Essentially, all models are wrong, but some are useful.

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BIOAVAILABILITY AND TOXICITY OF NICKEL TO FRESHWATER ORGANISMS: A MODELING APPROACH

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Woord vooraf

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List of abbreviations

AAP	Algal Assay Procedure
ACR	Acute to Chronic Ratio
AFA	Active Fulvic Acid
AHA	Aldrich Humic Acid
ANZECC	Australia and New Zealand Environment and Conservation Council
ARMCANZ	Agriculture and Resource Management Council of Australia and New Zealand
ATP	adenosine triphosphate
B	Belgium
BC	Before Christ
BL	Biotic Ligand
BLM	Biotic Ligand Model
CCAP	Culture Collection of Algae and Protozoa
CCC	Criteria Continuous Concentration
CCME	Canadian Council of Ministers of the Environment
CCREM	Canadian Council of Resource and Environment Ministers
CHESS	Chemical Equilibria in Soils and Solutions
CMC	Criteria Maximum Concentration
CSA	Chemical Safety Assessment
CSR	Chemical Safety Report
DEFRA	Department for Environment, Food and Rural Affairs
DI50	Dose resulting in 50% inhibition
DIC	Dissolved Inorganic Carbon
DOC	Dissolved Organic Carbon
DTA	Direct Toxicity Assessment
EC	European Commission
EC10	Effect concentration resulting in 10% inhibition
EC20	Effect concentration resulting in 20% inhibition
EC30	Effect concentration resulting in 30% inhibition
EC50	Effect concentration resulting in 50% inhibition
ECB	European Chemicals Bureau
ECD	Environmental Concentration Distribution

ECOSAT	Equilibrium Calculation of Speciation and Transport
EDTA	ethylene diamine tetraacetic acid
ELS	Early Life Stage
EQS	Environmental Quality Standard
E_rC10	Effect concentration resulting in 10% growth inhibition (algae)
E_rC50	Effect concentration resulting in 50% growth inhibition (algae)
EU	European Union
F	France
FA	Fulvic Acid
F-AAS	Flame Atomic Absorption Spectrometry
FAV	Final Acute Value
FIAM	Free Ion Activity Model
FM	Fathead minnow
FOREGS	Forum of the European Geological Surveys Directors
GF-AAS	Graphite Furnace Atomic Absorption Spectrometry
GSIM	Gill Surface Interaction Model
HC_5	Hazardous Concentration (for 5% of all species)
HMTV	Hardness-Modified Trigger Value
I	Intercept
IC	Inorganic Carbon
ICMM	International Council on Mining and Metals
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrometer
JPC	Joint Probability Curve
LA50	Lethal Accumulation associated with 50% effect
LC50	Lethal concentration for 50% of exposed organisms
LOEC	Lowest Observed Effect Concentration
$LOEC_m$	Lowest Observed Effect Concentration for the endpoint mortality
$LOEC_r$	Lowest Observed Effect Concentration for the endpoint reproduction
LOEL	Lowest Observable Effect Level
LOE_rC	Lowest Observed Effect Concentration based on growth rate (algae)
MDL	Method Detection Limit
MERAG	Metals Environmental Risk Assessment Guidance
MLE	Maximum Likelihood Estimation
MOPS	3-N-morpholinopropanesulfonic acid

NERC	Natural Environment Research Council
NGSO	National Guidelines and Standards Office
NICA	Non-Ideal Competitive Adsorption
NIST	National Institute of Standards and Technology
NL	The Netherlands
NOEC	No Observed Effect Concentration
$NOEC_m$	No Observed Effect Concentration for the endpoint mortality
$NOEC_r$	No Observed Effect Concentration for the endpoint reproduction
NOE_rC	No Observed Effect Concentration based on growth rate (algae)
NOM	Natural Organic Matter
OCEE	Optimal Concentration range of Essential Elements
OECD	Organisation for Economic Co-operation and Development
PEC	Predicted Environmental Concentration
PNEC	Predicted No Effect Concentration
PSII	Photosystem II
RCR	Risk Characterization Ratio
REACH	Registration, Evaluation, Authorisation and Restriction of Chemical Substances
RIP	REACH Implementation Project
ROS	Reactive Oxygen Species
S	Slope
SCAMP	Surface Chemistry Assemblage Model for Particles
SCHER	Scientific Committee on Health and Environmental Risks
SCTEE	Scientific Committee on Toxicity, Ecotoxicity and the Environment
SD	Standard Deviation
SE	Standard Error
SSD	Species Sensitivity Distribution
SSE	Sum of Squared Errors
TGD	Technical Guidance Document
TOC	Total Organic Carbon
TV	Trigger Value
UK	United Kingdom
USA	United States of America
US EPA	United States Environmental Protection Agency

WER	Water Effect Ratio
WFD	Water Framework Directive
WHAM	Windermere Humic Aqueous Model
WQC	Water Quality Criteria
WQG	Water Quality Guideline
WQS	Water Quality Standard
YCT	Yeast-Cerophyll-Trout chow

Chapter 1

General introduction and conceptual framework of the study

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General introduction and conceptual framework of the study

1.1. Nickel in society and the environment

1.1.1. Nickel and its properties

The atomic number of nickel (Ni) is 28 and its atomic weight is 58.6934 g/mol. At a temperature of 20 °C, the density of nickel is 8.90 g/cm^3. Its electron configuration is [Ar]4s^{2}3d^8. Nickel belongs to the first row of the transition metals which runs from scandium to zinc and in which the 3d orbital is successively being filled. Together with iron, ruthenium, osmium, cobalt, rhodium, iridium, palladium and platinum, nickel belongs to the group VIII transition metals, located in the centre of the periodic system. These metals are also known as the platinum metals. Nickel is found almost exclusively in the +2 oxidation state in its compounds. The widespread use of nickel in society is due to its strength, high ductility, high electrical and thermal conductivity, its magnetic characteristics, catalytic properties and maybe most of all, to its resistance to corrosion at environmental temperatures between -20 and +30 °C (Chau and Kulikovsky-Cordeiro, 1995; Zumdahl, 1995).

In the past, nickel has been categorized into the class of the 'heavy metals'. This term has been frequently used as a group name for metals and metalloids that are associated with contamination and potential toxicity. However, the term 'heavy metals' has not been used consistently in scientific literature since the available definitions (based on density, atomic weight or mass, atomic number or other chemical properties) failed to (i) consistently define this disparate group of elements, and (ii) classify metallic elements according to their potential toxicity (Duffus, 2002).

A more sound classification system of metallic elements which gives indications about their potential toxicity is based on Lewis acid behaviour. This system, which was originally developed by Ahrland et al. (1958), describes metal ions as Class A (hard metals), Class B (soft metals), or borderline (intermediate metals), depending on their observed affinity for different ligands. First-row d-block transition metals, including nickel, mainly fall into the group of the borderline metals and show widely variable coordination chemistry. Borderline metals have no general preference for O-, N- or S-donating functional groups. According to

Nieboer and Richardson (1980), borderline metals are likely to cause harmful structural changes to membranes because of their affinity for phosphate groups and non-oxygen centres in membranes.

1.1.2. Historical and current use of nickel

The use of nickel can be traced back as far as 3500 BC. Bronzes from what is now known as Syria have been demonstrated to contain up to 2% nickel. Chinese manuscripts mention the use of 'white copper' in the Orient between 1700 and 1400 BC. The term 'white copper' was also used for the naturally occurring copper-nickel or copper-nickel-zinc alloys that were used in China as far back as 235 BC for weapons, utensils and other metal ware. Copper-nickel (75:25 ratio) coins were first used around 170 BC in parts of Central Asia (Nriagu, 1980).

Since nickel ores were easily mistaken for ores of other valuable metals such as silver or copper, any understanding of the existence of this metal and its use dates to more contemporary times. Nickel was discovered in 1751 by the Swedish chemist Axel Fredrik Cronstedt while he was attempting to extract copper from niccolite, i.e. a nickel ore with a deceptive reddish cupric colour. Before the discovery of nickel, niccolite was named 'kupfernickel'. This name, which literally means 'devil's copper' (nickel = demon, goblin) was first used by German miners who attributed their inability to extract copper from this 'inferior' ore to the work of the devil (Sevin, 1980).

Despite its discovery, it was not until the early 1800s that purified nickel was obtained (Nriagu, 1980). The commercial exploitation of nickel was started in the 1850s after the development of technology to remove copper and other impurities (Sevin, 1980). From then on, nickel mining and production as well as the number of its applications have steadily increased. Despite a sharp increase in demand since the 1950s, nickel production is still far below that of other common metals such as iron, copper, zinc, aluminium, manganese and chromium (Kelly, 1988). World mine production has increased from 7500 ton/year in 1900 (Eisler, 1998) to approximately 1 449 000 ton/year in 2005 (Hetherington et al., 2007).

An overview of today's primary use of nickel is given in Figure 1.1 (Nickel Development Institute, 1996).

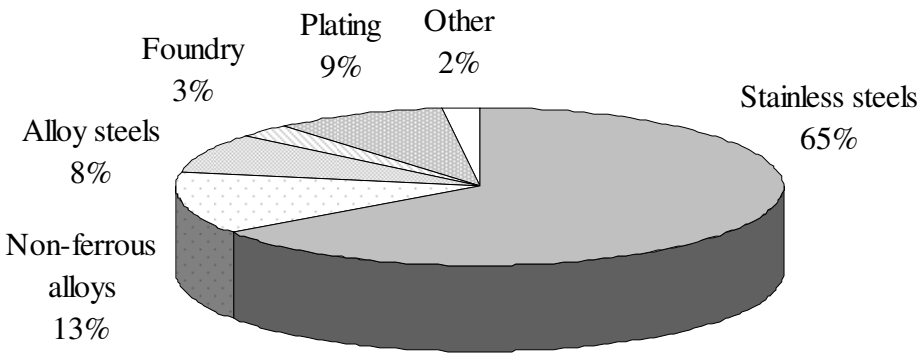

Figure 1.1. Primary use of nickel (Nickel Development Institute, 1996).

Nearly 90% of all nickel is consumed in the production of alloys and foundry products. There are over 3000 different nickel-bearing alloys in use (Kelly, 1988), of which stainless steel is the most important, followed by non-ferrous alloys (alloys with metals other than steel) and alloy steels (e.g., superalloys, which are metal mixtures designed to withstand extremely high temperatures and/or pressures, or to have high electrical conductivity). About 9% of primary nickel is used in plated products (i.e. metal products covered by a thin layer of nickel to decrease susceptibility to corrosion). The remaining 2% of primary nickel is used in a number of relatively small applications, including chemicals, catalysts (e.g., for hydrogenating vegetable oils), batteries (e.g., nickel-cadmium and nickel-metal hydride batteries), magnets, coins, pigments (e.g., for use in green glass), and powders (e.g., for use in powder metallurgy).

Most of the plating, foundry and 'other' applications already represent 'end-uses' of nickel. The steels and other nickel alloys, on the other hand, are 'intermediate' products that must be further processed into end-use commercial products in a number of industrial applications. An overview of the end-use of nickel is therefore given in Figure 1.2 (Nickel Development Institute, 1996).

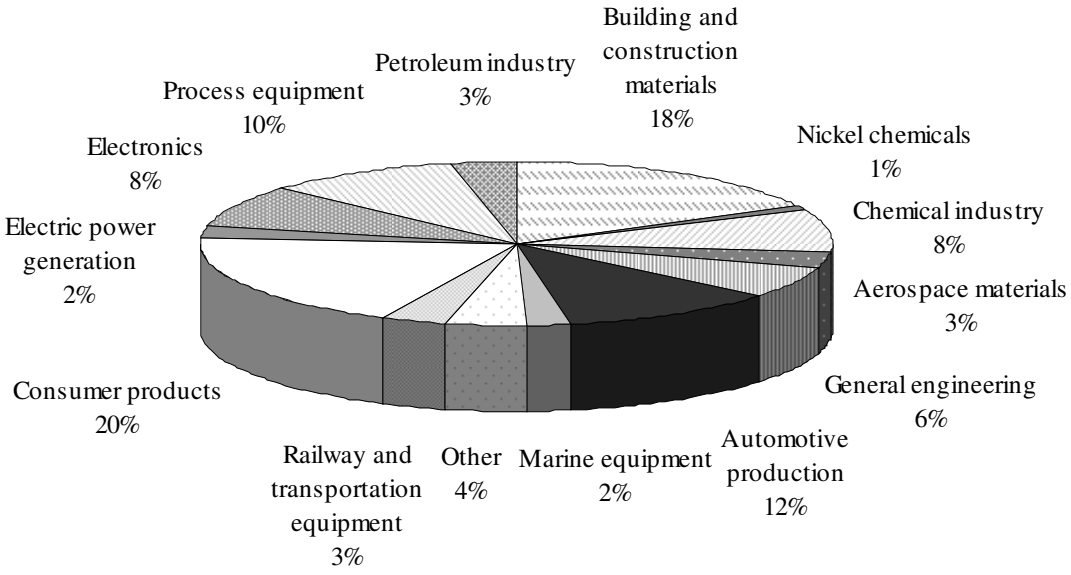

Figure 1.2. Distribution of the end-use of nickel (Nickel Development Institute, 1996).

1.1.3. Sources of nickel in the environment

1.1.3.1. Natural sources

Taking the entire earth into consideration (core + mantle + crust), nickel is the fifth most common element (after iron, oxygen, silicon and magnesium) (Cunningham and Saigo, 1999). However, in order of decreasing elemental abundance in the earth's crust, nickel is ranked number 24 (Zumdahl, 1995). The earth's crust contains 0.0075% nickel. There are two sources of nickel: sulphide ores which account for 60% of the world's output and laterite ores which account for the remainder. Nickel sulphide deposits usually contain between 1 and 4% nickel and may contain significant amounts of copper and platinum group metals. The nickel to copper ratio in these ores is usually in the range of 10:1 to 1:3. Laterite deposits may contain up to 3% nickel (Kelly, 1988).

There are 165 scientifically named minerals containing nickel and approximately 60 to 70 nickel-bearing minerals which remain unnamed. The ones that are most commonly mined are pentlandite (composition: $(Ni,Fe)_9S_8$) and garnierite (composition: $(Ni,Mg)_3Si_2O_5(OH)_4$). The annual world mine production of nickel is still increasing and was estimated at 1 449 000

ton/year in 2005. The current major producers are Russia, Canada, Australia, New Caledonia and Indonesia (Hetherington et al., 2007).

Nickel enters surface waters from three natural sources: as particulate matter in rainwater, through dissolution of primary bedrock materials and from secondary soil phases. Nickel tends to accumulate in the oceans and leaves the ocean as seaspray aerosols which release nickel-containing particles in the atmosphere (Eisler, 1998). Natural background concentrations (dissolved nickel) in uncontaminated freshwater ecosystems have been reported to be typically < 15 µg/L (Nriagu, 1980; Stokes, 1981; Zuurdeeg, 1992; Eisler, 1998). Geochemical baseline mapping in Europe yielded a distribution of dissolved nickel concentrations in stream water of which the 10th, 50th and 90th percentile were 0.39, 1.91 and 4.72 µg/L, respectively (Salminen et al., 2005, http://www.gtk.fi/publ/foregsatlas, Forum of the European Geological Surveys Directors). Naturally elevated concentrations may however occur due to volcanic activity or erosion of nickeliferous deposits (Chau and Kulikovsky-Cordeiro, 1995). This is also the case for background nickel concentrations in the atmosphere, sediment and soil.

1.1.3.2. Anthropogenic sources

While natural sources of airborne nickel (soil dust, sea salt, volcanoes, forest fires and vegetation exudates) account for about 16% of the atmospheric nickel burden, the remainder originates from anthropogenic sources. Through atmospheric deposition, the anthropogenically increased atmospheric nickel burden also affects nickel concentrations in surface waters, sediments and – mainly – soils (Eisler, 1998). Further, leaching of nickel from contaminated soils contributes to nickel concentrations in groundwater and surface water. For an analysis of nickel fluxes from society to the environment and between environmental media one can refer to the studies of Walterson (1998) and Landner and Reuther (2004).

A recent study (Callebaut and Van Hyfte, 2005) estimated the total emissions of nickel to air, water and soil in Europe (EU-15) at 642 074, 631 570 and 382 964 kg Ni/year, respectively. About 86% of the emissions to air are due to industrial processes (mainly emissions from power production plants and oil refineries). Another 10% can be attributed to traffic (mainly emissions from road transport and navigation). Waste management (mainly emissions from sewage treatment plants, but also from landfills and waste incineration plants)

is responsible for about 70% of the nickel emissions to water. The other main contributor to nickel emissions to the aquatic environment is industry (about 25%, due to manufacture of petrochemical products, other organic based chemicals, plastics in primary form, basic precious and non-ferrous metals, other basic metals, dyes and pigments, fertilizers, refined petroleum products, vegetable and animal oils and fats, etc.). Agriculture is responsible for about 96% of the emissions of nickel to soil. These emissions are mainly due to the use of mineral fertilizers, manure and sewage sludge (Callebaut and Van Hyfte, 2005).

Nickel concentration measurements in surface waters all around the world clearly indicate anthropogenic nickel input in an important number of monitored freshwater bodies. Waters receiving municipal waste waters show increased nickel concentrations compared to reference waters (e.g., average dissolved nickel concentration of 28 µg/L (range: 11-84 µg/L) in River Ivel versus 3.7 µg/L (1.3-11.5 µg/L) in River Yare, UK, Eisler, 1998). According to Chau and Kulikovsky-Cordeiro (1995), natural waters near industrial sites may contain 50 to 2000 µg Ni/L (dissolved concentrations). Relatively high aquatic nickel concentrations are also generally measured in areas currently or previously engaged in mining-related activities (e.g., average dissolved nickel concentration of 131 µg/L (8-2700 µg/L) in the Sudbury area, Ontario, Canada, Eisler, 1998). In Flanders, the 10th and 90th percentile of the total nickel concentration distribution observed in surface waters are 5 and 26 µg/L, respectively. However, concentrations up to 2883 µg/L have been measured in the vicinity of industrial locations, with the main contributors to the contamination being metal producing and metallurgic industry (Surface Water Database, Heijerick et al., unpublished data).

1.2. Nickel essentiality and toxicity

1.2.1. Essentiality (Muyssen et al., 2004; Nordberg et al., 2007)

A number of metals have been demonstrated to play a role in various enzymatic reactions and metabolic processes. As a result, uptake of these essential metals is necessary to meet the requirements of various biological functions. An element is considered essential when the following three conditions are fulfilled: (i) it has been demonstrated to be present in healthy living organisms; (ii) deficiency symptoms are noted upon depletion or removal of the element from the environment; and (iii) the deficiency symptoms are linked to a distinct biochemical defect (Wittmann, 1979). Some metals such as iron, manganese, zinc, copper,

cobalt and molybdenum are essential for all living organisms. However, evidence for the essentiality of nickel is still lacking for several groups of organisms.

Nickel has been demonstrated to be essential for a wide variety of animal species including chickens, rats, pigs, cows, sheep and goats (for a review on terrestrial organisms see Phipps et al., 2002). The basis for the essentiality of nickel in humans has been more difficult to define. Since nickel supply generally exceeds its requirement, a nickel deficiency disease has not been identified in humans. Because of the evidence for beneficial effects of nickel as nutritional supplement (Anke et al., 1995; Nielsen, 1996), the nutritional terminology 'apparent beneficial intake' has been deemed more appropriate for nickel.

The essentiality of nickel has also been demonstrated for bacteria. At least nine nickel-containing bacterial enzymes are know to date: urease, NiFe-hydrogenase, carbon monoxide dehydrogenase, acetyl-CoA decarboxylase/synthase, methyl coenzyme M reductase, certain superoxide dismutases, some glyoxalases, aci-reductone dioxygenase and methylenediurease (Kanai et al., 2003; Mulrooney and Hausinger, 2003; Lindahl, 2004).

Studies on the essentiality of nickel in aquatic organisms are limited to cyanobacteria, phytoplankton and aquatic higher plants and are related to the role of nickel in urease and hydrogenase (e.g., Gordon et al., 1978; Van Baalen and O'Donnell, 1978; Pederson et al., 1986; Price and Morel, 1991). Urease plays a key role in nitrogen metabolism of plants and microbial organisms by metabolizing urea to ammonium and carbon dioxide. Hydrogenase catalyzes the reversible oxidation of molecular hydrogen (H_2). At this point in time, no report has been made of nickel essentiality or deficiency in aquatic invertebrates and fish. However, the inverse relationships between bioconcentration factor and exposure concentration observed for fish and aquatic invertebrates (e.g., *Salmo gairdneri*, Calamari et al., 1982; *Daphnia magna*, Hall, 1982) suggest that these organisms are capable of actively regulating internal nickel concentrations. However, since active regulation does not necessarily prove essentiality, further research is needed to confirm this hypothesis.

Each species has an optimal concentration range for each essential element. Within this range it can satisfy its metabolic requirements and develop and perform in an optimal way (Hopkin, 1989). Van Assche et al. (1997) termed this range the optimal concentration range of essential elements (OCEE). The OCEE for a species may shift through adaptation or

acclimation. For nickel, the information on optimal and deficient concentrations is very limited. Calculations by Price and Morel (1991) demonstrate that nickel limitation may occur in the ocean. However, it remains to be investigated whether nickel deficiency is an environmental reality in freshwater ecosystems, since nickel concentrations in freshwater bodies are generally much higher than in the oceans.

1.2.2. Toxicity

Metal-organism interactions in the aquatic environment generally involve the following steps (Rainbow and Dallinger, 1993; Campbell, 1995):

(i) advection or diffusion of the metal from the bulk solution to the biological surface;
(ii) diffusion of the metal through the outer 'protective layer' (e.g., cell wall for microorganisms and plants, mucus for animal cells);
(iii) sorption or surface complexation of the metal at passive binding sites within the protective layer or at sites on the outer surface of the plasma membrane;
(iv) uptake or 'internalization' of the metal;
(v) transport, distribution and sequestration within the organism;
(vi) excretion.

Depending on the metal and the organism, the latter step may be absent.

All trace metals eventually become toxic at increased environmental concentrations. According to Ochiai (1977), the toxicity of metals is due to non-specific binding of metal ions resulting in (i) blockage of essential functional groups of biomolecules, (ii) displacement of essential metal ions in biomolecules, or (iii) modification of the active conformation of biomolecules. Sites of toxic action may be located at the organism-water interface (e.g., physiologically active sites in the plasma membrane such as transport sites and membrane-bound enzymes) as well as inside the organism (biomolecules involved in all kinds of metabolic processes). The location of the sites of toxic action may be linked to the route of metal exposure (waterborne or dietborne). In this study, we focused on the toxicity of nickel through waterborne exposure.

According to Paquin et al. (2002), the physiological mechanisms of toxicity for most metals can generally be divided into three categories: (i) monovalent metals (e.g., Ag^+ and Cu^+) affecting Na^+ transport, (ii) divalent metals (e.g., Cd^{2+} and Zn^{2+}) disrupting Ca^{2+} metabolism, and (iii) metals that cross the gill and act centrally (e.g., Pb^{2+} and Hg^{2+}). Recent research on fish however suggests that Pb^{2+} is primarily an antagonist of active Ca^{2+} uptake (see Niyogi and Wood, 2004).

Mechanisms of metal toxicity are very diverse and because of their complexity often not entirely understood. Compared to other common trace metals such as copper, silver, cadmium and zinc, relatively few studies have investigated nickel toxicity to freshwater organisms. At the time the present study was initiated, the most critical knowledge gaps with regard to nickel toxicity to freshwater organisms concerned chronic toxicity, mechanisms of toxicity and bioavailability. In the following sections an overview is given of the knowledge on nickel toxicity (to fish, cladocerans and green microalgae) that was available at that time, including research results published up to one year after the start of the present study.

1.2.2.1. Fish

Most available nickel toxicity studies have, without any doubt, investigated acute nickel toxicity to freshwater fish. 96-h LC50s for nickel have been published for rainbow trout (*Oncorhynchus mykiss*), fathead minnow (*Pimephales promelas*), goldfish (*Carassius auratus*), guppy (*Poecilia reticulata*), bluegill (*Lepomis macrochirus*), pumpkinseed (*Lepomis gibbosus*), nile tilapia (*Oreochromis niloticus*), banded killifish (*Fundulus diaphanus*), striped bass (*Morone saxatilis*), white perch (*Morone americana*), American eel (*Anguilla rostrata*), carp (*Cyprinus carpio*) and three-spined stickleback (*Gasterosteus aculeatus*) (e.g., Pickering and Henderson, 1966; Rehwoldt et al., 1971; Pickering, 1974; Hale, 1977; Blaylock and Frank, 1979; Nebeker et al., 1985; Atchison et al., 1987; Schubauer-Berigan et al., 1993; Alkahem, 1994; Meyer et al., 1999; Pyle et al., 2002a; Pane et al., 2003a; Brix et al., 2004; Hoang et al., 2004). The reported 96-h LC50s range from 230 µg/L (*O. mykiss*, low water hardness, high pH and alkalinity, larvae < 1 day post-hatch, Hoang et al., 2004) to 88 mg/L (*P. promelas*, high water hardness, subadult fish, Meyer et al., 1999). Comparison of the reported toxicity data is hampered by the use of different test media as well as the use of different developmental stages.

The gill can be considered the main route of metal uptake and toxicity when fish are acutely exposed to waterborne nickel (Pane et al., 2004a). The gill is a multifunctional organ which serves many purposes such as respiration, osmoregulation (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, Cl^{-}, H^{+}), nitrogenous waste excretion and acid-base balance. It has also been demonstrated to be involved in trace element absorption (Spry et al., 1988; Kamunde et al., 2002, Pane et al., 2004a).

The acute mechanism of waterborne nickel toxicity to fish was first investigated by Pane et al. (2003a, 2004a) using rainbow trout. Ion flux experiments revealed no marked impact on average unidirectional or net fluxes of Na^{+}, Cl^{-} or Ca^{2+}. Overall, acute exposure to waterborne nickel did not result in significant plasma ion concentration changes of Na^{+}, Cl^{-}, Ca^{2+} and Mg^{2+}. Measurement of blood gases and acid/base balance during cannulation experiments with adult fish revealed a drop of mean arterial oxygen tension, an increase in hematocrit and plasma lactate and a decrease of spleen haemoglobin. Ventilation experiments demonstrated an increase of ventilation rate, ventilation volume, opercular stroke volume and resting oxygen consumption. Ultrastructural damage to the respiratory epithelium of the gill impaired gas exchange leading to eventual suffocation.

These experiments indicated that acutely, nickel primarily acts as a respiratory toxicant. In this manner, nickel is quite similar to aluminium (Playle et al., 1989) and different from other waterborne metals such as copper, silver, cadmium and zinc, whose acute mechanisms of toxic action are primarily ionoregulatory. These findings suggest that a fourth category may be added to the classification proposed by Paquin et al. (2002): a category represented by metals that act primarily as respiratory toxicants.

Chronic nickel toxicity to fish has been studied less frequently than acute toxicity. Most available studies focused on nickel toxicity to early life stages (ELS). Pickering (1974) observed a decrease of the mean number of eggs per female and per spawning as well as decreased egg hatchability for fathead minnow. He reported a no observed effect concentration (NOEC) of 380 µg/L for survival, growth and reproduction combined. Blaylock and Frank (1979) observed a 50% decrease of the hatchability of carp eggs at a nickel concentration of 6 mg/L and a significantly increased amount of abnormal larvae at a nickel concentration of 3 mg/L. The ELS study conducted by Nebeker (1985) demonstrated that exposure to nickel decreased the hatchability of rainbow trout eggs. He also observed

significant mortality at 134 µg/L and significant effects on growth (weight and length) at 35 µg/L. In another ELS study with rainbow trout embryos, Birge et al. (1978) calculated a 28-d LC50 of 50 µg/L. Pane et al. (2004b) observed 33% mortality among juvenile rainbow trout (20-50 g) exposed for 42 days to 2034 µg/L. It should be noted that comparison of the reported toxicity data is almost impossible because of the large differences in test medium composition, test duration and developmental stages of the embryos or fish used in the tests.

Pane et al. (2004b) chronically (42 days) exposed rainbow trout to sublethal waterborne nickel. This kind of exposure did not significantly affect plasma ion concentrations of Na^+, Cl^-, Ca^{2+} and Mg^{2+}. However, as demonstrated for acute exposure to waterborne nickel (Pane et al., 2004a), chronic exposure also resulted in ultrastructural changes in the gill epithelium, hereby limiting branchial diffusing capacity and consequently the maximal rates of oxygen consumption during strenuous exercise. This clearly has environmental implications, since overall fitness of fish in a population would decrease as a result of impaired predator avoidance, prey capture and migration success.

Fish can also be exposed to nickel through the ingestion of contaminated food items and – for benthic-feeding fish – sediments. Ptashynski et al. (2001, 2002) exposed lake whitefish (*Coregonus clupeaformis*) and/or lake trout (*Salvelinus namaycush*) to nickel contaminated food and investigated the effect of dietary nickel at a physiological level (e.g., haematological parameters, metallothionein and plasma peroxide concentrations, histological alterations). The most sensitive endpoint following long-term exposure to dietary nickel was the occurrence of histological alterations in kidney and liver (significantly higher frequency of alterations observed in lake whitefish fed low dose diets, i.e. 10 $\mu g/g_{wetweight}$, for 10 to 104 days). This could be attributed to the role of these organs in accumulation, detoxification and excretion of contaminants in fish. So far, the relative importance of waterborne and dietborne nickel toxicity has not been studied in fish.

In contaminated ecosystems, fish may present a source of dietary nickel to pescivorous animals in the food chain. Depending on the route of exposure, nickel has been demonstrated to accumulate in significant amounts in kidney, liver, muscle, gill, intestine, pyloric caeca, stomach, heart, liver, gall bladder, gonad, bone, skin and scales (e.g., Ghazaly, 1992; Ptashynski and Klaverkamp, 2002; Pane et al., 2004a,b). An overview of bioaccumulation studies is given by Ptashynski et al. (2001).

With regard to the environmental implications of nickel contamination it may also be interesting to mention the study of Giattina et al. (1982), who reported a nickel avoidance threshold value of 23.9 µg/L for rainbow trout (low water hardness). Nickel has also been demonstrated to affect the agonistic behaviour of fish. Alkahem (1994) observed an increased frequency of agonistic acts such as threats, nips and chases among nile tilapia which were acutely exposed to nickel. However, Sloman et al. (2003) did not observe any significant behavioural changes between pairs of rainbow trout juveniles exposed for 24 hours to sublethal nickel concentrations (15% of the 96-h LC50). Possibly, behavioural changes are only consistently observed during longer exposures. However, the chronic effect of nickel on fish behaviour remains to be investigated.

Scott and Sloman (2004) discussed the possible mechanisms through which metals may affect fish behaviour. These mechanisms include disruption of normal olfactory function, endocrine disruption, neurological dysfunction or metabolic disruption. Nickel has already been demonstrated to enter the olfactory system (reviewed by Tjälve and Henriksson, 1999). Adverse effects of nickel on protein and carbohydrate metabolism have also been observed in fish (e.g., Chaudhry and Nath, 1984; Ghazaly, 1992; Sreedevi et al., 1992).

From the abovementioned laboratory studies it is clear that fish populations living in nickel contaminated water bodies may suffer from the adverse effects of short- as well as long-term exposure to nickel. The occurrence of adverse effects of nickel to fish in contaminated lakes has been demonstrated by several authors. For example, Gauthier et al. (2006) and Pyle et al. (2002b) observed that time to hatch was affected by nickel in Sudbury lakes and lakes in the neighbourhood of Saskatchewan uranium mines. They also reported that larval mortality was positively associated with nickel.

Since this study will focus on the effect of water chemistry on nickel toxicity to freshwater organisms, it is useful to summarize the available scientific literature on this topic. It should be kept in mind that all these studies concern acute toxicity, while data on chronic toxicity are required for in-depth risk assessment exercises (European Commission, 2005).

Schubauer-Berigan et al. (1993) demonstrated that nickel toxicity to fathead minnow (< 1 day post-hatch) increased with increasing pH. However, Pyle et al. (2002a) did not observe any effect of pH on nickel toxicity to larval fathead minnows between pH 5.5 and

7.0, while a further increase of pH to 8.5 resulted in a reduction of toxicity. Using a multivariate test design, Hoang et al. (2004) observed that nickel toxicity to fathead minnow increased with increasing pH, be it only at high levels of water hardness and alkalinity. The authors mentioned that the effect was confounded by water hardness, alkalinity and dissolved organic matter. Although pH affects nickel speciation, especially at higher pH levels (see further), none of these studies evaluated the observed effect of pH using Ni^{2+}-based toxicity data.

Water hardness has been demonstrated to protect against acute nickel toxicity to fathead minnow (Meyer et al., 1999; Pyle et al., 2002a; Hoang et al., 2004). In these studies, water hardness was adjusted by simultaneously increasing calcium and magnesium concentrations or by increasing calcium concentrations alone. Consequently, the individual effect of magnesium remains to be investigated.

Finally, Hoang et al. (2004) investigated the effect of dissolved organic matter (DOC) and reported that nickel toxicity decreased with increasing DOC concentrations. This effect appeared to be saturated above DOC concentrations of 5 mg/L. However, here also, the effect of DOC was not evaluated using toxicity data based on nickel speciation. Since DOC affects nickel speciation, this is needed for correct interpretation of the observed effects.

1.2.2.2. Cladocerans

The acute toxicity of nickel to cladocerans has been studied by Biesinger and Christensen (1972), Baudouin and Scoppa (1974), Chapman et al. (1980), Kühn et al. (1989), Schubauer-Berigan et al. (1993), Pane et al. (2003b) and Keithly et al. (2004). For *D. magna*, 48-h EC50s have been reported between 510 µg/L (Biesinger and Christensen, 1972) and 4970 µg/L (Chapman et al., 1980). For *Ceriodaphnia dubia*, reported 48-h EC50s range from 13 µg/L (Schubauer-Berigan et al., 1993) to 400 µg/L (Keithly et al., 2004). Baudouin and Scoppa (1974) reported a 48-h EC50 of 1.0 mg/L for *Daphnia hyalina*. Medium composition has without any doubt been the main factor contributing to within-species variation in toxicity among these studies. Overall, the reported toxicity data indicate that cladocerans are much more sensitive to acute nickel exposure than fish.

In fish, the gill has been demonstrated to be the main route of metal uptake and toxicity during acute exposure to waterborne metals, including nickel (Pane et al., 2004a). There is growing evidence that the gill-like structures in crustaceans have similar functions as the gills in fish (Kikuchi, 1983; Kikuchi and Shiraishi, 1997). Therefore, it is often assumed that the underlying mechanisms of metal toxicity to cladocerans are similar to those observed in fish. However, although Pane et al. (2003a, 2004a) demonstrated that acute nickel toxicity to fish occurs by a respiratory rather than ionoregulatory mechanism, no toxic effect on respiratory parameters was observed in acutely exposed *D. magna* (Pane et al., 2003b). A significant reduction of whole body Mg^{2+} concentration and unidirectional Mg^{2+} uptake rate indicated that Mg^{2+} antagonism is one possible mechanism for acute toxicity of waterborne nickel to *D. magna*. Apparently, mechanisms of acute nickel toxicity to cladocerans are different from those observed in fish.

The adverse effects of chronic nickel exposure on *D. magna* and/or *C. dubia* have been studied by Biesinger and Christensen (1972), Chapman et al. (1980), Kühn et al. (1989), Münzinger (1990), Münzinger and Monicelli (1991), Enserink et al. (1991), Kszos et al. (1992), Pane et al. (2003b, 2004c) and Keithly et al. (2004). Comparison of the reported toxicity data is hampered by the use of different test media and the fact that there is no consistency among these studies with regard to considered endpoints and reported effect data.

For *D. magna*, the reported 21-d LC50s varied between 71.9 µg/L (Pane et al., 2004c) and 360 µg/L (Enserink et al., 1991). Biesinger and Christensen (1972) – who reported a 21-d EC50 of 95 µg/L and a 21-d LC50 of 130 µg/L – demonstrated that reproduction is a more sensitive endpoint than mortality. This is supported by the findings of Kszos et al. (1992). The lowest effect concentration reported for *D. magna* is a 21-d NOEC of 10.2 µg/L for a non-specified endpoint (Chapman et al., 1980). Overall, *C. dubia* appeared to be more sensitive to nickel than *D. magna*. Kszos et al. (1992) and Keithly et al. (2004) reported NOECs for the endpoints mortality and reproduction of $\leq$ 3.8 µg/L. As observed for acute nickel exposure, cladocerans are clearly more sensitive to chronic nickel exposure than fish.

Other endpoints that have been demonstrated to be adversely affected as a result of chronic exposure to waterborne nickel include growth (Biesinger and Christensen, 1972; Münzinger, 1990; Enserink et al., 1991; Münzinger and Monicelli, 1991; Pane et al., 2004c), population yield (Enserink et al., 1991), intrinsic rate of natural increase (Münzinger, 1990;

Enserink et al., 1991) and brood size of primiparous females (Münzinger, 1990; Münzinger and Monicelli, 1991).

The effects of sublethal nickel concentrations on successive generations of *D. magna* have been studied by Münzinger (1990) and Pane et al. (2004c). Münzinger (1990) reported a significant decrease of the mean life span and length of primiparous animals when exposing seven successive generations to 160 µg/L. The progeny of exposed generations exhibited no adaptation towards nickel except an altered reproduction pattern (increase of intrinsic rate of population growth). Transfer to nickel free water did not entirely reverse the observed adverse effects on progeny.

Pane et al. (2004c) reported a 21-d NOEC of 42 µg/L for survival, growth and reproduction of the first generation of females exposed to nickel. However, the progeny of this generation showed increased nickel sensitivity, since a concentration of 42 µg/L resulted in lower weight and decreased whole body levels of glycogen and ATP. This was not the case for the progeny of females exposed to 21 µg/L. These offspring even showed a significantly increased resistance to acute nickel challenge. The results of this study indicate that the occurrence of increased or decreased resistance in successively exposed generations strongly depends on the concentration to which the organisms are exposed.

From the abovementioned laboratory studies it is clear that cladoceran populations living in nickel contaminated water bodies may suffer from the adverse effects of chronic exposure to relatively low nickel concentrations. Kszos et al. (1992) demonstrated that low survival and fecundity of both *C. dubia* and *D. magna* in water from industrially contaminated streams was linked to nickel concentrations well below US EPA (United States Environmental Protection Agency) water quality criteria (WQC).

Pane et al. (2003b) investigated the chronic mechanism of waterborne nickel toxicity in *D. magna*. Chronic nickel exposure was observed to result in a significant reduction of unidirectional Mg^{2+} uptake rate and whole body Mg^{2+} concentration. However, it also impaired respiratory function, as evidenced by a significant decrease of both oxygen consumption rate and whole body haemoglobin concentration. Apparently, the underlying mechanisms of chronic nickel toxicity to cladocerans are both ionoregulatory and respiratory and consequently show some similarity to the identified mechanism of acute nickel toxicity to

cladocerans (ionoregulatory, Pane et al., 2003b) on the one hand, and the identified mechanisms of acute and chronic nickel toxicity to fish (respiratory, Pane et al., 2003a, 2004a,b) on the other hand.

During the past few years, metals such as zinc (De Schamphelaere et al., 2004a) and copper (De Schamphelaere et al., 2007a) have been demonstrated to cause adverse effects in *D. magna* when taken up via food. However, it remains to be investigated whether dietborne exposure contributes to chronic nickel toxicity in cladocerans or not. Since significant nickel accumulation has been observed in *D. magna* by several authors (e.g., Hall, 1982; Pane et al., 2003b), cladocerans living in nickel contaminated water bodies may in turn present a source of dietary nickel toxicity to their predators.

The effect of water chemistry on nickel toxicity to cladocerans has been studied by Chapman et al., (1980), Kszos et al. (1992), Schubauer-Berigan et al. (1993) and Keithly et al. (2004). Water hardness has been demonstrated to protect against acute and chronic nickel toxicity to *D. magna* and *C. dubia* (Chapman et al., 1980; Kszos et al., 1992; Keithly et al., 2004). Schubauer-Berigan et al. (1993) investigated the effect of pH on the acute toxicity of nickel to *C. dubia* and observed an increase in toxicity with increasing pH. The results of these studies are more or less similar to the results obtained for fish (see above). However, not enough information is provided to come to a thorough understanding of the effects of water chemistry on nickel toxicity to cladocerans.

The abovementioned studies investigated the effects of only two water chemistry parameters on nickel toxicity: pH and water hardness. No effort was made to discriminate between possible individual effects of calcium and magnesium. Also, none of these studies evaluated the observed effects using toxicity data based on nickel speciation, which is especially important for the evaluation of the effect of pH (see further). Other factors that considerably increase the uncertainty around the reported toxicity data were: (i) the covariance of pH and water hardness in the studies of Chapman et al. (1980) and Kszos et al. (1992), (ii) acclimation of test organisms to the water hardness levels of the test solutions (Chapman et al., 1980), and (iii) feeding of test organisms during acute exposures (Schubauer-Berigan et al., 1993). Similar to what was concluded for fish, the effects of water chemistry on nickel toxicity to cladocerans needs to be more thoroughly investigated.

1.2.2.3. Algae

In fish and aquatic invertebrates, the epithelial cells of the gills represent the interface between the water column and the organism's plasma/hemolymph. As explained in the previous sections, the interaction of metals with the gills may result in toxicity through impairment of their respiratory and/or ionoregulatory functions. Overall, the gill can be considered the main route of metal uptake and toxicity when fish and aquatic invertebrates are exposed to waterborne nickel. In algae however, metals will first encounter a cell wall, through which they must migrate before reaching the plasma membrane. Because of this fundamental difference between plants and animals, mechanisms of metal toxicity in algae have often been assumed to be very different from those observed in fish and aquatic invertebrates.

The cell wall is a protective polysaccharide and glycoprotein layer, the composition of which depends on the type of cell, tissue, and plant species. The macromolecules making up this external layer contain a variety of functional groups, resulting in a matrix of negatively charged sites at which the metal can accumulate and through which the metal must migrate before eventually reaching the plasma membrane and being transported into the cytoplasm. It is commonly accepted that binding to the cell wall and the plasma membrane is kinetically fast and that – in the case of facilitated diffusion – uptake into the cytoplasm is the rate-limiting step of the uptake process (Campbell, 1995).

The cell walls of freshwater algae typically contain acidic polysaccharides such as pectin. At neutral pH the plentiful carboxylate groups of pectin and the present glycoproteins provide anionic sites which have a strong binding affinity for metal ions (Crist et al., 1994). The nature of binding between metal ions and the cell wall can be electrostatic, ionic or covalent (Haynes, 1980). According to Nieboer and Richardson (1980), nickel is a borderline metal that is close in character to Class B metals, which have a high affinity for carboxylate groups and are predicted to bind readily to the cell wall.

Adsorption and/or uptake of nickel by microalgae have been studied by Jin et al. (1996a), Fargašová et al. (1999), Macfie and Welbourn (2000) and Mehta et al. (2000). Macfie and Welbourn (2000) studied adsorption and uptake of nickel in a wild type as well as a wall-less strain of *Chlamydomonas reinhardtii*. They reported that total nickel

concentrations in the cells increased rapidly and leveled off after 12 hours. The total nickel burden (= adsorbed + absorbed nickel) of the walled cells was not significantly different from that of the wall-less cells. The walled strain had significantly less intracellular nickel since 50% of the total nickel burden was adsorbed to the cell wall. In a previous study, Macfie et al. (1994) reported similar EC30s for both the walled and the wall-less strain of *C. reinhardtii* between pH 5.0 and 6.8, indicating that the presence of a cell wall does not offer increased protection against nickel toxicity. The results of this study were supported by Jin et al. (1996a), who investigated adsorption and uptake of nickel by four strains of *Scenedesmus acutus* f. *alternans* and concluded that extracellular binding contributes only slightly to nickel resistance.

The adverse effects of nickel to freshwater microalgae have been studied by numerous authors. However, it is very difficult to quantitatively compare the observed effects among studies since (i) there is little consistency in the reported studies with regard to test conditions (e.g., exposure duration, composition of the test medium), (ii) a wide variety of endpoints has been investigated, (iii) effect concentrations have not been reported in all studies, and (iv) indispensable information is missing in the materials and methods section of several studies. In the following paragraph a chronological overview is given of the available studies.

- Shehata and Badr (1980) exposed *Scenedesmus* sp. to nickel for 8 to 10 days and reported a gradual decrease of growth rate (based on chlorophyll a concentration measurements) between 250 and 1500 µg/L. Growth was observed to cease at 2000 µg/L.
- Spencer and Greene (1981) investigated biomass increase of *Pediastrum tetras, Ankistrodesmus falcatus, A. falcatus* var. *acicularis, S. quadricauda* and *Scenedesmus dimorphus* during an exposure period of 14 days. The increase of algal biomass was negatively affected by the lowest exposure concentration (99.8 µg/L) in all cases.
- Michnowicz and Weaks (1984) studied the effect of nickel on growth (based on dry weight measurements) of *Selenastrum capricornutum* during 14 days of exposure to nickel but did not observe any adverse effects at the highest concentration tested (400 µg/L). Interestingly, they reported an increase of growth at low nickel concentrations.
- Wong and Chang (1991) observed a slight inhibition of growth (based on optical density measurements at λ = 650 nm) of *Chlorella pyrenoidosa* at a nickel concentration of 1000 µg/L. Photosynthesis was reported to be slightly inhibited at 500 µg/L.

- Macfie et al. (1994) investigated the effect of nickel on growth (based on cell counts) of *C. reinhardtii* during 5 days of exposure to nickel and reported EC30s (expressed as Ni^{2+} concentration) of 123-129 and 393-399 µg/L at pH 6.8 and 5.0, respectively.
- Lustigman et al. (1995) observed an increase of growth (based on cell counts and optical density measurements at λ = 750 nm) of *Chlorella vulgaris* at a nickel concentration of 10 µg/L. Growth was severely inhibited at 50 µg/L and 100 µg/L. At these concentrations, chlorophyll content decreased down to negligible and was not restored when transferring cells to nickel free medium, indicating permanent damage.
- Issa et al. (1995) reported growth rate inhibition (based on optical density measurements at λ = 750 nm), decrease of cell dry weight and chlorophyll a, b and carotenoid content, reduction of photosynthesis, increase of protein content and increase of respiration during a 7-day exposure of *Kirchneriella lunaris* to nickel.
- Jin et al. (1996b) observed an inhibitory effect of nickel on growth (based on cell counts) and photosynthesis of four strains of *S. acutus* f. *alternans* exposed to nickel for 14 days. However, effect concentrations are expressed as DI50s (dose – mol/cell dry weight – resulting in 50% inhibition).
- Fargašová (1998), Fargašová et al. (1999) and Fargašová (2001) demonstrated that nickel adversely affects growth (based on cell counts and optical density measurements at λ = 750 nm) and chlorophyll content (a, b, total) of *S. quadricauda* during a 12-day exposure to nickel. Respiratory rate was observed to increase. Fargašová et al. (1999) reported EC50s of 580, 440, 570 and 390 µg/L based on growth inhibition, total chlorophyll content, chlorophyll a content and chlorophyll b content, respectively.
- Pereira et al. (2005) calculated 96-h EC50s for growth (based on cell counts) of 1070 µg/L for *Pseudokirchneriella subcapitata* and 669 µg/L for *Gonium pectorale*.

