 0.8 mg DOC/L x 40% x 2 (mg FA/mg DOC) = 0.64 mg FA/L. This would result in a point estimate of $NOEC_{Ni2+}$ for each of the three test waters.

A second possibility is the following. We know that at Ni concentrations between 1.8 and 5.2 µg/L, at a pH of 7.9-8.1, and at a hardness of 76 mg $CaCO_3$/L, 30-40% of the Ni was complexed to YTC-ligands (see section 4.3.2.2), using exactly the same concentration of YTC as present in the standard chronic *C. dubia* tests of Keithly et al. (2004), i.e. about 12 mg solids/L. Now, we also know that increasing Ni, reducing pH, and increasing hardness result in a lower fraction of Ni being organically complexed (see section 3.1). Now, the 'dissolved' chronic NOECs reported by Keithly et al. (2004) are between 4.0 and 6.9, i.e. similar to the range for which Ni speciation was determined with YTC. However, pH and hardness in Keithly et al. (2004) are lower (7.6 to 7.8) and higher (113-253), respectively, and thus a lower fraction of Ni is expected to be bound to YTC ligands than in our experiments. Thus, one could suggest that $NOEC_{dissolved} \geq NOEC_{inorganic} \geq 0.6 \times NOEC_{dissolved}$ and use the lower boundary, i.e. $0.6 \times NOEC_{dissolved}$ as a worst case Ni-dissolved input for WHAM VI speciation calculations, while assuming that DOC = 0. This would result in a reasonable worst case assessment of $NOEC_{Ni2+}$ for this dataset.