Although comparison of the abovementioned toxicity data is hampered by the lack of consistency with regard to the test methods used in these studies, it is clear that nickel affects all tested microalgae in a similar way.

Patrick et al. (1975) investigated the effect of nickel on algal communities in experimental streams and reported changes in species composition and a decrease in species diversity of algal communities exposed to nickel concentrations ranging from 2 to 1000 µg/L. The same authors reported that diatoms decreased in diversity and abundance while the abundance and diversity of filamentous green and blue-green algae increased. According to

Nyholm and Peterson (1997), the initial response of a natural phytoplankton community to toxic pollution is typically an alteration of its community structure. Such early changes in community structure may be the most sensitive response of an aquatic ecosystem to toxic pollution. Only at higher toxic doses, functional parameters such as overall productivity will be affected.

Although nickel toxicity to algae may be partly due to interactions of nickel with transport systems in the plasma membrane, research concerning the mechanisms of nickel toxicity in algae has mainly focused on intracellular processes so far. For instance, nickel has been observed to inhibit electron flow in chloroplasts of *C. reinhardtii* mainly at the reducing side of photosystem II (PSII) (El-Naggar, 1998). Similar observations have been made in higher plants (e.g., *Hordeum vulgare*, Tripathy et al., 1981; *Pisum sativum*, Mohanty et al., 1989). According to Tripathy et al. (1981) and Singh et al. (1989), nickel induces changes in chlorophyll a emission characteristics and brings about a lowering of the chlorophyll a fluorescence yield. Clearly, nickel induces alterations in the chloroplast photosynthetic apparatus resulting in an irreversible loss of electron transport activity.

The biochemical and physiological consequences of the inhibition of electron transport activity have been studied by Rai et al. (1994) in *C. vulgaris*. They reported a decrease of final yield (based on chlorophyll a content), carbon fixation, oxygen evolution, ATP content and nutrient uptake (NH_4^+, Na^+, K^+, Ca^{2+}) during an exposure period of 15 days. Further, nickel has been observed to cause lipid peroxidation through increased formation of reactive oxygen species (ROS). Randhawa et al. (2001) investigated nickel resistance of several strains of the green alga *S. acutus* f. *alternans* and demonstrated that thiol antioxidants (e.g., glutathione) and antioxidant enzymes play a role in protecting cells against nickel toxicity.

Compared to fish and cladocerans, much less studies have investigated the effect of water chemistry on nickel toxicity to algae. Mehta et al. (2000) studied the effect of pH and cations on adsorption and uptake of nickel by *C. vulgaris*. They reported that nickel adsorption and uptake decreased with decreasing pH and increasing cation concentrations (e.g., Na^+, K^+, Ca^{2+}, Mg^{2+}). Issa et al. (1995) demonstrated that calcium protects against nickel-induced growth reduction in *K. lunaris*. However, the protective effect of calcium was evaluated based on a comparison of toxicity test results obtained at only one elevated calcium concentration (5 mM) with those obtained in a control treatment. Macfie et al. (1994)

investigated the effect of pH on nickel toxicity to *C. reinhardtii* and observed a 3-fold increase of toxicity between pH 5.0 and 6.8 for both the walled and the wall-less strain tested (EC30s based on Ni^{2+} concentration decreased from 393 to 123 µg/L for the walled strain and from 399 to 129 µg/L for the wall-less strain). These results suggest that competition between H^+ and Ni^{2+} for ionic binding sites is not restricted to the cell wall but must also occur at the plasma membrane.

From the abovementioned studies it is clear that the effect of water chemistry on nickel toxicity to algae has only marginally been studied. Several factors that may affect nickel toxicity (e.g., magnesium, DOC, alkalinity) have remained unstudied so far. On the other hand, the effects of factors that have been considered in the abovementioned studies (such as calcium and pH) should be investigated in more detail and within environmentally relevant ranges to allow them to be taken into account in risk assessment exercises and water quality criteria setting procedures.

1.3. Bioavailability

The term 'bioavailability' was once strictly used in pharmacology. Bioavailability is one of the principal pharmacokinetic properties of therapeutically active drugs, representing a measurement of the rate and extent to which a drug is absorbed and becomes available at the site of toxic action (Wagner, 1979). Because of the growing evidence that the toxic effect of metals in aquatic organisms is strongly dependent on the physicochemical characteristics of the surrounding environment as well as on several biological factors, the term has also become broadly used in the field of aquatic metal toxicology (and later on also for metal toxicology in sediments and soils).

During the past decades, it has become clear that neither total nor dissolved aqueous metal concentrations are good predictors of metal toxicity to aquatic organisms (e.g., Campbell, 1995; Bergman and Dorward-King, 1997; Janssen et al., 2000). First, metal toxicity is strongly dependent on water chemistry because water chemistry affects metal speciation and because different metal species have different potencies to adversely affect living organisms. Knowledge of metal speciation is therefore indispensable for predicting toxicity. Second, water chemistry can also affect metal toxicity by interfering with the metal under consideration at sites of metal uptake and/or toxic action. A mechanistic and/or

physiological understanding of these interactions is therefore also needed for predicting metal toxicity. Finally, metal toxicity may be influenced by several biological factors, such as the developmental stage of the organism (e.g., Hoang et al., 2004), genetic differences (e.g., Baird and Barata, 1998) and acclimation (e.g., Bossuyt and Janssen, 2005a).

In the following sections, we will explain how water chemistry affects metal bioavailability and toxicity to aquatic organisms and how the effects of water chemistry can be dealt with in view of the development of models for predicting metal toxicity to these organisms.

1.3.1. Effect of water chemistry on metal speciation

Metal speciation can be defined as "the distribution of an element amongst defined chemical species in a system" (Templeton et al., 2000). Metal speciation can be subdivided in physical speciation (i.e. dissolved metal versus metal adsorbed to particulate material) and chemical speciation (i.e. free metal ions versus metal complexed to dissolved organic and inorganic ligands). It has long been recognized that the formation of organic and inorganic metal complexes as well as metal sorption to particulate material reduces metal bioavailability and toxicity in the aquatic compartment (Pagenkopf et al., 1974; Sunda and Guillard, 1976; Sunda and Hansen, 1979; Pagenkopf, 1983).

Research concerning the bioavailability of metal species revealed that metal species have very different potencies to adversely affect living organisms. The order of toxic potential is generally Me^{2+} (free metal ion) > inorganic complexes > organic complexes (reviewed by Campbell, 1995; Paquin et al., 2002). However, several exceptions have been described to this general rule (e.g., Campbell, 1995; Campbell et al., 1997, 2002).

The distribution of a metal between the dissolved and the particulate phase can be assumed to determine the relative importance of the waterborne and dietary route of metal uptake. The adverse effects of dietborne exposure have been studied for several metals, including zinc (Clearwater et al., 2002, fish; De Schamphelaere et al., 2004a, *D. magna*), copper (Clearwater et al., 2002, fish; De Schamphelaere et al., 2007a, *D. magna*) and nickel (Ptashynski et al., 2001, 2002, fish). Dissolved and particulate metal concentrations can be measured directly (e.g., for monitoring purposes) but can nowadays also be computed with

geochemical speciation models (e.g., SCAMP, the Surface Chemistry Assemblage Model for Particles, Lofts and Tipping, 1998, 2003).

Within the dissolved phase, metal speciation is mainly determined by pH and the relative presence of inorganic (e.g., Cl^-, SO_4^{2-}, OH^-, CO_3^{2-}, HCO_3^-) and organic (e.g., humic, fulvic and hydrophilic acids) ligands. A decrease of pH results in an increase of the free metal ion concentration due to competition between protons and metal ions for binding to organic and inorganic ligands. On the other hand, the free metal ion concentration decreases with increasing pH as a result of increased metal complexation. The specific distribution of metal species as a function of pH is highly dependent on the metal under consideration.

Several techniques are available to directly measure metal speciation (e.g., ion-selective electrodes, voltammetry). For example, nickel speciation has been determined using ligand exchange adsorptive cathodic stripping voltammetry (Turner and Martino, 2006). However, the available methods for determining the speciation of nickel and other metals in environmental samples are difficult to apply on a routine basis and are therefore not widespread in use.

Alternatively, computer models can be used to calculate metal speciation in the dissolved phase. Models such as MINTEQA2 (Allison et al., 1991) and MINEQL+ (Schecher and McAvoy, 1992) can be used for calculating chemical activities and aqueous concentrations of the free metal ion as well as of inorganic metal complexes. The computations of these models are based on extensive thermodynamic databases containing stability constants for inorganic complexes which are updated from time to time (e.g., update of MINTEQA2 database in Visual MINTEQ with the most recent data from the NIST – National Institute of Standards and Technology – standard reference database).

Since binding of metals to natural organic matter (NOM) is often the dominant process in natural waters, it is essential that chemical speciation models include an accurate description of the reactions of trace metals with NOM. This is an immense challenge due to the heterogeneous nature of NOM (MacCarthy, 2001). The Windermere Humic Aqueous Model – Model V (WHAM V, Tipping, 1994) was one of the first models capable of calculating equilibrium chemical speciation in surface waters, ground waters, sediments and soils containing NOM.

WHAM V is a combination of several submodels. These are Humic Ion Binding Model V (Tipping and Hurley, 1992; Tipping, 1993a,b) and models of inorganic solution chemistry, precipitation of aluminium and iron oxyhydroxides, cation exchange on a representative clay and adsorption-desorption reactions of fulvic acids (Tipping and Woof, 1990, 1991). The interactions of metals with NOM are described in terms of intrinsic equilibrium constants and electrostatic terms. Competitive metal binding is assumed to take place at single proton dissociating sites (monodentate) and at bidentate sites formed by pairs of proton dissociating sites. Further, the importance of ion accumulation in the diffuse layers surrounding organic molecules is taken into account by incorporating a Donnan type diffuse layer model for non-specific electrostatic binding. WHAM V has been successfully calibrated to a large number of datasets including acid-base titrations and metal titrations of different types of organic matter (such as humic and fulvic acid) originating from different sources and under a large range of environmentally relevant conditions.

Together with the CHESS model (Chemical Equilibria in Soils and Solutions) developed by Santore and Driscoll (1995), the Humic Ion Binding Model V and the models of inorganic solution chemistry of WHAM V have been incorporated in the Biotic Ligand Model (BLM) software (latest version = Version 2.2.3., HydroQual, 2007) for calculating metal speciation. This software has been developed for calculating metal toxicity as a function of water chemistry and integrates both metal speciation and interactions of metals and other ions at the organism-water interface (see further).

Although WHAM V as well as the BLM software developed by Hydroqual are very useful and become more and more widespread in use, the more recently developed WHAM VI (Tipping, 1998) offers an even more advanced and better calibrated chemical speciation model. Humic Ion Binding Model VI is more sophisticated than Model V because of the incorporation of tridentate binding sites and more detailed descriptions of binding heterogeneity (Tipping, 1998). The Windows Interface developed by the Natural Environment Research Council of the UK (NERC, 2001) also incorporates a model for cation exchange on clay and a surface complexation model (SCAMP, Lofts and Tipping, 1998, 2003) with parameters for ion binding to oxides of iron, aluminium, manganese and silicon. WHAM VI is believed to increase the reliability of speciation calculations at lower metal concentrations than those for which WHAM V was originally calibrated. This may be of particular importance for modeling chronic exposures.

The Non-Ideal Competitive Adsorption (NICA) model developed by Benedetti et al. (1995) offers an alternative to the WHAM models for calculating metal speciation. It is a multiple site model that accounts for non-ideal binding of metal ions to heterogeneous organic matter. Whereas the WHAM models use discrete affinity constants to describe binding to different sites, the NICA model allows continuous binding and uses distributions of affinity constants. Like the WHAM models, the NICA model has also been refined to include a Donnan type formulation for non-specific electrostatic binding (Kinniburgh et al., 1996). The NICA model has been incorporated in ECOSAT (Equilibrium Calculation of Speciation and Transport, Keizer and Van Riemsdijk, 1998), a Windows Interface for calculation of (i) metal speciation, (ii) 1-dimensional and semi-2-dimensional stationary water or gas transport, and (iii) slow mass transfer (reaction kinetics).

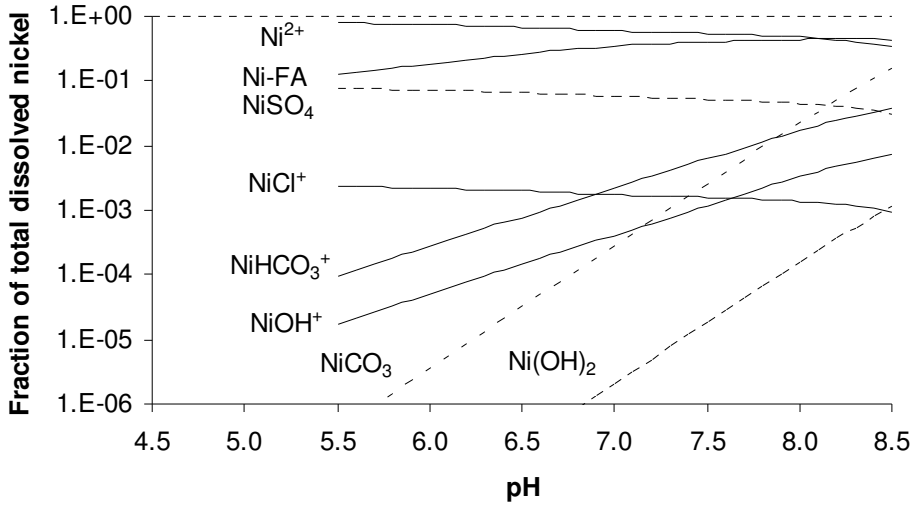

Figure 1.3. Nickel species distribution as a function of pH. Composition of medium = 0.75 mM Ca, 0.25 mM Mg, 1.5-3.0 mM Na (varied to maintain charge balance), 0.05 mM K, 1.0 mM SO_4^{2-}, 1.55 mM Cl^-, 2.0 µM Ni and 5 mg DOC/L. Dissolved inorganic carbon (DIC) concentrations were estimated assuming an open system in equilibrium with the atmosphere using thermodynamic stability constants taken from Stumm and Morgan (1996). Nickel speciation was simulated at a temperature of 20 °C using WHAM VI software (NERC, 2001). Stability constants for inorganic complexes were taken from Smith et al. (2004). Model parameters describing the interactions between nickel and fulvic acid (FA) were optimized as described in chapter 2.

In the present study, all speciation calculations were conducted using WHAM VI software (NERC, 2001). Figure 1.3 presents an example of nickel speciation as a function of pH over a pH range relevant for most European surface waters (5.5 to 8.5). Stability constants for inorganic complexes were taken from Smith et al. (2004). Model parameters describing

the interactions between nickel and fulvic acid (FA) were optimized as described in chapter 2. The free nickel ion concentration clearly decreases with increasing pH due to increased complexation to organic (FA) and inorganic ligands. This could have important implications for nickel toxicity and will be thoroughly discussed in the following chapters.

1.3.2. Modeling metal bioavailability and toxicity

The development of models for predicting metal bioavailability and toxicity to aquatic organisms not only requires accurate prediction of metal speciation in the aquatic environment, it also requires a quantitative description of (i) how water chemistry affects metal binding to sites of toxic action at the organism-water interface, and (ii) how metal binding to these sites of toxic action is related to the toxic response under consideration. In this section we will therefore give an overview of the key findings that allowed researchers to fulfil these two requirements and to bring bioavailability modeling to its current state of development.

One of the most important findings in early bioavailability research was without any doubt that the activity of the free metal ion is directly related to metal toxicity. This was first observed for copper by Zitko et al. (1973), with numerous examples to follow for copper (e.g., Pagenkopf et al., 1974; Sunda and Guillard, 1976) as well as for other metals, such as cadmium (e.g., Sunda et al., 1978), zinc (e.g., Allen et al., 1980) and silver (e.g., LeBlanc et al., 1984; Bury et al., 1999). Although this finding has been confirmed ever since in scientific literature, a few exceptions have also been reported, be it mostly under very particular conditions (e.g., Campbell, 1995; Campbell et al., 1997, 2002).

Another important early finding was that the mechanism by which water hardness protects against metal toxicity can be attributed to competition of water hardness cations (Ca^{2+} and/or Mg^{2+}) with the free metal ion for binding at sites of toxic action (Zitko and Carson, 1976). Several years later, researchers began to appreciate that protons can also protect against metal toxicity through competition (e.g., Campbell and Stokes, 1985; Cusimano et al., 1986). This finding implies that acidification can affect metal toxicity in two opposite ways: (i) an increase of free metal ion concentration due to the effect of pH on metal speciation, implying an increase in toxicity, and (ii) a decrease of metal binding to sites of toxic action due to proton competition, implying a decrease of toxicity.

These early findings led to the formulation of the free ion activity model (FIAM, Morel, 1983) and the gill surface interaction model (GSIM, Pagenkopf, 1983). Both models assumed that the degree of toxic response is related to the fraction of available toxic action sites occupied by the reactive metal species. Despite some minor differences, both the FIAM and the GSIM used chemical equilibrium formulations and accounted for competition between cations (Ca^{2+}, Mg^{2+} and/or H^{+}) and the metal stressor for binding to physiologically active sites at the organism-water surface.

Although conceptually, both the FIAM and the GSIM contained many of the features of bioavailability models that have recently been accepted by regulatory agencies, the full potential of both models as tools for use in the regulatory arena was not realized at the time they were presented. Nevertheless, in the years following the introduction of the FIAM and the GSIM, these concepts became increasingly supported by further research. Therefore, these primarily conceptual descriptions of metal-organism interactions formed an ideal basis for the development of more refined bioavailability models, such as the BLM.

A series of physiological studies conducted in the 1980s and 1990s demonstrated that, at the levels of concern for acute toxicity, many cationic metals had specific inhibitory effects on ion transport functions in fish gills. For example, Cu^{2+} (or Cu^{+}) (e.g., Laurén and McDonald, 1985, 1986) and Ag^{+} (e.g., Wood et al., 1996; Morgan et al., 1997) were demonstrated to block active Na^{+} (and Cl^{-}) uptake at the gills, while active Ca^{2+} uptake was observed to be blocked by Cd^{2+} (e.g., Verbost et al., 1987, 1989), Zn^{2+} (e.g., Spry and Wood, 1989; Hogstrand et al., 1996) and Co^{2+} (e.g., Comhaire et al., 1998). Although these studies yielded a lot of mechanistic information that helped guide the conceptual development of the BLM, they did not yet fulfil the requirements for a quantitative description of metal-organism interactions and their relation to toxicity.

In this respect, a major breakthrough came from a range of experiments conducted by Playle and colleagues (e.g., Playle et al., 1992, 1993; Janes and Playle, 1995). These experiments on metal-gill interactions in fish showed that cations in solution actually compete with metal ions for a limited number of binding sites on the gill, hereby affecting the degree of metal accumulation on the gill. Moreover, these studies were the first to determine gill binding site densities and (conditional) stability constants for binding of metal ions and competing cations to the gill. These parameters could easily be incorporated into chemical

equilibrium models and made it possible to predict metal accumulation in fish gills as a function of water chemistry.

Finally, it remained to be investigated how gill metal binding is related to toxicity. To this end, fish needed to be exposed to a range of metal concentrations – preferably in a range of different water chemistries – for simultaneous measurement of gill metal burdens and percentage mortality. The results of such experiments could then be used for calculating the gill metal burden that is predictive of 50% mortality in acute exposures. This parameter was first termed the LA50 (LA = lethal accumulation) and was (at least at that time) the final missing parameter for the practical development of bioavailability models.

In this respect, the most important studies to mention are those of MacRae et al. (1999) and Meyer et al. (1999). In their study on the effect of water hardness on short-term nickel accumulation on the gills of fathead minnow, Meyer et al. (1999) demonstrated that the LA50 was constant over the entire water hardness range tested, while 96-h LC50s based on dissolved nickel as well as on free nickel ion concentrations varied by a factor of 10. MacRae et al. (1999) determined the LA50 for copper and, most importantly, concluded that the measurement of gill copper accumulation is an acceptable alternative for determining a toxicity-based gill copper binding affinity (or vice versa).

The results of the study of MacRae et al. (1999) also suggested that metal complexation by biological ligands may be adequately simulated by chemical speciation models simply by including the biological ligands in the speciation calculations and using a single conditional stability constant for calculating the complexation of metals by these ligands. Assuming that the LA50 is independent of water chemistry, as was first demonstrated by Meyer et al. (1999), it was now possible to (i) predict metal toxicity from dissolved metal concentrations and physicochemical characteristics of the surrounding solution, and (ii) predict dissolved metal concentrations resulting in a certain toxic effect (e.g., 50% mortality) in aquatic systems with known water chemistry.

The integration of all the abovementioned key findings into modeling practice was first realized by Di Toro et al. (2001), who developed a bioavailability model for predicting acute copper toxicity to fish and introduced a new conceptual framework: the BLM (Figure 1.4, example for nickel). Since then, increasing research efforts have been made to further

refine the BLM framework and extend it to other metals and other organisms. Critical reviews of BLM research have been provided by Paquin et al. (2002) and Niyogi and Wood (2004).

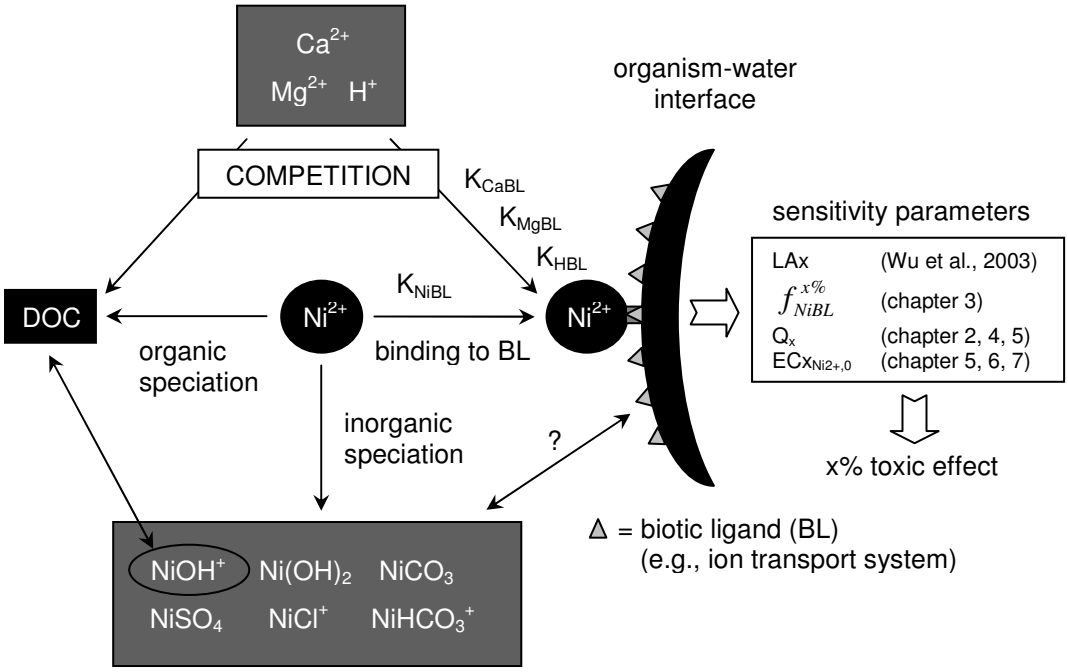

Figure 1.4. Schematic overview of the biotic ligand model (BLM) concept, adapted for nickel. The speciation part of the model includes (i) complexation of Ni^{2+} by inorganic ligands (e.g., Cl^-, SO_4^{2-}, OH^-, CO_3^{2-}, HCO_3^-), (ii) complexation of Ni^{2+} and $NiOH^+$ by organic ligands (dissolved organic carbon, DOC), and (iii) competition between Ni^{2+} and other cations (e.g., Ca^{2+}, Mg^{2+}, H^+) for binding to both inorganic and organic ligands. Throughout this study, nickel speciation has been calculated using WHAM VI software (NERC, 2001). Stability constants for inorganic complexes were taken from Smith et al. (2004). Model parameters describing the interactions between nickel and fulvic acid (FA) were optimized as described in chapter 2. The other part of the model concerns the interactions of Ni^{2+} and possibly other nickel species with active metal sites (the biotic ligand, BL) at the organism-water interface. Cations such as Ca^{2+}, Mg^{2+} and H^+ can compete with Ni^{2+} for binding to BL sites, hereby decreasing the amount of Ni^{2+} bound to the BL. K_{NiBL}, K_{CaBL}, K_{MgBL} and K_{HBL} represent affinities of Ni^{2+}, Ca^{2+}, Mg^{2+} and H^+ for binding to the BL. It is assumed that the fraction of BL sites occupied by Ni^{2+} at x% toxic effect is constant and independent of water chemistry. The LAx (LA = lethal accumulation, x = % mortality, unit = $nmol/g_{wetweight}$) was the model parameter originally proposed by Di Toro et al. (2001) to link Ni^{2+} complexation by the BL to toxicity. For nickel, LA50s have been proposed by Wu et al. (2003). The $f_{NiBL}^{x\%}$ is a similar type of sensitivity parameter (proposed by De Schamphelaere and Janssen, 2002) representing the fraction of BL sites that have to be occupied by Ni^{2+} to result in x% toxic effect. This parameter is used in chapter 3. The Q_x and $ECx_{Ni2+,0}$ are new types of sensitivity parameters that are proposed and/or used in chapter 2, 4, 5, 6 and 7.

Since the BLM framework was originally developed for fish, the first challenge was to extend its use to aquatic invertebrates. Due to their small size, it is extremely difficult to obtain gill metal accumulation data for invertebrate test organisms (e.g., daphnids). Furthermore, until a few years ago (e.g., Bianchini and Wood, 2003; Pane et al., 2003b), there had been almost no mechanistic research on metal toxicity in daphnids. A first modeling approach was therefore to recalibrate fish-based BLMs using daphnid toxicity data, assuming that the LA50 (representing the organism's sensitivity) is the only parameter that has to be changed in order to explain differences in metal toxicity between different types of living organisms (e.g., Santore et al., 2001, 2002).

A second modeling approach was introduced by De Schamphelaere and Janssen (2002) and allowed the derivation of all model parameters directly from toxicity data. The advantage of this approach is that it recognizes the possible existence of species-specific differences in mechanisms of metal toxicity and/or in the toxicity modifying capacity of certain physicochemical parameters.

The development of a BLM according to the method of de Schamphelaere and Janssen (2002) requires assessment of the individual effects of possible competing cations. This is usually done in univariate test series, i.e. series of toxicity tests in which the concentration of one cation is varied at a time. If the BLM concept is correct, a univariate test design should reveal linear relationships between effect concentrations expressed as free metal ion activity (e.g., $EC50_{Me2+}$) and competing cation activities. These linear relationships then serve as a basis for calculating species-specific stability constants for competing cations. In the situation in which the toxicity of a divalent metal is determined by competition between the free metal ion (Me^{2+}) and Ca^{2+}, Mg^{2+} and H^+ for binding to the biotic ligand (BL), the BLM equation for predicting toxicity of the metal under consideration can be written as follows (De Schamphelaere and Janssen, 2002):

$$EC50_{Me^{2+}} = \frac{f_{MeBL}^{50\%}}{(1-f_{MeBL}^{50\%}).K_{MeBL}}.\left\{1 + K_{CaBL}.(Ca^{2+}) + K_{MgBL}.(Mg^{2+}) + K_{HBL}.(H^{+})\right\} \qquad \text{Eq. 1.1}$$

where $EC50_{Me2+}$ is the predicted EC50 expressed as free metal ion activity, K_{MeBL}, K_{CaBL}, K_{MgBL} and K_{HBL} are the stability constants for the complexes formed between the BL and the free metal ion, Ca^{2+}, Mg^{2+} and H^+, respectively, and (Ca^{2+}), (Mg^{2+}) and (H^+) are the activities

of the respective cations. The remaining parameter ($f_{MeBL}^{50\%}$) represents the fraction of the available BL sites that are occupied by the free metal ion at the 50% effect level. This parameter is the sensitivity parameter of the model (similar in function to the LA50 used in the models developed by Di Toro and colleagues) and is assumed to be independent of water quality characteristics.

Since the relationship between acute copper toxicity to *D. magna* (based on free copper ion activity) and H^+ activity appeared to be curvilinear rather than linear, De Schamphelaere and Janssen (2002) suggested that, next to H^+ competition, other factors play a role in determining the effect of pH on copper toxicity. The accuracy of the model increased notably when $CuOH^+$ (i.e. a copper species which becomes more abundant with increasing pH) was included in the model as metal species contributing to copper toxicity. Later on, De Schamphelaere et al. (2002) further increased the model's accuracy by incorporating $CuCO_3$ in the model formulation.

Nonlinear relationships between free metal ion toxicity and H^+ activity have also been observed for other organisms and other metals (e.g., Peterson et al., 1984; Parent and Campbell, 1994; Heijerick et al., 2002a, 2005a; De Schamphelaere et al., 2003; Borgmann et al., 2005; De Schamphelaere and Janssen, 2006), including nickel (this study). How one can deal with such relationships when developing bioavailability models is discussed in chapter 2, 4 and 5.

In their review on metal bioavailability research, Paquin et al. (2002) gave an overview of ongoing and future developments that would significantly extend the application field of bioavailability modeling. These included modeling efforts with regard to (i) chronic exposures, (ii) algae, (iii) marine organisms, (iv) dietary toxicity, (v) multiple metal binding sites on the organism-water interface, (vi) the possible effect of water chemistry on the stability of cation-BL complexes, (vii) the possible contribution of metal species other than the free metal ion to metal toxicity, (viii) protective effects of cations through membrane stabilization, (ix) the possible effect of gill microenvironment physicochemistry on metal speciation and toxicity, (x) multi-metal exposures, and (xi) non-equilibrium circumstances. Many of these topics (of which the major topics are mentioned below) have been examined in the present study and are thoroughly discussed in the following chapters.

A first major topic that has been addressed in the present study was the development of chronic nickel bioavailability models. The BLM framework was originally developed for predicting acute toxicity. The extension of its applicability to chronic effects has been questioned because of the fact that a variety of processes involved in chronic toxicity (e.g., dietary exposure, physiological acclimation, physiological disturbance through deleterious effects on tissues, behavioural changes) may hamper a simple description of metal-organism interactions. Nevertheless, using the BLM framework as a basis, several authors have developed bioavailability models that are capable of accurately predicting chronic metal toxicity to fish and aquatic invertebrates (e.g., zinc, De Schamphelaere and Janssen, 2004a; De Schamphelaere et al., 2005a; Heijerick et al., 2005a; copper, De Schamphelaere and Janssen, 2004b). The chronic nickel bioavailability models for fish and *D. magna* that have been developed in the present study are described in chapter 2 and 4, respectively.

The models presented in chapter 2 and 4 are the first chronic nickel bioavailability models reported in literature. Before, only acute models had been developed for fish and aquatic invertebrates (Wu et al., 2003; Keithly et al., 2004). The models proposed by these authors are all based on the study of Meyer et al. (1999). However, this concise dataset only justifies the derivation of a model parameter describing the effect of calcium on acute nickel toxicity to fish. Therefore it was considered necessary to develop a new acute nickel bioavailability model for an aquatic invertebrate (*D. magna*). The new model is presented in chapter 3. The drawbacks of the formerly proposed models (Wu et al., 2003; Keithly et al., 2004) are also discussed in this chapter.

A second major topic was the development of a nickel bioavailability model for microalgae. The potential for application of the BLM framework to microalgae has been explored by several authors (e.g., Campbell et al., 2002; Heijerick et al., 2002a, 2005b; De Schamphelaere et al., 2003, 2005a,b; Hassler and Wilkinson, 2003; Kola and Wilkinson, 2005; De Schamphelaere and Janssen, 2006; Fortin et al., 2007). Although all of these authors encountered difficulties that questioned the applicability of the original BLM concept, some of them have proposed solutions that allow the development of bioavailability models for microalgae (e.g., Heijerick et al., 2002a; De Schamphelaere et al., 2003, 2005a,b; De Schamphelaere and Janssen, 2006). However, the proposed solutions deviate from the original BLM approach. In the present study, a chronic nickel bioavailability model has been developed for the unicellular green alga *P. subcapitata*. This is discussed in chapter 5.

A final major topic was addressed in chapter 6 and 7. In these chapters, it was investigated (i) whether organisms living in soft waters are intrinsically more sensitive to nickel than organisms living in hard waters, and (ii) whether a single bioavailability model can be used to predict the toxicity of nickel as a function of water hardness in both soft and hard waters. The finding of Snavely et al. (1991) that there exist water hardness-dependent high affinity transport systems for Ni^{2+} and Mg^{2+} (and to a lesser extent, Ca^{2+}) represented one of the motivations for this research.

1.4. Regulation – Water Quality Standards and Risk Assessment

All over the world, authorities have developed or are developing methods for the derivation of water quality standards (WQS) and/or the evaluation of the risks posed by metals and other pollutants of which environmental concentrations are increased as a result of human activities. Since metal bioavailability and toxicity are highly dependent on water chemistry, regulatory actions concerning metals require special attention. In different parts of the world, metal bioavailability is already taken into account in risk assessment exercises or in the derivation of WQS. Regulatory approaches with regard to metals have typically been very diverse. However, the development and worldwide acceptance of bioavailability models may bring more uniformity. To illustrate the existing diversity and resemblance, the regulatory approaches in the USA, Canada, Australia and New Zealand and Europe are described in the following sections.

1.4.1. USA

Under the Clean Water Act, water quality criteria (WQC) have been developed for 150 pollutants. The WQC for nickel at a water hardness of 100 mg $CaCO_3$/L are shown in Table 1.1 (US EPA, 1987, 1996). These WQC are expressed as dissolved nickel concentration. Hardness-based equations (see Table 1.1) allow the calculation of site-specific WQC as a function of water hardness. Two different WQC are formulated: the CMC (criteria maximum concentration), which is based on acute toxicity data and represents the highest nickel concentration to which an aquatic community can be exposed briefly without resulting in an unacceptable effect, and the CCC (criteria continuous concentration), which is the chronic equivalent of the CMC. The CMC should never be exceeded, while the 4-day average nickel concentration may exceed the CCC once in three years.

For nickel, the CMC was calculated by dividing the final acute value (FAV) by a safety factor of 2. The FAV is calculated as the 5th percentile of the statistical distribution of genus mean acute values (algae not considered). Due to the limited availability of chronic toxicity data for nickel, the CCC was calculated by dividing the FAV by the geometric mean of the acute-to-chronic ratios that were available for three species (ACR = acute value divided by chronic value, acute value = acute EC50, chronic value = geometric mean of chronic NOEC and LOEC).

Most WQC for metals were first developed in the 1980s. During this period, the protective effect of water hardness on metal toxicity was increasingly reported in scientific literature, while less was known about the effects of other physicochemical parameters such as pH and DOC. The hardness-based approach followed in several countries, such as the USA, has therefore serious drawbacks and may result in WQC that may be over- as well as under-protective.

This drawback was addressed by introducing the WER (water effect ratio) approach (US EPA, 1994, 2001). The WER is calculated as the acute LC50 in site water divided by the acute LC50 in standard laboratory water. The outcome of this evaluation can be used for the derivation of site-specific WQC that also take into account toxicity mitigating effects other than those caused by water hardness. However, this approach was soon considered costly, time consuming, inefficient and sometimes even inaccurate (Allen and Hansen, 1996; US EPA, 2001).

Recently, the Environmental Protection Agency of the USA (US EPA) has developed a Framework for Metals Risk Assessment (US EPA, 2007a). This document describes the key principles of metal risk assessment and serves as a guideline for risk assessors. An entire chapter of this document is dedicated to the use of bioavailability models in metal risk assessment exercises and the derivation of site-specific WQC for metals. The first actual use of bioavailability models for regulatory purposes in the USA is the use of the acute copper BLM for the derivation of WQC for copper. This is described in the document on the revised aquatic life ambient freshwater quality criteria for copper (US EPA, 2007b).

1.4.2. Canada

In Canada, water quality guidelines (WQG) have been developed by the National Guidelines and Standards Office (NGSO) in cooperation with the Canadian Council of Resource and Environment Ministers (CCREM). The WQG for nickel are shown in Table 1.1 (CCREM, 1987). These WQG are expressed as total nickel concentration. Four different WQG are proposed for four different water hardness ranges. According to the protocol for the derivation of WQG for the protection of aquatic life (most recent version: Canadian Council of Ministers of the Environment, CCME, 1999), a minimum aquatic toxicological dataset should be available to allow the derivation of full freshwater life guidelines. Data on metal toxicity to algae are also required, although interim guidelines may be proposed when algal toxicity data are missing. The WQG should preferably be based on chronic toxicity data (lowest LOEL (lowest observable effect level) divided by a safety factor of 10), but can also be calculated using acute toxicity data (lowest EC50 or LC50 divided by an ACR or an application factor).

Currently, the NGSO is revising the WQG for nickel as a test case for the development of a new protocol for the derivation of WQG for the protection of aquatic organisms. This new protocol will be based on newer scientific principles and a weight-of-evidence approach. It will address issues such as bioavailability, exposure and toxicity modifying factors, natural background concentrations and essentiality. The derivation of WQG will be based on the statistical distribution of chronic EC20s instead of directly on chronic LOELs or chronic EC50s or LC50s. Such statistical distributions are called species sensitivity distributions (SSD) and are also used in Australia and New Zealand and Europe for the derivation of WQG (see further).

1.4.3. Australia and New Zealand

In Australia and New Zealand, WQC have been developed by the Australian and New Zealand Environment and Conservation Council (ANZECC) and the Agriculture and Resource Management Council of Australia and New Zealand (ARMCANZ). Depending on the required level of protection, different trigger values (TV) have been defined. Each TV_x value in Table 1.1 represents the total nickel concentration at which 100-x% of the species are not affected. The TV_x values are calculated as the xth percentile of the statistical distribution

of chronic NOECs (i.e. SSD approach). Hardness-modified trigger values (HMTV) can be calculated using the equation mentioned in Table 1.1 (ANZECC/ARMCANZ, 2000). The TV_x values presented in Table 1.1 are applicable to waters with a hardness of 30 mg $CaCO_3$/L.

Table 1.1. Examples of water quality standards (WQS) in several countries/regions.

Country	WQS		Hardness	References
	Terms used	Value (µg/L)	(mg $CaCO_3$/L)	
USA	CMC [a]	470 [c]	100	US EPA, 1987, 1996
	CCC [a]	52 [c]		
Canada	WQG [b]	25	< 60	CCREM, 1987
		65	60-120	
		110	120-180	
		150	> 180	
Australia and New Zealand	TV_1 [b]	8 [d]	30	ANZECC/ARMCANZ, 2000
	TV_5 [b]	11 [d]		
	TV_{10} [b]	13 [d]		
	TV_{20} [b]	17 [d]		
Europe				
Current national EQS	Various [b]	1.8-1000	Various	EC, 2006a
Interim EU EQS	EQS [a]	20	Not specified	

EU = European Union, EQS = environmental quality standard(s), CMC = criteria maximum concentration, CCC = criteria continuous concentration, WQG = water quality guideline, TV_x = trigger value – x = percentile of statistical distribution of chronic NOECs (no observed effect concentrations), US EPA = United States Environmental Protection Agency, CCREM = Canadian Council of Resource and Environment Ministers, ANZECC = Australia and New Zealand Environment and Conservation Council, ARMCANZ = Agriculture and Resource Management Council of Australia and New Zealand, EC = European Commission.

[a] Expressed as dissolved nickel concentration.

[b] Expressed as total nickel concentration.

[c] Equations for hardness-correction:

$CMC = 0.998 \times e^{(0.8460 \times \ln(\text{hardness}) + 2.255)}$

$CCC = 0.997 \times e^{(0.8460 \times \ln(\text{hardness}) + 0.0584)}$

[d] Equation for calculating hardness-modified trigger value (HMTV):

$HMTV = TV \times (\text{hardness}/30)^{0.85}$

The guideline document developed by ANZECC/ARMCANZ (2000) proposes the use of TV_1 values as WQC for ecosystems with a high conservation value. TV_5 values may apply to slightly or moderately disturbed systems, while TV_{10} and TV_{20} values should only be used as criteria for highly disturbed systems. When the total metal concentrations measured in the aquatic system under consideration exceed the chosen TV, the next tier is triggered, which involves measurement of dissolved metal concentrations. If the dissolved metal concentrations still exceed the TV, the potential risk is further investigated in the next tiers. These tiers include modeling (e.g., MINTEQ, MINEQL, WHAM) or measurement of metal

speciation, the use of bioavailability models (e.g., FIAM, BLM), recalculation of the TV taking account of locally important species/genera, and direct toxicity assessment (DTA), in which the toxicity of ambient water is directly assessed with standard test organisms (cfr. WER approach in the USA) and/or local species. A major advantage of the tiered approach is that costly evaluation methods (e.g., speciation measurements and DTA) are only applied when necessary instead of on a routine basis.

1.4.4. European Union (EU)

1.4.4.1. Water Framework Directive

Under the Water Framework Directive (EC, 2000) (EC = European Commission), the EC was required to establish a list of priority substances. The first list of 33 substances, including nickel and three other metals (i.e. cadmium, mercury and lead), was published in 2001 (EC, 2001). For these substances, EU-wide environmental quality standards (EQS) are to be formulated. Once EQS are established at EU level, it is a legal obligation for all member states to ensure that all surface water bodies (other than those covered by the exemptions and extensions provided for in the WFD) comply with these standards by 2015. Exceptions (e.g., application of lower or higher national, regional or local EQS) will be discussed on a case-by-case basis by the European Commission.

For nickel, the range of existing national EQS (expressed as total nickel concentration) in EU member states is shown in Table 1.1 (EC, 2006a). In several countries, site-specific EQS are calculated as a function of water hardness. The proposed European EQS for nickel (expressed as dissolved nickel concentration) is also shown in Table 1.1 (EC, 2006a). For the determination of EQS, the WFD refers to the methods described in the technical guidance document (TGD) on risk assessment of existing substances (later extended to new notified substances and biocidal products, most recent version: EC, 2003). For priority substances for which a risk assessment is or was required by the Existing Chemicals Regulation (EC, 1993) or the Plant Protection Products Directive (EC, 1991), the outcome of the risk assessment was or will be taken into consideration for the determination of an EQS. This is done because the paramount objective of the EC in proposing EQS is consistency between the derivation of EQS and the outcome of risk assessments. The EQS proposed for nickel is an interim EQS since the nickel risk assessment has not been finalized yet.

All proposed EQS were reviewed by the Scientific Committee on Health and Environmental Risks (SCHER, formerly the Scientific Committee on Toxicity, Ecotoxicity and the Environment, SCTEE) (SCTEE, 2004a). Important comments of the SCTEE with regard to the determination of EQS for metals were that background concentrations and metal bioavailability should be considered. The latter recommendation was addressed by including an option for the member states to take into account metal bioavailability when evaluating the compliance of their water bodies with the EQS for the metal under consideration. Further, it was mentioned that the required methodology to do so will be provided by the EC. This methodology could be based on bioavailability models or relationships between metal toxicity and toxicity modifying factors. One of the aims of the present study was to develop nickel bioavailability models that support the development of such a methodology for nickel.

1.4.4.2. REACH

REACH is the new European regulation for Registration, Evaluation, Authorisation and Restriction of Chemical substances (EC, 2006b). It entered into force on the 1st of June 2007 and replaces about 40 pieces of European legislation concerning chemical substances, including the Existing Chemicals Regulation (EC, 1993), under which risk assessments have been performed for several metals, including nickel. Under the REACH legislation, a chemical safety report (CSR) should be provided by industry for all chemical substances that are manufactured and/or imported in the EU at a total quantity of 10 tonnes or more per year. This CSR should include a chemical safety assessment (CSA) in which the risks posed by the substance to man and the environment are assessed over its entire life cycle.

Guidance on how to perform a CSA has been provided in a new technical guidance document (EC, 2005 – final version expected early 2008) that was developed under REACH Implementation Project 3.2-1B (RIP 3.2-1B). The methods described in the new TGD were largely adopted from the TGD on risk assessment of existing substances, new notified substances and biocidal products (EC, 2003) and were further refined.

The TGD describes exposure and effects assessment procedures leading to predicted environmental concentrations (PECs) and predicted no effect concentrations (PNECs). When the PEC exceeds the PNEC for a given substance in a given environmental compartment, the risk characterization ratio (RCR = PEC/PNEC) is larger than 1, indicating that the substance

is likely to pose a risk to the organisms living in the environmental compartment under consideration.

To assess the exposure of the environment to a substance, it is necessary to (i) estimate and/or evaluate the emissions of the substance to each environmental compartment, and (ii) account for the environmental fate and behaviour of the substance (e.g., transport, transformation, bioconcentration, biomagnification, degradation). For some types of substances, previously developed emission scenarios have been included in the TGD, whereas for other substances, new emission scenarios are to be developed by industry. For data-rich substances monitoring data may also be used.

The tiered approach proposed by the REACH legislation allows the exposure assessment to become an iterative process. The lowest tier includes a qualitative approach while in the highest tier a probabilistic approach is followed. Methods based on environmental concentration distributions (ECDs) allow for instance the probabilistic quantification of the difference between average and worst case scenarios. Exposure assessments have to be performed for each environmental compartment. They can be performed at different scales (e.g., local scale, only taking into account point sources; regional scale, taking into account both point and diffuse sources).

The methods proposed for PNEC derivation are dependent on the data-richness of the substance. For data-poor substances, PNECs are determined by applying an assessment factor to the lowest acute EC50 or LC50 or the lowest chronic NOEC. The assessment factors range from 10 to 1000 depending on the type and amount of available toxicity data. For data-rich substances, probabilistic methods based on species sensitivity distributions (SSDs) are recommended. A SSD is a cumulative distribution function of the geometric means of the available NOECs for each tested species. It is assumed that the available toxicity data represent a reflection of the sensitivity of the entire ecosystem. Based on a statistical distribution function fitted through the data, the HC_5 can be determined, representing the hazardous concentration for 5% of the species. The PNEC is subsequently calculated by dividing the HC_5 by an additional safety factor between 1 and 5. However, it can be argued that there is “neither scientific rationale nor evidence which allows an objective selection of the additional safety factor magnitudes” (SCTEE, 2004b).

Since metal bioavailability and toxicity are known to be affected by water chemistry, a substantial part of the intraspecies variation in observed toxicity can typically be attributed to the use of test media with varying physicochemical composition. This variation brings about a lot of uncertainty around the outcome of the effects assessment and should therefore be dealt with in high-tier risk assessment. Guidance on this issue and other metal-specific issues is provided in the MERAG document (Metals Environmental Risk Assessment Guidance, ICMM, 2007) which has been developed by ICMM (International Council on Mining and Metals), Eurometaux and the UK government (DEFRA, Department for Environment, Food and Rural Affairs). The MERAG project has gained the support of a number of authorities including those in Canada, the USA, the EU and several EU member states. This support has already resulted in the use of MERAG concepts in national and regional risk assessment frameworks.

The MERAG fact sheet on the incorporation of bioavailability for water, soils and sediments describes how bioavailability models can be used for the normalization of SSDs. It should be investigated on a case-by-case basis whether bioavailability models developed for a specific species may be applied to other species belonging to the same trophic level (this is termed ‘read-across’). The basic idea of read-across is that a single bioavailability model may be used for all species belonging to the same trophic level, provided that the sensitivity parameter of the model is adjusted to account for species-specific sensitivity differences.

Since most metals are data-rich substances, probabilistic methods may be used for both the effects and exposure assessment. Consequently, risk characterization should not be confined to the calculation of a single RCR (PEC/PNEC). The probabilistic risk quotient method offers an interesting alternative to the PEC/PNEC approach. Basically, random samples out of the ECD and the SSD are divided to obtain a risk quotient distribution (e.g., using Monte Carlo techniques). This distribution allows to determine the probability for the RCR to be larger than 1 and hence the probability that the environmental compartment under consideration will be affected. Further, joint probability curves (JPC) can be derived from the ECD and the SSD. These curves represent the fraction of species affected for each exposure exceedance or vice versa. The outcome of probabilistic risk assessments may be further evaluated by performing uncertainty analyses. Such analyses may help competent authorities to identify needs for further research or to formulate risk management measures.

The objectives of the present study were mainly related to the effects assessment of nickel for the aquatic compartment. In this study, toxicity data have been generated for three standard test species (*D. magna, O. mykiss, P. subcapitata*) belonging to different trophic levels (chapters 2 to 5) as well as for several cladocerans and green microalgae collected in the field (chapter 6 and 7). These data provided a lot of new information for the aquatic nickel effects assessment. Furthermore, the bioavailability models that have been developed for the abovementioned organisms (chapters 2 to 7) present useful tools for SSD normalization, which is necessary to reduce the uncertainty around the outcome of the effects assessment.

1.5. Introduction to the standard test species

1.5.1. Rainbow trout (*Oncorhynchus mykiss*)

Within the phylum of the Chordata, *O. mykiss* (Figure 1.5) belongs to the class of the Actinopterygii (i.e. ray-finned fishes), the order of the Salmoniformes and the family of the Salmonidae. There are two forms within the species *O. mykiss*. The first form is anadromous, i.e. it migrates to the ocean and then returns to its freshwater body of origin for reproducing. The other form, which is called 'rainbow trout' based on the broad red band along its side, never leaves freshwater. However, rainbow trout have also been demonstrated to migrate away from their hatching place for growing up and back to their hatching place for spawning (which is called 'homing').

Figure 1.5. *Oncorhynchus mykiss* – rainbow trout.

Rainbow trout is native to tributaries of the Pacific Ocean in Asia and North America as well as much of the central, western, eastern and especially the northern regions of the

USA. They are typically found in well-oxygenated (mostly clear) lakes and streams. The optimal water temperature for wild rainbow trout is 12 °C although the species has been observed in waters with temperatures ranging from 6 to 23 °C. Rainbow trout have been introduced in several other countries for food and sport fishing and are therefore now present in every continent except Antarctica. In some of the locations where they have been introduced, rainbow trout have had serious negative impacts on freshwater ecosystems.

Rainbow trout are predators with a highly diverse diet and belong to the top levels of the food chain in most freshwater environments. Although insects, insect larvae, crayfish and other crustaceans (e.g., cladocerans) make up a large portion of their diet, they also feed on smaller fish. Their natural enemies are larger fish (even from the same species), water shrew, mink, the common rat, and to some extent otters and herons. Mainly due to predation, about 94% of fry does not survive the first three to four months of its life. Later on, another 20% of juvenile fish dies before reaching maturity. Next to predation, trout mortality may also be due to food competition with other species, lack of oxygen, and anthropogenic activities (Burton et al., 1975).

The fish species that are currently most commonly used for toxicity testing are rainbow trout (*O. mykiss* – cold water species) and fathead minnow (*P. promelas* – temperate to warm water species). The most frequently used test methods are those described by the OECD (1996) and the US EPA (2002a,b). In acute toxicity tests, juvenile fish are exposed for 96 hours to a series of toxicant concentrations to investigate the effect of the toxicant on fish survival. The output of an acute toxicity test with fish is usually a LC50 value. Chronic toxicity tests should normally include reproduction as an endpoint. However, since fish chronic exposures may take about a year to complete, short-cut methods have been developed that focus on the most sensitive life stages (Hoffman et al., 1995). These long-term toxicity tests include early life stage (ELS) toxicity tests, in which egg hatchability, larval deformation and larval growth and survival are studied, and toxicity tests with somewhat older juvenile fish, in which growth and survival represent the most important endpoints.

In the present study, long-term juvenile growth tests have been performed in accordance with the test guideline described by the OECD (1996). More details on the followed procedure, studied endpoints and calculated effect concentrations are given in the materials and methods section of chapter 2.

1.5.2. *Daphnia magna*

One of the subphyla of the phylum Arthropoda is the Crustacea. This subphylum represents the most abundant and diverse group of invertebrate organisms in aquatic ecosystems. Within this subphylum, *D. magna* (Figure 1.6) belongs to the order (or suborder, depending on the classification system) of the Cladocera within the class of the Branchiopoda. Branchiopods are characterized by a ventral groove, useful for suspension and filter feeding. The water current in the ventral groove, in most species used for feeding, is produced by a battery of unspecialised trunk appendages called 'legs'. These appendages carry numerous setae which are used for filter feeding. The vasicular epipodites of the appendages are called gills (branchia) and have been demonstrated to be involved in both respiration and ionoregulation (Kikuchi, 1983; Kikuchi and Shiraishi, 1997).

Figure 1.6. *Daphnia magna.*

Cladocerans (water fleas) are small planktonic organisms. Adults of species occurring in Europe are typically between 0.3 and 18 mm, with *Leptodora kindtii*, a predating water flea, being the largest species (Flößner, 2000). With adults of up to 6 mm in length, *D. magna* also represents one of the largest European species. The thorax and the abdomen of cladocerans are generally covered by a chitinous carapace. The head carries several appendages, some of which form the mouth parts, and some of which function as sensory (antennula) and/or swimming organs (antennae). Among the cladocerans there are free-swimming species (e.g., *D. magna*) but also epibenthic species and species that are attached to living plants. All together, cladocerans generally dominate the zooplankton community in freshwater habitats (Scourfield and Harding, 1966; Flößner, 2000).

Freshwater cladocerans inhabit all types of water bodies all over the world (e.g., springs, groundwater, marshes, rivers, lakes). The largest densities are found in small lakes and big ponds with rich submersed and emerged vegetation. *D. magna* is a typical inhabitant of small polluted water bodies in which no fish are present. Cladocerans are mainly non-selective suspension feeders that collect food particles using their trunk appendages. The distance between the numerous setae on these appendages determines the size of the food particles suited for consumption. Cladocerans may feed on bacteria, protozoans, planktonic microalgae (e.g., *Daphnia*), periphyton (e.g., *Simocephalus*), detritus (e.g., Chydoridae) or other zooplankton (e.g., Polyphemidae) (Scourfield and Harding, 1966; Flößner, 2000).

In the cladoceran life cycle, parthenogenetic generations typically alternate with bisexual generations. At the onset of the spring, females hatch from fertilized diapausing eggs (ephippia). As long as conditions remain favourable, these females reproduce parthenogenetically. During this type of reproduction, eggs are released in the brood chamber under the female's carapace. When the young have hatched from the eggs, the skeleton is moulted, releasing the young from the brood chamber. Certain factors, such as a change in water temperature or decreased food supply, may induce the release of males. When males are present, ephippia will be produced allowing the population to survive the unfavourable period (Scourfield and Harding, 1966; Flößner, 2000).

The most frequently used aquatic invertebrates in standard toxicity testing belong to the Daphniidae (e.g., *D. magna, Daphnia pulex, C. dubia*). The reasons for the selection and success of daphnids as standard test organisms are (i) their worldwide distribution in various freshwater habitats, (ii) their key function in aquatic food chains (they graze on primary producers and are consumed by many fish species), (iii) their small size, (iv) their relatively short life cycle, (v) their parthenogenetic reproduction under favourable conditions, and (vi) their sensitivity to a broad range of aquatic contaminants (Clesceri et al., 1998).

Standard methods for toxicity testing with daphnid species have been developed by several standardizing organisations and authorities. Currently, the most frequently used standard test methods are those developed by the OECD (1996) and the US EPA (2002a,b). In acute toxicity tests, neonates (< 24 h old) are exposed to a range of toxicant concentrations for 24 to 48 hours. Based on the results of these tests, EC50s or LC50s are calculated, representing the concentrations that cause 50% immobilization or mortality, respectively.

In chronic tests, juveniles are exposed individually to the test medium for longer periods, during which the animals are fed and survival and reproduction are monitored daily. The *D. magna* reproduction test developed by the OECD (1996) lasts 21 days, while the *C. dubia* test developed by the US EPA (2002b) is finished after 7 days of exposure or earlier if at least 60% of the control females have released their third brood. The outcomes of these tests are EC50s for reproduction, LC50s for survival, and NOECs and/or LOECs (no observed effect concentrations and lowest observed effect concentrations, respectively) for both endpoints.

Detailed information on the procedures followed in the present study is given in the materials and methods section of chapter 3 and 4. In chapter 6, the results of toxicity tests with field-collected cladocerans are discussed. The tests with these non-standard test species were based on the abovementioned test methods and slightly modified where necessary.

1.5.3. *Pseudokirchneriella subcapitata*

P. subcapitata (Figure 1.7) is a unicellular green alga which belongs to the phylum of the Chlorophyta. This phylum contains mostly aquatic photosynthetic eukaryotic algae, both unicellular and multicellular. Within the Chlorophyta, *P. subcapitata* belongs to the class of the Chlorophyceae, the order of the Chlorococcales and the family of the Chlorellaceae. The species was formerly known as *Selenastrum capricornutum* and *Raphidocelis subcapitata* (Hindák, 1990).

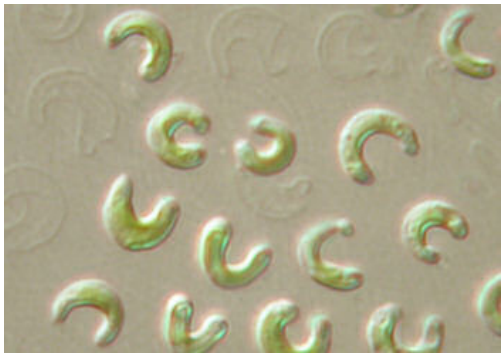

Figure 1.7. *Pseudokirchneriella subcapitata.*

Although *P. subcapitata* is a unicellular species (5-7 µm), it may occur in colonies of several cells embedded in a structureless mucilaginous envelope. Cells reproduce asexually by autosporulation. The autospores are released by longitudinal rupture of the parental cell wall. Sexual reproduction has not been observed yet. *P. subcapitata* is a planktonic species living in freshwater ponds, lakes and pools. It has been reported to occur in Europe and North America (Hindák, 1990).

As primary producers, planktonic microalgae represent a key component of food chains in aquatic systems. Many species serve directly as food source for zooplanktonic organisms, which are subsequently consumed by other invertebrates, fish or birds. Changes in the structure (relative species abundance) and functional characteristics (e.g., productivity, standing crop) of the algal community may induce direct structural changes in the rest of the ecosystem and/or indirectly affect the ecosystem by affecting water quality (Nyholm and Peterson, 1997).

Although algae were used as early as 1910 in toxicity tests (Allen and Nelson, 1910), it was not until the 1960s that a standardized assay with freshwater algae was developed (e.g., Skulberg, 1964). The method introduced by Skulberg (1964) was further developed into a comprehensive protocol by the US EPA (1971). The Algal Assay Procedure Bottle Test (AAP test) was originally developed for the determination of the algal growth potential in natural waters and effluents. Algal toxicity test methods were later developed based on the AAP test method (for a historical overview, see Nyholm and Peterson, 1997; Janssen and Heijerick, 2003).

The usefulness of the results from early algal toxicity test studies has been the subject of considerable controversy. Kenaga and Moolenaar (1979) even concluded that algal toxicity tests were insensitive and proposed the use of fish and *Daphnia* toxicity tests as surrogates for algal bioassays. This has been reflected in the fact that several authorities (e.g., USA) do not take algal toxicity data into account for the derivation of WQS.

Although most standard algal bioassays that have been used for regulatory purposes are similar in design, subtle differences in all aspects of the design may have contributed to the large variability in test results obtained for individual species. Potential uses and limitations of the different algal toxicity test procedures have been discussed by several

authors (e.g., Hörnström, 1990; Lewis, 1995). Janssen and Heijerick (2003) specifically discussed the applicability of standard algal bioassays for toxicity testing of metals. Some of their recommendations were given special attention in the recently reviewed algal growth inhibition test method of the OECD (2006).

Most standard methods for algal toxicity testing that are currently used for regulatory purposes (e.g., US EPA, 2002b; OECD, 2006) represent tests in which the adverse effects of a toxicant on a rapidly growing algal population is investigated in a nutrient-enriched test medium during an exposure period of 3 to 4 days. Usually, biomass or cell density is monitored during the test, although other relevant measurements may be made (e.g., optical density), as long as they can be related to biomass or cell density. The results of algal growth inhibition tests allow the calculation of growth rate-based EC50s, NOECs and LOECs. Further details on the procedures followed in the present study are given in the materials and methods section of chapter 5 (*P. subcapitata*, i.e. the most commonly used standard test alga) and chapter 7 (field-collected green microalgae, i.e. non-standard test organisms).

1.6. Conceptual framework of the study

In the European Union as well as in several countries all over the world, policy makers have provided or are interested to provide the possibility to take into account bioavailability in risk assessment exercises as well as in the development of environmental quality standards for metals. However, to do so, tools have to be developed that are capable of predicting metal bioavailability and toxicity as a function of the physicochemical composition of the environmental compartment under consideration.

At the time this study was started, the effect of water chemistry on the toxicity of nickel to freshwater organisms had insufficiently been studied (see section 1.2). Moreover, close investigation of preliminary BLM-type bioavailability models developed by Wu et al. (2003) and Keithly et al. (2004) revealed serious drawbacks which would inevitably result in inaccurate predictions (see section 1.3). Consequently, further research was considered indispensable for the development of bioavailability models for regulatory purposes.

In a first part of this study, the effect of water chemistry on nickel bioavailability and toxicity has therefore been thoroughly investigated using standard test species belonging to

three different trophic levels (i.e. the unicellular green alga *P. subcapitata*, the aquatic invertebrate *D. magna* and the fish *O. mykiss*). Based on the results of these toxicity studies, a nickel bioavailability model was developed for each of these test species. Since chronic (or long-term) data are recommended for regulatory purposes, the main focus of this study was on the development of chronic bioavailability models. For *D. magna*, both an acute and a chronic model were developed.

Chapter 2 describes the development of a nickel bioavailability model for predicting long-term nickel toxicity to rainbow trout (*O. mykiss*). The model was developed based on the results of a univariate test design which was used for investigating how calcium, magnesium and pH affect nickel toxicity individually. The toxicity mitigating effects of calcium and magnesium were studied individually since magnesium was expected to play a central role in the mechanism of nickel toxicity. During the first year of this study already, this was supported by findings of Pane et al. (2003b) for *D. magna*.

The modeling approach presented in **chapter 2** allows accounting for nonlinear effects (e.g., pH effect) next to BLM-type linear effects that are generally assumed to represent single-site competition (e.g., effects of calcium and magnesium). Finally, it was evaluated whether the model was capable of accurately predicting (i) long-term nickel toxicity to rainbow trout in surface waters with varying physicochemical composition, and (ii) acute nickel toxicity to fathead minnow as reported in literature (provided that the model was calibrated to account for sensitivity differences between species, life stages and/or exposure durations).

In **chapter 3**, a new acute nickel bioavailability model was developed for *D. magna*. Therefore, the individual effects of calcium, magnesium, sodium and pH were studied using a univariate experimental design. The applicability of the model to surface waters was evaluated using the results of a series of tests in nickel-spiked surface waters with varying physicochemical composition. It was also evaluated whether the model was able to accurately predict 48-h EC50s reported in literature for *D. magna* as well as for *C. dubia*. Finally, the new model was compared with the previously proposed models of Wu et al. (2003) and Keithly et al. (2004).

Chapter 4 describes the development of a chronic nickel bioavailability model for *D. magna*. Here also, the individual effects of calcium, magnesium and pH were studied using a univariate test design. The same approach as proposed in chapter 2 was followed for model development. The model was validated using the results from tests in nickel-spiked field waters. These waters also allowed the evaluation of the importance of DOC as nickel toxicity mitigating factor at the typically low nickel concentrations at which chronic effects are observed in *D. magna*.

The fourth and last bioavailability model that was developed during the first part of this study was the chronic nickel bioavailability model for the unicellular green alga *P. subcapitata*, which is described in **chapter 5**. The model was developed based on the results of a similar experimental design as in the former chapters. Next to individual effects, possible interactive effects of magnesium and pH (i.e. the two most important factors affecting nickel toxicity to algae) were studied using a bivariate test design. The model was first developed following the original BLM approach. In a second phase, the modeling approach proposed in chapter 2 and adopted in chapter 4 was followed to increase the model's accuracy in both artificial and natural waters.

Although the bioavailability models described in chapters 2 to 5 can readily be used for regulatory purposes, their use is theoretically limited to aquatic systems of which the physicochemical composition falls within the water chemistry ranges for which the models were developed and validated. For instance, the models developed in this study for fish, crustaceans and algae have a lower water hardness boundary of 22, 42 and 20 mg $CaCO_3$/L, respectively. Since surface waters in large geographic areas (e.g., Scandinavia, Scotland, Northern Portugal) are characterized by lower water hardness levels (Salminen et al., 2005), it was considered necessary to investigate the models' accuracy in these waters before actually using them for regulatory purposes in these areas.

The two most important research questions that were addressed to investigate this issue were (i) whether organisms living in 'soft' water (operationally defined water hardness < 10 mg $CaCO_3$/L) are intrinsically more sensitive to nickel than those living in 'hard' water (operationally defined water hardness > 25 mg $CaCO_3$/L), and (ii) whether a single bioavailability model can be used to predict the protective effect of water hardness on the toxicity of nickel to organisms living in both soft and hard water. These research questions

were addressed in **chapter 6 and 7**, using cladocerans and algae that were collected in soft and hard water lakes in Sweden. These two chapters form the second part of this study.

Finally, **chapter 8** presents an overview of general conclusions and future research needs that have been identified during this study. The application of the developed models in risk assessment exercises and water quality standard development is also shortly discussed in this final chapter.

Chapter 2

Development of a bioavailability model for predicting nickel toxicity to fish

Redrafted from

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Chapter 2

Development of a bioavailability model for predicting nickel toxicity to fish

Abstract. The effects of calcium, magnesium and pH on the toxicity of nickel to juvenile rainbow trout (*Oncorhynchus mykiss*) were examined during 17- to 26-day exposures to nickel in 15 synthetic test solutions. Higher chemical activities of Ca^{2+}, Mg^{2+} and H^+ reduced nickel toxicity, as demonstrated by increased 17-day median lethal concentrations expressed as Ni^{2+} activity (17-d $LC50_{Ni2+}$). A nonlinear increase of the 17-d $LC50_{Ni2+}$ with increasing H^+ suggested that the effect of pH could not be appropriately described by single-site competition between Ni^{2+} and H^+ for sensitive sites on the fish gill. Instead, a linear increase of pNi^{2+} (= – log 17-d $LC50_{Ni2+}$) with increasing pH was observed with a slope of 0.32. This slope was used as the basis for modeling the effect of pH. The effects of calcium and magnesium were modeled according to single-site competition with log K_{CaBL} = log K_{MgBL} = 3.6, both assumed to be independent of pH. The effect of pH was superimposed on this competition effect and was also assumed to be independent of calcium and magnesium concentrations. The model was able to predict 17-d LC50s (expressed as dissolved nickel) in most synthetic test waters within a factor 2 deviation from observed toxicity. The model's predictive capacity was also evaluated using results of similar laboratory toxicity tests with juvenile rainbow trout in nickel-spiked European surface waters. For most of these waters, predicted 17-d LC50s did not deviate more than a factor 2 from observed toxicity. The same model, calibrated to account for sensitivity differences between species, life stages and/or exposure durations, was able to accurately predict 96-h LC50s for larval, juvenile and subadult fathead minnow (*Pimephales promelas*) previously reported in literature. Although the developed model seems very promising, the uncertainty around the role of alkalinity and the exact mechanisms by which calcium, magnesium and pH modify nickel toxicity need to be further explored.

2.1. Introduction

Recently, both water quality criteria and ecological risk assessment of metals in the aquatic environment have evolved from being based on total or dissolved metal concentrations to taking metal bioavailability into account. A breakthrough in this direction was the incorporation of the protective effect of water hardness against toxicity of several metals in water quality criteria setting (Alabaster and Lloyd, 1980; US EPA, 1986). More

recently however, the importance of accounting for the modifying effects of other physicochemical parameters on metal toxicity, such as pH, alkalinity, calcium, magnesium, sodium and dissolved organic carbon (DOC), is increasingly being recognized by several organizations (for a review, see Paquin et al., 2002 and Niyogi and Wood, 2004). In this context, bioavailability models such as the biotic ligand model (BLM) have been developed to predict acute and chronic (or long-term) metal toxicity to fish, invertebrates and algae (Santore et al., 2001, 2002; De Schamphelaere and Janssen, 2002, 2004a,b; De Schamphelaere et al., 2002; Heijerick et al., 2002a,b, 2005a; Hoang et al., 2004; Keithly et al., 2004). Such models are currently being used in the EU risk assessment on zinc and zinc compounds (ECB, 2003; Bodar et al., 2005; Van Assche, 2006) and for the derivation of site-specific water quality criteria for copper in the USA (US EPA, 2007b).

Although nickel is an important aquatic contaminant, knowledge on its bioavailability is relatively limited compared to copper or zinc. Consequently, the currently available nickel BLMs are far less advanced (for an overview, see Niyogi and Wood, 2004). Nickel toxicity modifying parameters have been reported to include pH, DOC, calcium, water hardness and alkalinity (Schubauer-Berigan et al., 1993; Meyer et al., 1999; Pyle et al., 2002a; Hoang et al., 2004; Keithly et al., 2004). Provisional acute nickel BLMs used by Keithly et al. (2004) and Hoang et al. (2004) were not capable of accurately predicting nickel toxicity to *Ceriodaphnia dubia* and *Pimephales promelas*, respectively, over a broad range of water chemistry. These authors mainly attributed this to an incomplete understanding of the effects of pH and/or alkalinity on nickel toxicity. Indeed, contradictory observations concerning the effect of pH on nickel toxicity are reported in literature. Using *P. promelas* as test species, both Schubauer-Berigan et al. (1993) and Hoang et al. (2004) demonstrated nickel toxicity to increase with increasing pH, while Pyle et al. (2002a) observed a decrease of nickel toxicity to the same species above pH 7.0. The protective effects of calcium (Meyer et al., 1999) or water hardness (Pyle et al., 2002a; Hoang et al., 2004; Keithly et al., 2004) seem to be more consistent across studies.

It is important to note that all studies described above are acute toxicity studies using mortality as endpoint. In the context of the EU risk assessment on nickel and nickel compounds and the EU Water Framework Directive (EC, 2000), data are needed on the bioavailability and long-term toxicity of nickel to fish and other species. In this study, we examined the individual effects of calcium, magnesium and pH on long-term (17-day)

toxicity of nickel to juvenile rainbow trout (*Oncorhynchus mykiss*), using both mortality and growth as toxicological endpoints. The ultimate aim of the present study was to develop a bioavailability model that could accurately predict long-term nickel toxicity not only in assays using synthetic test media, but also in natural waters. It was expected that the newly generated data as well as the developed model could help to explain observations on nickel toxicity to fish reported in literature.

2.2. Materials and methods

2.2.1. Toxicity tests in synthetic test solutions

To investigate the individual effects of calcium, magnesium and pH on the long-term toxicity of nickel to juvenile rainbow trout, three sets of toxicity assays were performed in each of which the concentration of one cation was varied in a univariate manner. Each set consisted of five assays and in each assay, a control treatment and five nickel concentrations were tested. Tests belonging to the same test series were conducted simultaneously. In order to allow comparison of the sensitivity of fish across test series, each test series contained one assay in a test solution with similar pH and similar, low concentrations of calcium and magnesium. This test solution was termed the 'basic' test medium. The calcium test series contained two tests in basic medium (Table 2.1).

2.2.2. Preparation of synthetic test solutions

All test solutions were prepared using carbon filtered, deionized water (conductivity < 2 µS/cm) and reagent grade chemicals purchased from VWR International (Leuven, Belgium). The synthetic basic medium was composed of $CaCl_2$ (0.12 mM), $MgCl_2$ (0.06 mM), $MgSO_4$ (0.06 mM), KCl (0.078 mM), $NaHCO_3$ (0.595 mM), $FeCl_3$ (0.3 µM), H_3BO_3 (3 µM), $MnCl_2$ (2.1 µM), $ZnCl_2$ (22 nM), Na_2MoO_4 (29 nM), $CoCl_2$ (6 nM) and Cu (background ≈ 10 nM). Instead of EDTA, Aldrich Humic Acid (AHA, 50 µg DOC/L final concentration) was added to keep iron in solution (Heijerick et al., 2002a).

Table 2.1. Main physicochemical characteristics of synthetic test media. Numbers between brackets indicate the standard error.

Test medium [a]	T (°C)	pH	Ca (mg/L)	Mg (mg/L)	Hardness [b] (mg $CaCO_3$/L)	Na [c] (mg/L)	Cl [c] (mg/L)	Alkalinity [d] (mg $CaCO_3$/L)
pH 5.8	13.9 (0.21)	5.48 (0.02)	4.59 (0.10)	2.96 (0.01)	23.7	82.3	140	0.0462
pH 6.4	13.9 (0.22)	6.76 (0.01)	4.42 (0.02)	2.95 (0.01)	23.2	82.3	139	1.01
pH 7.0	14.4 (0.23)	7.19 (0.01)	4.59 (0.11)	2.94 (0.01)	23.6	82.3	136	5.68
pH 7.6	14.7 (0.15)	7.67 (0.01)	4.48 (0.03)	2.97 (0.01)	23.4	82.3	120	26.9
pH 8.2	14.7 (0.20)	8.47 (0.01)	4.49 (0.02)	2.98 (0.01)	23.5	82.3	13.6	180
Mg 0.12 mM	13.5 (0.08)	7.53 (0.03)	3.86 (0.09)	3.04 (0.02)	22.2	13.7	13.6	28.0
Mg 0.5 mM	13.5 (0.14)	7.53 (0.03)	3.83 (0.05)	11.4 (0.07)	56.4	13.7	41.2	28.0
Mg 1.0 mM	13.9 (0.12)	7.58 (0.04)	4.54 (0.25)	22.4 (0.21)	104	13.7	76.3	28.2
Mg 2.0 mM	14.4 (0.06)	7.55 (0.04)	3.80 (0.08)	47.6 (0.56)	206	13.7	148	28.0
Mg 3.0 mM	14.3 (0.05)	7.54 (0.03)	3.64 (0.05)	72.0 (1.02)	305	13.7	218	28.0
Ca 0.12 mM (1)	13.7 (0.12)	7.59 (0.01)	4.20 (0.12)	2.85 (0.04)	22.2	13.7	13.6	28.2
Ca 0.12 mM (2)	14.1 (0.17)	7.52 (0.01)	4.30 (0.10)	2.94 (0.04)	22.8	13.7	13.6	27.9
Ca 0.5 mM	13.5 (0.18)	7.63 (0.01)	17.0 (0.10)	2.92 (0.04)	54.4	13.7	41.1	28.3
Ca 1.0 mM	14.6 (0.12)	7.62 (0.01)	40.1 (0.07)	2.90 (0.02)	112	13.7	76.5	28.3
Ca 3.0 mM	14.1 (0.19)	7.52 (0.02)	110 (1.91)	2.94 (0.01)	286	13.7	218	27.9

[a] In the calcium test series, the test in basic medium was conducted in duplicate.

[b] Hardness was calculated from measured calcium and magnesium concentrations.

[c] No measurements were conducted for Na, K, SO_4 and Cl, hence nominal values were used for calculations. In all tests, nominal K and SO_4 concentrations were 3.1 and 5.9 mg/L, respectively.

[d] Alkalinity was calculated from nominal added inorganic carbon (IC) and measured pH, using thermodynamic stability constants taken from Stumm and Morgan (1996).

Calcium and magnesium concentrations were adjusted by adding $CaCl_2$ or $MgCl_2$. All test solutions were in equilibrium with the surrounding air. Therefore they are termed 'ambient' test solutions throughout this chapter. The pH of ambient test solutions was 7.56 ± 0.04 (mean ± standard deviation, SD) in the calcium and the magnesium test series. In the pH test series, different pH levels were obtained by adding different amounts of $NaHCO_3$. Although sodium was not expected to have an important influence on nickel toxicity, sodium was set to the same concentration through addition of NaCl in all test solutions of the pH test series. In the test conducted at pH 5.8, 0.75 g/L MOPS (3-N-morpholinopropanesulfonic acid) was added to maintain this pH throughout the test. MOPS was observed not to affect metal speciation (Kandegedara and Rorabacher, 1999). Additionally, De Schamphelaere et al. (2004b) demonstrated that MOPS was not toxic and did not affect copper and zinc toxicity to freshwater species.

For each bioassay, a nickel concentration series was prepared by adding $NiCl_2$. Each bioassay consisted of a control treatment and five nickel concentrations somewhere within the range of 50 to 4800 µg/L. All solutions were allowed to equilibrate for 24 hours at 14.5 °C prior to being used in the toxicity tests. The physicochemical composition of all synthetic test solutions is given in Table 2.1.

2.2.3. Toxicity tests in European surface waters

Surface waters with differing physicochemical characteristics (Table 2.2) were sampled at five locations in Europe, which were selected based on a data compilation containing physicochemical properties and metal concentrations of European surface waters that are relatively undisturbed by anthropogenic inputs. The variation in these five waters with regard to the main factors affecting nickel bioavailability (calcium, magnesium, water hardness, DOC, pH, alkalinity) reasonably covered the physicochemical variation in European surface waters. Samples were taken in two countries (Belgium and The Netherlands) between March and October 2004, and the samples were given the following labels: Ankeveen, Bihain, Brisy, Eppe and Markermeer (Table 2.2). A short description of the site type: Ankeveen – ditch in lake system in lowland peat (NL), Bihain – small creek in highland peat (B), Brisy – river in mixed forest (B), Eppe – small stream near protected forested area 'Le Val Joly' (B), Markermeer – part of large lake cut off from the North Sea by a dam (NL).

Table 2.2. Main physicochemical characteristics of sampled surface waters.

Site	pH	Ca (mg/L)	Mg (mg/L)	Hardness [a] (mg $CaCO_3$/L)	Na (mg/L)	K (mg/L)	Cl (mg/L)	SO_4 (mg/L)	Alkalinity [b] (mg $CaCO_3$/L)	DOC (mg C/L)	Ni_{diss} [c] (µg/L)
	7.55	83.0	13.7	263	15.3	2.65	29.1	81.1	36.3	18.4	4.1
Ankeveen											
Bihain	5.63	3.75	1.12	14.0	4.48	0.880	9.41	4.73	1.16	6.32	2.6
Brisy	7.39	7.53	4.54	37.5	6.80	2.29	16.2	8.65	23.4	3.83	1.7
Eppe	8.05	28.1	6.90	98.6	8.99	4.52	15.5	18.5	91.0	4.87	1.2
Markermeer	8.19	54.7	16.6	205	70.4	8.53	63.4	100	121	4.50	2.2

[a] Hardness was calculated from measured calcium and magnesium concentrations.

[b] Alkalinity was calculated from measured inorganic carbon (IC) concentrations and pH, using thermodynamic stability constants taken from Stumm and Morgan (1996).

[c] Background dissolved nickel concentration. Iron, aluminium, copper and zinc concentrations were 56-262 µg/L, 20-120 µg/L, < 2.0 µg/L and 2.1-11 µg/L, respectively.

At each site, 1500 L of water was membrane filtered (0.45 µm) and collected in 60-L metal-free polyethylene vessels. The samples were immediately transferred to the laboratory, where they were stored at 15 °C in total darkness. Before testing, the following parameters were measured: pH (pH-meter P407, Consort, Turnhout, Belgium), DOC, inorganic carbon (IC) (TOC-5000, Shimadzu, Duisburg, Germany), Ca, Mg, Na, K, Fe, Al, Mn, Ni, Cu, Zn, Pb and Cd (ICP-OES, Perkin Elmer 3300 DV) and Cl, NO_3 and SO_4 (Ion Chromatography, Dionex QIC analyser, IONPAC AS4A).

With each of the five sampled waters, a long-term toxicity test was performed under the same conditions as described for the tests with synthetic test solutions (see further). To obtain a nickel concentration series (control treatment + five nickel concentrations), the waters were spiked with $NiCl_2$ and allowed to equilibrate for 24 hours prior to testing. MOPS buffer (0.75 g/L) was added to the Bihain surface water to maintain its low pH throughout the test.

2.2.4. Experimental animals

Juvenile (28 to 35 days post hatch, swim-up fry) rainbow trout (*O. mykiss*) were obtained from Houghton Springs Fish Farm (UK). Characteristics of the culture medium at the fish farm are as follows: pH ~ 6.5, hardness ~ 240 mg $CaCO_3$/L and dissolved nickel < 3 µg/L, which is the method detection limit (MDL) of the graphite furnace atomic absorption spectrophotometer used in this study (GF-AAS, SpectrAA800 with Zeeman background correction, Varian, Mulgrave, Australia). Upon arrival in our laboratory the fish were held in 200-L aquaria (at a density of about 400 fish per aquarium) for four days in an aerated 1:1 mixture of deionized water and carbon filtered city tap water (Gent, Belgium). Temperature was maintained between 14 and 16 °C. Water hardness was 180 mg $CaCO_3$/L, pH 7.8 and dissolved nickel < 3 µg/L (MDL).

NH_4^+, NO_2^- and NO_3^- (Spectroquant, Merck, Darmstadt, Germany) and oxygen saturation (immersion electrode) were monitored daily and were always below 0.1, 0.1 and 5.0 mg/L and higher than 90%, respectively. The juveniles were fed ad libitum once a day with a commercial food for juvenile salmonids (TroCo Crumble HE EX, Coppens International, Helmond, The Netherlands) with the following composition: 56% crude protein, 15% crude fat, 11.5% nitrogen-free extract (mostly digestible carbohydrates), 0.5%

crude fibre and 9% ash. Every day, faeces and detritus were removed manually from the bottom of the aquaria. A light cycle of 12 hours light and 12 hours dark was maintained. Gradual changes from light to dark and vice versa were spread over half an hour in the morning as well as in the evening.

2.2.5. Long-term testing

Before testing, fish were acclimated for one week to the control test solution. This was done under exactly the same conditions as during toxicity testing. During acclimation, mortality was less than 5% in all cases. The fish were subsequently exposed in a flow-through system to five different nickel concentrations and a control for 17 to 26 days. For each nickel concentration, 20 fish were exposed in a 15-L polyethylene aquarium. At all times, water temperature was within 1 °C of the target temperature of 14.5 °C. Aquaria were vigorously aerated (O_2 concentration > 90% of saturation) and a light cycle of 12 hours light and 12 hours dark was maintained, with gradual changes (spread over half an hour in the morning as well as in the evening) from dark to light and vice versa.

During the first two weeks of the exposure to nickel, fish were fed daily at 4% of the average wet body weight measured at test initiation. For the remainder of the test, the feeding rate was increased to 6%. The same commercial fish food as described above was used. The range of average wet body weight at test initiation was 207 to 354 mg. A medium renewal rate of 8 L per aquarium per day (i.e. about 1.1 to 1.9 L per g fish per day at test initiation) was maintained using peristaltic pumps (Smith & Nephew Watson-Marlow, Falmouth, Cornwall, United Kingdom). Uneaten food and faeces were siphoned from the bottom of the aquaria every other day. All experiments were conducted in accordance with national and institutional guidelines for the protection of animal welfare.

2.2.6. Endpoints

Mortality was recorded daily and dead fish were removed as soon as possible. Effects on growth rate were investigated by measuring wet weight at test initiation and termination. At the end of the 1-week acclimation period, a random sample of 10 fish was taken from each test solution for determination of the average initial weight. At the end of exposure, surviving fish were flash-killed in a 0.1% 2-phenoxy-ethanol solution (Sigma-Aldrich, Steinheim,

Germany) and weighed individually. Weights were recorded to the nearest 0.1 mg. These data were used to calculate pseudo-specific growth rates (see further).

2.2.7. Chemical analyses

Water temperature and oxygen saturation were measured daily and pH was measured twice a week using an immersion electrode. NH_4^+, NO_2^- and NO_3^- were monitored at least once a week using spectrophotometric test kits (Spectroquant, Merck, Darmstadt, Germany) and were always below 0.1, 0.1 and 5.0 mg/L, respectively. Total calcium and magnesium concentrations were measured once (pH test series and tests in natural waters) or twice a week (calcium and magnesium test series). Dissolved nickel concentrations were measured twice a week in each aquarium after filtration through a 0.45 µm filter (Gelman Sciences, Ann Arbor, MI, USA). Samples for calcium, magnesium and nickel were acidified with 0.14 N HNO_3.

Calcium and magnesium were measured using flame atomic absorption spectrometry (F-AAS, SpectrAA100, Varian, Mulgrave, Australia). Nickel was measured using F-AAS at nickel concentrations above 100 µg/L, and graphite furnace AAS (GF-AAS, SpectrAA800 with Zeeman background correction, Varian, Mulgrave, Australia) at nickel concentrations below 100 µg/L. Calibration standards (Sigma-Aldrich, Steinheim, Germany) and a reagent blank were analyzed with every ten samples. The MDL of the GF-AAS was approximately 3 µg/L throughout the study. Two certified reference samples, TMDA-62 and TM-25.2 (National Water Research Institute, Burlington, ON, Canada) with certified nickel concentrations (mean ± 95% confidence interval) of 97.7 ± 8.5 µg/L and 10.0 ± 1.7 µg/L, respectively, were analyzed at the beginning and at the end of each series of nickel measurements. Measured nickel concentrations were always within 10% of the certified value.

Means of all nickel, calcium and magnesium measurements made during a test were used for data analysis and model development. For Na, K, Cl, SO_4 and IC, nominal concentrations were used. Previous studies at our laboratory indicated that measured values of these parameters typically deviate less than 10% from nominal values. Alkalinity was calculated from nominal IC concentrations and pH using thermodynamic stability constants from Stumm and Morgan (1996). Dissolved organic carbon concentrations were not measured in the synthetic test solutions because measurements during previous fish experiments in our

laboratory demonstrated that DOC concentrations remained below 1 mg/L (De Schamphelaere and Janssen, 2004a) and because we maintained a slightly higher renewal rate in our experiments compared to these earlier experiments. Such low DOC concentrations have no substantial effect on nickel speciation (Wu et al., 2003). The same types of analyses were conducted during the tests in nickel-spiked surface waters. Additionally, for the waters sampled in Ankeveen, Brisy and Eppe, DOC and IC concentrations (TOC-5000, Shimadzu, Duisburg, Germany) were measured weekly in each aquarium.

2.2.8. Data treatment and statistics

NOECs (no observed effect concentrations) based on mortality ($NOEC_m$) were calculated using Fisher's Exact Test (Finney, 1948; Pearson and Hartley, 1962) ($p < 0.05$). 17-d LC50s (median lethal concentrations) and their 95% confidence intervals were calculated using the trimmed Spearman-Karber method (Hamilton, 1977). Observed mortalities at each measured nickel concentration were used as input for the calculations.

Pseudo-specific growth rates (r_3, d^{-1}) were calculated for each surviving fish at test termination according to the following equation (OECD, 1996):

$$r_3 = \frac{\ln W_2 - \overline{\ln W_1}}{t_2 - t_1} \qquad \text{Eq. 2.1}$$

where W_1, W_2 = weight of fish at t_1 (0 days) and t_2 (x days at end of test), respectively; ln W_2 = natural logarithm of the weight of an individual fish at t_2; $\overline{\ln W_1}$ = average of the natural logarithms of W_1 of 10 randomly selected fish weighed at t_1. Growth rates in each exposure concentration were statistically compared to control growth rates using the Mann Whitney U test ($p < 0.05$).

2.2.9. Equilibrium chemical speciation calculations and LC50 predictions

Chemical speciation of nickel and other ions was calculated using WHAM VI software (Tipping, 1998; Natural Environment Research Council, 2001). Stability constants for inorganic complexes were taken from Smith et al. (2004) and used to replace the constants

in the default thermodynamic database of the software. For MOPS, a pK_a of 7.2 was added to the database (Kandegedara and Rorabacher, 1999). Speciation calculations were performed for each individual exposure concentration of nickel as well as for all 17-d LC50s. For the synthetic test solutions, the input variables were the average pH and calcium and magnesium concentrations measured during the tests, nominal concentrations of Na, K, SO_4, Cl, IC and MOPS, and the measured dissolved nickel concentration (or the LC50 based on dissolved nickel). Since background DOC concentrations, which were assumed to be < 1 mg/L (see above), were calculated not to have a substantial effect on nickel speciation, fulvic acid (FA) and humic acid concentrations were set to zero.

For the natural waters, similar inputs were used, except that measured IC was used when available, organic complexation was taken into account and dissolved trace metal concentrations of zinc, aluminium and iron were also considered. Fe^{3+} and Al^{3+} were allowed to form colloidal hydroxide precipitates (i.e. $Fe(OH)_3$ and $Al(OH)_3$) when their solubility product was exceeded (methodology and solubility products are given by Cheng et al., 2005). Binding to DOC was modeled by assuming a fraction to behave as FA with the remainder being inert for ion binding (Cheng et al., 2005). The former fraction is termed the 'active fulvic acid' (AFA) fraction.

Binding to DOC was modeled using an AFA fraction of 0.4 and a binding constant representing the binding of nickel to FA (log K_{NiFA}) of 1.75. These values were taken from a WHAM VI calibration exercise with nickel-spiked surface waters from the same sources as the ones used in the present study (Van Laer et al., 2006). This calibration exercise was performed since the original WHAM VI model was only calibrated using data on organic nickel complexation in soil and ground water. The accuracy of WHAM VI in predicting nickel complexation to organic matter substantially increased using the abovementioned model parameters. Since the AFA fraction was assumed to be 0.4, the concentration of FA (mg/L) for input into the model software equals 0.8 x DOC (mg C/L) (Cheng et al., 2005).

The model software allows inputs of nickel as total or dissolved nickel concentration, but also as Ni^{2+} activity. The first option was used to calculate nickel speciation (e.g., Ni^{2+} activity) from dissolved nickel concentrations. The second option was used when $LC50_{Nidiss}$ (expressed as dissolved nickel concentration) had to be calculated from $LC50_{Ni2+}$ (expressed as Ni^{2+} activity), predicted with Equation 2.3 (see further).

2.3. Results

2.3.1. Effect of nickel on growth

Growth rate data are reported in Table 2.3. According to OECD test guideline 215 (OECD, 1996), one should only investigate the effect of a toxicant on juvenile fish growth at test concentrations that do not affect mortality by more than 10%. This is because higher mortality reduces stocking density of the fish, which may in turn enhance territorial behaviour and eventually affect growth rate. Alternatively, we calculated growth rates for all fish exposed to nickel concentrations ≤ $NOEC_m$, i.e. exposure concentrations not significantly affecting fish mortality. As indicated in Table 2.3, a statistically significant growth rate reduction was only observed after 17-d exposure to 170 µg/L in the test 'Ca 0.12 mM (1)'.

2.3.2. Effect of nickel on mortality

Only the mortality data of up to day 17 of the exposure will be discussed in this chapter, since 17 days was the maximum exposure duration for which data from all test solutions were available for model development. An analysis of longer-term mortality data resulted in similar observations, interpretations and conclusions (not discussed in this chapter). 17-d LC50s, expressed as dissolved nickel concentration and Ni^{2+} activity, are reported in Table 2.3. 17-d $LC50_{Nidiss}$ were similar in all basic test solutions (Ca 0.12 mM (1), Ca 0.12 mM (2) and Mg 0.12 mM), i.e. between 496 and 673 µg/L. This indicates that the sensitivity of juvenile fish to nickel did not substantially vary between different test series.

2.3.2.1. Effects of calcium and magnesium on nickel toxicity

Calcium clearly protects rainbow trout against nickel toxicity, as demonstrated by the 4-fold increase of 17-d $LC50_{Nidiss}$ (496 to 1950 µg/L) between 0.12 and 1.0 mM calcium (Table 2.3). A further increase of calcium concentration to 3.0 mM did not result in a further reduction of toxicity. At the highest exposure concentrations investigated at 0.5, 1.0, 2.0 and 3.0 mM magnesium, 17-d mortality was lower than 50%. For the tests conducted at 0.5, 1.0 and 3.0 mM magnesium, no 17-d $LC50_{Nidiss}$ could be derived, while for the test conducted at 2.0 mM magnesium, an extrapolated 17-d $LC50_{Nidiss}$ could be calculated using the logistic model described by De Schamphelaere and Janssen (2004a).

Table 2.3. Effect data for all test solutions: 17-d LC50s expressed as dissolved nickel concentration (17-d $LC50_{Nidiss}$) and their 95% confidence intervals (95% CI), 17-d LC50s expressed as Ni^{2+} activity (17-d $LC50_{Ni2+}$) and -log (Ni^{2+} activity) (17-d $LC50_{pNi2+}$), full-test NOECs for the endpoint mortality ($NOEC_m$), and average growth rates ± SE (standard error) of fish in the control treatment and fish exposed at the $NOEC_m$. Growth rates significantly different from control growth rates are printed in bold.

Test solution	17-d $LC50_{Nidiss}$ (µg/L)	95% CI (µg/L)	17-d $LC50_{Ni2+}$ (µM)	17-d $LC50_{pNi2+}$	Full-test $NOEC_m$ (µg/L)	Control growth rate ± SE (d^{-1})	Growth rate at $NOEC_m$ ± SE (d^{-1})
pH 5.8	2440	2180-2730	31.6	4.50	1390	0.027 ± 0.005	0.027 ± 0.005
pH 6.4	1020	939-1110	13.1	4.88	352	0.029 ± 0.004	0.026 ± 0.005
pH 7.0	781	713-855	9.91	5.00	173	0.030 ± 0.004	0.031 ± 0.005
pH 7.6	614	570-662	7.43	5.13	350	0.030 ± 0.005	0.026 ± 0.003
pH 8.2	558	500-622	3.19	5.50	225	0.029 ± 0.003	0.030 ± 0.002
Mg 0.12 mM	673	485-935	9.36	5.03	140	0.010 ± 0.004	0.006 ± 0.002
Mg 0.5 mM	> 796 [a]		> 10.3	< 4.99	431	0.014 ± 0.005	0.009 ± 0.005
Mg 1.0 mM	> 1480 [a]		> 18.2	< 4.74	– [c]	0.012 ± 0.003	0.006 ± 0.004 [c]
Mg 2.0 mM	2910 [b]	1370-6180	33.3	4.48	788	0.013 ± 0.002	0.026 ± 0.005
Mg 3.0 mM	> 1620 [a]		> 17.7	< 4.75	– [c]	0.015 ± 0.010	0.001 ± 0.003 [c]
Ca 0.12 mM (1)	496	438-562	6.68	5.18	170	0.025 ± 0.004	**0.012 ± 0.004**
Ca 0.12 mM (2)	519	466-577	7.01	5.16	172	0.039 ± 0.006	0.040 ± 0.006
Ca 0.5 mM	662	566-775	8.53	5.07	182	0.023 ± 0.006	0.017 ± 0.003
Ca 1.0 mM	1950	1500-2550	23.8	4.62	340	0.030 ± 0.006	0.021 ± 0.006
Ca 3.0 mM	1990	1870-2120	21.9	4.66	1420	0.028 ± 0.006	0.027 ± 0.007
Ankeveen	3200	2760-3710	27.2	4.57	1770	0.041 ± 0.003	0.041 ± 0.005
Bihain	640	566-723	7.68	5.11	265	0.026 ± 0.008	0.029 ± 0.008
Brisy	1010	875-1160	11.4	4.94	582	0.033 ± 0.004	0.020 ± 0.006
Eppe	4140 [b]	1140-15100	37.8	4.42	576	0.030 ± 0.005	0.029 ± 0.004
Markermeer	1890	1720-2070	13.1	4.88	560	0.035 ± 0.007	0.032 ± 0.008

[a] Calculation of LC50 not possible due to no or limited mortality at the highest exposure concentration.

[b] Extrapolated LC50 calculated using the log-logistic model described by De Schamphelaere and Janssen (2004a). Parameter estimation and calculation of the 95% CI was carried out using the Levenberg-Marquardt algorithm (Levenberg, 1944; Marquardt, 1963).

[c] No significant mortality observed. Reported growth rates are for the highest exposure concentration.

Nevertheless, the protective effect of magnesium is obvious. First, the 17-d $LC50_{Nidiss}$ at 2.0 mM magnesium is about 4-fold higher than that obtained in the basic test solution at 0.12 mM magnesium (2910 versus 673 µg/L). Second, at concentrations of 1.0 and 3.0 mM magnesium, less than 50% mortality was observed at nickel concentrations higher than the 17-d $LC50_{Nidiss}$ in basic test medium (673 µg/L). The nature of the magnesium data makes it difficult to compare the effects of calcium and magnesium quantitatively. However, based on the approximately 4-fold increase of 17-d $LC50_{Nidiss}$ between 0.12 mM calcium and 1.0 or 3.0 mM calcium, and the similar 4-fold increase of 17-d $LC50_{Nidiss}$ between 0.12 mM magnesium and 2.0 mM magnesium, it is clear that magnesium protects fish at least equally well against nickel toxicity as calcium.

2.3.2.2. Effect of pH on nickel toxicity

A pH increase from 5.5 to 8.5 (measured pH levels, Table 2.1) resulted in a 4.5-fold increase of nickel toxicity, based on the reduction of the 17-d $LC50_{Nidiss}$ from 2440 to 558 µg/L (Table 2.3). This seems to suggest that H^+ protects against nickel toxicity. However, calculation of nickel speciation is indispensable for evaluation of the pH effect (see further).

2.3.2.3. Nickel toxicity in natural waters

The 17-d $LC50_{Nidiss}$ obtained in nickel-spiked surface waters varied between 640 and 3200 µg/L. For 'Eppe' only an extrapolated 17-d $LC50_{Nidiss}$ (4140 µg/L) with a large 95% confidence interval could be derived (Table 2.3).

2.4. Discussion

2.4.1. Effect of nickel on growth rate

Overall, nickel was observed not to affect growth of juvenile rainbow trout in our experiments. This seems to be in contradiction with Nebeker et al. (1985), who observed significant effects of nickel on rainbow trout growth at concentrations below those significantly affecting mortality, more specifically at 35 µg/L for newly fertilized eggs (75-d exposure), 431 µg/L for eyed eggs (52-d exposure) and 700 µg/L for pre-swim-up larvae (38-d exposure). However, their conclusion was based on longer experiments in one single test

water with fish that were in earlier life stages than the juvenile fish we used in our experiments. Since we did not observe significant growth effects at exposure concentrations ≤ $NOEC_m$ in almost all test solutions, further analysis, interpretation and modeling will be performed on the basis of mortality data.

2.4.2. Effect of nickel on mortality

The experiment at pH 7.6 in the pH test series has, compared to the basic test medium, a similar pH and calcium and magnesium concentration but a 6-fold higher sodium concentration (3.6 versus 0.6 mM). The experiment at the high sodium concentration yielded a 17-d $LC50_{Nidiss}$ of 614 µg/L and the tests in basic medium yielded similar 17-d $LC50_{Nidiss}$ (i.e., 496-673 µg/L). This indicates that sodium does not affect nickel toxicity in rainbow trout. This appears to contradict a preliminary set of nickel BLM parameters presented by Wu et al. (2003) (see also the BLM review by Niyogi and Wood, 2004), which suggests that sodium competes with nickel for binding sites on the gill and that sodium protects against nickel toxicity. However, closer investigation of the model proposed by Wu et al. (2003) revealed that their BLM parameters were calibrated to the gill nickel binding dataset of Meyer et al. (1999), which only contained information on the effect of calcium on nickel binding to fathead minnow gills. Hence, only the binding parameters for calcium and nickel are supported by these data. Wu et al. (2003) seem to recognize this by stating that their presented set of model parameters was 'not unique in terms of its predictive capacity'. Our data suggest that the inclusion of a sodium binding constant in the nickel BLM is not appropriate.

2.4.2.1. Effects of calcium and magnesium on nickel toxicity

Different studies have reported the protective effect of water hardness (calcium and magnesium combined) or calcium (individually) on nickel toxicity to fish (Meyer et al., 1999; Pyle et al., 2002a; Hoang et al., 2004). The present study is the first to demonstrate an important protective effect of magnesium. However, as opposed to metals like copper, zinc, silver and lead (see the review of Niyogi and Wood, 2004), nickel has been demonstrated not to act as an ionoregulatory toxicant to fish, but rather as a respiratory toxicant, in acute as well as in chronic exposures (Pane et al., 2003a, 2004a,b; Brix et al., 2004). As discussed by Pane et al. (2004a,b), exposure of rainbow trout to nickel results in damage to the ultra-structure of the respiratory epithelium, which in turn leads to reduced gas exchange capacity, oxygen

uptake and, eventually, mortality. The protective effects of calcium and magnesium may hence result from any effect that these ions have on nickel accumulation on or uptake by the gill.

Both calcium and magnesium have been demonstrated to be involved in the regulation of membrane permeability (Ebel and Günther, 1980; McWilliams, 1983; Hunn, 1985). In this capacity, they may reduce the uptake and toxicity of nickel indirectly. Nickel toxicity may also be reduced through direct competition of calcium and magnesium with nickel for binding to negative sites on the gill surface. In this respect, magnesium might be expected to protect to a somewhat lesser degree than calcium. Indeed, the Irving-Williams order of cation binding to ligands indicates that magnesium would bind less well to negative sites on the gill surface (Schwartz and Playle, 2001). However, our modeling approach (see further) will lead to similar values for log K_{CaBL} and log K_{MgBL}, describing the binding strength of complexes between calcium or magnesium and the biotic ligand (BL).

The higher than expected log K_{MgBL} value may be the result of direct competition between Mg^{2+} and Ni^{2+} for a shared uptake pathway. This is supported by the fact that (i) Ni^{2+} and Mg^{2+} have similar dehydrated ionic radii (0.066 and 0.069 nm, respectively) (Weast, 1973), (ii) Ni^{2+} is a competitive inhibitor of Mg^{2+} uptake via three different types of magnesium transporters in the prokaryote *Salmonella typhimurium* (Snavely et al., 1991), (iii) Ni^{2+} inhibits unidirectional Mg^{2+} uptake in *Daphnia magna* (Pane et al., 2003b), (iv) Mg^{2+} inhibits Ni^{2+} uptake into brush border membrane vesicles isolated from the kidney of rainbow trout (Pane et al., 2006a,b), and (v) nickel-magnesium interactions are also well known from mammalian literature (discussed by Pane et al., 2003b), suggesting that Mg^{2+} transport systems are highly conserved throughout evolution.

Although nickel transporters have not been identified in fish species yet, we postulate that the protective effect of magnesium on nickel toxicity to rainbow trout is partly due to the existence of a shared transport system. If so, the protective effect of increased calcium concentrations may also be the result of the inhibiting effect of calcium on the transcription rate of Ni^{2+}/Mg^{2+} transporters, which has already been demonstrated in *S. typhimurium* (Snavely et al., 1991).

2.4.2.2. Effect of pH on nickel toxicity

Three other studies (Schubauer-Berigan et al., 1993; Pyle et al., 2002a; Hoang et al., 2004) have investigated the effect of pH on nickel toxicity to fish. All of these studies used shorter exposures (96 hours), another fish species (fathead minnow) and a younger life stage (< 24-h old larvae). Schubauer-Berigan et al. (1993) observed nickel toxicity to be higher at pH 8.0-8.5 than at pH 7.0-7.5. Conversely, Pyle et al. (2002a) reported a 4.1-fold decrease of nickel toxicity between pH 7.0 and 8.5. Hoang et al. (2004) observed no effect of pH at low levels of hardness and alkalinity (20 mg $CaCO_3$/L), but a 1.5-fold increase of nickel toxicity over a pH range of 7.3 to 8.5 at higher levels of hardness and alkalinity (100 mg $CaCO_3$/L). It is important to note that in all these studies, the authors claimed to have modified pH univariately while maintaining alkalinity at a relatively constant level, although only Hoang et al. (2004) provided analytical verification for this.

Additional experiments by Hoang et al. (2004) in which alkalinity was varied while effort was made to keep pH constant, revealed a significant (approximately 2.5-fold) increase of nickel toxicity with increasing alkalinity, the variation mainly occurring between 3 and 27 mg $CaCO_3$/L. However, increased alkalinity in these experiments appeared to be generally accompanied by an increase in pH. This was justified by the fact that the effect of alkalinity was only observed in the low hardness test solutions (20 mg $CaCO_3$/L), in which pH was reported not to affect nickel toxicity. The data of the present study, which were also obtained under co-varying pH and alkalinity, are in agreement with Hoang et al. (2004) in that the main increase of nickel toxicity was also observed up to an alkalinity of 27 mg $CaCO_3$/L (see Table 2.1 and Table 2.3).

Summarizing, the acute toxicity studies with larval fathead minnows seem to suggest that the effect of pH and/or alkalinity on nickel toxicity is relatively complex and not yet completely understood. The exact roles of pH and alkalinity in determining nickel toxicity therefore require additional investigation. It also remains to be determined whether the same type of pH and/or alkalinity effects also applies to older life stages of this species. Indeed, while the mode of action of nickel toxicity in juvenile fish has been shown to be respiratory distress at the gills (Pane et al., 2003a, 2004b), gills in larval fish are far less developed and up to a certain age, oxygen uptake as well as ionoregulation occurs through their entire body surface (Rombough, 2002). Hence, the mechanism of toxic action of nickel and,

consequently, the toxicity modifying effects of water chemistry do not necessarily need to be identical in larval and juvenile fish.

Despite this uncertainty, it is intriguing that a simultaneous variation of pH and alkalinity invoked similar modifications to 96-h nickel toxicity to larval fathead minnows and 17-d nickel toxicity to juvenile rainbow trout. This is especially relevant for the incorporation of bioavailability into regulatory frameworks because the covariance between pH and alkalinity is typical for many surface waters. This will be taken forward in model development (see further).

2.4.3. Development of a nickel bioavailability model

Because knowledge of metal speciation is crucial for the enhanced understanding and modeling of bioavailability and toxicity of metals, all 17-d $LC50_{Nidiss}$ and investigated nickel concentrations were converted to Ni^{2+} activity using WHAM VI. In the calcium and magnesium test series, conducted at a pH of 7.5 to 7.6, the following average species distribution was calculated at the 17-d LC50 levels: 93.1% Ni^{2+}, 3.9% $NiHCO_3^+$, 1.9% $NiCO_3$, < 1% NiCl, $NiOH^+$, $Ni(OH)_2$ and $NiSO_4$. In the pH test series, at and below pH 7.7, between 92.7% and 98.6% of the dissolved nickel was calculated to be Ni^{2+}. At pH 8.5, only 43.9% was present as Ni^{2+}, $NiOH^+$ and $Ni(OH)_2$ were still below 1%, but $NiHCO_3^+$ and $NiCO_3$ represented 11.3% and 43.8% of the dissolved nickel, respectively.

Increased activities of Ca^{2+} and Mg^{2+} resulted in an increased 17-d $LC50_{Ni2+}$ (Figure 2.1). Since calcium and magnesium do not affect nickel speciation to a large extent, the interpretation of these Ni^{2+}-based data results in the same conclusions as those based on dissolved nickel concentrations (see above). Although the presently available data do not shed light on the exact mechanisms of the protective effects of calcium and magnesium, we will model them as single-site BLM-type competition effects.

The effect of pH (or H^+) on the toxicity of Ni^{2+} is presented in Figure 2.2. The data suggest a curvilinear relationship between 17-d $LC50_{Ni2+}$ and H^+ activity. Since a single-site competitive effect of H^+ would yield a linear relationship (De Schamphelaere and Janssen, 2002), this observation suggests that other mechanisms are (also) at play.

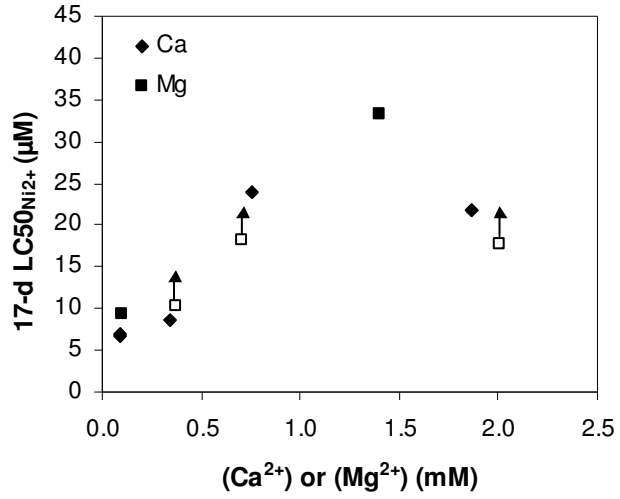

Figure 2.1. 17-d $LC50_{Ni2+}$ (µM) as a function of Ca^{2+} and Mg^{2+} activity (mM). Open symbols with upwardly pointed arrows indicate that the 17-d $LC50_{Ni2+}$ is higher than the plotted value, which represents the highest exposure concentration tested.

Without excluding the potential role of competition between H^+ and Ni^{2+} and acknowledging the co-variance between pH and alkalinity in our study, a number of processes may be proposed which may, at least partly, explain this nonlinearity. First, instead of one binding site, several binding sites with different protonation constants may be involved in the binding and uptake of Ni^{2+} (sensu Borgmann et al., 2005). Second, differences in pH may result in a change of membrane permeability and ion transport. Third, low pH levels result in increased mucus secretion in the gill microenvironment (Tao et al., 2001). Since metal ions can bind to ligands in the secreted mucus, this may result in additional protection against nickel toxicity with decreasing pH. Third, Günther et al. (1986) have reported the existence of an electro-neutral Mg^{2+}/HCO_3^- symporter in Yoshida ascites tumor cells. If such a symporter also exists in fish gills and Ni^{2+} could be transported by it as a Mg^{2+} analogue, increased uptake and toxicity of Ni^{2+} would be expected at increased HCO_3^- concentrations, which occur at increased pH and alkalinity. Fourth, as suggested by Hoang et al. (2004), $NiHCO_3^+$ and $NiCO_3$, which both become more abundant with increasing pH and alkalinity, might contribute to nickel toxicity.

According to the calculation method described by De Schamphelaere and Janssen (2002) and De Schamphelaere et al. (2002), $NiHCO_3^+$ and $NiCO_3$ should be approximately 9.1-fold and 2.3-fold more toxic than Ni^{2+}, respectively. Chemically, this is not very likely because metal complexes typically have lower affinity for ligands than free metal ions.

However, these complexes may only 'appear' to be more toxic than Ni^{2+}. Indeed, since rainbow trout can reduce pH in the gill microenvironment by 1 to 2 pH units at a pH of 8.5 in the bulk solution (Playle, 1998), nickel-carbonate complexes may be dissociated at such high pH levels. Thus, the relative presence of Ni^{2+}, carbonate and bicarbonate ions would increase in the gill microenvironment, which could in turn favour the uptake of Ni^{2+}. This would explain the downward curvature of the relationship between the 17-d $LC50_{Ni2+}$ and H^+ activity with decreasing H^+ in the surrounding medium (Figure 2.2, upper panel). It is clear that several mechanisms and theories could explain the nonlinear nature of the H^+ effect. Further research is however needed to elucidate which mechanisms actually determine the observed effect.

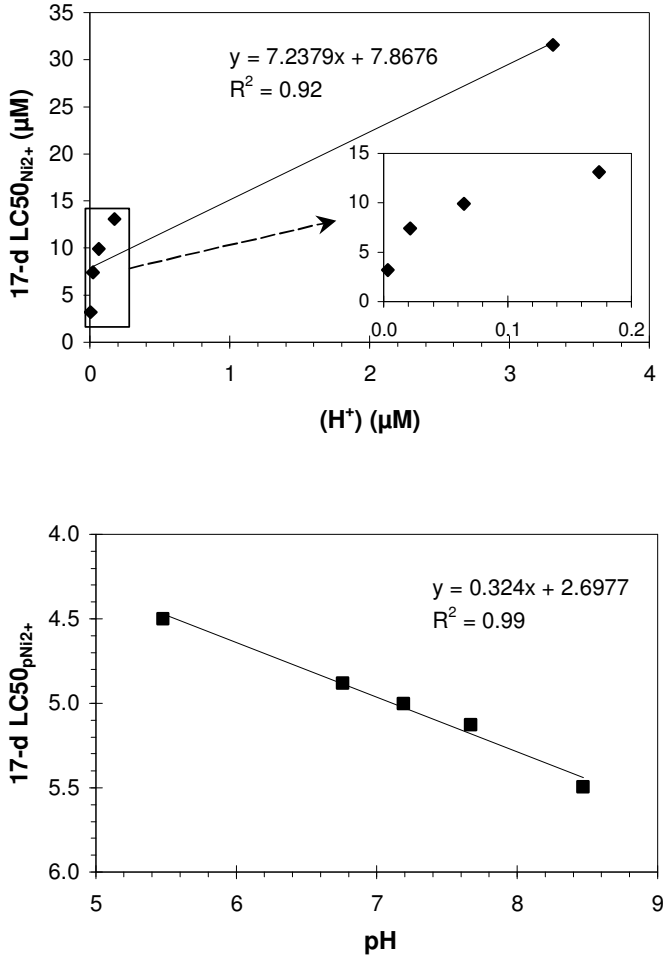

Figure 2.2. The effect of pH on 17-d toxicity of nickel to juvenile rainbow trout. Upper panel: 17-d $LC50_{Ni2+}$ (µM) as a function of H^+ activity (µM); lower panel: 17-d $LC50_{pNi2+}$ (= – log (17-d $LC50_{Ni2+}$)) as a function of pH. The inset in the upper panel is an enlargement of the left part of the main figure and illustrates that a linear relation between 17-d $LC50_{Ni2+}$ and H^+ activity is not applicable over the entire H^+ range. The regression line in the lower panel is used as a basis for modeling the effect of pH on nickel toxicity (see Equation 2.3).

The results of this study indicate that it would be inappropriate to model the effect of pH as a single-site competition effect between H^+ and Ni^{2+}. Instead, it is proposed to model the pH effect based on an empirical linear relationship between pH and $LC50_{pNi2+}$, where pNi^{2+} = – log (Ni^{2+} activity) (Figure 2.2, lower panel). The effect was superimposed on the traditional BLM-type competition effects of calcium and magnesium. It is noted though that this model may only be valid in cases where pH and alkalinity are co-varying according to a system in equilibrium with the atmosphere or a system close enough to this situation. An important model assumption is that the effects of calcium and magnesium are independent of pH and vice versa. In the model, Q is considered a measure of bioavailable nickel and is dependent on Ni^{2+}, Ca^{2+}, Mg^{2+} and pH in test solution i at dissolved nickel concentration j:

$$Q_{i,j} = -\log \frac{\left(Ni^{2+}\right)_{i,j}}{\left\{1 + K_{CaBL} \cdot \left(Ca^{2+}\right)_i + K_{MgBL} \cdot \left(Mg^{2+}\right)_i\right\}} - S_{pH} \cdot pH_i \qquad \text{Eq. 2.2}$$

In this equation, (Ni^{2+}), (Ca^{2+}), and (Mg^{2+}) represent the chemical activities of these ions, K_{CaBL} and K_{MgBL} are the parameters describing the protective effects of calcium and magnesium on nickel toxicity as typical BLM-type competition effects (De Schamphelaere and Janssen, 2002) and S_{pH} is the slope of the linear relationship between 17-d $LC50_{pNi2+}$ and pH (Figure 2.2, lower panel). The lower the value of $Q_{i,j}$, the higher the toxicological bioavailability of nickel. The eventual model parameter Q_x can be regarded as a measure of the amount of nickel that has to be bioavailable in order to cause x% mortality. As predictor of the toxic effect, Q_x represents a similar function as the gill metal concentration in the BLM (Di Toro et al., 2001; Santore et al., 2001, 2002), yielding:

$$LCx_{Ni2+,i} = 10^{-\left(S_{pH} \cdot pH_i + Q_x\right)} \cdot \left\{1 + K_{CaBL} \cdot \left(Ca^{2+}\right)_i + K_{MgBL} \cdot \left(Mg^{2+}\right)_i\right\} \qquad \text{Eq. 2.3}$$

In this model, Q_x is the intercept of the linear relationship between LCx_{pNi2+} and pH, after correction for calcium and magnesium competition. Rather than the LC50 values only, all concentration response data were used to optimize the model parameters. Therefore, a logistic regression was fitted to a plot of % survival data ($y_{i,j}$) versus $Q_{i,j}$:

$$y_{i,j} = \frac{100}{1 + \left(\frac{\exp(Q_{i,j})}{\exp(Q_{50})} \right)^{\beta}} \qquad \text{Eq. 2.4}$$

Note that, after optimization of all model parameters, this equation can be rearranged in order to derive a Q_x for predicting other effect levels than 50% mortality. This chapter will only discuss the model's performance in predicting LC50s. For the fitting of Equation 2.4 to our data, we assumed log K_{CaBL} = log K_{MgBL}, based on a comparison of the observed effects of calcium and magnesium (see above). The best fit was obtained for S_{pH} = 0.3240, log K_{CaBL} = log K_{MgBL} = 3.6, Q_{50} = 2.946 and β = -4.477, with an R^2 of 0.74 (Figure 2.3, lower panel). For comparison, we also provided a plot of % survival versus pNi^{2+} in Figure 2.3 (upper panel). The latter plot yielded a cloud of data points without any obvious relation between pNi^{2+} and % survival, suggesting that pNi^{2+} is a much worse predictor of nickel toxicity than Q. Using different values for log K_{CaBL} and log K_{MgBL} or higher values for both of them did not result in an improvement of the fit. Therefore, the abovementioned parameter values were retained for the final model.

According to Equation 2.3, the 17-d $LC50_{Ni2+}$ can now be predicted for any test solution. By inserting the 17-d $LC50_{Ni2+}$ into WHAM VI, the corresponding 17-d $LC50_{Nidiss}$ can be calculated. Figure 2.4 (upper panel) visualizes how well the model is calibrated to the dataset used for model development: all 17-d $LC50_{Nidiss}$ were predicted by an error of less than factor 2, with an average prediction error of factor 1.2 and a maximum prediction error of factor 1.7. The latter error represents an overestimation of the 17-d $LC50_{Nidiss}$ in the experiment conducted at the highest calcium concentration (3.0 mM). This is due to the observed plateau in the calcium effect, which is not predicted by the single-site competition between nickel and calcium assumed in our model.

2.4.4. Model validation – natural waters

Speciation calculations demonstrated that at the 17-d LC50 levels only 8 to 20% of the total dissolved nickel was complexed by DOC and between 59 and 81% occurred as free Ni^{2+}. Apparently, as suggested by Wu et al. (2003), organic complexation only plays a minor role

in nickel speciation at these dissolved nickel concentrations, suggesting that the protective effect of DOC on nickel toxicity to juvenile rainbow trout is limited.

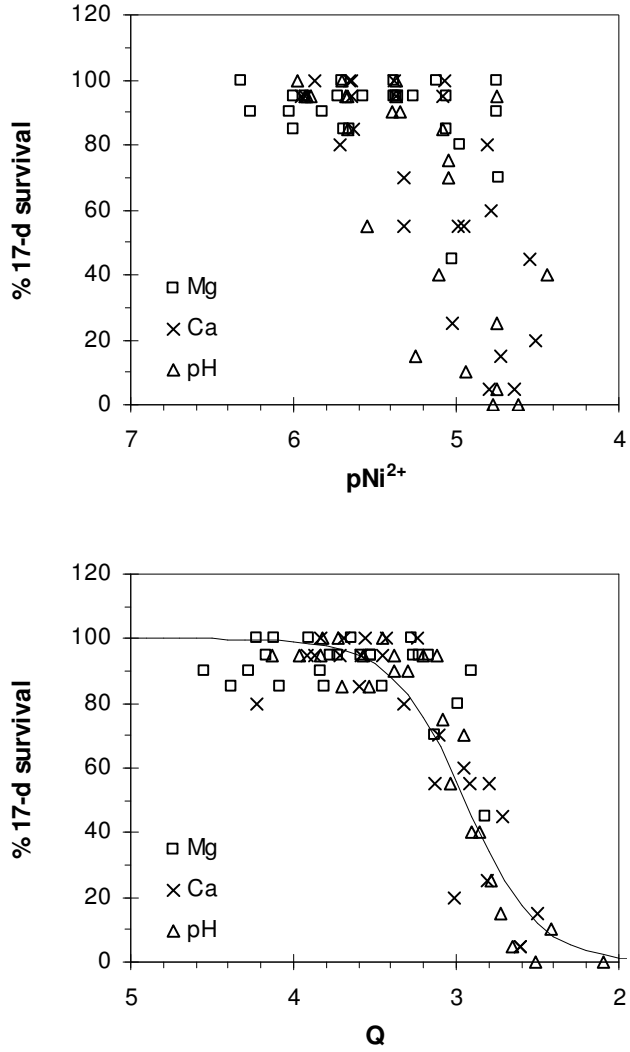

Figure 2.3. % 17-d survival of juvenile rainbow trout as a function of pNi^{2+} = – log (Ni^{2+} activity) (upper panel) and Q, which is a measure of the bioavailable pNi^{2+} (lower panel). Concentration response data from all test series (Table 2.1) were used. The full line represents the best-fit logistic response curve (Equation 2.4) with optimized parameter values (Table 2.4).

The results of the validation exercise are shown in Figure 2.4 (upper panel). Since the 17-d $LC50_{Nidiss}$ for 'Eppe' was extrapolated, model performance should be evaluated based on predictions for the other four natural surface waters. For 'Ankeveen', 'Brisy' and 'Markermeer', 17-d $LC50_{Nidiss}$ were predicted by an average error of factor 1.1. The 17-d $LC50_{Nidiss}$ in the very soft, acidic 'Bihain' test water was overestimated by a factor of 2.9. The latter may be due to the fact that this test water had a pH close to and water hardness clearly

below the minima for which our model was developed. The combination of these rather 'extreme' conditions may have resulted in a higher than expected sensitivity of juvenile rainbow trout in this test water.

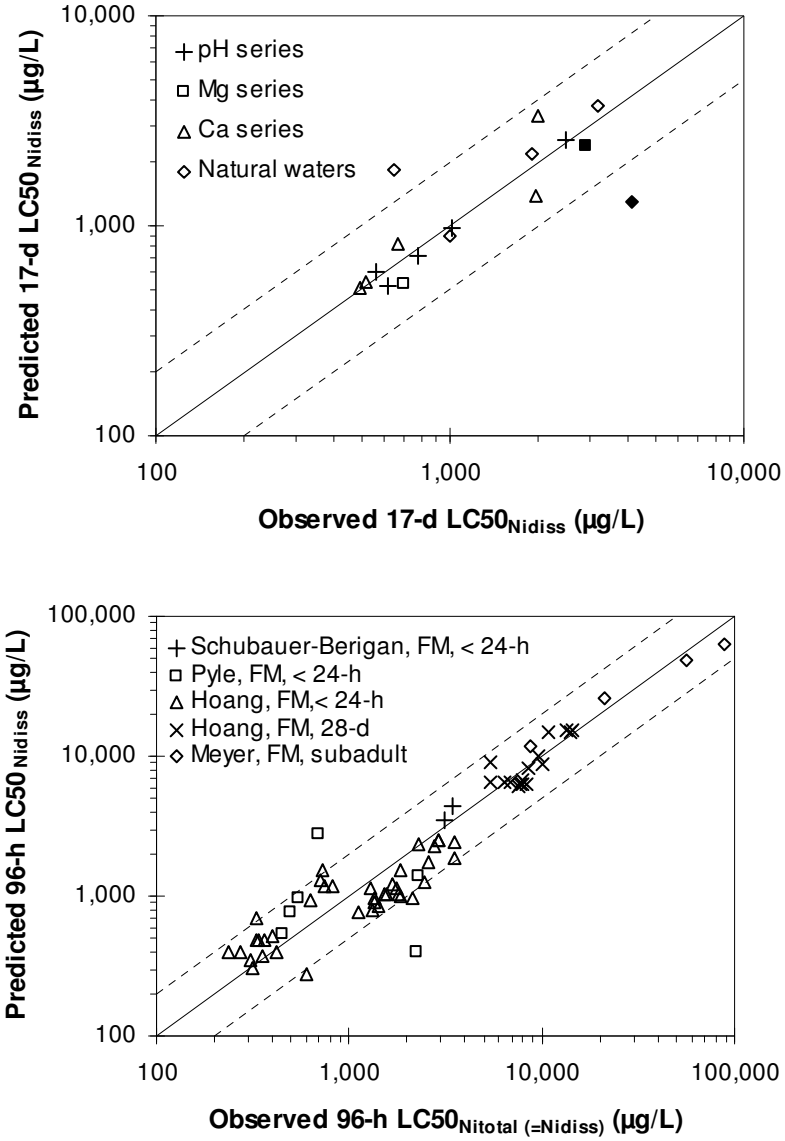

Figure 2.4. Predicted $LC50_{Nidiss}$ versus observed $LC50_{Nidiss}$ (µg/L). 96-h LC50s (lower panel) were reported in literature as total nickel concentrations but were assumed to be similar based on dissolved nickel concentrations (as mentioned by Hoang et al., 2004). Predictions were made using optimized model parameters (Table 2.4) and Equation 2.3 linked to WHAM VI. The solid line indicates a perfect match between predicted and observed LC50s; the dashed lines indicate ratios of 0.5 and 2 between observed and predicted LC50s. Upper panel: 17-d LC50s for rainbow trout (present study). Filled symbols represent extrapolated 17-d LC50s (Table 2.3). Lower panel: 96-h LC50s for fathead minnow (FM) taken from several studies (Schubauer-Berigan et al., 1993; Meyer et al., 1999; Pyle et al., 2002a; Hoang et al., 2004).

2.4.5. Model validation – acute 96-h LC50s for fathead minnow

In this section, the proposed model is validated using acute toxicity data for fathead minnow (Schubauer-Berigan et al., 1993; Meyer et al., 1999; Pyle et al., 2002a; Hoang et al., 2004). In these tests, 96-h LC50s were reported based on total nickel concentrations (96-h $LC50_{Nitotal}$). However, Hoang et al. (2004) mentioned that there was no difference between 96-h $LC50_{Nidiss}$ and 96-h $LC50_{Nitotal}$. The same model parameters as those derived in the present study were used to describe the effects of pH, calcium and magnesium on nickel toxicity (i.e., S_{pH}, log K_{CaBL} and log K_{MgBL}). Only the Q_{50} value was optimized to reflect the potential difference in sensitivity between different species, life stages, and exposure durations. Assumptions about DOC that were made during model development (see materials and methods section) were left unchanged.

First, a single Q_{50} was calibrated for all three studies conducted with larval fathead minnows (Schubauer-Berigan et al., 1993; Pyle et al., 2002a; Hoang et al., 2004). The calibration had to be based on data from tests conducted at ambient pH (open system). This excluded several larval fathead minnow tests of Hoang et al. (test numbers 7 to 16 in Table 1 in Hoang et al., 2004), Pyle et al. (tests at pH 5.5 and 8.5) and Schubauer-Berigan et al. (test at pH 7.5) from the calibration. In all these tests, pH was modified without modifying alkalinity. Eventually, data from 35 out of 49 test waters remained for the calibration. These test waters had a pH between 6.1 and 8.8, water hardness between 12 and 235 mg $CaCO_3$/L, alkalinity between 3.3 and 375 mg $CaCO_3$/L and DOC concentration between 0.5 and 10.3 mg/L. The 96-h LC50s reported for these tests ranged from 239 to 3500 µg/L.

Second, a Q_{50} was determined for 16 tests with 28-d old fathead minnows described by Hoang et al. (2004). These tests were conducted at a pH between 6.0 and 8.6, water hardness between 19 and 101 mg $CaCO_3$/L, alkalinity between 4 and 386 mg $CaCO_3$/L and DOC between 0.5 and 9.8 mg/L. The 96-h LC50s obtained in these tests varied between 5450 and 14360 µg/L. A third Q_{50} was calibrated to the four tests with subadult fathead minnows (weighing 1 to 6 g) conducted by Meyer et al. (1999) at different calcium concentrations (0.15 to 2.4 mM).

The results of all these calibrations are reported in Table 2.4. We obtained 96-h Q_{50} values of 3.009 for larval (< 24-h old) fathead minnows (Schubauer-Berigan et al., 1993; Pyle

et al., 2002a; Hoang et al., 2004), 2.141 for 28-d old juvenile fathead minnows (Hoang et al., 2004) and 1.644 for subadult fathead minnows (weighing 1 to 6 g) (Meyer et al., 1999). Apparently, larval minnows are markedly more sensitive to nickel than juvenile minnows. This was already demonstrated by Hoang et al. (2004). Overall, fathead minnows seem to become less sensitive to nickel as they grow up. This confirms the general assumption that organisms are more sensitive to chemicals when they are smaller or in an earlier developmental stage.

Table 2.4. Parameter values of the bioavailability model for predicting nickel toxicity to fish. The Q_{50} values were optimized to take account of differences in sensitivity between different fish species, life stages and exposure durations.

Model parameter	Parameter value	Study
Log K_{CaBL}	3.6	This study
Log K_{MgBL}	3.6	This study
S_{pH}	0.3240	This study
Species, life stage, exposure duration	Q_{50} values	Study
Rainbow trout, juvenile (200-300 mg), 17-d	2.946	This study
Fathead minnow, larval (< 24-h old), 96-h	3.009	Schubauer-Berigan et al., 1993; Pyle et al., 2002a; Hoang et al., 2004
Fathead minnow, juvenile (28-d old), 96-h	2.141	Hoang et al., 2004
Fathead minnow, subadult (1-6 g), 96-h	1.644	Meyer et al., 1999

Finally, the reported parameter values were used to predict 96-h LC50s in all test solutions (including the ones excluded from calibration of Q_{50} values). The predictive capacity of the calibrated models is depicted in Figure 2.4 (lower panel). The 96-h LC50s for 28-d old (Hoang et al., 2004) and subadult (Meyer et al., 1999) fathead minnows were predicted by an average error of factor 1.2 and 1.3, respectively, with maximum prediction errors being 1.7 and 1.4, respectively. When the larval fathead minnow data are considered, an average prediction error of factor 1.6 is noted. Considering only the data from test solutions with ambient pH, prediction errors averaged a factor of 1.5, which is reasonably accurate given the fact that these data were taken from three different studies.

The developed model is definitely more accurate than the nickel BLM proposed by Wu et al. (2003) in predicting 96-h LC50s from the fathead minnow dataset of Hoang et al. (2004). The latter model predicted a large number of 96-h LC50s by an error higher than factor 2 or even higher than factor 5 (see Figure 3 in Hoang et al., 2004). Using our newly developed model, extremely large prediction errors (factor 4.2 and 5.7) are only observed for

the data from Pyle et al. (2002a) that were obtained in test solutions in which pH was intended to be modified independently from alkalinity, but for which no analytical verification was provided. The inaccuracy of the model is due to the fact that Pyle et al. (2002a) observed an increase of 96-h LC50s with increasing pH, while our model (Equation 2.3) predicts the opposite. It is interesting to mention that similar pH modifications made by Hoang et al. (2004) did not yield 96-h LC50s for which model predictions were equally poor as for the data reported by Pyle et al. (2002a). Leaving out the data taken from Pyle et al. (2002a), predictions did not deviate more than a factor 2.2 from the observed 96-h LC50s.

Overall, the developed model reasonably reflected the effects of water chemistry on nickel toxicity to fish, even in a number of test solutions in which pH was modified independently from alkalinity. Although the exact mechanisms by which pH and/or alkalinity affect nickel toxicity to fish are currently not known, it can be concluded that our empirical model is capable of accurately predicting nickel toxicity to fish within a large range of conditions.

2.5. Conclusion

Univariate experiments in synthetic test solutions demonstrated that increasing calcium and magnesium concentrations and decreasing pH reduced the 17-d toxicity of nickel to juvenile rainbow trout. Based on data from these experiments a bioavailability model was developed. The effects of calcium and magnesium were modeled as BLM-type single-site competition effects and a log-linear pH effect was superimposed on these effects. The model parameters representing the observed effects are log K_{CaBL} = log K_{MgBL} = 3.6 and S_{pH} = 0.3240. A new sensitivity parameter Q_{50} (= 2.946 for 17-d exposure of juvenile rainbow trout) was introduced as a measure of the amount of bioavailable nickel that is needed to cause 50% mortality among exposed fish.

The model was calibrated to the toxicity data obtained in all synthetic test solutions and independently validated using toxicity data obtained in a series of nickel-spiked surface waters. For all waters except one – a natural water that had pH at the border of and water hardness outside the chemistry ranges for which the model was developed – the model predicted 17-d $LC50_{Nidiss}$ within a factor 2 deviation from the observed 17-d $LC50_{Nidiss}$.

Using literature data, the model was also calibrated to account for differences in sensitivity between different species, life stages and/or exposure durations by adjusting the sensitivity parameter Q_{50}. The calibrated models demonstrated good performance in predicting acute nickel toxicity to larval, juvenile and subadult fathead minnow (Schubauer-Berigan et al., 1993; Meyer et al., 1999; Pyle et al., 2002a; Hoang et al., 2004).

Although the exact mechanisms by which calcium, magnesium, pH and/or alkalinity affect nickel toxicity are currently not known, the developed model seems reasonably able to reflect the effects of these nickel toxicity modifying factors. Further research, especially into the interaction between Mg^{2+} and Ni^{2+} and the effects of pH and alkalinity on nickel toxicity, is recommended to allow further refinement of the developed bioavailability model.

Chapter 3

Development of an acute nickel bioavailability model for *Daphnia magna*

Redrafted from

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Chapter 3

Development of an acute nickel bioavailability model for *Daphnia magna*

Abstract. The effects of calcium, magnesium, sodium and pH on the acute toxicity of nickel to *Daphnia magna* were investigated in a series of 48-h immobilization assays in synthetic test solutions. Both calcium and magnesium reduced nickel toxicity, while sodium did not. Nickel toxicity was not affected in the pH range of 5.7 to 7.5, but a further increase of pH up to 8.1 resulted in an increase of toxicity of the free Ni^{2+} ion. Based on the results of these experiments, a biotic ligand model (BLM) was developed in which the effects of calcium and magnesium were modeled as single-site competition effects. Stability constants representing the binding strength between Ca^{2+} and Mg^{2+} and the biotic ligand (BL) were log K_{CaBL} = 3.10 and log K_{MgBL} = 2.47, respectively. The effect of pH could not be appropriately described by single-site competition between Ni^{2+} and H^{+}. Since the overall variation of toxicity within the tested pH range was relatively small, we decided not to incorporate the effect of pH in the current model. The model was able to predict 48-h EC50s in all synthetic test solutions by an error less than factor 2. The model's predictive capacity was also evaluated using results of toxicity tests in nickel-spiked surface waters. For 15 out of 16 tested waters, 48-h EC50s were predicted by an error less than factor 2. Additionally, after calibration to account for interclonal or interspecies sensitivity differences, the model was able to accurately predict previously published 48-h EC50s for another *D. magna* clone as well as for *Ceriodaphnia dubia*. Finally, the predictive capacity of the model was demonstrated to be better than that of previously proposed models that include a log K_{NaBL}, a log K_{HBL} and a log K_{CaBL}, but did not incorporate a log K_{MgBL}. An in-depth comparison of these models demonstrated that (i) there is no need to incorporate a log K_{NaBL}, (ii) it is important to recognize the protective effect of magnesium, and (iii) the incorporation of a log K_{HBL} does not adequately describe the effect of pH. Although the model seems very promising, further research, especially into the effects pH and alkalinity, would be needed to allow further refinement.

3.1. Introduction

It is increasingly being recognized that metal bioavailability and toxicity to aquatic organisms is dependent on the physicochemical composition of the surrounding medium. The results of research into the effects of physicochemical parameters such as pH, alkalinity, water

hardness, calcium, magnesium, sodium and dissolved organic carbon (DOC) on the toxicity of metals to freshwater organisms are continually being published (see reviews of Paquin et al., 2002; Niyogi and Wood, 2004). Di Toro et al. (2001) integrated the available knowledge into the biotic ligand model (BLM) concept, which has become the foundation for a whole range of bioavailability models, all attempting to predict metal toxicity as a function of water chemistry (Paquin et al., 1999; Santore et al., 2001, 2002; De Schamphelaere and Janssen, 2002, 2004a,b; De Schamphelaere et al., 2002; Heijerick et al., 2002a,b, 2005a; Wu et al., 2003; Hoang et al., 2004; Keithly et al., 2004). Such models are currently being used in the EU risk assessment on zinc and zinc compounds (ECB, 2003; Bodar et al., 2005; Van Assche, 2006) and for the derivation of site-specific water quality criteria for copper in the USA (US EPA, 2007b).

Initial effort towards the development of nickel bioavailability models for freshwater organisms came from Wu et al. (2003). They presented a preliminary set of gill binding constants for nickel, calcium, sodium and protons, based on the fathead minnow gill accumulation data of Meyer et al. (1999). In the same report, Wu et al. (2003) calibrated the fathead minnow model to acute toxicity data for the aquatic invertebrates *Daphnia magna* (Chapman et al., 1980) and *Ceriodaphnia dubia* (Schubauer-Berigan et al., 1993). Binding site density and binding constants were kept the same as for fathead minnow, and the LA50 (lethal accumulation level associated with 50% effect) was adjusted to account for species-specific sensitivity. Keithly et al. (2004) evaluated the applicability of a slightly modified version of this model to the hardness-dependent acute toxicity of nickel to *C. dubia*.

Although the models presented by Wu et al. (2003) and Keithly et al. (2004) provide a reasonably good simulation of the data concerned, there is a clear need for improvement. First, the data used for model calibration only cover two nickel toxicity modifying factors, i.e. water hardness (Chapman et al., 1980; Keithly et al., 2004) and pH (Schubauer-Berigan et al., 1993). Second, the set of binding constants (for nickel, calcium, sodium and protons) was based on gill accumulation data for fathead minnow, taken from a study in which only the effect of calcium on nickel accumulation on the gill was studied (Meyer et al., 1999). Considering these drawbacks, it is clear that further testing with standard test organisms (e.g., *D. magna*, *C. dubia*) is required for further development of a robust acute nickel bioavailability model for freshwater invertebrates.

In the present study, the individual effects of calcium, magnesium, sodium and pH on the acute toxicity of nickel to *D. magna* were investigated. Based on the results of these experiments, binding constants for competing cations were derived and a species-specific bioavailability model was developed. The predictive capacity of the new model was evaluated using results from acute toxicity tests in nickel-spiked surface waters. Finally, it was investigated if the model could help in explaining literature data on the acute toxicity of nickel to *D. magna* and *C. dubia*.

3.2. Materials and methods

3.2.1. Toxicity tests in synthetic test solutions

Five sets of toxicity assays were performed to investigate the individual effects of calcium, magnesium, sodium and pH on the acute toxicity of nickel to *D. magna*. The calcium, magnesium and sodium test series each consisted of nine assays. The effect of pH was studied in two separate test series: pH test series 1 (six assays), in which pH was controlled by adding $NaHCO_3$, and pH test series 2 (three assays), in which MOPS (3-N-morpholinopropanesulfonic acid) was used as pH buffer (see further). In each assay, a control treatment and six nickel concentrations were tested. Assays belonging to the same test series were conducted simultaneously.

3.2.2. Preparation of synthetic test solutions

All test solutions were prepared using carbon filtered, deionized water (conductivity < 2 µS/cm) and reagent grade chemicals purchased from VWR International (Leuven, Belgium). The synthetic basic medium was composed of 0.25 mM $CaCl_2$, 0.25 mM $MgSO_4$ and 0.078 mM KCl. All test solutions (except those used in the pH test series) were adjusted to pH 6.8 by adding 0.078 mM $NaHCO_3$. Calcium, magnesium and sodium concentrations were adjusted by adding $CaCl_2$, $MgCl_2$ and NaCl to the basic medium, respectively. In pH test series 1, different pH levels were obtained by adding different amounts of $NaHCO_3$. In this test series, sodium was set to the same concentration through addition of NaCl. In pH test series 2, pH was controlled by adding MOPS (3.6 mM) and the required amount of dilute NaOH or HCl solution. For each bioassay, a nickel concentration series was prepared by adding $NiCl_2$. Each bioassay consisted of a control treatment and six nickel concentrations.

All solutions were allowed to equilibrate for 24 hours at 20 °C prior to being used in the toxicity tests. The physicochemical composition of all synthetic test solutions is given in Table 3.1.

Table 3.1. Overview of the physicochemical characteristics of the synthetic test solutions used in the acute immobilization assays with juvenile *D. magna* and the observed 48-h $EC50_{Nidiss}$ (expressed as dissolved nickel, mg/L) and 48-h $EC50_{Ni2+}$ (expressed as Ni^{2+} activity, µM).

Test medium [a]	pH	Major ions (mg/L)				Alkalinity [c]	48-h $EC50_{Nidiss}$	48-h $EC50_{Ni2+}$
		Ca	Mg	Na [b]	Cl [b]	(mg $CaCO_3$/L)	(mg/L) [d]	(µM)
Ca 0.25 mM	6.50	8.86	5.86	2.31	23.8	2.28	1.82 (1.71-1.94)	24.8
Ca 0.5 mM	6.63	18.6	5.69	2.16	41.5	2.55	2.08 (1.78-2.42)	27.6
Ca 1.0 mM	6.71	34.8	5.49	2.13	77.0	2.71	2.53 (2.29-2.79)	32.2
Ca 1.5 mM	6.77	52.9	5.25	2.11	112	2.82	3.41 (3.15-3.69)	41.9
Ca 2.0 mM	6.89	71.1	5.18	2.08	149	3.03	3.56 (3.25-3.90)	42.4
Ca 2.5 mM	6.89	87.8	5.57	1.79	185	3.03	4.49 (3.83-5.26)	52.0
Ca 3.0 mM	6.86	103	5.40	1.79	220	2.98	5.50 (4.78-6.33)	62.3
Ca 4.0 mM	6.90	144	5.32	2.05	291	3.04	3.40 (2.70-4.29)	36.8
Ca 5.0 mM	6.92	181	5.30	2.02	362	3.07	2.28 (1.82-2.87)	23.7
Mg 0.25 mM	6.62	9.12	6.05	1.79	23.8	2.53	2.26 (2.09-2.44)	30.8
Mg 0.5 mM	6.58	9.03	11.7	1.79	41.5	2.45	2.61 (2.40-2.84)	34.6
Mg 1.0 mM	6.60	8.92	22.9	1.79	77.0	2.49	2.96 (2.73-3.22)	37.5
Mg 1.5 mM	6.48	8.23	33.7	1.79	112	2.24	3.24 (2.94-3.57)	39.7
Mg 2.0 mM	6.59	8.70	41.8	1.79	149	2.47	3.54 (3.26-3.84)	42.2
Mg 2.5 mM	6.79	8.55	52.5	1.79	185	2.86	3.77 (3.38-4.21)	43.7
Mg 3.0 mM	6.82	8.56	66.1	1.79	220	2.91	4.06 (3.72-4.44)	45.7
Mg 4.0 mM	6.85	8.10	84.6	1.79	291	2.96	3.63 (3.29-4.01)	39.3
Mg 5.0 mM	6.75	7.87	111	1.79	362	2.79	3.51 (3.20-3.86)	36.5
Na 0.078 mM	6.80	8.80	5.82	1.79	23.8	2.88	3.17 (2.62-3.83)	43.2
Na 1.0 mM	6.80	8.74	5.84	23.0	56.5	2.88	3.49 (3.12-3.90)	45.8
Na 2.0 mM	6.80	8.84	5.77	46.0	92.0	2.88	3.19 (2.81-3.61)	40.5
Na 4.0 mM	6.80	8.74	5.85	92.0	163	2.88	3.32 (2.97-3.72)	40.0
Na 6.0 mM	6.80	8.35	5.55	138	234	2.88	3.61 (3.02-4.30)	41.7
Na 8.0 mM	6.80	8.27	5.59	184	306	2.88	3.64 (3.23-4.10)	40.2
Na 10 mM	6.80	9.10	5.11	230	377	2.88	3.40 (3.08-3.75)	36.7
Na 12 mM	6.80	8.51	5.17	276	448	2.88	3.23 (2.96-3.53)	33.8
Na 14 mM	6.80	8.36	5.46	322	519	2.88	3.55 (3.18-3.96)	36.2
pH 5.8 (1)	5.95	7.04	6.32	92.0	145	0.110	2.52 (2.36-2.68)	30.8
pH 6.3 (1)	6.28	7.03	6.26	92.0	145	0.570	2.65 (2.50-2.80)	32.3
pH 6.8 (1)	6.74	7.10	6.26	92.0	143	2.77	2.81 (2.61-3.02)	34.2
pH 7.3 (1)	7.24	7.09	6.33	92.0	156	14.2	2.61 (2.40-2.84)	30.8
pH 7.8 (1)	7.53	6.98	6.31	92.0	116	46.9	3.07 (2.75-3.43)	33.9
pH 8.3 (1)	8.13	6.89	6.29	92.0	23.0	198	3.27 (2.90-3.68)	21.6
pH 5.8 (2)	5.72	7.08	6.35	41.7	78.4	0.740	2.32 (2.13-2.54)	29.9
pH 6.3 (2)	6.07	6.63	6.29	47.7	72.5	1.34	2.57 (2.36-2.78)	33.0
pH 6.8 (2)	6.63	6.75	6.33	60.4	58.9	2.55	2.72 (2.46-3.00)	34.5

[a] pH test series 1 differs from pH test series 2 in the way pH was controlled: $NaHCO_3$ (1) versus MOPS + NaOH/HCl (2).

[b] No measurements were conducted for Na, K, SO_4 and Cl. In all tests, nominal K and SO_4 concentrations were 3.05 and 24.0 mg/L, respectively.

[c] Alkalinity was calculated from nominal added inorganic carbon (IC) and measured pH, using thermodynamic stability constants taken from Stumm and Morgan (1996).

[d] Numbers between brackets indicate 95% confidence intervals.

3.2.3. Toxicity tests in European surface waters

Surface waters with varying physicochemical characteristics (Table 3.2) were sampled at eight locations in Europe that are relatively undisturbed by anthropogenic inputs. The variation in the sampled waters with regard to the main factors affecting nickel bioavailability (calcium, magnesium, water hardness, DOC, pH, alkalinity) reasonably covered the physicochemical variation observed in European surface waters. Samples were taken in four different countries (Belgium, France, the Netherlands and the United Kingdom) between May 2001 and March 2003, and the samples were given the following labels: Ankeveen, Bihain, Brisy, Clywedog, Markermeer, Mole, Regge and Voyon. Ankeveen, Bihain and Markermeer were sampled twice. At each location, the required amount of water was membrane filtered (0.45 µm) and collected in metal-free polyethylene vessels. The samples were immediately transferred to the laboratory, where they were stored at 4°C in total darkness.

Before testing, the following parameters were measured: pH (pH-meter P407, Consort, Turnhout, Belgium), DOC, inorganic carbon (IC) (TOC-5000, Shimadzu, Duisburg, Germany), Ca, Mg, Na, K, Fe, Al, Mn, Ni, Cu, Zn, Pb and Cd (ICP-OES, Perkin Elmer 3300 DV) and Cl, NO_3 and SO_4 (Ion Chromatography, Dionex QIC analyzer, IONPAC AS4A). With each of the sampled waters, an acute toxicity test was performed under the same conditions as described for the tests in synthetic test solutions (see further). To obtain a nickel concentration series (control treatment + six nickel concentrations), the waters were spiked with $NiCl_2$ and allowed to equilibrate for 24 hours. MOPS (3.6 mM) and NaOH/HCl were used for setting pH in all test waters except for those with pH > 7.8 (Voyon and Markermeer). An overview of sampling locations, sampling dates and main physicochemical parameters (pH, calcium, magnesium, water hardness, DOC, alkalinity, background nickel concentration) is given in Table 3.2.

3.2.4. Testing

Acute 48-h immobilization assays with juvenile *D. magna* (< 24 hours old) were performed according to OECD guideline 202 (OECD, 1996). Test organisms originated from a healthy *D. magna* clone (K6), which has been cultured under controlled laboratory conditions in M4 medium (Elendt and Bias, 1990) for more then ten years.

Table 3.2. Overview of sampling locations, sampling dates, main physicochemical characteristics (pH, dissolved organic carbon (DOC), calcium, magnesium, water hardness, alkalinity, background dissolved nickel concentration) and 48-h $EC50_{Nidiss}$ (expressed as dissolved nickel, mg/L).

Date (MM/YY)	Site ID (country)	Site type	pH [b]	DOC (mg C/L)	Ca (mg/L)	Mg (mg/L)	Hardness (mg $CaCO_3$/L)	Alkalinity [c] (mg $CaCO_3$/L)	Ni_{diss} [d] (µg/L)	48-h $EC50_{Nidiss}$ [e] (mg/L)
05/01	Ankeveen (NL) [a]	Lake system in	7.14	25.8	48.1	8.19	154	23.3	4.6	5.25 (4.69-5.87)
		lowland peat	7.14	25.8	48.1	8.19	154	23.3	4.6	5.44 (4.77-6.20)
03/03	Ankeveen (NL)		6.79	17.3	38.3	6.54	123	28.2	4.0	5.72 (5.41-6.06)
05/01	Bihain (B) [a]	Small creek in	6.23	6.62	34.5	1.47	92.1	0.390	2.5	2.23 (2.06-2.52)
		highland peat	6.21	6.62	34.5	1.47	92.1	0.390	2.5	2.11 (1.90-2.35)
03/03	Bihain (B)		6.15	5.37	3.68	1.07	13.6	8.36	2.5	0.86 (0.75-0.98)
03/03	Brisy (B)	River in mixed forest	7.09	2.53	4.99	3.38	26.4	12.8	2.0	2.01 (1.89-2.13)
05/01	Clywedog (UK) [a]	Large artificial lake in	5.94	1.75	3.03	1.38	13.2	0.590	1.5	1.04 (0.87-1.23)
		Welsh highlands	5.96	1.75	3.03	1.38	13.2	0.590	1.5	0.98 (0.82-1.17)
05/01	Markermeer (NL) [a]	Part of large lake	7.92	9.20	72.7	20.6	266	118	2.1	5.49 (4.88-6.17)
		cut off from the North	7.96	9.20	72.7	20.6	266	118	2.1	6.13 (5.41-6.94)
03/03	Markermeer (NL)	Sea by a dam	8.09	7.49	52.7	14.0	189	127	1.0	4.52 (3.95-5.17)
05/01	Mole (UK) [a]	River in chalk cliff	7.58	5.14	51.4	8.33	163	89.5	3.9	5.01 (4.39-5.71)
		landscape	7.62	5.14	51.4	8.33	163	89.5	3.9	5.13 (4.60-5.72)
03/03	Regge (NL)	River in brook valley landscape	7.70	9.87	60.1	7.96	183	161	4.0	6.30 (5.65-7.04)
03/03	Voyon (F)	Small stream in forested area 'Le Val Joly'	8.02	4.17	37.1	7.13	122	122	6.0	3.84 (3.48-4.23)

[a] Tests were conducted in duplicate.

[b] Average pH measured in each single test.

[c] Alkalinity was calculated from measured inorganic carbon (IC) concentrations and pH, using thermodynamic stability constants taken from Stumm and Morgan (1996).

[d] Background dissolved nickel concentration.

[e] Numbers between brackets indicate 95% confidence intervals.

Dissolved nickel concentrations in the culture medium were always below 3 µg/L, i.e. the method detection limit (MDL) of the graphite furnace atomic absorption spectrometer used in this study (GF-AAS, SpectrAA800 with Zeeman background correction, Varian, Mulgrave, Australia). Each experiment consisted of a control treatment and six nickel concentrations. The difference between successive nickel concentrations was 0.11-0.15 log-units. Each treatment was performed with three replicates using ten organisms per replicate. The number of 'immobilized' juveniles in each cup was recorded after 24 and 48 hours.

3.2.5. Chemical analyses

Water temperature, oxygen saturation and pH were measured at the beginning and at the end of each test. The glass electrode for pH measurements was calibrated with pH 4, pH 7 and pH 10 buffers (Merck, Darmstadt, Germany). A maximum difference of 0.2 pH units between the beginning and the end of testing was allowed. Samples for measurement of total calcium and magnesium and dissolved nickel concentrations (filtration through a 0.45 µm filter, Gelman Sciences, Ann Arbor, MI, USA) were taken at the end of testing and acidified with 0.14 N HNO_3. Calcium, magnesium and nickel concentrations were measured using flame atomic absorption spectrometry (F-AAS, SpectrAA100, Varian, Mulgrave, Australia). Nickel concentrations in control treatments were measured using GF-AAS. Measurement procedures were the same as described in chapter 2.

For data analysis and model development, average measured pH and measured concentrations of calcium, magnesium and nickel were used. For Na, K, Cl, SO_4 and IC, nominal concentrations were considered adequate since previous studies at our laboratory indicated that measured values of these parameters typically deviate less than 10% from nominal values. Alkalinity was calculated from nominal IC concentrations and measured pH using thermodynamic stability constants taken from Stumm and Morgan (1996). No samples were taken for measurement of DOC concentrations. For the tests conducted in the nickel-spiked surface waters shown in Table 3.2., Ca, Mg, Na, K, Cl, SO_4, IC and DOC concentrations measured prior to testing were used for data treatment and model evaluation.

3.2.6. Data treatment and statistics

48-h EC50 values (concentrations resulting in 50% of the exposed juveniles being immobilized) and their 95% confidence intervals were calculated using the trimmed Spearman-Karber method (Hamilton, 1977). Observed 'immobilizations' at each measured nickel concentration were used as input for the calculations.

3.2.7. Equilibrium chemical speciation calculations

Speciation of nickel and other ions was calculated using WHAM VI software (Tipping, 1998; NERC, 2001) as described in chapter 2. Speciation calculations were performed for all 48-h EC50s. For the synthetic test solutions, the input variables were the average measured pH, the average measured calcium and magnesium concentration, nominal concentrations of Na, K, SO_4, Cl, IC and MOPS, and the 48-h EC50 based on dissolved nickel (48-h $EC50_{Nidiss}$). Since De Schamphelaere et al. (2006) demonstrated that background DOC does not significantly contribute to nickel speciation in synthetic waters, fulvic (FA) and humic acid concentrations were set to zero.

For the natural waters, similar inputs were used, except that for Ca, Mg, Na, K, SO_4, Cl and IC, measured values determined prior to testing were used. Dissolved trace metal concentrations of zinc, aluminium and iron (measured prior to testing) were also considered (see chapter 2 for computational details). The concentration of FA for input into WHAM VI was calculated as 0.8 x DOC (mg C/L), using the DOC concentration measured prior to testing. Details on the modeling of organic nickel complexation are given in chapter 2.

3.2.8. Model development

The protective effects of calcium and magnesium on the acute toxicity of nickel to *D. magna* were modeled as single-site BLM-type competition effects. The model parameters representing these effects (log K_{CaBL} and log K_{MgBL}) were determined according to the method described by De Schamphelaere and Janssen (2002), using the regression equations of the linear relationships between Ca^{2+} and Mg^{2+} activity and the 48-h $EC50_{Ni2+}$ (48-h EC50 expressed as Ni^{2+} activity). Only the effects of calcium and magnesium were included in the

model (see further). Therefore, the BLM equation for prediction of the $EC50_{Ni2+,i}$ for any test solution *i* can be written as follows (sensu De Schamphelaere and Janssen, 2002):

$$EC50_{Ni^{2+},i} = \frac{f_{NiBL}^{50\%}}{\left(1-f_{NiBL}^{50\%}\right).K_{NiBL}}.\left\{1+K_{CaBL}.\left(Ca^{2+}\right)_i + K_{MgBL}.\left(Mg^{2+}\right)_i\right\} \quad \text{Eq. 3.1}$$

In this model, $(Ca^{2+})_i$ and $(Mg^{2+})_i$ are the chemical activities of these ions in solution *i*, K_{CaBL} and K_{MgBL} are the parameters describing the protective effects of calcium and magnesium on nickel toxicity, $f_{NiBL}^{50\%}$ represents the fraction of the BL sites occupied by nickel at 50% effect and K_{NiBL} is the stability constant for binding of nickel to the BL. K_{NiBL} and $f_{NiBL}^{50\%}$ cannot be unambiguously derived from EC50 data alone. For reasons of comparability with other models (see further), it was considered appropriate to adopt the log K_{NiBL} of 4.0 proposed by Wu et al. (2003) and Keithly et al. (2004). The sensitivity parameter $f_{NiBL}^{50\%}$ was calculated as the geometric mean of $f_{NiBL,i}^{50\%}$ for each test solution *i* that was used for model development:

$$f_{NiBL,i}^{50\%} = \frac{K_{NiBL}.(Ni^{2+})_i}{1+K_{NiBL}.(Ni^{2+})_i + K_{CaBL}.(Ca^{2+})_i + K_{MgBL}.(Mg^{2+})_i} \quad \text{Eq. 3.2}$$

In this equation, $(Ni^{2+})_i$ is the 48-h $EC50_{Ni2+}$ observed in test solution *i* (calculated using WHAM VI software).

3.3. Results

3.3.1. Effects of calcium and magnesium on nickel toxicity

An increase of (nominal) calcium and magnesium concentration from 0.25 to 3.0 mM (total hardness = 50 to 325 mg $CaCO_3$/L) resulted in a 3.0-fold (1.82 to 5.50 mg/L) and 1.8-fold (2.26 to 4.06 mg/L) increase of 48-h $EC50_{Nidiss}$, respectively (Table 3.1). A further increase of (nominal) calcium and magnesium concentration to 5.0 mM (total hardness = 525 mg $CaCO_3$/L) resulted in a decrease of the 48-h $EC50_{Nidiss}$ to 2.28 mg/L and 3.51 mg/L, respectively. Speciation calculations (WHAM VI) demonstrated that the average nickel species distribution at the 48-h EC50 levels observed in the calcium and magnesium test

series was as follows: 96.7% Ni^{2+}, 2.1% $NiSO_4$ and < 1% $NiHCO_3^+$, $NiCO_3$, $Ni(OH)_2$, $NiOH^+$ and $NiCl^+$.

Figure 3.1 shows the linear relationships between 48-h $EC50_{Ni2+}$ and Ca^{2+} and Mg^{2+} activity. The data points of the tests conducted at the two highest calcium and magnesium concentrations were not taken into account in the regression. Similar to what was observed based on 48-h $EC50_{Nidiss}$ and total calcium and magnesium concentrations, 48-h $EC50_{Ni2+}$ increased with increasing Ca^{2+} and Mg^{2+} activities (Table 3.1). This could be expected, since calcium and magnesium do not affect nickel speciation to a large extent.

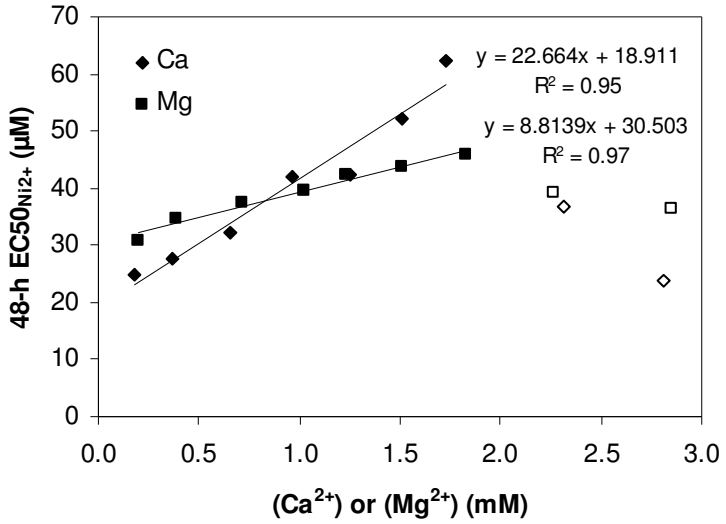

Figure 3.1. 48-h $EC50_{Ni2+}$ (expressed as Ni^{2+} activity, µM) as a function of Ca^{2+} and Mg^{2+} activity (mM). Open symbols represent tests in which nickel toxicity may have been influenced by additional stress due to elevated calcium and magnesium concentrations. These tests were not used for model development. The regression lines through the remaining points were used for modeling the effects of calcium and magnesium on nickel toxicity.

3.3.2. Effect of sodium on nickel toxicity

Within the investigated concentration range of 0.078 to 14 mM, sodium did not significantly affect nickel toxicity to *D. magna* (Table 3.1).

3.3.3. Effect of pH on nickel toxicity

Comparison of the 48-h EC50s obtained in $NaHCO_3$- versus MOPS-buffered test solutions demonstrates that, within the pH range tested, the buffering method did not significantly alter nickel toxicity to *D. magna*.

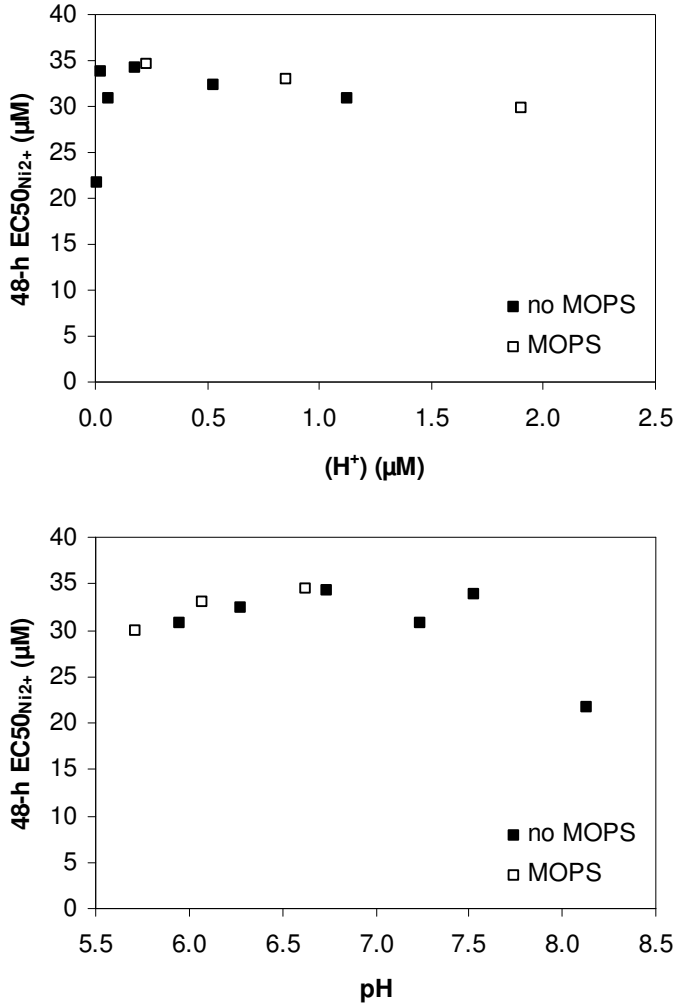

Figure 3.2. The effect of pH on the acute toxicity of nickel to *D. magna*. Upper panel: 48-h $EC50_{Ni2+}$ (expressed as Ni^{2+} activity, µM) as a function of H^+ activity (µM); lower panel: 48-h $EC50_{Ni2+}$ (µM) as a function of pH.

Overall, an increase of pH from 5.7 to 8.1 resulted in a gradual increase of 48-h $EC50_{Nidiss}$ from 2.32 to 3.27 mg/L (Table 3.1). In the tests conducted at a pH between 5.7 and 7.5 (Table 3.1), between 87.5 and 96.8% of the dissolved nickel was calculated to occur as Ni^{2+}. $NiHCO_3^+$, $NiCO_3$, $Ni(OH)_2$ and $NiOH^+$ all represented less than 2% of the dissolved nickel. However, in the test conducted at pH 8.1 (Table 3.1), nickel-carbonate complexes

became increasingly important at the expense of Ni^{2+}. $NiHCO_3^+$ and $NiCO_3$ represented 16.5 and 29.0% of the dissolved nickel, respectively, while the contribution of Ni^{2+} in the nickel species distribution decreased to 52.5%. Because of the decreased relative occurrence of Ni^{2+}, the 48-h $EC50_{Ni2+}$ of the test conducted at pH 8.1 was the lowest of all 48-h $EC50_{Ni2+}$ observed in the pH test series, while the 48-h $EC50_{Nidiss}$ for this test was the highest (Table 3.1).

The nonlinear relationship between 48-h $EC50_{Ni2+}$ and H^+ activity is shown in Figure 3.2 (upper panel). Nickel toxicity was almost not affected within the pH range of 5.7 to 7.5. Only a further increase of pH up to 8.1 resulted in increased toxicity. Overall, the effect of pH appears to be of limited importance, as reflected by a factor 1.6 difference between the highest (pH 6.6) and the lowest (pH 8.1) 48-h $EC50_{Ni2+}$.

3.3.4. Model development

Based on the regression lines shown in Figure 3.1, a log K_{CaBL} and log K_{MgBL} of 3.10 and 2.47 were calculated, respectively. As mentioned in the materials and methods section, the log K_{NiBL} was assumed to be 4.0. The sensitivity parameter $f_{NiBL}^{50\%}$ was determined to be 0.205 (theoretically meaning that 20.5% of the BL sites have to be occupied by nickel to cause 50% effect). All model parameters are summarized in Table 3.3 (see further). To investigate how well the model is calibrated to the dataset used for model development, the 48-h $EC50_{Ni2+}$ for each test solution was predicted using Equation 3.1, translated to the 48-h $EC50_{Nidiss}$ using the WHAM VI software, and compared to the observed 48-h $EC50_{Nidiss}$ (Figure 3.3). All 48-h $EC50_{Nidiss}$ were predicted by an error of less than factor 2 (average and maximum prediction error = factor 1.2 and 1.6, respectively).

3.3.5. Model validation – natural waters

The 48-h $EC50_{Nidiss}$ obtained in the nickel-spiked surface waters varied between 0.86 and 6.30 mg/L (Table 3.2). These waters cover a large range of DOC (1.75 to 25.8 mg C/L), water hardness (13.2 to 266 mg $CaCO_3$/L), calcium (3.03 to 72.7 mg/L), magnesium (1.07 to 20.6 mg/L), pH (5.94 to 8.09) and alkalinity (0.390 to 161 mg $CaCO_3$/L) (Table 3.2). Speciation calculations (WHAM VI) demonstrated that at the 48-h EC50 levels, between 59.1

and 94.6% of the dissolved nickel occurred as free Ni^{2+}, the remainder being primarily complexed by DOC (5.2 to 23.1%) and – at pH levels > 7.5 – carbonate (up to 15.0 % $NiHCO_3^+$ and 17.1% $NiCO_3$). The predictive capacity of the model described above is illustrated in Figure 3.3. For 15 out of 16 test waters, the 48-h $EC50_{Nidiss}$ was predicted by an error of less than factor 2 (average and maximum prediction error = factor 1.5 and 2.5, respectively).

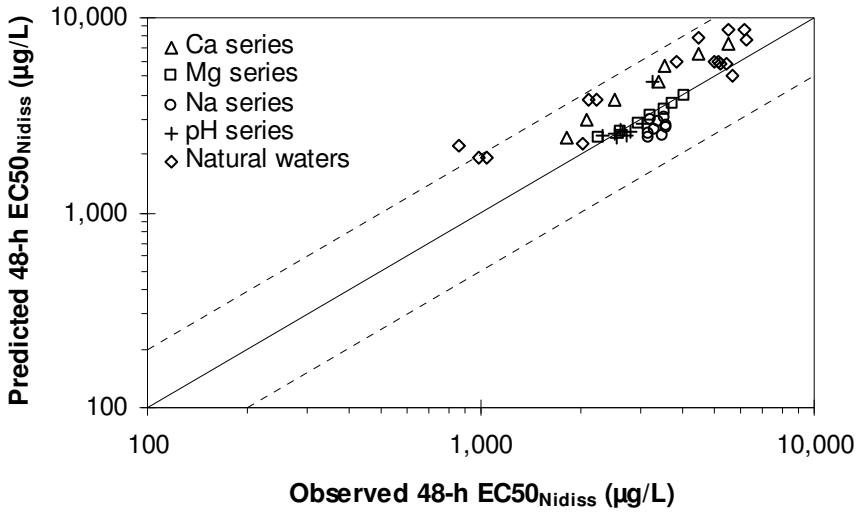

Figure 3.3. Predicted 48-h $EC50_{Nidiss}$ versus observed 48-h $EC50_{Nidiss}$ (expressed as dissolved nickel, µg/L) for the synthetic test solutions used for model development and the nickel-spiked surface waters used for model validation. Predictions were made using the model parameters shown in Table 3.3 and Equation 3.1 linked to WHAM VI. The solid line indicates a perfect match between predicted and observed EC50s; the dashed lines indicate ratios of 0.5 and 2 between observed and predicted EC50s.

3.4. Discussion

3.4.1. Effects of calcium and magnesium on nickel toxicity

Although calcium and magnesium concentrations up to 3.0 mM increasingly protected *D. magna* against acute nickel exposure, this protection seemed to decrease when calcium and magnesium concentrations further increased to 5.0 mM. For zinc, Heijerick et al. (2002b) observed a similar decrease of 48-h EC50s at calcium and magnesium concentrations higher than 3.0 and 2.0 mM, respectively. Further, Heijerick et al. (2005a) noted an increase of reproductive toxicity of zinc to *D. magna* at calcium concentrations above 3.0 mM and at 5.0 mM the net reproduction of control organisms was significantly reduced.

In this study, survival of control animals was not affected in the toxicity tests conducted at the highest water hardness levels. This is in agreement with the findings of Cowgill and Milazzo (1990) that *D. magna* was not adversely effected at these water hardness levels. However, for *D. magna* exposed to additional stress factors, such as increased nickel concentrations, the costs for coping with both high hardness levels and increased nickel concentrations may be higher than the beneficial effect of water hardness on nickel toxicity. This may explain the lower than expected 48-h EC50s at 4.0 and 5.0 mM calcium and magnesium. Consequently, the results of these tests were not used for model development.

Chapman et al. (1980) observed increasing 48-h $EC50_{Nidiss}$ (0.63 to 4.97 mg/L) for *D. magna* in well waters with adjusted waters hardness levels ranging from 50 to 200 mg $CaCO_3$/L. For four out of five tests, the 48-h $EC50_{Nidiss}$ were within a factor 2 of the values we observed at similar water hardness levels. Keithly et al. (2004) exposed *C. dubia* to nickel-spiked synthetic test solutions with water hardness levels between 50 and 253 mg $CaCO_3$/L and observed an increase of 48-h $EC50_{Nidiss}$ from 81 to 400 µg/L. The higher sensitivity of *C. dubia* to nickel corroborates with earlier observations that smaller organisms tend to be more sensitive to metals than larger ones (Grosell et al., 2002). Bossuyt and Janssen (2005) and Muyssen et al. (2005) provided evidence for a size-sensitivity relationship within the Cladocera (the family of the Chydoridae – sediment dwellers – not included) for copper and zinc, respectively.

Both Chapman et al. (1980) and Keithly et al. (2004) demonstrated the protective effect of water hardness (calcium and magnesium combined) on the toxicity of nickel to daphnids. However, the present study is the first to demonstrate that both calcium and magnesium offer protection to *D. magna*, individually. The protective effect of magnesium is most likely due to its central role in the mechanism of nickel toxicity. Pane et al. (2003b) demonstrated that acute nickel exposure reduces both whole body Mg^{2+} concentration and unidirectional Mg^{2+} uptake rate in *D. magna*. These observations suggest the existence of a shared uptake pathway for Mg^{2+} and Ni^{2+}.

This is supported by the fact that (i) Ni^{2+} and Mg^{2+} have similar dehydrated ionic radii (0.066 and 0.069 nm, respectively) (Weast, 1973), (ii) Ni^{2+} is a competitive inhibitor of Mg^{2+} uptake via three different types of magnesium transporters in the prokaryote *Salmonella typhimurium* (Snavely et al., 1991), (iii) Mg^{2+} inhibits Ni^{2+} uptake into brush border

membrane vesicles isolated from the kidney of rainbow trout (Pane et al., 2006a,b), and (iv) nickel-magnesium interactions are also well known from the mammalian literature (discussed by Pane et al., 2003b), suggesting that Mg^{2+} transport systems are highly conserved throughout evolution.

Snavely et al. (1991) demonstrated that Ca^{2+} (although to a lesser extent than Mg^{2+}) also competes with Ni^{2+} for uptake through all identified magnesium transport systems in *S. typhimurium*. Consequently, one may expect that calcium also protects directly against nickel toxicity. However, the protective effect of calcium may be primarily due to its function in the regulation of membrane permeability and ion transport (e.g., McWilliams, 1983; Hunn, 1985). Through its role in the tightening of paracellular junctions (e.g., Evans, 1987), calcium may protect indirectly against nickel toxicity, by reducing the efflux of magnesium through these junctions. Although magnesium also plays a role in the regulation of membrane permeability (Ebel and Günther, 1980), from a purely chemical point of view (e.g., the Irving-Williams order of cation binding to ligands), it is expected that the protection offered by magnesium through this mechanism will be weaker than that of calcium (Schwartz and Playle, 2001).

3.4.2. Effect of sodium on nickel toxicity

The observation that sodium did not modify nickel toxicity to *D. magna* is in line with the fact that there are no known indications for sodium to be involved in the mechanism of nickel uptake or toxicity.

3.4.3. Effect of pH on nickel toxicity

As De Schamphelaere et al. (2004b) already demonstrated for copper and zinc, the buffering method ($NaHCO_3$ versus MOPS) did not significantly alter nickel toxicity to *D. magna*. Consequently, the results of both pH test series were pooled for evaluation of the pH effect.

Speciation calculations demonstrated that acute nickel toxicity to *D. magna* remained relatively unaffected within the pH range of 5.7 to 7.5, and that a further increase of pH up to 8.1 resulted in increased toxicity (Figure 3.2). Nonlinear relationships between metal toxicity

and H^+ activity were also observed for long-term nickel toxicity to rainbow trout (chapter 2), acute copper toxicity to *D. magna* (De Schamphelaere and Janssen, 2002; De Schamphelaere et al., 2002) and *Hyalella azteca* (Borgmann et al., 2005) and chronic zinc toxicity to *D. magna* (Heijerick et al., 2005a). Such relationships can not be modeled as single-site competitive effects of H^+ and have to be dealt with in another suitable way.

To explain copper bioavailability at pH > 8, De Schamphelaere et al. (2002) had to account for toxicity of $CuCO_3$ in their acute copper BLM for *D. magna*. For nickel, Hoang et al. (2004) suggested that $NiHCO_3^+$ and $NiCO_3$ (both increasingly present at increasing pH, see results section) might be bioavailable. However, according to the method described by De Schamphelaere et al. (2002), $NiHCO_3^+$ and $NiCO_3$ were calculated to be about 1.1-fold more and 1.5-fold less toxic than Ni^{2+}, respectively. This is rather unlikely if only chemical binding to the biotic ligand (BL) would be involved. Based on chemical complex formation theory, $NiHCO_3^+$ and $NiCO_3$ should have a much lower affinity for ligands than the Ni^{2+} ion.

A possible explanation for the apparent toxicity of nickel-carbonate complexes may be that they dissociate in the physicochemically different gill microenvironment, hereby increasing the concentration of Ni^{2+} available for binding to the BL. It is also worth mentioning the study of Günther et al. (1986), in which a magnesium transporter in Yoshida ascites tumor cells was identified to be an electro-neutral Mg^{2+}/HCO_3^- symporter. If such a transporter also exists in the gill structures of Crustacea (Kikuchi, 1983) and Ni^{2+} could be transported by it, dissociation of $NiHCO_3^+$ in the gill microenvironment would definitely result in a higher than expected uptake of Ni^{2+}. Overall, increased HCO_3^- concentrations, occurring at increased pH and/or alkalinity, would enhance the uptake and toxicity of Ni^{2+}.

It is clear that more detailed research in this direction is needed to improve our understanding of the direct cause of the higher than expected nickel toxicity at elevated pH levels. Because of the need for further research and the fact that the overall variation in toxicity within the investigated pH range (5.7 to 8.1) was relatively small (factor 1.6), it was decided not to incorporate the effect of pH into the model yet.

Although pH appeared to be of limited importance in modifying acute nickel toxicity to *D. magna*, Schubauer-Berigan et al. (1993) observed a decrease of 48-h $EC50_{Nitotal}$ (expressed as total recoverable nickel) for *C. dubia* from > 200 to 13 µg/L when pH was

increased from 6.0-6.5 to 8.5-8.7 (representing an increase of toxicity by a factor of at least 15). Although it is possible that the effect of pH on acute nickel toxicity is more important for *C. dubia*, comparison of the data reported by Schubauer-Berigan et al. (1993) with our data for *D. magna* is hampered by several factors (see further).

3.4.4. Effect of DOC on nickel toxicity

The low fraction of Ni^{2+} bound to DOC suggests that DOC is of minor importance in determining acute nickel toxicity to *D. magna*. According to Wu et al. (2003), this would overall be the case at acutely toxic nickel concentrations, which are relatively high compared to those reported for metals such as copper and zinc. For *D. magna*, the weak correlation (R^2 = 0.33) between 48-h $EC50_{Nidiss}$ and DOC concentration confirms this suggestion.

3.4.5. Development of a nickel bioavailability model

Model performance was very good both in the artificial waters used for model development and in the nickel-spiked surface waters used for the validation experiments (Figure 3.3). Toxicity was underestimated by a factor > 2 (2.5) for Bihain (03/03) and by factors close to 2 for Bihain (05/01) and Clywedog. Compared to the other test waters, waters from Bihain and Clywedog were characterized by very low magnesium concentrations (between 1.1 and 1.5 mg/L, Table 3.2), which were much lower than the lowest magnesium concentration in the synthetic test solutions used for model development (i.e. 6.05 mg/L, Table 3.1).

Since acute exposure to nickel is known to reduce both whole body Mg^{2+} and the unidirectional Mg^{2+} uptake rate in *D. magna* (Pane et al., 2003b), the extremely low magnesium concentrations of these surface waters may have caused the daphnids to be overly sensitive to nickel stress. Moreover, according to Cowgill and Milazzo (1991), *D. magna* may have experienced stress anyhow when exposed to water hardness levels as low as in Bihain (03/03) and Clywedog (water hardness = 13.6 and 13.2 mg $CaCO_3$/L, respectively). Clearly, care should be taken when using the model outside its physicochemical boundaries. This is especially the case for use in soft waters (water hardness < 25 mg $CaCO_3$/L) and/or waters with extremely low magnesium concentrations.

3.4.6. Model application – literature data for *D. magna* and *C. dubia*

To evaluate the applicability to literature data (Chapman et al., 1980; Schubauer-Berigan et al., 1993; Keithly et al., 2004) the model was calibrated to each dataset by optimizing the sensitivity parameter $f_{NiBL}^{50\%}$ (to account for interclonal or interspecies sensitivity differences) and the accuracy of the model in predicting the reported 48-h EC50s was thoroughly examined. For each dataset, speciation calculations were conducted as described in the materials and methods section.

It should be mentioned that while analyzing the datasets of Chapman et al. (1980) and Schubauer-Berigan et al. (1993), several difficulties were encountered that may have increased the uncertainty around the predictions made by the model. For instance, in the study of Chapman et al. (1980), not all experiments were conducted simultaneously, other parameters (such as pH) co-varied with water hardness, and organisms were acclimated (for a non-reported duration) to the water hardness under consideration before being tested. In the study of Schubauer-Berigan et al. (1993), 48-h EC50s were based on total recoverable nickel instead of on dissolved nickel, and test organisms were fed (at 6 mg/L final concentration) using a YCT (Yeast-Cerophyll-Trout chow) suspension. The addition of food to acute toxicity tests with daphnids is not a common practice and may alter metal toxicity and bioavailability.

Although Chapman et al. (1980) also carried out experiments with *D. magna*, toxicity was systematically underpredicted when the $f_{NiBL}^{50\%}$ calculated for our *D. magna* clone (0.205, Table 3.3) was used (average and maximum prediction error = 2.7 and 5.1, respectively). Using an optimized $f_{NiBL}^{50\%}$ of 0.0950 (Table 3.3), the 48-h $EC50_{Nidiss}$ reported by Chapman et al. (1980) were predicted by an average error of factor 1.4, the maximum prediction error being 2.0 (Figure 3.4). Apparently, the *D. magna* clone used by Chapman et al. (1980) was significantly more sensitive than the clone used in the present study, requiring adjustment of $f_{NiBL}^{50\%}$ to account for interclonal sensitivity differences. Limited sensitivity differences between different clones of the same species are not unusual (e.g., Soares et al., 1992).

Table 3.3. Model parameters of the acute nickel bioavailability models for *D. magna* and *C. dubia* presented in this study, the Wu et al. (2003) study and the Keithly et al. (2004) study.

Model parameters	This study	Wu et al. (2003)	Keithly et al. (2004)
log K_{NiBL}	4.0 [a]	4.0	4.0
log K_{CaBL}	3.10	4.0	4.0
log K_{MgBL}	2.47	–	–
log K_{NaBL}	–	3.0	3.0
log K_{HBL}	–	7.5	6.7
Sensitivity parameter $f_{NiBL}^{50\%}$ for calibration to the following datasets:			
Present study – *D. magna*	0.205	0.0284	0.0652
Chapman et al. (1980) – *D. magna*	0.0950	0.00874 [b]	0.0185 [b]
Schubauer-Berigan et al. (1993) – *C. dubia*	0.00469	? [c]	0.000210 [b]
Keithly et al. (2004) – *C. dubia*	0.0108	– [d]	0.00192 [b]

[a] In the present study, the log K_{NiBL} was assumed to be equal to the log K_{NiBL} proposed by Wu et al. (2003) and adopted by Keithly et al. (2004). This assumption does not affect model predictions.

[b] This parameter was derived using the reported LA50 (lethal accumulation at the 50% effect level, expressed as nmol/$g_{wetweight}$) and the gill binding site density of 1000 nmol/$g_{wetweight}$, which was assumed by both Wu et al. (2003) and Keithly et al. (2004).

[c] No LA50 was mentioned in the report of Wu et al. (2003).

[d] No model calibration was performed.

Using an optimized $f_{NiBL}^{50\%}$ of 0.0108 (Table 3.3), the 48-h $EC50_{Nidiss}$ for *C. dubia* that were reported by Keithly et al. (2004) were predicted by an error between factor 1.2 and 1.6 (Figure 3.4). However, the effect of water hardness appears to be slightly underestimated by the model. For all predictions to be situated within the 95% confidence intervals of the observed 48-h $EC50_{Nidiss}$, an increase of both log K_{CaBL} and log K_{MgBL} to at least 3.3 would be required. Unfortunately, the available data do not allow derivation of reliable optimized binding constants. Nevertheless, although *C. dubia* appears to experience a slightly larger protection from increased water hardness compared to *D. magna*, this difference did not result in unacceptable prediction errors.

For the study of Schubauer-Berigan et al. (1993), the optimized $f_{NiBL}^{50\%}$ was calculated to be 0.00469 (Table 3.3). For comparison with earlier proposed models (Wu et al., 2003; Keithly et al., 2004; see further), the 48-h $EC50_{Nitotal}$ at pH 6.0-6.5 was assumed to be 200 µg/L instead of > 200 µg/L. For the tests conducted at pH 6.0-6.5 and 7.0-7.3, model predictions were within a factor 2 from observed toxicity. However, at pH 8.5-8.7, toxicity was underestimated 22-fold (Figure 3.4). This could be expected, since we did not incorporate the effect of pH into the model. To accurately predict the markedly higher toxicity of nickel at

elevated pH (and alkalinity) levels, the mechanisms determining nickel toxicity under these circumstances should be studied in further detail (see above).

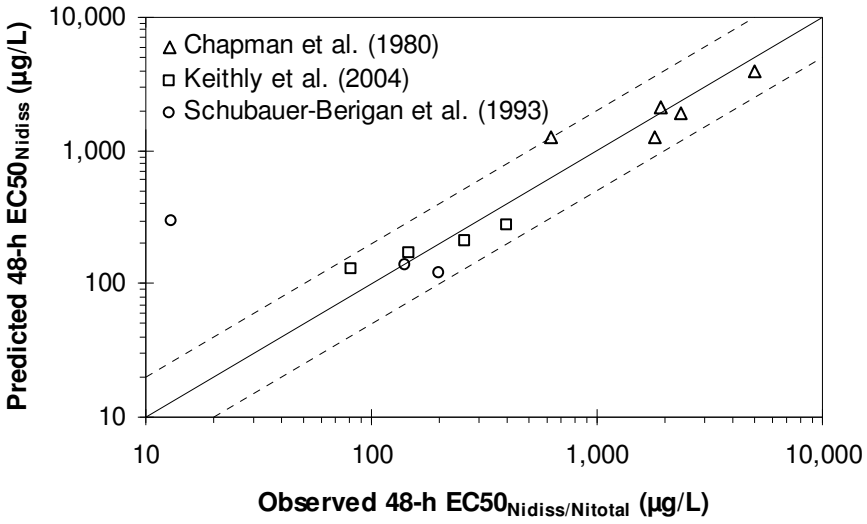

Figure 3.4. Applicability of the acute *D. magna* bioavailability model developed in this study to literature data on acute nickel toxicity to *D. magna* (Chapman et al., 1980) and *C. dubia* (Schubauer-Berigan et al., 1993; Keithly et al., 2004). Predicted 48-h $EC50_{Nidiss}$ (expressed as dissolved nickel, µg/L) were plotted against observed 48-h $EC50_{Nidiss}$ (µg/L). The 48-h EC50s reported by Schubauer-Berigan et al. (1993) were based on total recoverable nickel instead of on dissolved nickel concentrations (48-h $EC50_{Nitotal}$). Predictions were made using optimized model parameters (Table 3.3) and Equation 3.1 linked to WHAM VI. The solid line indicates a perfect match between predicted and observed EC50s; the dashed lines indicate ratios of 0.5 and 2 between observed and predicted EC50s.

3.4.7. Comparison with the models proposed by Wu et al. (2003) and Keithly et al. (2004)

Wu et al. (2003) presented a preliminary set of gill binding constants for nickel, calcium, sodium and protons, based on the fathead minnow gill accumulation data of Meyer et al. (1999) (Table 3.3). Assuming binding site density and binding constants to be the same as for fathead minnow, they calibrated the model to acute toxicity data for *D. magna* (Chapman et al., 1980) and *C. dubia* (Schubauer-Berigan et al., 1993) by adjusting the sensitivity parameter LA50 (translated to $f_{NiBL}^{50\%}$ in Table 3.3). Keithly et al. (2004) lowered the log K_{HBL} (Table 3.3) and evaluated the applicability of the model to the same datasets as well as to their own dataset concerning the hardness-dependent acute toxicity of nickel to *C. dubia*.

Although the models presented by Wu et al. (2003) and Keithly et al. (2004) seem to provide a reasonably good simulation of the concerned data, two important remarks need to be made on these studies. First, the data used for model calibration only cover two nickel toxicity modifying factors, i.e. water hardness (calcium and magnesium combined, Chapman et al., 1980; Keithly et al., 2004) and pH (Schubauer-Berigan et al., 1993). Second, the set of binding constants (for nickel, calcium, sodium and protons) was based on gill nickel accumulation data for fathead minnow, taken from a study in which only the effect of calcium on nickel accumulation on the gill was studied (Meyer et al., 1999), hence not justifying the inclusion of a log K_{HBL} and a log K_{NaBL} into the models.

A comparison of the applicability of all models (present study; Wu et al., 2003; Keithly et al., 2004) to the datasets of Chapman et al. (1980) and Schubauer-Berigan et al. (1993) demonstrates that all models perform equally well for the data of Chapman et al. (1980), but that their performance exhibits slight differences for the data of Schubauer-Berigan et al. (1993). Although none of the models was capable of accurately predicting nickel toxicity in the test performed at pH 8.5-8.7, the models of Wu et al. (2003) and Keithly et al. (2004) performed slightly better due to the incorporation of a log K_{HBL}. However, the poor predictions at pH 8.5-8.7 demonstrate that modeling single-site competition between H^+ and Ni^{2+} is not appropriate to describe the effect of pH. It is recommended to investigate nickel toxicity at elevated pH and/or alkalinity levels more closely since clearly, mechanisms other than proton competition are involved in determining nickel toxicity at varying pH levels (see above).

The performance of the models proposed by Wu et al. (2003) and Keithly et al. (2004) was further evaluated by calibrating both models to the *D. magna* data generated in the present study (by adjusting the sensitivity parameter $f_{NiBL}^{50\%}$, see Table 3.3). Comparing the applicability of both models to the dataset generated in this study (Figure 3.5), the following observations were made.

First, a comparison of average prediction errors (Wu et al., 2003: 1.9; Keithly et al., 2004: 1.6; present study: 1.3), maximum prediction errors (7.3, 3.4 and 2.5, respectively) and number of 48-h $EC50_{Nidiss}$ predicted by an error > 2 (10/48, 12/48 and 1/48, respectively)

demonstrated that the models presented by Wu et al. (2003) and Keithly et al. (2004) are less accurate than the newly developed model.

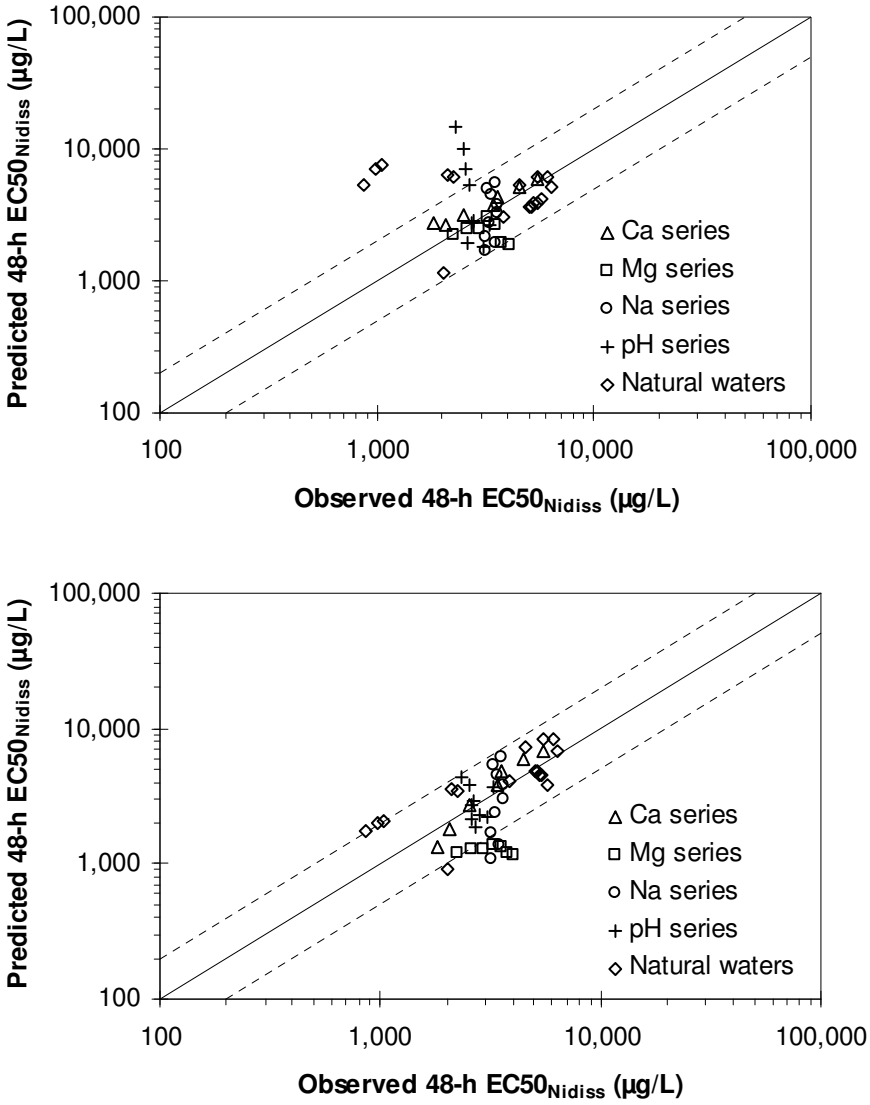

Figure 3.5. Applicability of the acute bioavailability models proposed by Wu et al. (2003) (upper panel) and Keithly et al. (2004) (lower panel) to the *D. magna* data generated in the present study. Predicted 48-h $EC50_{Nidiss}$ (expressed as dissolved nickel, µg/L) were plotted versus observed 48-h $EC50_{Nidiss}$ (µg/L). Predictions were made using optimized sensitivity parameters (Table 3.3) and Equation 3.1 linked to WHAM VI. The solid line indicates a perfect match between predicted and observed EC50s; the dashed lines indicate ratios of 0.5 and 2 between observed and predicted EC50s.

Second, the slightly better performance of the model developed by Keithly et al. (2004) versus the model developed by Wu et al. (2003) is due to a somewhat lower log K_{HBL}. Since almost no variation in toxicity was observed in the pH range of 5.7-7.5, modeling the

effect of pH as a single-site proton competition effect results in an overestimation of toxicity at higher pH levels and an underestimation at lower pH levels. Therefore, it was considered more appropriate (at least for *D. magna*) not to model the effect of pH at all until more detailed knowledge on the involved mechanisms has become available.

Third, the effect of calcium was fairly well predicted by both models. On the contrary, the variation in toxicity observed in the magnesium test series was poorly predicted due to the absence of a log K_{MgBL} in both models. This confirms the importance of taking into account the effect of magnesium and to investigate the toxicity mitigating effects of calcium and magnesium separately.

Fourth, since sodium was demonstrated not to influence the acute toxicity of nickel to *D. magna*, both the Wu et al. (2003) and the Keithly et al. (2004) model underestimated toxicity at higher sodium concentrations and overestimated toxicity at lower sodium concentrations.

Finally, the performance of both models in natural waters was quite variable. Most remarkable was that due to the incorporation of a log K_{HBL}, nickel toxicity was underestimated in all low-pH natural waters tested (e.g., Bihain, Clywedog).

The present calibration exercise draws the attention to some important shortcomings of the models proposed by Wu et al. (2003) and Keithly et al. (2004) that, however, could not have been detected by calibration of these models to the literature data available at the time they were developed. Overall, the present study has offered a solution to most of these shortcomings, as demonstrated by the good predictive capacity of the developed model in both synthetic and natural waters with a wide range of physicochemical characteristics.

3.5. Conclusion

In this study, a nickel bioavailability model was developed based on the results of a series of acute experiments with *D. magna* in which the individual effects of calcium, magnesium, sodium and pH were investigated. The protective effects of calcium and magnesium were modeled as BLM-type single-site competition effects represented by a log K_{CaBL} and a log K_{MgBL} of 3.10 and 2.47, respectively. Since sodium was not observed to affect

nickel toxicity, no log K_{NaBL} was incorporated into the model. The effect of pH was also not incorporated, because the overall variation in toxicity observed within the tested pH range was relatively small, and because the mechanisms by which pH and/or alkalinity affect nickel toxicity should be further studied to allow a true description of the observed toxicity at elevated pH and alkalinity levels.

The model was capable of predicting toxicity by an error less than factor 2 in all synthetic test solutions used for model development as well as in 15 out of 16 nickel-spiked surface waters covering a broad range of water chemistry. The model was also successfully calibrated to literature data concerning the effect of water hardness on acute nickel toxicity to *D. magna* (Chapman et al., 1980) and *C. dubia* (Keithly et al., 2004). The applicability of the model was demonstrated to be better than that of previously proposed models (Wu et al., 2003; Keithly et al., 2004) that include a log K_{NaBL}, a log K_{HBL} and a log K_{CaBL}, but no log K_{MgBL}. An in-depth comparison of these models demonstrated that (i) there is no need to incorporate a log K_{NaBL}, (ii) it is important to recognize the protective effect of magnesium, and (iii) the incorporation of a log K_{HBL} does not satisfactorily describe the effect of pH. Although the presented model seems very promising, further research, especially into the effects pH and alkalinity, would be needed to allow model refinement.

Chapter 4

Modeling chronic nickel bioavailability and toxicity to *Daphnia magna*

Redrafted from

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Chapter 4

Modeling chronic nickel bioavailability and toxicity to *Daphnia magna*

Abstract. In the present study, the individual effects of calcium, magnesium and pH on the chronic toxicity of nickel to *Daphnia magna* were examined in a series of 21-d reproduction tests in synthetic test solutions. Based on the linear increase of 21-d $EC50_{Ni2+}$ (21-d EC50 expressed as Ni^{2+} activity) with increasing activities of Ca^{2+} and Mg^{2+}, the effects of calcium and magnesium were modeled according to single-site competition with log K_{CaBL} = 3.53 and log K_{MgBL} = 3.57. Because the increase of 21-d $EC50_{Ni2+}$ with increasing H^+ activity was nonlinear, the effect of pH could not be appropriately described by single-site competition between Ni^{2+} and H^+. Instead, the effect of pH was modeled based on an empirical linear relationship between pH and 21-d $EC50_{pNi2+}{}^*$ (= – log (21-d $EC50_{Ni2+}$ corrected for the presence of calcium and magnesium)) and was superimposed on the effects of calcium and magnesium. For all test solutions used for model development, the developed model predicted the observed 21-d $EC50_{Nidiss}$ (21-d EC50 expressed as dissolved nickel concentration) by an error of less than factor 2. The importance of dissolved organic carbon (DOC) in protecting *D. magna* against chronic nickel toxicity was demonstrated by conducting 21-d reproduction tests in a series of nickel-spiked surface waters. These tests also allowed evaluating the predictive capacity of the model in natural waters. The model tended to overestimate chronic nickel toxicity in the natural waters tested. This may have been due to (i) temporal nickel sensitivity shifts in the *D. magna* clone used for testing, (ii) potential interactive effects of pH and calcium and/or magnesium, and/or (iii) a protective effect of DOC beyond its effect on nickel speciation. Although the initial model presents a useful tool for assessing the risk of nickel to aquatic organisms, the exact mechanisms underlying the effect of water chemistry on chronic nickel toxicity to *D. magna* should be further investigated to allow future model refinement.

4.1. Introduction

Crustaceans such as the standard test organisms *Ceriodaphnia dubia* and *Daphnia magna* have been reported to be much more sensitive to nickel than fish (Kszos et al., 1992). Chronic nickel toxicity data for crustaceans are therefore considered essential for risk assessment purposes. Although several studies have investigated chronic nickel toxicity to *D.*

magna (Biesinger and Christensen, 1972; Chapman et al., 1980; Kühn et al., 1989; Münzinger, 1990; Münzinger and Monicelli, 1991; Enserink et al., 1991; Kszos et al., 1992; Pane et al., 2003b, 2004c) and *C. dubia* (Kszos et al., 1992; Keithly et al., 2004), only three of these studies have dealt with nickel bioavailability (Chapman et al., 1980; Kszos et al., 1992; Keithly et al., 2004). These studies all investigated chronic nickel toxicity as a function of water hardness (calcium and magnesium combined). The individual effects of calcium and magnesium and possible other nickel toxicity modifying factors, such as pH and dissolved organic carbon (DOC), have not been studied so far.

The limited knowledge concerning nickel bioavailability clearly hampers the development of a chronic nickel bioavailability model for crustaceans. This type of model is needed for science-based risk assessments and site-specific water quality criteria setting. The major research goals of the present study were therefore (i) to investigate nickel bioavailability as a function of water chemistry in chronic exposures of *D. magna* to nickel, and (ii) to develop and validate a chronic nickel bioavailability model for *D. magna*.

First, the individual effects of calcium, magnesium and pH on chronic nickel toxicity to *D. magna* were investigated in 21-d reproduction tests conducted in artificial test solutions. Nickel toxicity has been reported to decrease with increasing water hardness (Chapman et al., 1980; Kszos et al., 1992; Keithly et al., 2004) but has not been studied as a function of individual increases of calcium and magnesium concentrations so far. Because of the central role of magnesium in the mechanism of nickel toxicity (Pane et al., 2003b) and the evidence that both acute nickel toxicity to *D. magna* and long-term nickel toxicity to rainbow trout decrease with increasing magnesium concentrations (chapter 2 and 3), we investigated the toxicity mitigating effects of calcium and magnesium separately.

Based on the results reported in chapter 3 and those reported by Schubauer-Berigan et al. (1993) on the effect of pH on acute nickel toxicity to *D. magna* and *C. dubia*, respectively, it was also considered necessary to investigate the effect of pH on chronic nickel toxicity to *D. magna*. The effect of sodium was not studied since there are no known indications for sodium to be involved in the mechanism of nickel uptake and/or toxicity. In chapter 3, evidence has already been provided that sodium does not affect acute nickel toxicity to *D. magna*.

In the second phase of this study, a chronic nickel bioavailability model was developed for *D. magna*. The predictive capacity of the model in natural waters was evaluated using results from chronic toxicity tests in nickel-spiked surface waters. These results also allowed us to evaluate the effect of DOC on nickel bioavailability, and the effect of simultaneous variation of calcium, magnesium and pH on chronic nickel toxicity to *D. magna*.

4.2. Materials and methods

4.2.1. Toxicity tests in synthetic test solutions

To investigate the individual effects of calcium, magnesium and pH on the chronic toxicity of nickel to *D. magna*, three test series were conducted. In each series the concentration of one cation was varied in a univariate matter (Table 4.1). Each test series consisted of six toxicity tests in which, next to a control treatment, animals were exposed to five different nickel concentrations. Tests of the same series were conducted simultaneously.

4.2.2. Preparation of synthetic test solutions

All test solutions were prepared using carbon filtered, deionized water (conductivity < 2 µS/cm) and reagent grade chemicals purchased from VWR International (Leuven, Belgium). The synthetic basic medium was composed of 0.25 mM $CaCl_2$, 0.25 mM $MgSO_4$, 0.078 mM KCl and 0.078 mM $NaHCO_3$. Calcium and magnesium concentrations were adjusted by adding $CaCl_2$ and $MgCl_2$ to the basic medium, respectively.

The pH was adjusted to pH 6.8 by adding 3.6 mM MOPS buffer (3-N-morpholinopropanesulfonic acid) and the required amount of dilute KOH / NaOH (magnesium test series / calcium test series) or HCl solution. MOPS is considered a suitable buffer for metal toxicity testing since it does not affect metal speciation (Kandegedara and Rorabacher, 1999) and it has been demonstrated not to be toxic and not to affect copper, zinc and nickel toxicity to freshwater species (De Schamphelaere et al., 2004b; chapter 3). In the tests conducted at pH 5.8, 6.4, 7.0 and 7.6, pH was controlled by adding MOPS buffer and NaOH or HCl solution. In the test conducted at pH 8.2 as well as in a second test conducted at pH 7.6 (both indicated as pH 7.6* and pH 8.2* throughout this chapter), pH was adjusted by

adding $NaHCO_3$. In all tests of the pH test series, sodium concentrations were adjusted to the same level through addition of NaCl.

For each toxicity test, a nickel concentration series was prepared by adding $NiCl_2$. Each test consisted of a control treatment and five nickel concentrations. All solutions were allowed to equilibrate for 24 hours at 20 °C prior to being used in the toxicity tests. The physicochemical composition of all synthetic test solutions is given in Table 4.1.

Table 4.1. Main physicochemical characteristics of the synthetic test solutions.

Test medium	pH	Major ions (mg/L)					Hardness [c]	Alkalinity [d]
		Ca	Mg	Na [b]	K [b]	Cl [b]	(mg $CaCO_3$/L)	(mg $CaCO_3$/L)
Ca 0.25 mM	6.85	7.23	5.74	46.0	3.05	20.5	41.7	2.96
Ca 0.5 mM	6.81	14.5	5.72	46.8	3.05	35.5	59.7	2.89
Ca 1.0 mM	6.81	29.6	5.66	47.7	3.05	70.9	97.1	2.89
Ca 1.5 mM	6.79	43.4	5.68	48.6	3.05	106	132	2.86
Ca 2.0 mM	6.79	58.4	5.65	49.4	3.05	142	169	2.86
Ca 3.0 mM	6.80	88.3	5.49	50.3	3.05	213	243	2.88
Mg 0.25 mM	6.79	7.29	5.97	1.79	86.9	20.5	42.8	2.86
Mg 0.5 mM	6.81	7.54	12.0	1.79	82.9	38.2	68.2	2.89
Mg 1.0 mM	6.82	7.54	23.8	1.79	79.8	73.7	117	2.91
Mg 1.5 mM	6.80	7.63	35.4	1.79	76.6	109	165	2.88
Mg 2.0 mM	6.80	7.37	47.4	1.79	72.0	145	213	2.88
Mg 3.0 mM	6.81	7.61	72.1	1.79	70.4	215	315	2.89
pH 5.8	5.87	10.0	6.91	80.5	3.05	132	53.4	0.652
pH 6.4	6.40	10.0	6.90	80.5	3.05	117	53.4	3.25
pH 7.0	6.97	10.1	6.88	80.5	3.05	68.4	53.5	11.0
pH 7.6	7.35	10.0	7.02	80.5	3.05	41.5	53.9	21.9
pH 7.6* [a]	7.62	9.92	7.11	80.5	3.05	123	54.0	36.5
pH 8.2* [a]	8.22	9.11	7.20	80.5	3.05	20.5	52.4	174

[a] In these tests pH was adjusted by adding $NaHCO_3$ instead of MOPS buffer and NaOH/HCl.

[b] No measurements were conducted for Na, K, SO_4 and Cl, hence nominal values are reported. Nominal SO_4 concentration was 24.0 mg/L in all tests.

[c] Water hardness was calculated from measured calcium and magnesium concentrations.

[d] Alkalinity was calculated from nominal added (calcium and magnesium test series) or measured (pH test series) inorganic carbon (IC) and measured pH, using thermodynamic stability constants taken from Stumm and Morgan (1996).

4.2.3. Toxicity tests in European surface waters

Surface waters with differing physicochemical characteristics (Table 4.2) were sampled at six locations in Europe. The variation in these six waters with regard to possible nickel toxicity modifying factors (calcium, magnesium, water hardness, DOC, pH) reasonably covered the physicochemical variation observed in European surface waters (Table 4.2). Sampling sites were not influenced by anthropogenic inputs. Samples were taken in March

2003 at the following locations: Ankeveen (NL), Bihain (B), Brisy (B), Markermeer (NL), Regge (NL) and Voyon (F). A short description of these locations is given in chapter 2 and 3.

At each location, the required amount of water was membrane filtered (0.45 µm) and collected in metal-free polyethylene vessels. The samples were immediately transferred to the laboratory, where they were stored at 4 °C in total darkness. Before testing, the following parameters were measured: pH (pH-meter P407, Consort, Turnhout, Belgium), DOC, inorganic carbon (IC) (TOC-5000, Shimadzu, Duisburg, Germany), Ca, Mg, Na, K, Al, Ni, Cu and Zn (ICP-OES, Perkin Elmer 3300 DV) and Cl, NO_3 and SO_4 (Ion Chromatography, Dionex QIC analyzer, IONPAC AS4A).

In all the surface waters with pH < 7.8, pH was controlled by adding MOPS (3.6 mM) and NaOH or HCl solution. In the waters from Markermeer and Voyon (pH > 7.8), the natural buffer capacity was observed to be sufficient to prevent large deviations from ambient pH. To obtain a nickel concentration series (control + five nickel concentrations), the waters were spiked with $NiCl_2$ and allowed to equilibrate for 24 hours before testing. This equilibration time was sufficiently long since it has been demonstrated that nickel speciation in natural waters reaches equilibrium after as little as 2 hours (Van Laer et al., 2006).

4.2.4. Testing

Chronic toxicity tests (21 days) were performed according to OECD guideline 211 (OECD, 1996). Test organisms originated from a healthy *D. magna* culture (clone K6), which has been maintained under controlled laboratory conditions in M4 medium (Elendt and Bias, 1990) for more than ten years. Dissolved nickel concentrations in the culture medium were always below 3 µg/L, i.e. the method detection limit (MDL) of the graphite furnace atomic absorption spectrometer used in this study (GF-AAS, SpectrAA800 with Zeeman background correction, Varian, Mulgrave, Australia). At the start of each test, ten juveniles (< 24 hours old) per concentration were individually transferred to polyethylene cups containing 50 mL of test medium. Animals were fed daily with an algal mix of *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* in a 3:1 ratio (on a cell number basis). Each organism received 8 x 10^6 cells per day in the first week, 16 x 10^6 cells per day in the second week and 24 x 10^6 cells per day in the third week of exposure. Every other day, the medium was renewed, parent mortality recorded, and the number of produced juveniles counted.

Table 4.2. Main physicochemical characteristics of the sampled surface waters.

Site ID (country)	pH	Major ions (mg/L)						Hardness [a]	Alkalinity [b]	DOC	Ni_{diss} [c]
		Ca	Mg	Na	K	Cl	SO_4	(mg $CaCO_3$/L)	(mg $CaCO_3$/L)	(mg C/L)	(µg/L)
Ankeveen (NL)	6.79	38.3	6.54	64.0	6.08	50.0	127	123	12.3	17.3	4.0
Bihain (B)	6.15	3.68	1.07	21.7	0.872	15.2	4.01	13.6	8.36	5.37	2.5
Brisy (B)	7.09	4.99	3.38	75.9	2.06	23.3	1.00	26.4	12.8	2.53	2.0
Markermeer (NL)	8.09	52.7	14.0	89.1	8.71	318	109	189	127	7.49	1.0
Regge (NL)	7.71	60.1	7.96	121	10.4	144	63.0	183	161	9.87	4.0
Voyon (F)	8.02	37.1	7.13	102	1.32	21.2	20.4	122	122	4.17	6.0

The variation in these six waters with regard to possible nickel toxicity modifying factors (pH, calcium, magnesium, water hardness, DOC) reasonably covered the physicochemical variation observed in European surface waters. According to the data included in the FOREGS database (Forum of the European Geological Surveys Directors, http://www.gtk.fi/publ/foregsatlas), the 10th-90th percentile ranges for these water chemistry variables are as follows in European surface waters → pH: 6.4-8.3, Ca: 2.78-118 mg/L, Mg: 0.753-27.1 mg/L, DOC: 0.924-17.0 mg C/L).

[a] Water hardness was calculated from measured calcium and magnesium concentrations.

[b] Alkalinity was calculated from measured inorganic carbon (IC) concentrations and pH, using thermodynamic stability constants taken from Stumm and Morgan (1996).

[c] Background dissolved nickel concentration.

4.2.5. Chemical analyses

Water temperature and oxygen saturation were measured daily using an immersion electrode. The pH (pH-meter P407, Consort, Turnhout, Belgium) of each test solution was measured at the start of testing and from then on before and after each medium renewal. The glass electrode was calibrated with pH 4, pH 7 and pH 10 buffers (Merck, Darmstadt, Germany). The lowest and the highest pH measurement made during a test were not allowed to deviate by more than 0.3 pH units from each other.

Dissolved nickel concentrations (filtration through a 0.45 µm filter, Gelman Sciences, Ann Arbor, MI, USA) and total calcium and magnesium concentrations were measured once in each solution prepared for medium renewal and once a week in 'used' test medium. Samples were acidified with 0.14 N HNO_3. Calcium and magnesium concentrations were measured using flame atomic absorption spectrometry (F-AAS, SpectrAA100, Varian, Mulgrave, Australia). Dissolved nickel concentrations were measured using F-AAS for nickel concentrations above 100 µg/L and GF-AAS for nickel concentrations below 100 µg/L. Measurement procedures were the same as described in chapter 2.

Na, K, Cl and SO_4 concentrations were not measured during the tests since previous studies at our laboratory indicated that measured values of these parameters typically deviate less than 10% from nominal values. Inorganic carbon concentrations were measured in the pH test series only. No samples were taken for measurement of DOC concentrations during the tests. Alkalinity was calculated from nominal (calcium and magnesium test series) or measured (during the test: pH test series; before testing: natural waters) IC concentrations and measured pH using thermodynamic stability constants taken from Stumm and Morgan (1996).

4.2.6. Data treatment and statistics

NOECs and LOECs (i.e. no observed and lowest observed effect concentrations, respectively) for the endpoint mortality ($NOEC_m$ and $LOEC_m$) were calculated using Fisher's Exact Test (Finney, 1948; Pearson and Hartley, 1962) ($p < 0.05$). LC50s (i.e. concentrations resulting in 50% mortality) and their 95% confidence intervals were calculated using the trimmed Spearman-Karber method (Hamilton, 1977). The observed mortality at each measured nickel concentration was used as input for the calculations. NOECs and LOECs for

the endpoint reproduction ($NOEC_r$ and $LOEC_r$) were derived using the Mann Whitney U test ($p < 0.05$). EC10s, EC20s, EC50s (i.e. concentrations resulting in 10, 20 and 50% reduction of reproduction, respectively) and their 95% confidence intervals were calculated with a log-logistic model described by De Schamphelaere and Janssen (2004a) using the Levenberg-Marquardt algorithm (Levenberg, 1944; Marquardt, 1963). The total individual reproduction of all organisms at each measured nickel concentration was used as input for the calculations.

4.2.7. Equilibrium chemical speciation calculations

Speciation of nickel and other ions was calculated using WHAM VI software (Tipping, 1998; NERC, 2001) as described in chapter 2. Speciation calculations were performed for all EC10s, EC20s, EC50s and LC50s. For the synthetic test solutions, the input variables were the average measured pH, the average measured calcium and magnesium concentration, the average measured (pH test series) or nominal (calcium and magnesium test series) IC concentration, nominal concentrations of Na, K, SO_4, Cl and MOPS, and the effect concentration under consideration, based on dissolved nickel concentration measurements. De Schamphelaere et al. (2006) demonstrated that both background DOC in synthetic test solutions based on deionized water and exudates originating from algal food added in chronic toxicity tests do not significantly contribute to nickel speciation. Therefore, fulvic (FA) and humic acid concentrations were set to zero.

For the natural waters, similar inputs were used for pH, calcium, magnesium, MOPS and effect concentrations. For Na, K, SO_4, Cl and IC, measured values determined prior to testing were used. Dissolved trace metal concentrations of zinc, aluminium and iron (measured prior to testing) were also considered (see chapter 2 for computational details). The concentration of FA for input into WHAM VI was calculated as 0.8 x DOC (mg C/L), using the DOC concentration measured prior to testing. Details on the modeling of organic nickel complexation are given in chapter 2.

4.2.8. Model development

The protective effects of calcium and magnesium on the chronic toxicity of nickel to *D. magna* were modeled as single-site BLM-type competition effects. The model parameters representing these effects (K_{CaBL} and K_{MgBL}) were determined according to the method

described by De Schamphelaere and Janssen (2002), using the linear regression equations of the relationships between Ca^{2+} and Mg^{2+} activity and the 21-d $EC50_{Ni2+}$ (21-d EC50 expressed as Ni^{2+} activity). According to the method described in chapter 2, the model parameter representing the effect of pH (i.e. S_{pH}) was determined as the slope of the empirical linear relationship between pH and 21-d $EC50_{pNi2+}{}^{*}$. For each test solution *i* of the pH test series, the 21-d $EC50_{pNi2+}{}^{*}$ was calculated using the following equation:

$$EC50_{pNi^{2+},i}{}^{*} = -\log \frac{EC50_{Ni^{2+},i,observed}}{\left\{1 + K_{CaBL}.(Ca^{2+})_i + K_{MgBL}.(Mg^{2+})_i\right\}} \qquad \text{Eq. 4.1}$$

In this formula, $EC50_{Ni2+,i,observed}$ is the observed $EC50_{Ni2+}$ in test solution *i*, $(Ca^{2+})_i$ and $(Mg^{2+})_i$ are the chemical activities of Ca^{2+} and Mg^{2+} in test solution *i*, and K_{CaBL} and K_{MgBL} are the parameters describing the protective effects of calcium and magnesium on nickel toxicity. The effect of pH was superimposed on the traditional BLM-type competition effects of calcium and magnesium. As a result, the model equation for prediction of the $EC50_{Ni2+,i}$ in any solution *i* can be written as follows:

$$EC50_{Ni^{2+},i,predicted} = 10^{-(S_{pH}.pH_i + Q_{50})}.\left\{1 + K_{CaBL}.(Ca^{2+})_i + K_{MgBL}.(Mg^{2+})_i\right\} \qquad \text{Eq. 4.2}$$

In this model, Q_{50} can be regarded as a measure of the amount of nickel that has to be bioavailable in order to cause 50% reduction of reproduction. The value of this model parameter was calculated as the mean of $Q_{50,i}$ for all test solutions *i* used for model development:

$$Q_{50,i} = -\log \frac{EC50_{Ni^{2+},i,observed}}{\left\{1 + K_{CaBL}.(Ca^{2+})_i + K_{MgBL}.(Mg^{2+})_i\right\}} - S_{pH}.pH_i = EC50_{pNi^{2+},i}{}^{*} - S_{pH}.pH_i \qquad \text{Eq. 4.3}$$

Graphically, Q_{50} is the intercept of the regression line through a plot of $EC50_{pNi2+}{}^{*}$ versus pH for all test solutions *i* used for model development. The lower Q_{50}, the more bioavailable nickel is needed to cause 50% effect. As predictor of the toxic effect, Q_{50} has a similar role as the gill metal concentration (LA50) in the traditional BLM concept (Di Toro et al., 2001; Santore et al., 2001, 2002).

4.3. Results

4.3.1. Effects of calcium and magnesium on nickel toxicity

An increase of calcium concentration from 0.25 to 2.0 mM (for measured calcium concentrations see Table 4.1) resulted in a 3.0-fold (46.1-138 µg/L) and 3.1-fold (23.6-72.4 µg/L) increase of 21-d $LC50_{Nidiss}$ and 21-d $EC50_{Nidiss}$ (21-d LC50 and 21-d EC50 expressed as dissolved nickel concentration), respectively (Table 4.3). Further increase of calcium concentration to 3.0 mM did not result in further decrease of toxicity.

For magnesium, the 21-d $LC50_{Nidiss}$ and 21-d $EC50_{Nidiss}$ increased by a factor of 2.3 (54.5-124 µg/L) and 3.2 (31.5-99.5 µg/L) between 0.25 and 2.0 mM magnesium (for measured magnesium concentrations see Table 4.1), respectively. A relatively small further decrease of nickel toxicity was observed when magnesium concentration increased to 3.0 mM.

Both calcium and magnesium clearly protect *D. magna* against the effects of chronic nickel exposure. The protective effect of calcium and magnesium is also clear from the other effect data given in Table 4.3 ($NOEC_m$, $LOEC_m$, $NOEC_r$, $LOEC_r$, EC10 and EC20).

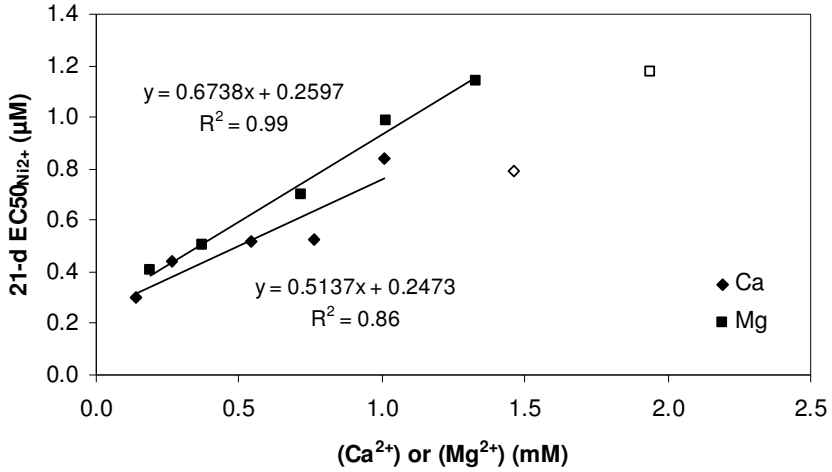

Figure 4.1. 21-d $EC50_{Ni2+}$ (expressed as Ni^{2+} activity, µM) as a function of Ca^{2+} and Mg^{2+} activity (mM). The data points represented by open symbols were not considered for model development. The regression lines through the remaining points were used for modeling the effects of calcium and magnesium on nickel toxicity.

Table 4.3. Effect data (µg Ni_{diss}/L) for all test solutions: $NOEC_m$, $LOEC_m$ and LC50 (mortality) and $NOEC_r$, $LOEC_r$, EC10, EC20 and EC50 (reproduction). For EC50s and LC50s, 95% confidence intervals are given between brackets.

Test solution	Mortality			Reproduction				
	$NOEC_m$	$LOEC_m$	LC50	$NOEC_r$	$LOEC_r$	EC10	EC20	EC50
Ca 0.25 mM	43.7	77.2	46.1 (37.6-56.5)	6.4	13.5	8.8 (5.87-13.0)	12.7 (9.42-17.0)	23.6 (20.0-27.9)
Ca 0.5 mM	44.4	74.2	52.1 (40.0-67.8)	13.9	26.1	16.4 (11.4-23.6)	21.7 (16.6-28.4)	34.9 (30.1-40.5)
Ca 1.0 mM	80.1	143	85.8 (67.3-109)	13.3	25.0	20.7 (15.4-27.8)	27.0 (21.6-33.6)	42.4 (37.2-48.4)
Ca 1.5 mM	74.2	141	109 (84.2-141)	15.6	25.3	13.0 (6.88-24.6) [e]	20.4 (12.7-32.9)	44.2 (33.7-58.0)
Ca 2.0 mM	139	245	138 (112-170)	43.3	78.1	34.0 (22.4-51.6)	45.0 (32.9-61.3)	72.4 (61.1-85.9)
Ca 3.0 mM	150	257	129 (99.7-166)	24.0	43.9	31.2 (19.5-50.0)	42.4 (29.7-60.5)	71.3 (57.9-87.8)
Mg 0.25 mM	48.5	80.4	54.5 (43.9-67.8)	15.3	27.1	16.4 (11.0-24.8)	20.9 (15.5-28.4)	31.5 (26.3-38.1)
Mg 0.5 mM	47.6	93.3	70.9 (58.1-86.6)	< 28.5 [d]	28.5	24.1 (20.1-28.5) [e]	29.0 (25.4-33.1)	40.0 (37.0-43.4)
Mg 1.0 mM	49.3	84.5	70.4 (59.4-83.4)	< 27.3 [d]	27.3	42.5 (37.0-48.9)	47.7 (43.0-53.0)	58.0 (52.5-64.1)
Mg 1.5 mM	84.7	160	110 (97.3-123)	< 46.0 [d]	46.0	47.0 (35.2-63.4)	58.3 (47.1-72.2)	83.9 (75.2-93.7)
Mg 2.0 mM	93.8	164	124 (-) [b]	48.2	93.8	76.7 (59.2-99.5)	84.3 (73.2-97.2)	99.5 (91.8-108)
Mg 3.0 mM	84.7	163	144 (114-182)	48.5	84.7	58.6 (42.5-79.8)	73.2 (57.8-92.8)	108 (92.8-126)
pH 5.8	64.5	135	77.2 (63.6-93.7)	< 11.2 [d]	11.2	24.1 (15.7-36.9)	33.2 (24.4-45.3)	57.8 (48.8-68.9)
pH 6.4	39.1	71.1	> 71.1 [c]	22.5	39.1	23.3 (12.9-42.1)	34.2 (23.0-50.7)	66.0 (53.0-82.2)
pH 7.0	37.0	75.4	53.9 (48.8-59.6)	< 6.1 [d]	6.1	34.7 (27.2-44.2)	40.6 (33.4-49.3)	53.1 (45.8-61.6)
pH 7.6	37.4	75.4	53.1 (-) [b]	11.8	21.4	32.0 (27.6-37.1)	37.3 (33.2-41.9)	48.6 (43.7-51.0)
pH 7.6*	38.5	68.3	46.0 (40.0-52.8)	22.8	38.5	25.6 (20.0-32.8)	29.7 (25.0-35.2)	38.2 (35.4-41.3)
pH 8.2*	> 37.4 [a]	> 37.4 [a]	> 37.4 [c]	20.4	37.4	16.9 (8.55-33.4)	25.8 (17.7-37.5)	53.1 (34.4-81.9) [g]
Ankeveen	292	507	358 (312-410)	293	507	256 (198-330)	291 (239-355)	365 (305-436)
Bihain	56.5	87.7	64.1 (56.3-72.9)	< 18.8 [d]	18.8	32.7 (21.7-49.2)	40.5 (30.1-54.6)	58.8 (49.4-69.9)
Brisy	60.1	96.6	80.9 (72.3-90.5)	< 60.1 [d]	60.1	52.3 (45.2-60.7) [e]	59.1 (52.6-66.3) [e]	72.7 (66.4-79.6)
Markermeer	164	274	249 (213-291)	90.0	164	139 (119-162)	160 (142-180)	203 (187-222)
Regge	281	518	377 (-) [b]	< 97.5 [d]	97.5	160 (-) [f]	230 (-) [f]	288 (-) [f]
Voyon	149	190	192 (158-233)	90.0	149	117 (94.5-145)	129 (111-150)	151 (141-161)

[a] No significant mortality observed at highest exposure concentration.

[b] No partial mortality observed.

[c] Mortality was less than 50% at highest exposure concentration.

[d] Reproduction at lowest exposure concentration was significantly different from control reproduction.

[e,g] Extrapolated ECx value (ECx lower than lowest or higher than highest exposure concentration, respectively).

[f] Confidence interval is not reliable due to steep concentration response curve.

Speciation calculations (WHAM VI) demonstrated that the average nickel species distribution in the calcium and magnesium test series (conducted at a pH of 6.8 to 6.9) at the 21-d EC50 level was as follows: 95.9% Ni^{2+}, 2.2% $NiSO_4$, 1.3% $NiHCO_3^+$ and < 1% NiCl, $NiOH^+$, $Ni(OH)_2$ and $NiCO_3$. Figure 4.1 shows the linear relationships between 21-d $EC50_{Ni2+}$ and Ca^{2+} and Mg^{2+} activity. The data points of the tests conducted at the highest calcium and magnesium concentration were not taken into account in the regression. Similar to what was observed based on dissolved nickel and total calcium and magnesium concentrations, 21-d $EC50_{Ni2+}$ increased with increasing Ca^{2+} and Mg^{2+} activities. This is logical, since calcium and magnesium do not affect nickel speciation to a large extent.

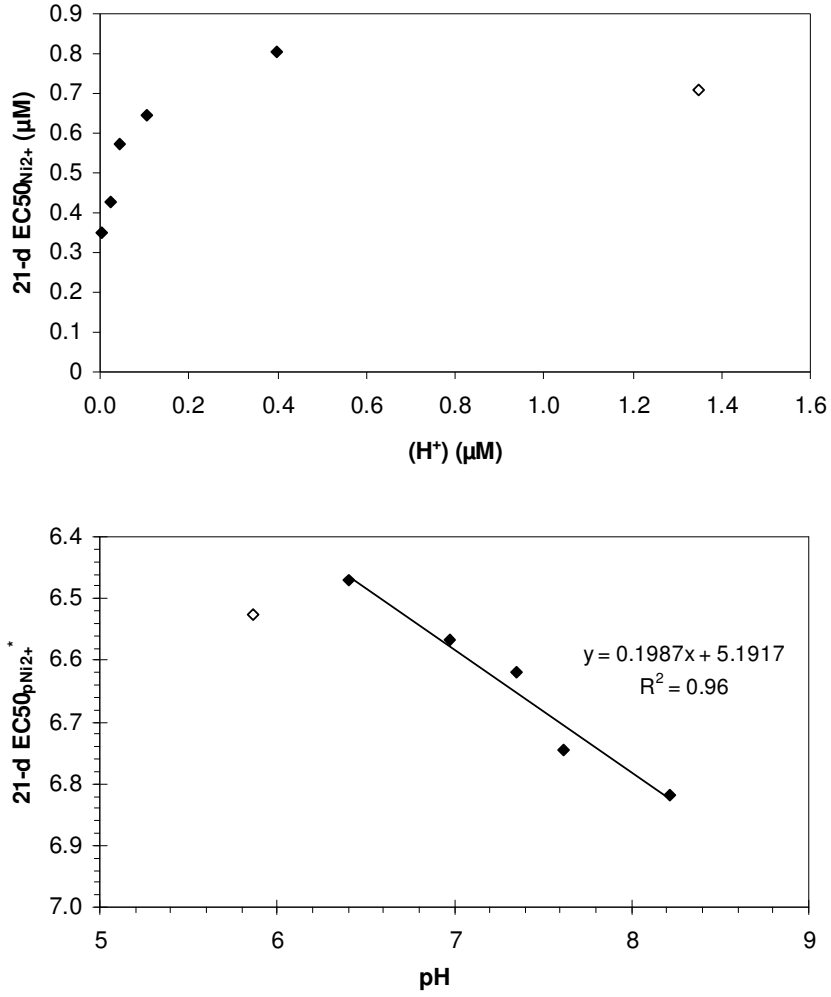

Figure 4.2. The effect of pH on 21-d reproductive toxicity of nickel to *D. magna*. Upper panel: 21-d $EC50_{Ni2+}$ (expressed as Ni^{2+} activity, µM) as a function of H^+ activity (µM); lower panel: 21-d $EC50_{pNi2+}{}^*$ (calculated using Equation 4.1) as a function of pH. The open symbol represents the test conducted at pH 5.87 (nominal pH = 5.8). The results of this test were not used for model development. The regression line in the lower panel was used for modeling the effect of pH on nickel toxicity.

4.3.2. Effect of pH on nickel toxicity

Based on the effect data reported in Table 4.3 (µg Ni_{diss}/L), no clear increase or decrease of nickel toxicity was observed within the tested pH range (pH 5.87 to 8.22, measured values, Table 4.1). Calculation of nickel speciation (WHAM VI) demonstrated that nickel-carbonate complexes became increasingly important with increasing pH. At the highest tested pH level (pH 8.22), $NiHCO_3^+$ and $NiCO_3$ represented 14.5 and 31.5% of the total dissolved nickel concentration, respectively. The contribution of Ni^{2+} in the nickel species distribution decreased to 52.0%.

The resulting curvilinear relationship between 21-d $EC50_{Ni2+}$ and H^+ activity is shown in Figure 4.2 (upper panel). Transformation of the 21-d $EC50_{Ni2+}$ according to Equation 4.1 yielded a linear relationship between 21-d $EC50_{pNi2+}{}^*$ and pH (Figure 4.2, lower panel). The data point for the test conducted at pH 5.87 was excluded from the regression. Consequently, the lower pH boundary of the model's applicability is 6.40. Overall, it is clear from Figure 4.2 that Ni^{2+} is more toxic at higher pH (lower H^+ activity).

4.3.3. Model development

Based on the regression lines shown in Figure 4.1 (calcium and magnesium) and Figure 4.2 (pH, lower panel), the following parameters were derived for the EC50 model: log K_{CaBL} = 3.53, log K_{MgBL} = 3.57, S_{pH} = 0.1987 and Q_{50} = 5.352. To investigate how well the model is calibrated to the dataset used for model development, the 21-d $EC50_{Ni2+}$ for each test solution was predicted using Equation 4.2, recalculated to the 21-d $EC50_{Nidiss}$ using WHAM VI software, and compared to the observed 21-d $EC50_{Nidiss}$ (Figure 4.3).

The 21-d $EC50_{Nidiss}$ were predicted by an average error of factor 1.3 (prediction error range = 1.1-1.6). Provided that the sensitivity parameter Q is adjusted, the model parameters derived for the EC50 model can also be used for predicting EC20s and EC10s. Q_{20} and Q_{10} were determined to be 5.537 and 5.646, respectively. The 21-d $EC20_{Nidiss}$ and 21-d $EC10_{Nidiss}$ were predicted by an average error of factor 1.4 (1.0-2.2) and 1.5 (1.0-2.7), respectively. Only one 21-d $EC20_{Nidiss}$ and two 21-d $EC10_{Nidiss}$ were predicted by an error higher than factor 2 (Figure 4.3).

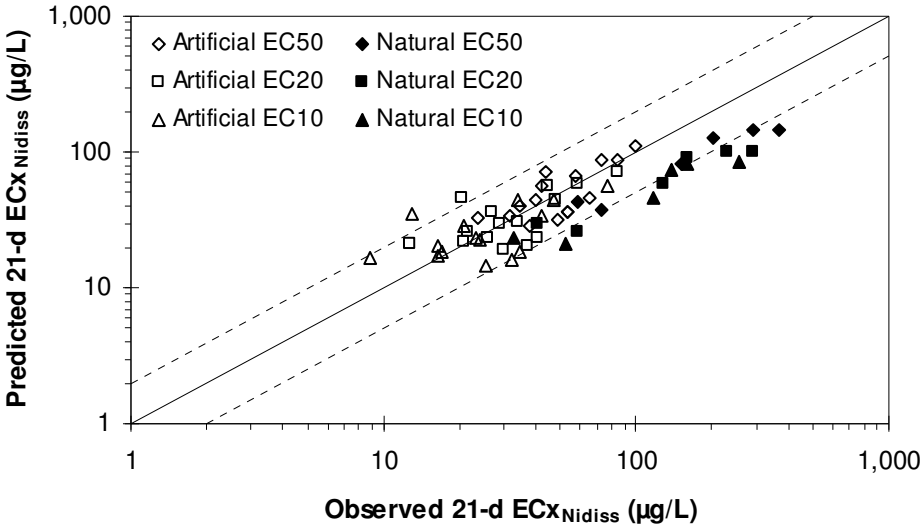

Figure 4.3. Predicted 21-d ECx_{Nidiss} versus observed 21-d ECx_{Nidiss} (expressed as dissolved nickel concentration, µg/L) for all artificial test solutions used for model development and all natural waters used for model validation. Predictions were made using Equation 4.2 linked to WHAM VI. The solid line indicates a perfect match between predicted and observed ECx values; the dashed lines indicate ratios of 0.5 and 2 between observed and predicted ECx values.

4.3.4. Model validation – European surface waters

The 21-d $LC50_{Nidiss}$ and the 21-d $EC50_{Nidiss}$ obtained in the nickel-spiked European surface waters (Table 4.2) varied between 64.1 and 377 µg/L and 58.8 and 365 µg/L, respectively (Table 4.3). The lowest effect concentrations were obtained in water from Bihain, which was characterized by very low hardness, low pH and low DOC concentration. Speciation calculations (WHAM VI) demonstrated that at the 21-d EC50 levels, between 42.9 and 67.4% of the dissolved nickel occurred as free Ni^{2+}, the remainder being primarily complexed by DOC (25.8 to 42.0%) and – at pH levels > 7.5 – by carbonate (up to 10.4 and 12.6% $NiHCO_3^+$ and $NiCO_3$, respectively).

The predictive capacity of the model described above is shown in Figure 4.3. The 21-d $EC50_{Nidiss}$, 21-d $EC20_{Nidiss}$ and 21-d $EC10_{Nidiss}$ were predicted by an average error of factor 1.9 (1.4-2.6), 2.1 (1.4-2.9) and 2.2 (1.4-3.1), respectively. Nickel toxicity in the water from Bihain, which had a pH below the lower pH boundary of the model, was predicted by an error of factor 1.4. In general, nickel toxicity was overestimated (EC10s, EC20s and EC50s were underestimated).

4.4. Discussion

The 21-d $LC50_{Nidiss}$ (range: 46.0-377 µg/L) and 21-d $EC50_{Nidiss}$ (range: 23.6-365 µg/L) observed in this study (Table 4.3) were more or less in the same range as those reported in literature, although the lowest 21-d $LC50_{Nidiss}$ (46.0 µg/L at pH 7.62 and a water hardness of 54.0 mg $CaCO_3$/L) and 21-d $EC50_{Nidiss}$ (23.6 µg/L at pH 6.85 and a water hardness of 41.7 mg $CaCO_3$/L) in our study were lower than the lowest 21-d LC50 and 21-d EC50 previously observed for *D. magna* at similar water hardness levels (71.9 µg/L at pH 7.3-7.6 and a water hardness of 45 mg $CaCO_3$/L (Pane et al., 2004c) and 95 µg/L at pH 7.74 and a water hardness of 45.3 mg $CaCO_3$/L (Biesinger and Christensen, 1972), respectively). This may be due to interclonal sensitivity differences (Münzinger and Monicelli, 1991) and/or to differences in the physicochemical composition of the test waters used in these studies (e.g., alkalinity, DOC).

Several field-collected cladoceran species (see chapter 6) as well as the standard test species *C. dubia* (Kszos et al., 1992; Keithly et al., 2004) have been reported to be more sensitive to chronic nickel exposure than *D. magna*. Overall, cladocerans appear to have a much higher chronic nickel sensitivity compared to fish (Kszos et al., 1992). A comparison of the chronic effect data for *D. magna* reported in this chapter to the long-term effect data for rainbow trout reported in chapter 2 supports this observation. The higher sensitivity of cladocerans to nickel is not surprising, since they are known to be one of the organism groups that are most sensitive to metals (Brix et al., 2001; Von Der Ohe and Liess, 2004).

Based on acute toxicity data and the relatively low metal gill binding affinity (log K_{NiBL} = 4.0), nickel has long been considered much less toxic than other metals such as copper, silver and zinc (Niyogi and Wood, 2004). Chronically however, the difference in toxicity appears to be much smaller. This is supported by the substantially higher acute to chronic ratios (ACR = 48-h EC50/chronic LC50) reported for nickel. Pane et al. (2004c) reported an ACR of 15 for *D. magna*. A comparison of the results obtained in the present study with those from the study on acute nickel toxicity to *D. magna* (chapter 3) yielded ACRs between 26 and 41 for the artificial waters and between 13 and 25 for the natural waters. For the field-collected cladocerans used in chapter 6, ACRs of 4 to 304 were calculated.

4.4.1. Effect of calcium and magnesium on nickel toxicity

Within a concentration range of 0.25 to 2.0 mM (for measured concentrations see Table 4.1), both calcium and magnesium increasingly protected *D. magna* against chronic nickel toxicity. A further increase of calcium or magnesium concentration up to 3.0 mM did not (calcium) or only slightly (magnesium) result in a further decrease of toxicity. Therefore, the tests conducted at 3.0 mM calcium and magnesium were not used for model development. Similar observations were made for (i) the effects of calcium and magnesium on the acute toxicity of nickel (chapter 3) and zinc (Heijerick et al., 2002b) to *D. magna*, (ii) the effect of calcium on the chronic toxicity of zinc to *D. magna* (Heijerick et al., 2005a), and (iii) the effect of sodium on the acute toxicity of copper to several cladocerans (De Schamphelaere et al., 2007b).

It should be mentioned that we did not observe a decrease in control survival or reproduction at the highest water hardness level used in this study (315 mg $CaCO_3$/L). This is in accordance with Cowgill and Milazzo (1990), who reported a NOEC of 650 mg $CaCO_3$/L for the effect of water hardness on *D. magna*. However, for *D. magna* exposed to additional stress factors, such as increased nickel concentrations, the NOEC for water hardness may be lower than the one reported by Cowgill and Milazzo (1990). This may explain the lower than expected effect concentrations at 3.0 mM calcium and magnesium.

One of the explanations given by De Schamphelaere et al. (2007b) for the observed plateau in the protective effect of sodium on acute copper toxicity to several cladocerans was that sodium-insensitive pathways of copper uptake and/or toxicity may start to dominate the organisms' response at elevated sodium concentrations. Although there are indications that Ni^{2+} is primarily taken up by *D. magna* via Mg^{2+} transport systems (see further), the existence of magnesium-insensitive nickel internalization sites should not a priori be excluded. Indeed, for some other organisms there is already evidence for the existence of multiple nickel internalization sites that are differently influenced by calcium and magnesium concentrations (e.g., *Salmonella typhimurium*, Snavely et al., 1991; *C. reinhardtii*, Worms and Wilkinson, 2007). If such multiple nickel internalization sites also exist in *D. magna*, a similar explanation as the one given by De Schamphelaere et al. (2007b) may also apply to the higher than expected chronic nickel toxicity at increased calcium and magnesium concentrations.

To date, the effect of water hardness (calcium and magnesium combined) on chronic nickel toxicity to *D. magna* has only been studied by Chapman et al. (1980). These authors observed LOECs (reported for non-specified endpoints) of 21.4, 150 and 578 µg/L at water hardness levels of 51, 105 and 205 mg $CaCO_3$/L, respectively. Two other studies investigated the effect of water hardness on the chronic toxicity of nickel to *C. dubia*. Upon an increase of water hardness from 42 to 117 mg $CaCO_3$/L, Kszos et al. (1992) observed an increase of 7-d LC50s and 7-d EC50s from 4.61 to 10.2 and from 3.92 to 8.85 µg/L, respectively (effect data were calculated using the methods mentioned in the materials and methods section of the present study and the mortality and reproduction data reported by Kszos et al., 1992). Keithly et al. (2004) reported LC20s and EC20s from less than 3.8 to 10.4 and from less than 3.8 to 6.9 µg/L within a water hardness range of 50 to 253 mg $CaCO_3$/L, respectively.

Keithly et al. (2004) claimed that the effect of water hardness on LC20s and EC20s was not significant. Their conclusion was based on the fact that the slopes of the regression lines describing the linear relationships between water hardness and the LC20s and EC20s were not significantly different from zero. However, this does not mean that there were no effects at all. Indeed, when applying the ratio test described by Wheeler et al. (2006) for one-by-one comparison of the LC20s and EC20s reported by Keithly et al. (2004), significant differences ($p < 0.05$) were observed (i) between the LC50 obtained at 113 mg $CaCO_3$/L and the LC50s obtained at 161 and 253 mg $CaCO_3$/L, and (ii) between the EC50s obtained at 113 and 253 mg $CaCO_3$/L. Hence, it is concluded that the Keithly et al. (2004) data are not in contradiction with the findings of the present study for *D. magna*.

In contrast to the studies of Chapman et al. (1980), Kszos et al. (1992) and Keithly et al. (2004), the present study investigated the individual effects of calcium and magnesium and demonstrated that both water hardness ions offer protection against chronic nickel toxicity. This was also demonstrated for acute nickel toxicity to *D. magna* and long-term nickel toxicity to rainbow trout (see chapter 2 and 3).

The protective effect of magnesium is most likely due to direct competition with Ni^{2+} for uptake at Mg^{2+} transport sites. The existence of a shared uptake pathway for Mg^{2+} and Ni^{2+} is supported by the observations that (i) Ni^{2+} and Mg^{2+} have similar dehydrated ionic radii (Weast, 1973), (ii) Ni^{2+} is a competitive inhibitor of Mg^{2+} uptake via three different types of Mg^{2+} transporters in the prokaryote *S. typhimurium* (Snavely et al., 1991), (iii)

chronic nickel exposure reduces whole body Mg^{2+} concentration and unidirectional Mg^{2+} uptake rate in *D. magna* (Pane et al., 2003b), (iv) Mg^{2+} inhibits Ni^{2+} uptake into brush border membrane vesicles isolated from the kidney of rainbow trout (Pane et al., 2006a,b), and (v) nickel-magnesium interactions are also well known from the mammalian literature (discussed by Pane et al., 2003b).

The protective effect of calcium is expected to be primarily due to its function as regulator of membrane permeability and ion transport (McWilliams, 1983; Hunn, 1985). Through its role in the tightening of paracellular junctions (Evans, 1987), calcium may protect indirectly against nickel toxicity by reducing the efflux of magnesium ions through these junctions. Based on the findings of Snavely et al. (1991) that Ca^{2+} (although to a lesser extent than Mg^{2+}) also competes directly with Ni^{2+} for uptake through all identified Mg^{2+} transport systems in *S. typhimurium*, one may expect that calcium also protects directly against nickel toxicity.

In this study, the protective effects of calcium and magnesium on chronic nickel toxicity to *D. magna* were observed to be of similar importance, which is reflected in the almost identical log K_{CaBL} and log K_{MgBL} (3.53 and 3.57, respectively). This contrasts with acute nickel toxicity to *D. magna*, for which the protective effect of calcium was observed to be more important than the toxicity reducing effect of magnesium (log K_{CaBL} and log K_{MgBL} = 3.10 and 2.47, respectively, see chapter 3). However, for other metals, such as copper (De Schamphelaere and Janssen, 2004b) and zinc (Heijerick et al., 2005a), it has also been observed that the toxicity mitigating effect of the cation most closely related to the mechanism of toxicity becomes more important with increasing exposure duration (i.e. sodium for copper and calcium for zinc).

4.4.2. Effect of pH on nickel toxicity

Analysis of the toxicity test results based on calculated nickel speciation demonstrated that chronic nickel toxicity increases with increasing pH, suggesting that H^+ ions compete with Ni^{2+} for uptake. However, the relationship between 21-d $EC50_{Ni2+}$ and H^+ activity was not linear (Figure 4.2, upper panel), indicating that factors other than H^+ competition are involved in determining nickel toxicity as a function of pH. More or less similar nonlinear relationships were also observed for acute nickel toxicity to *D. magna* (chapter 3), long-term

nickel toxicity to rainbow trout (chapter 2), acute copper toxicity to *D. magna* (De Schamphelaere and Janssen, 2002) and *Hyalella azteca* (Borgmann et al., 2005) and chronic zinc toxicity to *D. magna* (Heijerick et al., 2005a).

De Schamphelaere et al. (2002) demonstrated that for acute copper toxicity to *D. magna*, model performance increased when the contribution of $CuOH^+$ and $CuCO_3$ to copper toxicity was incorporated in the model. For nickel, Hoang et al. (2004) suggested that $NiHCO_3^+$ and $NiCO_3$ (both increasingly present at increasing pH) might be bioavailable. However, according to the method described by De Schamphelaere et al. (2002), $NiHCO_3^+$ and $NiCO_3$ were calculated to be more toxic than Ni^{2+}. Based on chemical complex formation theory this is not very likely, since complexed metal species should have a much lower affinity for ligands than the free metal ion. Possible explanations for the apparent toxicity of nickel-carbonate complexes may be that these complexes dissociate in the physicochemically different gill microenvironment and/or facilitate Ni^{2+} uptake through Mg^{2+} transport systems such as electro-neutral Mg^{2+}/HCO_3^- symporters (so far only identified in Yoshida ascites tumor cells (Günther et al., 1986). These possibilities were discussed in detail in chapter 2 and 3 on the long-term toxicity of nickel to rainbow trout and the acute toxicity of nickel to *D. magna*.

The effect of pH on chronic nickel toxicity to *D. magna* appeared to be more important than on acute nickel toxicity (see chapter 3). This can not be ascribed to speciation differences, since nickel speciation calculated for the pH levels used in the present study was very similar to that at the comparable pH levels used in the acute study. Possibly, dietborne toxicity is responsible for the larger effect of pH on chronic nickel toxicity. In chronic toxicity tests with *D. magna* live algae are added as food source for the test organisms. Since both metal adsorption to (Crist et al., 1988) and metal absorption in (Worms and Wilkinson, 2007) algal cells generally increase with increasing pH, exposure to dietborne nickel could be expected to increase with increasing pH. The adverse effects of dietborne exposure to *D. magna* have already been demonstrated for metals such as copper (De Schamphelaere, 2007a) and zinc (De Schamphelaere et al., 2004a). However, it remains to be investigated whether dietborne exposure contributes to chronic nickel toxicity or not.

Currently, the present study is the only study discussing the effect of pH on chronic nickel toxicity to *D. magna*. Further research is needed to improve our understanding of the

mechanisms responsible for the increase of nickel toxicity at elevated pH levels. The effect of pH was now modeled according to the method described in chapter 2 (i.e. based on an empirical linear relationship between 21-d $EC50_{pNi2+}{}^{*}$ and pH, Figure 4.2, lower panel), and superimposed on the traditional BLM-type competition effects of calcium and magnesium. Because the data point at pH 5.87 was excluded from model development, the pH range of the model's applicability is 6.40 to 8.22.

4.4.3. Effect of DOC on nickel toxicity

Speciation calculations for the nickel-spiked surface waters revealed that at the 21-d EC50 levels 25.8 to 42.0% of the dissolved nickel was complexed by DOC. Relatively more nickel is complexed at chronic effect concentrations for *D. magna* than at acute effect concentrations for *D. magna* (5-23%) and long-term effect concentrations for rainbow trout (8-20%) (see chapter 2 and 3). This is logical, since acute nickel toxicity to *D. magna* and long-term nickel toxicity to rainbow trout were observed at substantially higher nickel concentrations, at which the maximum Ni^{2+} complexation capacity of organic matter is more likely to be reached. Consequently, the protective effect of DOC is expected to be much larger upon exposure of *D. magna* to chronically toxic nickel concentrations. This is supported by the fact that the positive linear correlation between 21-d $EC50_{Nidiss}$ and DOC concentration was much stronger (r^2 = 0.84) than between 48-h $EC50_{Nidiss}$ and DOC concentration (r^2 = 0.33) (see chapter 3).

4.4.4. Model development and validation

The model was developed based on 21-d EC50s for reproduction because (i) reproduction was observed to be the most sensitive endpoint (supported by the results of Biesinger and Christensen, 1972 and Kszos et al., 1992), and (ii) median effect concentrations can be estimated with more precision than EC20s and EC10s (EC50s typically have smaller 95% confidence intervals). After adjustment of the sensitivity parameter *Q*, 21-d EC20s and 21-d EC10s were predicted using the same model parameters. Although model performance was very good in the artificial waters used for model development (see above), toxicity was less accurately predicted in the nickel-spiked surface waters used in this study. Moreover, there appeared to be a general tendency for overestimating nickel toxicity (underestimating ECx values). Several explanations may be given for this.

First, the tests in the nickel-spiked surface waters were conducted about a year earlier than those in the artificial waters used for model development. Possibly, the sensitivity of our *D. magna* clone has shifted during that period. Therefore, we evaluated the model's performance when optimized sensitivity parameters were used for predicting nickel toxicity in the tested surface waters. The optimized Q_{50}, Q_{20} and Q_{10} were calculated to be 5.002, 5.110 and 5.200, respectively. The results from the test in the water from Bihain (pH < 6.4 = lower pH boundary of model) were not used for optimization of these parameters. Using these optimized parameters, the 21-d $EC50_{Nidiss}$, 21-d $EC20_{Nidiss}$ and 21-d $EC10_{Nidiss}$ were predicted by an average error of factor 1.2 (prediction error range = 1.0-1.5), 1.2 (1.0-1.7) and 1.3 (1.0-1.8), respectively (Figure 4.4). This remarkable improvement of the model's accuracy, accomplished by optimizing the sensitivity parameters while keeping all other model parameters (S_{pH}, log K_{CaBL} and log K_{MgBL}) fixed at their original values, suggests that there may indeed have occurred a shift in the sensitivity of our *D. magna* clone.

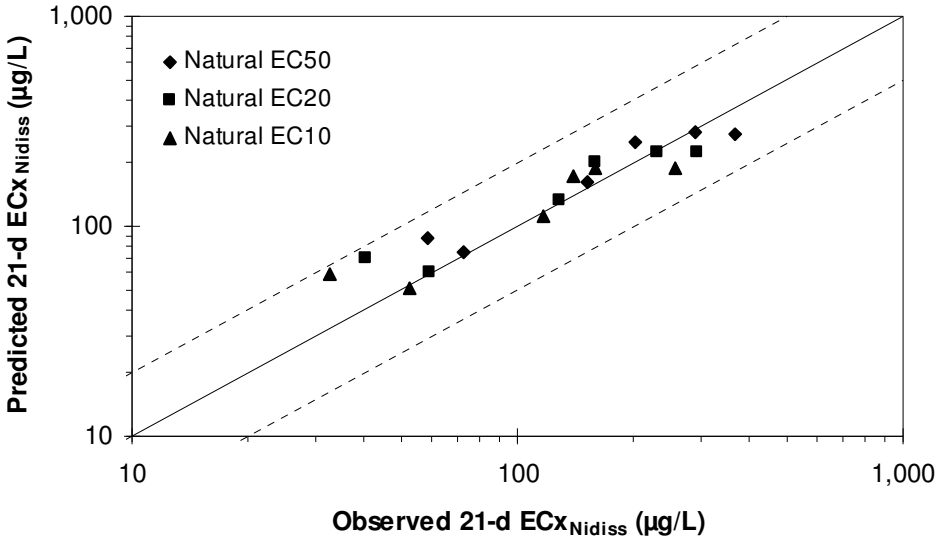

Figure 4.4. Effect of using optimized sensitivity parameters (Q_{50}, Q_{20} and Q_{10}) on the accuracy of the model's predictions in natural waters. Predicted 21-d ECx_{Nidiss} are plotted versus observed 21-d ECx_{Nidiss} (expressed as dissolved nickel concentration, µg/L). Predictions were made using Equation 4.2 linked to WHAM VI. The solid line indicates a perfect match between predicted and observed ECx values; the dashed lines indicate ratios of 0.5 and 2 between observed and predicted ECx values.

This possibility is corroborated by findings of Baird and Barata (1997). In their study on the constancy of clone performance, these authors observed that over relatively short periods of less than ten generations under constant conditions, the LC50 for cadmium varied

by more than one order of magnitude within a single *D. magna* clone. The same authors also reported a significant within-clone variation of sublethal sensitivity to copper, measured as feeding inhibition EC50 (Baird and Barata, 1998). Moreover, they suggested that some intraclonal variation in sensitivity is essentially uncontrollable.

Second, a plot of 21-d $EC50_{pNi2+}{}^{*}$ versus pH for the data obtained in the nickel-spiked surface waters (data point for Bihain excluded since pH < 6.4) revealed a regression line with a significantly steeper slope (S_{pH} = 0.3335) than that obtained with the artificial test waters used for model development (S_{pH} = 0.1987) (F-test, $p < 0.05$, according to Sokal and Rohlf, 1981). This does not necessarily mean that the effect of pH on nickel toxicity in natural waters is different from what is observed in artificial waters. It may also suggest that our model formulation (Equation 4.2), in which it was assumed that the protective effects of calcium and magnesium are independent of pH, is a simplification of reality. It must be kept in mind that the 21-d $EC50_{pNi2+}{}^{*}$ values were calculated (Equation 4.1) using fixed values of K_{CaBL} and K_{MgBL} that were determined based on the results from the univariate calcium and magnesium test series conducted in artificial test waters with a pH around 6.8. If in reality the protective effect of water hardness becomes less important at pH > 6.8, the S_{pH} for natural waters would only appear higher due to overcorrection for calcium and magnesium at higher pH, since pH and water hardness are positively correlated in natural waters. Multivariate test designs are needed in the future to investigate whether there are interactive effects of pH and calcium and/or magnesium on chronic nickel toxicity to *D. magna* or not.

Using the optimized S_{pH} of 0.3335 and the associated sensitivity parameters Q_{50} = 3.986, Q_{20} = 4.094 and Q_{10} = 4.183, the 21-d $EC50_{Nidiss}$, 21-d $EC20_{Nidiss}$ and 21-d $EC10_{Nidiss}$ for the natural waters used in this study were predicted by an average error of 1.3 (prediction error range = 1.1-2.2), 1.3 (1.1-2.6) and 1.4 (1.1-2.6), respectively. The least accurate predictions were obtained for the Bihain test water. This is logical, since the results obtained in this water were excluded from the calculation of S_{pH}. Although the overall accuracy of this model was lower than that of the model in which only the sensitivity parameters were adjusted (see above), it yielded the most accurate predictions for the natural waters with pH > 6.4. Until further research allows model refinement, we would therefore advise to use the model with the adjusted S_{pH} of 0.3335 for predicting chronic nickel toxicity to *D. magna* in natural waters with pH > 6.4.

A third explanation for the overestimation of nickel toxicity in natural waters may be that DOC (humic substances) ameliorates nickel toxicity in natural waters beyond its effect on nickel speciation. Glover et al. (2005) demonstrated that humic substances can influence Na^+ fluxes in *D. magna*, which could in turn have implications for the toxicity of metals affecting sodium metabolism. Similarly, if humic substances also influence Mg^{2+} fluxes in *D. magna*, increased DOC concentrations may protect against nickel toxicity in natural waters beyond the effect on nickel speciation. Because there are indications that our initial bioavailability model does not fully capture all variation in nickel toxicity, additional research will be required to allow further refinement of the model.

4.5. Conclusion

A series of univariate experiments in synthetic test solutions demonstrated that increasing calcium and magnesium concentrations and decreasing pH reduced the 21-d reproductive toxicity of nickel to *D. magna*. Based on the results of these experiments a chronic nickel bioavailability model was developed in which the effects of calcium and magnesium were modeled as BLM-type single-site competition effects and a log-linear pH effect was superimposed on these effects according to the method described in chapter 2. The model parameters representing these effects are log K_{CaBL} = 3.53, log K_{MgBL} = 3.57 and S_{pH} = 0.1987. The sensitivity parameters Q_{50}, Q_{20} and Q_{10} were calculated to be 5.352, 5.537 and 5.646, respectively.

The model's accuracy was evaluated using the results of the tests in artificial test waters used for model development as well as the results of similar toxicity tests in a series of nickel-spiked European surface waters. The model can clearly be used to improve nickel risk assessments beyond currently available approaches that depend on total dissolved nickel concentrations or estimates of nickel speciation. However, the validation exercise using the European surface waters indicated that the model may not fully capture all the effects of water chemistry on nickel toxicity. To allow model refinement, further research is required, especially into (i) the underlying mechanisms of increased nickel toxicity at elevated pH and/or alkalinity levels, (ii) the effect of DOC, and (iii) the possible existence of interactions between the effects of calcium, magnesium and pH.

Chapter 5

Modeling nickel bioavailability and toxicity to the unicellular green alga *Pseudokirchneriella subcapitata*

Redrafted from

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Chapter 5

Modeling nickel toxicity to the unicellular green alga *Pseudokirchneriella subcapitata*

Abstract. In this study, increasing calcium and magnesium concentrations and decreasing pH were observed to ameliorate nickel-induced growth inhibition of the green alga *Pseudokirchneriella subcapitata*. The toxicity reducing capacity of magnesium was observed to be much higher than that of calcium. To investigate to what extent the original biotic ligand model (BLM) concept could explain nickel toxicity as a function of water chemistry, the protective effects of Ca^{2+}, Mg^{2+} and H^+ were modeled as BLM-type single-site competition effects. The model parameters representing these effects were log K_{CaBL} = 2.0, log K_{MgBL} = 3.3 and log K_{HBL} = 6.5. Model performance was acceptable in both synthetic and natural waters. However, the fact that the relationship between 72-h E_rC50_{Ni2+} and H^+ activity – which was used for the derivation of the log K_{HBL} – was not linear over the entire tested pH range indicates that the effect of pH can probably not be attributed to H^+ competition with Ni^{2+} for a single site alone. The model's accuracy improved when the effect of pH was modeled based on the empirical relationship between 72-h $E_rC50_{pNi2+}{}^*$ (= – log (72-h E_rC50_{Ni2+} corrected for the presence of calcium and magnesium)) and pH. This method has been successfully used in our previous studies on the development of a chronic nickel bioavailability model for *Daphnia magna* and a long-term nickel bioavailability model for rainbow trout. Next to the individual effects of calcium, magnesium and pH, we also investigated possible interactive effects of pH and magnesium, the two most important factors affecting nickel toxicity to *P. subcapitata*. Although some interaction was observed, it seemed not important enough to be incorporated in the bioavailability model.

5.1. Introduction

As primary producers, planktonic microalgae are a key component of food chains in aquatic systems. Since changes in algal population density and diversity may affect the entire aquatic ecosystem, it is important to understand how algae are affected by aquatic contaminants, such as metals. As for test organisms belonging to higher trophic levels, such as daphnids and fish, metal toxicity to algae has been demonstrated to be dependent on water chemistry. Physicochemical parameters such as water hardness, calcium, magnesium, sodium, pH and/or dissolved organic carbon (DOC) have been reported to affect the toxicity of several

metals to algae (e.g., aluminium, Parent and Campbell, 1994; cadmium, Peterson et al., 1984; Kola and Wilkinson, 2005; Vigneault and Campbell, 2005; zinc, Heijerick et al., 2002a; copper, De Schamphelaere et al., 2003; Heijerick et al., 2005b; uranium, Franklin et al., 2000; Charles et al., 2002; Fortin et al., 2007).

For fish and daphnids, the biotic ligand model (BLM) concept has been demonstrated to be very successful for the development of bioavailability models capable of predicting metal toxicity as a function of water chemistry (reviewed by Niyogi and Wood, 2004). These models are useful (and indispensable) tools for regulatory exercises such as risk assessments and the derivation of water quality criteria. The applicability of the original BLM concept to algae has been discussed by several authors (e.g., Campbell et al., 2002; Heijerick et al., 2002a, 2005b; De Schamphelaere et al., 2003, 2005a,b; Hassler and Wilkinson, 2003; Kola and Wilkinson, 2005; De Schamphelaere and Janssen, 2006; Fortin et al., 2007). Although all of these authors encountered difficulties that questioned the applicability of the original BLM concept, some of them have proposed solutions that allow the development of bioavailability models using modified procedures (e.g., Heijerick et al., 2002a; De Schamphelaere et al., 2003, 2005a,b; De Schamphelaere and Janssen, 2006).

To date, almost all of the studies in which algae were exposed to nickel have focused on the mechanism by which nickel affects photosynthesis and respiration. To our knowledge, only a few studies have investigated the influence of water chemistry (e.g., cations and/or pH) on adsorption, uptake and/or toxicity of nickel (e.g., Macfie et al., 1994; Issa et al., 1995; Mehta et al., 2000; Worms and Wilkinson, 2007). No attempts have been made to develop a nickel bioavailability model for algae. Therefore, the objectives of this study were (i) to investigate how water chemistry affects the toxicity of nickel to a unicellular green alga, and (ii) to develop a bioavailability model capable of predicting nickel toxicity as a function of water chemistry. *Pseudokirchneriella subcapitata* was chosen as test species.

Using an approach similar to that applied in our previous studies on chronic nickel toxicity to *Daphnia magna* (chapter 4) and long-term nickel toxicity to rainbow trout (chapter 2) the individual effects of calcium, magnesium and pH on nickel-induced growth inhibition of *P. subcapitata* were examined. Additionally, it was investigated whether the effects of magnesium and pH on nickel toxicity interact with each other. Based on the results of these experiments, it was investigated to what extent the original BLM concept can be used to

explain the observed effects. The predictive capacity of the developed model was evaluated using results from toxicity tests conducted in both synthetic and natural waters. The latter also allowed us to evaluate the importance of DOC in reducing nickel toxicity.

5.2. Materials and methods

5.2.1. Individual effects of calcium, magnesium and pH on nickel toxicity

The individual effects of calcium, magnesium and pH were investigated using three univariate test series in synthetic test solutions. The calcium, magnesium and pH test series consisted of seven, nine and five bioassays, respectively. All test solutions were prepared using carbon filtered, deionized water (conductivity < 2 µS/cm) and reagent grade chemicals purchased from VWR International (Leuven, Belgium). The synthetic basic medium was the medium recommended by OECD guideline 201 (1996). Because of its strong metal complexing capacity, EDTA was replaced by artificial DOC according to Heijerick et al. (2002a).

In the calcium and magnesium test series, calcium and magnesium concentrations were adjusted by adding $CaCl_2$ and $MgCl_2$ to the basic medium, respectively. All test solutions were adjusted to the required pH (7.5 in the calcium and magnesium test series) by adding 3.6 mM MOPS buffer (3-N-morpholinopropanesulfonic acid) and the required amount of dilute KOH or HCl solution. MOPS has been demonstrated not to affect metal speciation (Kandegedara and Rorabacher, 1999). De Schamphelaere et al. (2004b) demonstrated that 3.6 mM MOPS is not toxic to *D. magna* and *P. subcapitata*. Moreover, MOPS does not affect the toxicity of copper and zinc to *D. magna* and *P. subcapitata* (De Schamphelaere et al., 2004b) or the toxicity of nickel to *D. magna* (chapter 3). In the pH test series, potassium concentrations were brought to the same level through addition of KCl.

For each growth inhibition test, a nickel concentration series was prepared by adding $NiCl_2$. Each bioassay consisted of a control treatment and five or six nickel concentrations. All solutions were allowed to equilibrate for 24 hours at 25 °C prior to being used in the toxicity tests. The physicochemical composition of all test solutions is given in Table 5.1.

Table 5.1. Main physicochemical characteristics of the synthetic test media used for model development (univariate calcium, magnesium and pH test series) and for the investigation of potential interactive effects of magnesium and pH (bivariate pH-magnesium test series).

Test medium	pH	Major ions (mg/L)				Hardness [b]	Alkalinity [c]
		Ca	Mg	K [a]	Cl [a]	(mg $CaCO_3$/L)	(mg $CaCO_3$/L)
Ca 0.12 mM	7.40	3.36	2.79	136	22.9	19.9	27.3
Ca 0.5 mM	7.38	14.0	2.81	132	49.7	46.4	27.2
Ca 1.0 mM	7.40	27.9	2.82	129	85.1	81.4	27.3
Ca 2.0 mM	7.39	58.0	2.77	125	156	156	27.3
Ca 3.0 mM	7.39	87.2	2.76	123	227	229	27.3
Ca 4.0 mM	7.41	115	2.76	123	298	299	27.4
Ca 5.0 mM	7.44	144	2.79	123	369	370	27.5
Mg 0.12 mM	7.48	3.11	2.99	140	25.0	20.1	27.7
Mg 0.5 mM	7.51	3.02	11.4	133	52.3	54.5	27.9
Mg 1.0 mM	7.50	3.14	20.5	129	86.2	92.2	27.8
Mg 1.5 mM	7.52	3.05	44.6	128	121	191	27.9
Mg 2.0 mM	7.53	3.08	46.3	128	159	198	28.0
Mg 2.5 mM	7.54	3.05	57.1	124	193	243	28.0
Mg 3.0 mM	7.53	2.99	71.2	120	228	300	28.0
Mg 4.0 mM	7.54	3.03	93.5	126	300	392	28.0
Mg 5.0 mM	7.52	2.97	115	124	371	481	27.9
pH 6.0	6.01	3.43	3.06	157	169	21.2	9.30
pH 6.4	6.45	3.49	3.10	157	141	21.5	16.6
pH 7.2	7.29	3.45	2.97	157	72.5	20.8	26.7
pH 7.6	7.65	3.58	2.85	157	46.6	20.7	28.4
pH 8.0	7.92	3.46	3.08	157	34.5	21.3	29.1
pH 6.0							
Mg 0.12 mM	6.23	4.61	3.15	144	141	24.5	12.8
Mg 1.5 mM	6.13	4.57	35.8	144	239	159	11.2
Mg 3.0 mM	6.08	4.18	73.2	144	346	312	10.4
pH 7.0							
Mg 0.12 mM	7.20	4.46	4.33	144	82.4	28.9	26.1
Mg 1.5 mM	7.16	4.42	33.4	144	181	149	25.8
Mg 3.0 mM	7.15	4.47	73.8	144	287	315	25.7
pH 7.8							
Mg 0.12 mM	7.95	4.34	2.96	144	22.9	23.0	29.2
Mg 1.5 mM	7.88	4.37	35.5	144	121	157	29.0
Mg 3.0 mM	7.85	4.28	73.3	144	227	312	28.9

[a] No measurements were conducted for Na, K, SO_4 and Cl, hence nominal values are reported. In all tests, nominal Na and SO_4 concentrations were 13.7 and 5.86 mg/L, respectively.

[b] Water hardness was calculated from measured calcium and magnesium concentrations.

[c] Alkalinity was calculated from nominal added inorganic carbon (IC) and measured pH, using thermodynamic stability constants taken from Stumm and Morgan (1996).

5.2.2. Combined effects of pH and magnesium on nickel toxicity

Using a bivariate 3 x 3 test design, nine additional toxicity tests were conducted to investigate potential interactive effects of pH and magnesium, which were observed to be the two most important factors affecting nickel toxicity to *P. subcapitata* (see further). In this test series, nickel toxicity was investigated at three different magnesium concentrations (0.12, 1.5

and 3.0 mM) and three different pH levels (6.0, 7.0 and 7.8). As in the univariate pH test series, potassium concentrations were brought to the same level through addition of KCl. The physicochemical composition of all test solutions used in this test series is also given in Table 5.1.

5.2.3. Toxicity tests conducted for model validation

Two different validation test series were conducted. In both test series, algal growth inhibition experiments were performed in nickel-spiked surface waters as well as in OECD test water (OECD, 1996). The natural waters were sampled at five locations in Europe. The variation in the sampled waters with regard to possible nickel toxicity modifying factors (calcium, magnesium, water hardness, DOC, pH, alkalinity) reflected the physicochemical variation typically observed in European surface waters. Samples were taken at the following locations: Ankeveen (NL), Bihain (B), Brisy (B), Regge (NL) and River Mole (UK). A short description of these locations is given in chapter 2 and 3.

At each location, the required amount of water was membrane filtered (0.45 µm) and collected in metal-free polyethylene vessels. The samples were immediately transferred to the laboratory, where they were stored at 4 °C in total darkness. Before testing, the following parameters were measured: pH (pH-meter P407, Consort, Turnhout, Belgium), DOC and inorganic carbon (IC) (TOC-5000, Shimadzu, Duisburg, Germany), Ca, Mg, Na, K, Al, Ni, Cu and Zn (ICP-OES, Perkin Elmer 3300 DV) and Cl, NO_3 and SO_4 (Ion Chromatography, Dionex QIC analyzer, IONPAC AS4A).

In the first validation test series, tests were conducted in water from Bihain, Ankeveen and River Mole and in basic OECD medium (OECD, 1996). All tests were performed in duplicate. In the second validation test series, tests were conducted in water from Ankeveen, Brisy and Regge, in basic OECD medium and in OECD medium with increased water hardness ('$OECD^+$', 250 mg $CaCO_3$/L). Before testing, the same nutrient concentrations as present in the basic OECD medium were added to each surface water sample. MOPS buffer (1.43 and 3.6 mM in test series 1 and 2, respectively) and NaOH/HCl were used for regulating and setting pH in all test solutions (except in the water from River Mole, which has a pH > 7.8). In the OECD and $OECD^+$ test water used in the second validation test series, sodium concentrations were brought to the same level through addition of NaCl.

Table 5.2. Main physicochemical characteristics of test media used for model validation (European surface waters + OECD test waters).

Test date / test water		pH	Major ions (mg/L) [c]						Hardness [e]	Alkalinity [f]	DOC	Ni_{diss}
			Ca	Mg	Na	K	Cl	SO_4	(mg $CaCO_3$/L)	(mg $CaCO_3$/L)	(mg/L)	(µg/L)
05/2001	Bihain (B) [a]	6.35	39.3	4.38	25.3	0.551	55.0	93.6	116	0.743	6.62	2.5
	Ankeveen (NL) [a]	7.37/7.47	52.9	11.1	36.1	0.551	59.2	105	178	29.6/30.2	25.8	4.6
	River Mole (UK) [a]	7.99/8.01	56.1	11.3	63.7	0.551	56.4	97.0	186	94.3/94.4	5.14	3.9
	OECD [a, b]	7.70	4.81	2.92	36.8	0.352	22.7	5.91	24.0	29.2	- [g]	- [h]
09/2005	Ankeveen (NL)	7.29	65.3	9.97	66.2	0.555	29.9	120	204	35.3	22.4	3.0
	Brisy (B)	6.53	8.82	3.82	31.7	2.79	21.9	9.99	37.7	5.98	4.10	2.0
	Regge (NL)	7.78	59.7	7.83	129	14.2	78.0	68.0	181	133	10.9	3.1
	OECD [b]	7.42	4.81	2.92	71.3 [d]	0.352	22.7	5.91	24.0	24.0	- [g]	- [h]
	$OECD^{+}$ [b]	7.42	80.2	12.2	71.3 [d]	0.352	182	5.91	250	23.9	- [g]	- [h]

[a] Tests in these waters were conducted in duplicate. Concentrations of major ions, inorganic carbon (IC), dissolved organic carbon (DOC) and background nickel were determined (measured and/or calculated) before testing. As a result, the same values were used for both tests. The pH was measured at the start and at the end of testing. The average measured pH is reported separately when different in both tests.

[b] Test medium recommended by the OECD (1996) and prepared and modified as described in the materials and methods section. The $OECD^{+}$ test water has increased water hardness.

[c] Natural waters: measured concentrations in water samples + nominal added concentrations (nutrients of OECD test water and NaOH/HCl solution added for pH setting). OECD test waters: nominal concentrations.

[d] In these two tests, sodium concentrations were brought to the same level through addition of NaCl.

[e] Water hardness was calculated using reported calcium and magnesium concentrations.

[f] Alkalinity was calculated from measured + nominal (natural waters) or nominal (OECD waters) IC concentrations and average measured pH, using thermodynamic stability constants taken from Stumm and Morgan (1996).

[g] DOC in synthetic test media was demonstrated not to complex significant amounts of nickel (De Schamphelaere et al., 2006).

[h] Background nickel concentration < 3 µg/L (i.e. the method detection limit (MDL) of the graphite furnace atomic absorption spectrometer used in this study). Background nickel concentrations in the natural waters were measured using ICP-OES (MDL = 1 µg/L).

After spiking with nickel, all test solutions were allowed to equilibrate for 24 hours at 25 °C prior to being used in the toxicity tests. The physicochemical composition of all test solutions used for model validation is given in Table 5.2.

5.2.4. Testing

P. subcapitata (CCAP 278/4) was obtained from the Culture Collection of Algae and Protozoa (CCAP, at the Scottish Association for Marine Science, Argyll, Scotland, UK) and was maintained at pH 8.3 in carbon filtered and 0.45 µm filtered aerated tap water (Gent, Belgium) to which the modified Provasoli's ES enrichment (Bold and Wynne, 1978) at 1/2 strength and, additionally, 1.4 mg/L $FeSO_4.7H_2O$, 15 mg/L $NaH_2PO_4.2H_2O$, 150 mg/L $NaNO_3$ and 2.35 mg/L $MnCl_2.4H_2O$ were added. The culture was kept at 25 °C under continuous light (240 µmol photons/m^2/s). Microscopic inspection at regular time intervals did not reveal any contamination of the culture throughout the study. Dissolved nickel concentrations in the culture medium were always below 3 µg/L, i.e. the method detection limit (MDL) of the graphite furnace atomic absorption spectrometer used in this study (GF-AAS, SpectrAA800 with Zeeman background correction, Varian, Mulgrave, Australia).

Prior to testing, two or three Erlenmeyer flasks containing the culture medium were inoculated with *P. subcapitata*. These flasks were continuously shaken for five days and aerated in order to provide sufficient CO_2 for algal growth and to maintain pH around 7.5. Algal growth was followed daily. By the fifth day, the algae were in the exponential growth phase and ready for use as inoculum for the tests.

The growth inhibition tests were performed according to OECD test guideline 201 (OECD, 1996). They were performed in 100 mL Erlenmeyer flasks containing 50 mL of test medium. Three replicates were used for the control treatment as well as for each of the five or six nickel concentrations. Each replicate was inoculated with 1 x 10^4 cells/mL (= cell density N_0 at the start (t_0) of testing). Test flasks were then incubated at 25 °C on a light table (24 h light, 120 µmol photons/m^2/s) and were manually shaken twice a day. Cell densities (N_2 and N_3) were measured after 48 (t_2) and 72 (t_3) hours of exposure using a particle counter (Coulter Counter Model DN, Coulter Electronics, UK).

5.2.5. Chemical analyses

The pH of each test solution was measured at the start and at the end of testing (pH-meter P407, Consort, Turnhout, Belgium). The glass electrode was calibrated with pH 4, pH 7 and pH 10 buffers (Merck, Darmstadt, Germany). A maximum difference of 0.3 pH units was allowed between the lowest and the highest measurement made in a single test. Samples for dissolved nickel analysis (filtration through a 0.45 µm filter, Gelman Sciences, Ann Arbor, MI, USA) were taken at the start and at the end of testing and were acidified with 0.14 N HNO_3. Nickel was measured using flame atomic absorption spectrometry (F-AAS, SpectrAA100, Varian, Mulgrave, Australia) for nickel concentrations above 100 µg/L and GF-AAS for nickel concentrations below 100 µg/L. Measurement procedures were the same as described in chapter 2.

In the test series used for model development as well as in the bivariate pH-magnesium test series, calcium and magnesium concentrations were measured at the start and at the end of testing (F-AAS). No calcium and magnesium measurements were performed during the tests of both validation test series. Na, K, Cl, SO_4, IC and DOC concentrations were not measured in any of the test series.

For data analysis, average measured values were used for those parameters that were measured at the start and at the end of testing. Nominal concentrations were used for all the other parameters. To determine the concentrations of Ca, Mg, Na, K, Cl, SO_4, IC and DOC in the tests conducted in natural waters, concentrations measured in the surface water samples (see above) were supplemented with nominal added concentrations (through addition of nutrient solutions and pH setting). Alkalinity was calculated from pH and IC concentrations (as determined above) using thermodynamic stability constants taken from Stumm and Morgan (1996).

5.2.6. Data treatment and statistics

All effect concentrations (72-h NOE_rC, LOE_rC, E_rC10 and E_rC50) were calculated using growth rate as an endpoint. In this study, the average specific growth rate μ (d^{-1}) in each replicate over 72 hours of exposure was calculated as the slope of the linear relationship between ln N_x and t_x (N_0 = cell density at the start of testing (t_0), N_2 and N_3 = measured cell

densities at day 2 (t_2) and 3 (t_3), respectively). NOE_rCs and LOE_rCs were determined using the Mann Whitney U test ($p < 0.05$). E_rC10s, E_rC50s and their 95% confidence intervals were calculated with a log-logistic model described by De Schamphelaere and Janssen (2004a) using the Levenberg-Marquardt algorithm (Levenberg, 1944; Marquardt, 1963).

5.2.7. Equilibrium chemical speciation calculations

Speciation of nickel and other ions was calculated using WHAM VI software (Tipping, 1998; NERC, 2001) as described in chapter 2. Speciation calculations were performed for all E_rC10s and E_rC50s. Since De Schamphelaere et al. (2006) demonstrated that both algal exudates and background DOC in synthetic waters based on deionized water do not significantly contribute to nickel speciation, fulvic (FA) and humic acid concentrations in the synthetic test solutions were set to zero. For calculation of nickel speciation in natural waters, the concentration of FA used as input into WHAM VI was calculated as 0.8 x DOC (mg C/L), using the DOC concentration measured prior to testing. Details on the modeling of organic nickel complexation are given in chapter 2. Dissolved trace metal concentrations of zinc, aluminium and iron, measured in the natural waters prior to testing, were also considered (see chapter 2 for computational details).

5.2.8. Model development

To investigate to what extent the original BLM concept can be used to explain nickel toxicity to *P. subcapitata* as a function of water chemistry, the protective effects of Ca^{2+}, Mg^{2+} and H^+ were modeled as single-site BLM-type competition effects. The model parameters representing these effects (K_{CaBL}, K_{MgBL} and K_{HBL}) were determined according to the method described by De Schamphelaere and Janssen (2002), using the linear regression equations between 72-h E_rC50_{Ni2+} (72-h E_rC50 expressed as Ni^{2+} activity) and Ca^{2+}, Mg^{2+} and H^+ activity, respectively. The BLM equation for prediction of the E_rC50_{Ni2+} in any test solution *i* can then be written as follows:

$$E_rC50_{Ni^{2+},i,predicted} = E_rC50_{Ni^{2+},0}.\left\{1 + K_{CaBL}.(Ca^{2+})_i + K_{MgBL}.(Mg^{2+})_i + K_{HBL}.(H^+)_i\right\} \qquad \text{Eq. 5.1}$$

In this model, $(Ca^{2+})_i$, $(Mg^{2+})_i$ and $(H^+)_i$ are the chemical activities of these ions in solution *i* and K_{CaBL}, K_{MgBL} and K_{HBL} are the stability constants for binding of Ca^{2+}, Mg^{2+} and H^+ to the biotic ligand (BL) on the algal cell surface, respectively. The model parameter $E_rC50_{Ni2+,0}$ can be regarded as the E_rC50_{Ni2+} in a hypothetical test water in which no competing cations are present. Within the proposed BLM concept, this model constant represents the 'inherent' sensitivity of the algae under consideration, i.e. the sensitivity which is independent of water chemistry. A comparison of Equation 5.1 with the original notation of the BLM equation as proposed by De Schamphelaere and Janssen (2002) demonstrates that the $E_rC50_{Ni2+,0}$ can also be written as follows:

$$E_rC50_{Ni^{2+},0} = \frac{f_{NiBL}^{50\%}}{(1-f_{NiBL}^{50\%}).K_{NiBL}} \qquad \text{Eq. 5.2}$$

where K_{NiBL} is the stability constant for binding of nickel to the BL and $f_{NiBL}^{50\%}$ is the sensitivity parameter, representing the fraction of BL sites occupied by nickel at 50% inhibition of growth rate. However, since K_{NiBL} and $f_{NiBL}^{50\%}$ can not be unambiguously derived from EC50 data alone, it was decided to use the $E_rC50_{Ni2+,0}$ as 'comprehensive' sensitivity parameter. The value of this model parameter was calculated as the geometric mean of the E_rC50_{Ni2+} observed in each test solution *i* used for model development and corrected for the presence of competing cations.

$$E_rC50_{Ni^{2+},0} = \sqrt[n]{\prod_i^n \frac{E_rC50_{Ni^{2+},i,observed}}{1+K_{CaBL}.(Ca^{2+})_i + K_{MgBL}.(Mg^{2+})_i + K_{HBL}.(H^+)_i}} \qquad \text{Eq. 5.3}$$

5.3. Results

5.3.1. Effects of calcium and magnesium on nickel toxicity

An increase of calcium and magnesium concentration from 0.12 to 5.0 mM (nominal concentrations) resulted in a 1.5-fold (93.7-141 µg/L) and 9.0-fold (124-1120 µg/L) increase of 72-h E_rC50_{Nidiss} (72-h E_rC50 expressed as dissolved nickel concentration), respectively (Table 5.3). The protective effects of calcium and magnesium are also clear from the other effect data given in Table 5.3 (NOE_rC, LOE_rC and E_rC10).

Table 5.3. Growth rate-based effect data (µg Ni_{diss}/L) for all test solutions: 72-h NOE_rC, LOE_rC, E_rC10 and E_rC50. For E_rC10s and E_rC50s, 95% confidence intervals are given between brackets.

	Test solution	72-h NOE_rC	72-h LOE_rC	72-h E_rC10	72-h E_rC50
	Ca 0.12 mM	18.0	32.3	30.3 (23.6-38.9)	93.7 (83.1-106)
	Ca 0.5 mM	21.2	36.7	36.6 (26.6-50.4)	108 (94.6-123)
	Ca 1.0 mM	34.5	48.4	37.3 (29.7-47.0)	114 (104-125)
	Ca 2.0 mM	54.0	102	51.9 (37.7-71.5)	136 (119-153)
	Ca 3.0 mM	31.9	49.3	31.5 (22.7-43.8)	122 (106-140)
	Ca 4.0 mM	26.2	51.0	42.1 (34.1-52.5)	144 (132-158)
	Ca 5.0 mM	48.6	97.2	40.5 (28.2-58.0)	141 (120-164)
	Mg 0.12 mM	- [c]	31.5	25.3 (17.6-35.9)	124 (107-144)
	Mg 0.5 mM	47.3	101	75.2 (55.7-101)	255 (226-284)
	Mg 1.0 mM	83.0	155	108 (81.5-144)	321 (284-361)
	Mg 1.5 mM	- [c]	89.4	124 (95.6-159)	399 (358-446)
	Mg 2.0 mM	88.9	163	162 (125-211)	596 (534-665)
	Mg 2.5 mM	154	280	252 (189-340)	742 (659-846)
	Mg 3.0 mM	285	953	213 (96.5-464)	812 (578-1140)
	Mg 4.0 mM	- [c]	278	284 (209-392)	821 (713-944)
	Mg 5.0 mM	292	537	365 (276-483)	1120 (992-1270)
	pH 6.0	- [c]	52.3	47.5 (42.1-53.0)	125 (119-133)
	pH 6.4	47.9	100	51.9 (43.8-61.6)	145 (136-156)
	pH 7.2	29.4	45.5	37.0 (30.9-44.3)	91.8 (84.8-98.5)
	pH 7.6	33.4	54.1	44.3 (39.7-48.9)	83.1 (79.8-87.4)
	pH 8.0	- [c]	16.6	35.9 (24.5-52.5)	81.5 (69.4-95.6)
	pH 6.0				
	Mg 0.12 mM	68.4	104	57.6 (45.6-72.8)	172 (155-190)
	Mg 1.5 mM	327	570	312 (221-440)	880 (776-999)
	Mg 3.0 mM	181	326	145 (76.8-275)	883 (705-1110)
	pH 7.0				
	Mg 0.12 mM	- [c]	55.7	26.5 (17.3-40.6)	108 (90.1-131)
	Mg 1.5 mM	104	186	180 (13.6-221)	601 (552-654)
	Mg 3.0 mM	187	320	292 (214-398)	914 (805-1040)
	pH 7.8				
	Mg 0.12 mM	26.9	42.5	31.5 (23.9-41.4)	98.3 (87.3-111)
	Mg 1.5 mM	- [c]	38.1	44.3 (21.7-90.4)	345 (268-444)
	Mg 3.0 mM	65.0	103	91.2 (64.3-129)	395 (346-452)
05/2001	Bihain [a]	67.1	116	90.0 (54.5-148)	483 (380-620)
		73.0	129	88.2 (58.0-136)	508 (437-596)
	Ankeveen [a]	145	390	314 (237-416)	1240 (1120-1350)
		132	217	219 (125-384)	1040 (854-1260)
	River Mole [a]	78.4	119	154 (113-211)	584 (473-728)
		- [c]	65.0	73.7 (53.5-100)	750 (665-854)
	OECD [a, b]	- [c]	52.1	67.1 (44.7-101)	339 (293-392)
		41.9	84.9	44.6 (33.0-60.2)	400 (353-453)
09/2005	Ankeveen	98.6	176	425 (330-546)	1630 (1490-1780)
	Brisy	97.7	168	154 (129-183)	506 (474-540)
	Regge	- [c]	93.8	301 (255-354)	1190 (1120-1260)
	OECD [b]	21.5	43.0	62.5 (49.0-79.8)	362 (328-400)
	$OECD^+$ [b]	26.4	59.6	98.5 (74.8-130)	669 (600-746)

[a] Tests in these waters were conducted in duplicate.

[b] Test medium recommended by the OECD (1996) and prepared and modified as described in the materials and methods section. The $OECD^+$ test water has increased water hardness.

[c] A statistically significant effect was observed at the lowest exposure concentration tested.

Speciation calculations (WHAM VI) demonstrated that the average nickel species distribution at the 72-h E_rC50 levels was as follows: 92.5% Ni^{2+}, 4.4% $NiHCO_3^+$, 1.6% $NiCO_3$ and < 1% $NiOH^+$, $Ni(OH)_2$, $NiSO_4$ and $NiCl^+$. Figure 5.1 shows the linear relationships between 72-h E_rC50_{Ni2+} and Ca^{2+} (upper panel) and Mg^{2+} (lower panel, solid line) activity. Since calcium and magnesium do not affect nickel speciation to a large extent, 72-h E_rC50_{Ni2+} increased with increasing Ca^{2+} and Mg^{2+} activities in a similar way to what was observed based on 72-h E_rC50_{Nidiss} and total calcium and magnesium concentrations.

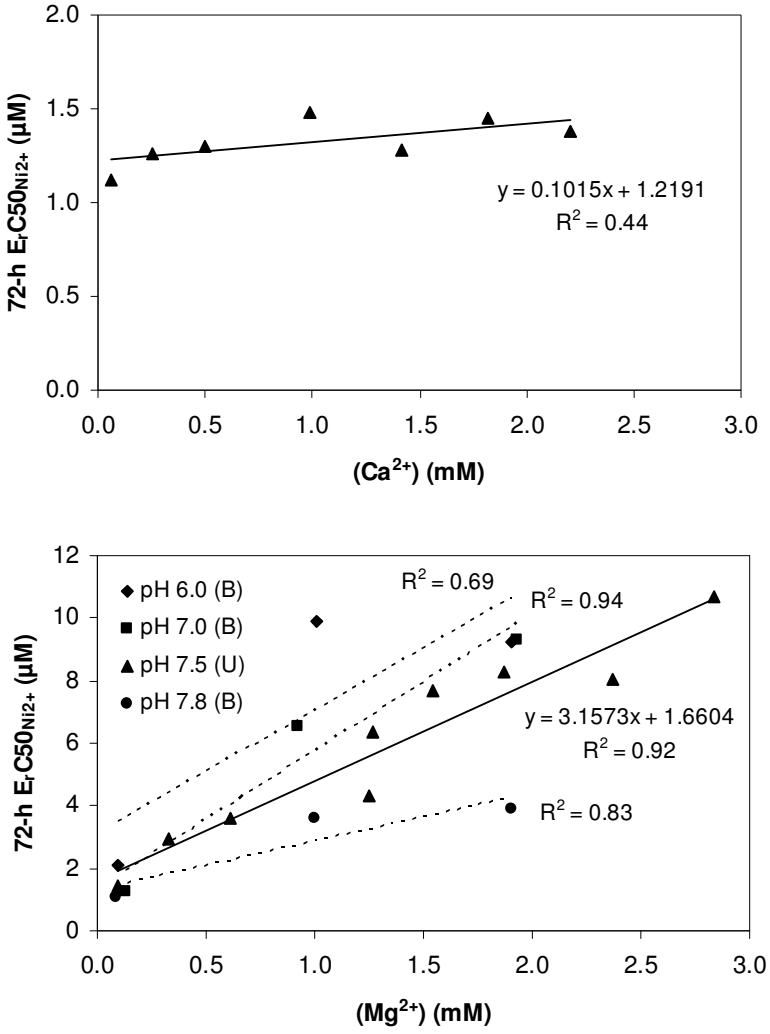

Figure 5.1. 72-h E_rC50_{Ni2+} (expressed as Ni^{2+} activity, µM) as a function of Ca^{2+} activity (mM, upper panel) and Mg^{2+} activity (mM, lower panel). The equations of the regression lines were used for modeling the effects of calcium and magnesium on nickel toxicity. In the lower panel the results are shown from both the univariate (U) magnesium test series (solid regression line, used for model development) and the bivariate (B) pH-magnesium test series (dashed regression lines).

5.3.2. Effect of pH on nickel toxicity

When pH decreased from 7.92 to 6.45 (measured pH levels), the 72-h E_rC50_{Nidiss} increased from 81.5 to 145 µg/L. A further decrease of pH down to 6.01 did not result in a further increase of the 72-h E_rC50_{Nidiss} (Table 5.3). Speciation calculations (WHAM VI) demonstrated that Ni^{2+} was the most abundant nickel species over the entire pH range tested (6.01-7.92). Nevertheless, the contribution of Ni^{2+} in the nickel species distribution gradually decreased from 96.7% at pH 6.01 to 88.6% at pH 7.92. On the other hand, the relative presence of $NiHCO_3^+$ and $NiCO_3$ increased from 1.7 to 4.8% and from 0.023 to 5.2% between pH 6.01 and 7.92, respectively. The remaining nickel species all represented < 1% of the dissolved nickel concentration. Figure 5.2 shows the relationship between 72-h E_rC50_{Ni2+} and H^+ activity (upper panel). Although the relationship can not be considered linear over the entire pH range tested, it is clear that Ni^{2+} is more toxic at high pH levels (low H^+ activities).

5.3.3. Combined effects of pH and magnesium on nickel toxicity

The results of the bivariate test design in which nickel toxicity was investigated at three different pH levels (6.0, 7.0 and 7.8, nominal values) and three different magnesium concentrations (0.12, 1.5 and 3.0 mM, nominal concentrations) are reported in Table 5.3. At pH 6.0, 7.0 and 7.8, an increase of magnesium concentration from 0.12 to 3.0 mM resulted in a 5.1- (172-883 µg/L), 8.5- (108-914 µg/L) and 4.0-fold (96.3-395 µg/L) increase of the 72-h E_rC50_{Nidiss}, respectively. This is more or less in agreement with the 6.6-fold increase of the 72-h E_rC50_{Nidiss} over the same magnesium concentration range in the univariate magnesium test series conducted at pH 7.5. The relationships between 72-h E_rC50_{Ni2+} and Mg^{2+} activity are shown in Figure 5.1 (lower panel, dashed lines).

At each magnesium concentration tested (0.12, 1.5 and 3.0 mM), the 72-h E_rC50_{Nidiss} at pH 7.8 was lower than the 72-h E_rC50_{Nidiss} at pH 6.0 (Table 5.3). Since Ni^{2+} was the most abundant nickel species in all these tests, the same observation holds for the 72-h E_rC50_{Ni2+}. However, a more pronounced plateau-like relationship between 72-h E_rC50_{Ni2+} and H^+ activity is observed with increasing magnesium concentrations (Figure 5.2, lower panel, dashed lines). This observation holds when the results from the univariate pH test series (conducted at 0.12 mM magnesium) are included in the analysis (Figure 5.2, lower panel, solid line).

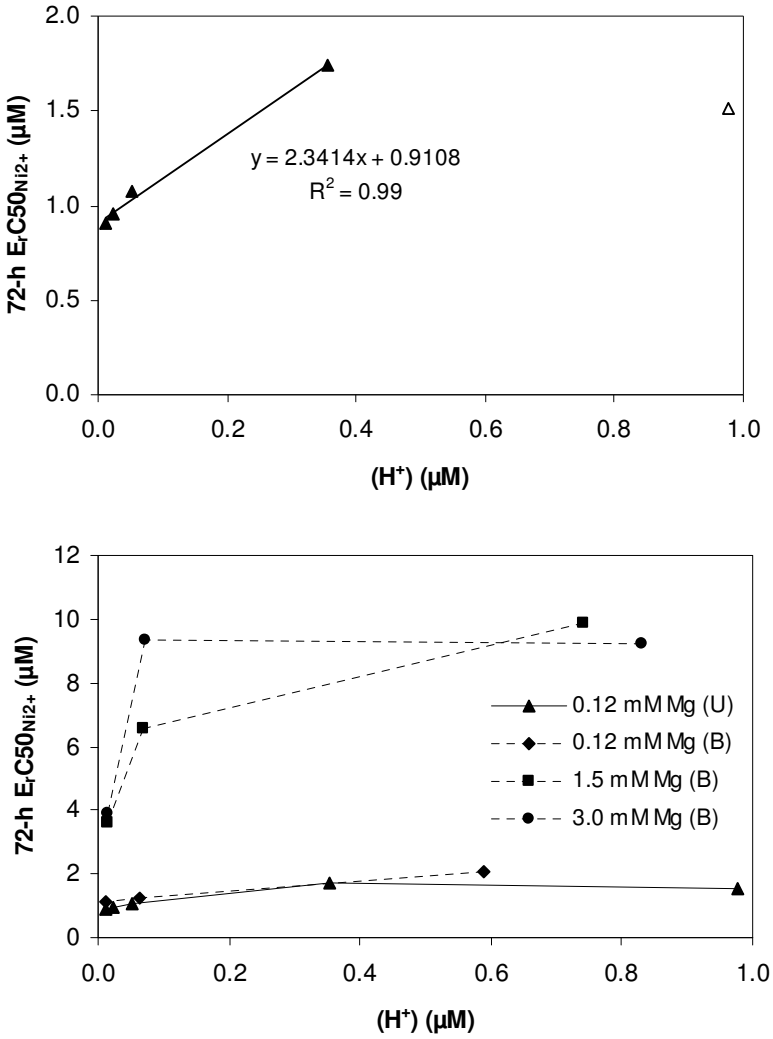

Figure 5.2. 72-h E_rC50_{Ni2+} (expressed as Ni^{2+} activity, µM) as a function of H^+ activity (µM). In the upper panel the results are shown from the univariate pH test series. The open symbol represents the test conducted at pH 6.01 (H^+ activity = 0.98 µM). The regression line through the remaining points was used as a basis for modeling the effect of pH as BLM-type single-site competition effect. In the lower panel the results are shown from both the univariate (U) pH test series (solid line) and the bivariate (B) pH-magnesium test series (dashed lines).

5.3.4. Model development

Based on the E_rC50 data from the univariate test series, the observed effects of calcium, magnesium and pH were modeled as BLM-type single-site competition effects. Based on the equations of the regression lines shown in Figure 5.1 (upper panel: Ca^{2+}; lower panel: Mg^{2+}, solid line) and Figure 5.2 (upper panel: H^+), log K_{CaBL}, log K_{MgBL} and log K_{HBL} were calculated to be 2.0, 3.3 and 6.5, respectively. Since the relationship between 72-h E_rC50_{Ni2+} and H^+ activity could only be considered linear up to an H^+ activity of 0.35 µM (pH 6.45), the data point from the test conducted at an H^+ activity of 0.98 µM (pH 6.01) was

excluded from model development (Figure 5.2, upper panel, open symbol). This sets the lower pH boundary of the model's applicability to 6.45. Using Equation 5.3, the sensitivity parameter $E_rC50_{Ni2+,0}$ was calculated to be 1.12 µM. An overview of all model parameters is given in Table 5.4 (see further).

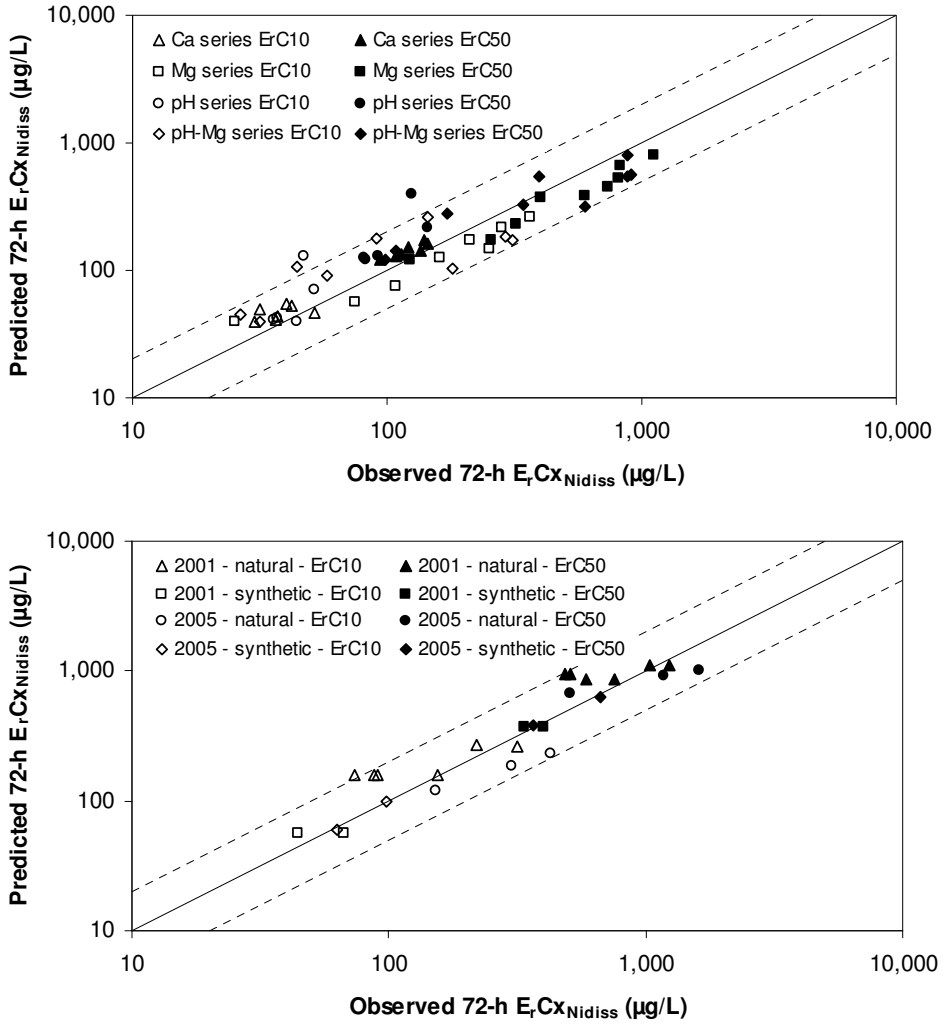

Figure 5.3. Predicted 72-h E_rCx_{Nidiss} versus observed 72-h E_rCx_{Nidiss} (µg/L). Predictions were made using Equation 5.1 linked to WHAM VI. The solid line indicates a perfect match between predicted and observed E_rCx values; the dashed lines indicate ratios of 0.5 and 2 between observed and predicted E_rCx values. Upper panel: model performance for the univariate calcium, magnesium and pH test series (used for model development) and the bivariate pH-magnesium test series; lower panel: model performance for the OECD test waters and natural waters of the validation test series (see Table 5.2). The values for the sensitivity parameters $E_rC50_{Ni2+,0}$ and $E_rC10_{Ni2+,0}$ are given in Table 5.4.

To investigate how well the model is calibrated to the dataset used for model development, the 72-h E_rC50_{Ni2+} for each test solution was predicted using Equation 5.1,

translated to the 72-h E_rC50_{Nidiss} using WHAM VI software, and compared to the observed 72-h E_rC50_{Nidiss}. The 72-h E_rC50_{Nidiss} were predicted by an average error of factor 1.3 (prediction error range = 1.0-1.7). Provided that the sensitivity parameter is adjusted, the model parameters of the E_rC50 model can also be used for predicting E_rC10s. Using an $E_rC10_{Ni2+,0}$ of 0.365 µM (calculated using Equation 5.3), the 72-h E_rC10_{Nidiss} were also predicted by an average error of factor 1.3 (1.0-1.7). For the test conducted at pH 6.01, which was not used for model development, the 72-h E_rC50_{Nidiss} and 72-h E_rC10_{Nidiss} were predicted by an error > factor 2 (3.2 and 2.7, respectively).

To investigate whether the developed model could accurately predict nickel toxicity in synthetic solutions in which pH and magnesium concentrations were varied simultaneously, the model was used to predict nickel toxicity in each test solution used in the bivariate pH-magnesium test series. The 72-h E_rC50_{Nidiss} and 72-h E_rC10_{Nidiss} were predicted by an average error of factor 1.4 (1.1-1.9) and 1.8 (1.3-2.4), respectively. The prediction errors were all < factor 2 except for the 72-h E_rC10_{Nidiss} in the test conducted at 1.5 mM Mg and pH 7.8. Remarkably, all 72-h E_rC50_{Nidiss} and 72-h E_rC10_{Nidiss} obtained at pH 6.0 (pH < lower pH boundary of model) were predicted by an error < factor 2. As mentioned above, this was not the case in the univariate pH test series. Model performance in all synthetic test solutions discussed in this paragraph is visualized in Figure 5.3, upper panel.

5.3.5. Model validation

The 72-h E_rC50_{Nidiss} obtained in the nickel-spiked surface waters (Table 5.2) varied between 483 and 1630 µg/L (Table 5.3). Speciation calculations (WHAM VI) demonstrated that at the 72-h E_rC50 levels, between 45.7 and 83.5% of the dissolved nickel occurred as free Ni^{2+}, the remainder being primarily complexed by DOC (12.5-46.6%), sulphate (up to 7.5% $NiSO_4$) and – at pH levels > 7.5 – carbonate (up to 11.6 and 11.9% $NiHCO_3^+$ and $NiCO_3$, respectively). The 72-h E_rC50_{Nidiss} observed in the $OECD^+$ medium (water hardness = 250 mg $CaCO_3$/L) was 1.8-fold higher (669 µg/L) than that observed in the basic OECD medium (water hardness = 24.0 mg $CaCO_3$/L) in the same test series (362 µg/L). This clearly reflects the protective effect of water hardness.

The 72-h E_rC50_{Nidiss} observed in the three tests in basic OECD medium were 2.7- to 4.3-fold higher (339-400 µg/L) than those observed in the two tests conducted in the same

medium in the univariate calcium and magnesium test series (93.7 and 124 µg/L, respectively). This may point at a significant sensitivity difference between the test series used for model development and the validation test series. A comparison of the 'test series-specific' $E_rC50_{Ni2+,0}$ values reported in Table 5.4 demonstrates that the 'test series-specific' $E_rC50_{Ni2+,0}$ values for both validation test series ($E_rC50_{Ni2+,0}$ = 3.27 and 3.97 µM) were substantially higher than those obtained for the different test series used for model development (0.762-1.52 µM). The fact that similar $E_rC50_{Ni2+,0}$ were obtained for the natural waters and the simultaneously tested OECD test waters indicates that the observed differences may indeed be ascribed to sensitivity shifts of the cultured algae over time. Similar though less pronounced differences were observed based on the 72-h E_rC10_{Nidiss}. Not surprisingly, all 72-h E_rC50_{Nidiss} were underestimated by more than a factor 2 when the model was not calibrated for the observed sensitivity difference.

To maintain the possibility to independently investigate the model's applicability to natural waters, the sensitivity parameters for the validation test series were optimized based on the results from the tests conducted in OECD medium ($E_rC50_{Ni2+,0}$ and $E_rC10_{Ni2+,0}$ = 3.57 and 0.549 µM, respectively, Table 5.4). Using these parameters, the 72-h E_rC50_{Nidiss} and 72-h E_rC10_{Nidiss} were predicted by an average error of factor 1.3 (prediction error range = 1.0-1.9) and 1.4 (1.0-2.1), respectively (Figure 5.3, lower panel). Remarkably, the prediction errors for the two tests conducted in Bihain test water (pH < lower pH boundary of model) were < factor 2.

Table 5.4. 'Test series-specific' and final values for the sensitivity parameters $E_rC50_{Ni2+,0}$ and $E_rC10_{Ni2+,0}$ (µM).

Test series	Test series-specific $E_rC50_{Ni2+,0}$ (µM)	Final $E_rC50_{Ni2+,0}$ (µM)	Test series-specific $E_rC10_{Ni2+,0}$ (µM)	Final $E_rC10_{Ni2+,0}$ (µM)
Ca	0.953	1.12 [b]	0.299	0.365 [b]
Mg	1.52		0.448	
pH [a]	0.762		0.327	
pH-Mg [a]	1.19		0.294	
Validation 2001 [a]	3.27	3.57 [c]	0.465	0.549 [c]
Natural [a]	3.12		0.434	
OECD	3.59		0.532	
Validation 2005	3.97		0.772	
Natural	4.28		0.949	
OECD	3.55		0.567	

[a] Data points from test solutions with pH < 6.45 (lower pH boundary of model) not included.

[b] Based on the results from the univariate calcium, magnesium and pH test series.

[c] Based on test results obtained in OECD test medium (three data points obtained in basic OECD test medium and one data point obtained in OECD medium with increased water hardness, see Table 5.2).

5.4. Discussion

5.4.1. Effects of calcium and magnesium on nickel toxicity

In this study, both calcium and magnesium were observed to protect *P. subcapitata* against nickel toxicity. With a 9.0-fold reduction of nickel toxicity (based on 72-h E_rC50_{Nidiss}) over a magnesium concentration range of 0.12 to 5.0 mM (nominal concentrations), magnesium appeared to offer the highest protection. To our knowledge, this study is the first to demonstrate an important nickel toxicity mitigating effect of magnesium for algae. Previously, we also demonstrated the importance of magnesium in reducing nickel toxicity to rainbow trout (chapter 2) and *D. magna* (chapter 3 and 4).

To our knowledge, only a few studies have observed nickel-magnesium interactions in algae, be it without investigating the effect of increasing magnesium concentrations on nickel toxicity. Mehta et al. (2002) reported a decrease of nickel adsorption to and uptake by *Chlorella vulgaris* with increasing magnesium concentrations. Recent Ni^{2+} uptake experiments conducted by Worms and Wilkinson (2007) indicated that the presence of 0.1 mM Mg^{2+} significantly reduced nickel uptake by *Chlamydomonas reinhardtii* over a wide range of Ni^{2+} concentrations (see further) compared to that observed in Mg^{2+}-free solutions. Finally, it has been reported that Ni^{2+} can substitute Mg^{2+} in the porphyrin ring of chlorophyll molecules (Küpper et al., 2006). However, metal-chlorophyll formation is not a nickel-specific phenomenon and has also been reported for several other metals (e.g., copper, cadmium and zinc, see Küpper et al., 2006).

The interaction between nickel and magnesium appears to be a lot better documented for organisms other than algae. For *D. magna* and rainbow trout we can refer to chapter 2, 3 and 5 of this study as well as to the studies of Pane et al. (2003b, 2006a,b). Nickel-magnesium interactions are also well known from mammalian literature (discussed by Pane et al., 2003b). However, the largest source of information on this topic is found in microbiological literature. For example, Abelson and Aldous (1950) demonstrated that increasing magnesium concentrations reduced nickel uptake by and toxicity to *Escherichia coli* and *Aerobacter aerogenes* (bacteria), *Candida utilis* (yeast) and *Aspergillus niger* (mould). Haavik (1976) and Joho et al. (1991) made similar observations for the bacterium *Bacillus licheniformis* and the yeast *Saccharomyces cerevisiae*, respectively.

All these observations suggest the existence of a shared uptake pathway for Ni^{2+} and Mg^{2+}. This is supported by the fact that Ni^{2+} and Mg^{2+} have similar dehydrated ionic radii (Weast, 1973). Evidence for Ni^{2+} uptake via Mg^{2+} transport systems has been provided for very different types of organisms: Archaea (e.g., *Methanococcus jannaschii*, Smith et al., 1998), Eubacteria (e.g., *A. aerogenes* and *E. coli*, Webb, 1970; *Bradyrhizobium japonicum*, Fu and Maier, 1991; *Salmonella typhimurium*, Snavely et al., 1991; Smith and Maguire, 1998), Fungi (e.g., *S. cerevisiae*, MacDiarmid and Gardner, 1998), Plantae (e.g., *Arabidopsis thaliana*, Li et al., 2001) and Animalia (e.g., human and mouse, Goytain and Quamme, 2005). Moreover, in several of these studies (MacDiarmid and Gardner, 1998; Smith et al., 1998; Li et al., 2001), the identified Mg^{2+} transport proteins were demonstrated to show an important structural and functional homology to the well-studied bacterial Mg^{2+} transport system CorA (Snavely et al., 1991; Smith and Maguire, 1998), suggesting at least some degree of conservation throughout evolution.

A recent study conducted by Worms and Wilkinson (2007) provided strong evidence for the existence of a shared uptake pathway for Ni^{2+} and Mg^{2+} in algae. These authors not only demonstrated that Mg^{2+} was capable of reducing Ni^{2+} uptake by *C. reinhardtii*, they also obtained similar log K values for the interaction of Ni^{2+} and Mg^{2+} with the Ni^{2+} internalization site (log K values both equalled 5.1). These observations strongly suggest that one or more magnesium transport systems represent a major pathway for nickel to enter the cell.

Although the protective effect of calcium was observed to be much less important than that of magnesium, nickel-calcium interactions have also been reported in algal (*Kirchneriella lunaris*, Issa et al., 1995; *C. vulgaris*, Mehta et al., 2000, *C. reinhardtii*, Worms and Wilkinson, 2007) and microbiological literature (e.g., *Nostoc muscorum*, blue-green alga, Rai and Raizada, 1985; *S. cerevisiae*, yeast, Joho et al., 1991). All these studies reported a decrease of nickel uptake and/or toxicity with increasing calcium concentrations. In this study, calcium was also observed to ameliorate nickel toxicity to rainbow trout (chapter 2) and *D. magna* (chapter 3 and 4).

Compared to magnesium, Worms and Wilkinson (2007) observed a much smaller decrease of Ni^{2+} internalization fluxes with increasing calcium concentrations (a 3-fold decrease upon a 1000-fold increase in calcium). The log K value obtained for the interaction of Ca^{2+} with the Ni^{2+} internalization site was much lower than the log K values obtained for

Mg^{2+} and Ni^{2+}. This is consistent with the findings of Snavely et al. (1991) for *S. typhimurium* and Joho et al. (1991) for *S. cerevisiae* that Ca^{2+} protects against nickel toxicity through direct competition with Ni^{2+} for uptake by Mg^{2+} transport systems, although to a much lesser extent than Mg^{2+} itself.

Further, the possibility should not be excluded that calcium, as a regulator of membrane permeability, also protects indirectly against nickel toxicity. In algae, calcium has been observed to induce membrane stabilization under stressful conditions (e.g., *Chlorella fosca*, Abdel-Basset and Issa, 1994). Increased membrane stabilization may for instance limit nickel-induced loss of ions, such as Mg^{2+}. In this respect, it must be noticed that so far, the adverse effects of nickel on Mg^{2+} efflux have only been demonstrated for *D. magna* (Pane et al., 2003b). Increased membrane stabilization may reduce Ni^{2+} efflux as well, as suggested by Worms and Wilkinson (2007) to explain increased Ni^{2+} internalization fluxes upon addition of low concentrations of calcium (< 0.05 mM). This would however counteract the protective effect of calcium. As for rainbow trout and *D. magna* (chapter 2, 3 and 4), it may be concluded that further research is needed to elucidate the mechanisms that are involved in determining the effect of calcium on nickel toxicity.

5.4.2. Effect of pH on nickel toxicity

Overall, the toxicity of Ni^{2+} was observed to increase with increasing pH. A similar observation was made by Macfie et al. (1994) for *C. reinhardtii*. However, the relationship between 72-h E_rC50_{Ni2+} and H^+ activity (Figure 5.2, upper panel) could not be considered linear over the entire pH range tested (pH 6.01-7.92, H^+ activity = 0.012-0.98 µM). Moreover, a more pronounced plateau-like relationship was observed with increasing magnesium concentrations (Figure 5.2, lower panel).

More or less similar nonlinear relationships have also been observed for other metals and/or algae, such as cadmium (*Scenedesmus quadricauda*, Peterson et al., 1984), aluminium (*Chlorella pyrenoidosa*, Parent and Campbell, 1994), zinc (*P. subcapitata*, Heijerick et al., 2002a) and copper (*S. quadricauda*, Peterson et al., 1984; *P. subcapitata*, De Schamphelaere et al., 2003; *C. vulgaris*, De Schamphelaere and Janssen, 2006).

The decrease of metal toxicity with decreasing pH levels is generally attributed to competition between protons and free metal ions for binding to ligands on the cell surface (Campbell and Stokes, 1985; Parent and Campbell, 1994). Macfie et al. (1994) demonstrated that there is also considerable competition for binding sites on the plasma membrane. This is supported by recent findings of Worms and Wilkinson (2007) that H^+ competes directly with Ni^{2+} for uptake by *C. reinhardtii*.

However, nonlinear relationships between H^+ activity and metal toxicity (expressed as free metal ion activity) indicate that the effect of pH can not be explained by H^+ competition for a single site alone. Overall, it has been observed that at high pH levels, less free metal ion is needed to cause a certain toxic effect in algae. In the model language used in this study this would be translated to a lower $EC50_{Me2+,0}$ at high pH levels than at lower pH levels. From equation 5.2 it is clear that a decrease of $EC50_{Me2+,0}$ could be the result of an increase of K_{MeBL}. Several authors have already discussed the possibility that K_{MeBL} increases with increasing pH (e.g., Parent and Campbell, 1994; Heijerick et al., 2002a; Fortin et al., 2007). According to Parent and Campbell (1994), pH changes may induce an allosteric change in the conformation and affinity of the BL.

It must be noted that, in the case of multiple metal internalization systems with different affinities for the free metal ion, the use of a single K_{MeBL} can only represent the average affinity of these systems. An increase of K_{MeBL} with increasing pH may hence be attributed to an increased importance of high-affinity transport systems with increasing pH. Observations made by Crist et al. (1990) and Xue and Sigg (1990) suggest that H^+ competition may occur at multiple binding sites with different pKa values on the cell wall. It remains to be investigated whether H^+ competition also occurs at multiple metal binding sites on the plasma membrane.

To explain the curvilinear relationship between H^+ activity and copper toxicity to *P. subcapitata* (expressed as free copper ion activity), De Schamphelaere et al. (2003) considered the possibility that $CuOH^+$ and $CuCO_3$ (the two most abundant inorganic copper species at higher pH levels) may also be toxic in addition to Cu^{2+}. This has also been demonstrated for fish and daphnids (Erickson et al., 1996; De Schamphelaere and Janssen, 2002; De Schamphelaere et al., 2002). The toxicity of $CuOH^+$ is assumed to occur mainly through direct binding to the BL, whereas $CuCO_3$ is suggested to contribute indirectly to

copper toxicity as a result of pH and alkalinity differences between the bulk solution and the organism's microenvironment.

As for *P. subcapitata*, we also observed nonlinear relationships between nickel toxicity (expressed as Ni^{2+} activity) and H^+ activity for rainbow trout (chapter 2) and *D. magna* (chapter 3 and 4). Interestingly, some of our explanations for the observed nonlinearity, such as the existence of multiple binding sites and the apparent toxicity of nickel-carbonate complexes, also turn up – be it for other metals – in algal literature (see above).

For rainbow trout and *D. magna*, we suggested that nickel-carbonate complexes may increase nickel toxicity at high pH levels through dissociation in the physicochemically different gill microenvironment and/or facilitation of Ni^{2+} uptake through Mg^{2+} transport systems similar to the electro-neutral Mg^{2+}/HCO_3^- symporter identified by Günther et al. (1986) in Yoshida ascites tumor cells.

As discussed above, Mg^{2+} transport systems appear to be quite well conserved throughout evolution. Possibly, similar Mg^{2+} transport systems also exist in algae and other organisms. It is clear that uptake of Ni^{2+} by a Mg^{2+}/HCO_3^- transporter would be enhanced by (i) increasing HCO_3^- concentrations, which normally occur at increased pH and alkalinity levels, and (ii) dissociation of nickel-carbonate complexes (increasingly present with increasing pH and alkalinity) in the phycosphere, which is assumed to be physicochemically different from the bulk solution. Interestingly, Lustigman et al. (1995) demonstrated that *C. vulgaris* can modify culture pH and ascribed this to the release of buffering substances (such as amino acids) or the uptake of H^+ ions. This was also suggested by Parent and Campbell (1994). If algae are capable of modifying pH and/or alkalinity in the phycosphere, this clearly would affect metal speciation and hence toxicity.

5.4.3. Combined effects of pH and magnesium on nickel toxicity

At all pH levels tested, magnesium was observed to protect against nickel toxicity (Figure 5.1, lower panel). The slopes of the regression lines through the data points obtained at pH 6.0, 7.0 and 7.5 were not significantly different from each other (F-test, $p < 0.05$, according to Sokal and Rohlf, 1981). However, to investigate whether pH affects the extent to

which magnesium protects against nickel toxicity, it is recommended to calculate the logarithm of the ratio of slope (S) and intercept (I) for the regression lines obtained at pH 6.0, 7.0 and 7.8 (Figure 5.1, lower panel, dashed lines). The log (S/I) values, which represent indicative values for log K_{MgBL}, were calculated to be 3.1, 3.5 and 3.1 at pH 6.0, 7.0 and 7.8, respectively. The average log (S/I) was 3.3, which is equal to the log K_{MgBL} determined based on the results of the univariate magnesium test series conducted at pH 7.5 (Figure 5.1, lower panel, solid regression line). Therefore, and because of the limited variation between the log (S/I) values obtained at the different pH levels tested, it was considered not necessary to incorporate a pH-dependent log K_{MgBL} in the model until further research confirms our observations and provides a better mechanistic understanding of the possible interactive effects of pH and magnesium on nickel toxicity.

With increasing magnesium concentrations, a more pronounced plateau-like relationship was observed between 72-h E_rC50_{Ni2+} and H^+ activity (Figure 5.2, lower panel). However, a plot of 72-h $E_rC50_{pNi2+}{}^*$ (= – log (72-h E_rC50_{Ni2+} corrected for the presence of calcium and magnesium)) versus pH yielded linear regression lines with similar slopes at each magnesium concentration tested (Figure 5.4). The slopes of the regression lines were calculated not to be significantly different from each other (F-test, $p < 0.05$, according to Sokal and Rohlf, 1981).

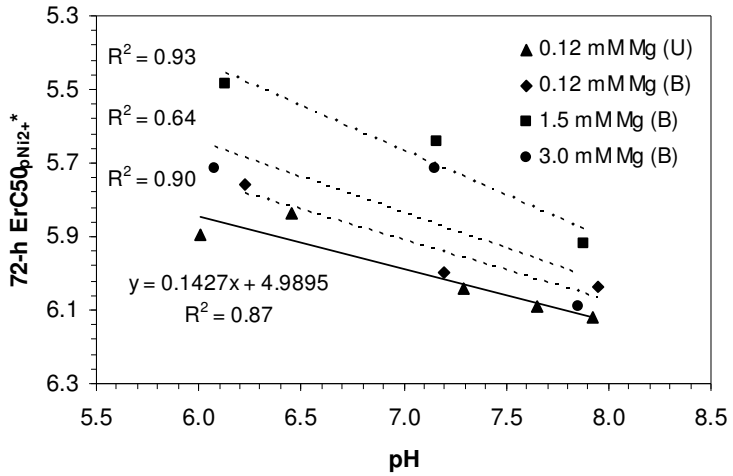

Figure 5.4. 72-h $E_rC50_{pNi2+}{}^*$ (= – log (72-h E_rC50_{Ni2+} corrected for the presence of calcium and magnesium)) as a function of pH. The solid line and the dashed lines represent the regression lines for the results from the univariate (U) pH test series and the bivariate (B) pH-magnesium test series, respectively. The regression line for the results from the univariate (U) pH test series (solid line) was used for modeling the effect of pH according to the method described in chapter 2.

Interestingly, similar log-linear pH-toxicity relationships have been reported for other metals in algal literature (cadmium, Peterson et al., 1984; copper, De Schamphelaere et al., 2003; zinc, De Schamphelaere et al., 2005a). In chapter 2 and chapter 4, such relationships have also been used as a basis for modeling the effect of pH on long-term nickel toxicity to rainbow trout and chronic nickel toxicity to *D. magna*, respectively. This clearly opens perspectives for model refinement (see further).

Since the 72-h $E_rC50_{pNi2+}^{*}$ are corrected for the presence of calcium and magnesium (using the earlier determined log K_{CaBL} and log K_{MgBL}), one would expect the regression lines to coincide. Theoretically, differences between the intercepts of parallel regression lines point at different sensitivities. There was indeed a slight sensitivity difference between the univariate pH test series and the bivariate pH-magnesium test series (Table 5.4). However, the different intercepts within the bivariate pH-magnesium test series may indicate that some variation in toxicity is not captured by the model. However, the possible unexplained variation in toxicity is very limited, since the model was demonstrated to accurately predict nickel toxicity in both synthetic and natural waters.

The existence of interactive effects of pH and water hardness has been suggested previously for the toxicity of other metals to algae (copper, De Schamphelaere and Janssen, 2006; uranium, Fortin et al., 2007). The results from our study on chronic nickel toxicity to *D. magna* (chapter 4) may also indicate that there are interactions between the effects of water hardness and pH. Although these interactions may be subtle and not important enough to be incorporated in bioavailability models, from a purely physiological point of view they are worth to be investigated more closely.

5.4.4. Effect of DOC on nickel toxicity

Speciation calculations for the nickel-spiked surface waters revealed that at the 72-h E_rC50 levels 12.5 to 46.6% of the dissolved nickel was complexed by DOC. The 72-h E_rC50_{Nidiss} observed in the natural waters were positively correlated to their DOC content (R^2 = 0.64), indicating the importance of DOC as a nickel toxicity reducing factor. The model developed in this study only takes into account the effect of DOC on nickel speciation. The results of the model validation exercise do not suggest that DOC may affect nickel toxicity beyond its effect on speciation. This is supported by recent findings of Worms et al. (2007)

that nickel uptake by *C. reinhardtii* and *Chlorella kesslerii* in the presence of humic substances can be quantitatively predicted on the basis of Ni^{2+} concentrations. However, certain studies on other metals have reported a higher metal uptake than expected based on free metal ion concentrations in the presence of organic substances. For lead, Slaveykova et al. (2003) attributed the increased uptake in the presence of fulvic acid to an increase in surface charge resulting from fulvic acid sorption to the algal surface.

5.4.5. Model development and validation

To investigate to what extent the original BLM concept could explain nickel toxicity as a function of water chemistry, the observed effects of Ca^{2+}, Mg^{2+} and H^+ were modeled as BLM-type single-site competition effects. Since the relationship between 72-h E_rC50_{Ni2+} and H^+ activity could only be considered linear up to an H^+ activity of 0.35 µM (pH 6.45), the data point obtained at an H^+ activity of 0.98 µM (pH 6.01) was excluded from model development. This sets the lower pH boundary of the model's applicability to 6.45. Nickel toxicity was predicted by an error < factor 2 in all synthetic test solutions used for model development. As expected, prediction errors were > factor 2 for the test conducted at pH 6.01.

For the bivariate pH-magnesium test series, all prediction errors (except for one 72-h E_rC10) were < factor 2. Clearly, the limited interaction observed in this study between the effects of pH and magnesium is not important enough to result in unacceptably inaccurate predictions of the model. Therefore, it was decided not to incorporate any interaction in the model until the underlying mechanisms of possible interactive effects are better understood.

Provided that the model was calibrated for the lower sensitivity of the test organisms used in both validation test series, nickel toxicity was also accurately predicted in the natural waters. Only one 72-h E_rC10 was predicted by an error > 2. Both in the validation test series and the bivariate pH-magnesium test series, prediction errors for tests conducted at pH < 6.45 (the lower pH boundary of the model) were < factor 2, indicating that the pH range of the model's applicability may possibly be extended without resulting in unacceptable prediction errors.

The results of this study indicated that the sensitivity of the test algae may have shifted over time. This is not unlikely, since the different test series have been conducted over an

overall period of almost five years. The toxicity tests of the first and the second validation test series have been conducted in 2001 and 2005, respectively, whereas the tests used for model development were performed between the end of 2003 and the beginning of 2004. In our study on the chronic toxicity of nickel to *D. magna* (chapter 4), we also found indications for a sensitivity shift of our *D. magna* clone over time. Interclonal sensitivity shifts are not exceptional and have been studied by several authors (e.g., Soares et al., 1992; Baird and Barata, 1997, 1998). Baird and Barata (1998) even suggested that some within-clone variation in sensitivity observed in laboratory cultures is essentially uncontrollable. For algae exposed to nickel, within-clonal sensitivity differences may for instance be due to differences in the magnesium status of the algal cells. This is strongly supported by recent findings of Worms and Wilkinson (2007).

Although the accuracy of the BLM-type model could be considered acceptable, the curvilinear relationship between 72-h E_rC50_{Ni2+} and H^+ activity clearly leaves room for model refinement. Several ways have been described in literature to model such relationships. These include (i) the use of a pH-dependent $f_{MeBL}^{50\%}$ and/or K_{MeBL} (Heijerick et al., 2002a), (ii) taking into account toxicity of pH-related metal species other than the free metal ion (De Schamphelaere and Janssen, 2002; De Schamphelaere et al., 2002), (iii) extension to a multiple-binding site model (Borgmann et al., 2005), and (iv) modeling the effect based on a log-linear relationship between pH and toxicity (see chapter 2 and 4).

As discussed above, the last option is applicable to our dataset. A new set of model parameters was therefore determined following the method described in chapter 2. In this method, the effect of pH was modeled based on the empirical linear relationship between 72-h $E_rC50_{pNi2+}{}^*$ and pH and was then superimposed on the traditional BLM-type competition effects of calcium and magnesium, assuming that the protective effects of calcium and magnesium are not affected by pH and vice versa. According to this method the model equation for prediction of the $E_rC50_{Ni2+,i}$ in any solution *i* can be written as follows:

$$E_rC50_{Ni^{2+},i,predicted} = 10^{-(S_{pH}.pH_i+Q_{50})}.\left\{1 + K_{CaBL}.(Ca^{2+})_i + K_{MgBL}.(Mg^{2+})_i\right\} \qquad \text{Eq. 5.4}$$

In this formula, $(Ca^{2+})_i$ and $(Mg^{2+})_i$ are the chemical activities of Ca^{2+} and Mg^{2+} in test solution *i*, K_{CaBL} and K_{MgBL} are the parameters describing the protective effects of calcium

and magnesium on nickel toxicity, S_{pH} is the slope of the linear relationship between 72-h $E_rC50_{pNi2+}{}^*$ and pH, and pH_i is the pH of test solution i. Q_{50} can be regarded as a measure of the amount of nickel that has to be bioavailable in order to cause 50% reduction of algal growth. The value of this model parameter was calculated as the mean of $Q_{50,i}$ for all test solutions i used for model development:

$$Q_{50,i} = -\log \frac{E_rC50_{Ni^{2+},i,observed}}{\left\{1 + K_{CaBL}.(Ca^{2+})_i + K_{MgBL}.(Mg^{2+})_i\right\}} - S_{pH}.pH_i \qquad \text{Eq. 5.5}$$

The model parameter S_{pH} was calculated to be 0.1427 using the results from the univariate pH test series (Figure 5.4, solid regression line). Based on the model assumption that pH does not affect the protective effects of calcium and magnesium, the same K_{MgBL} and K_{CaBL} were used as in the BLM-type model developed in this study. For the K_{MgBL}, justification for this model assumption has been provided by the results of the bivariate pH-magnesium test series, which indicate that the extent to which magnesium protects against nickel toxicity is quite similar at different pH levels. Using Equation 5.5, the sensitivity parameter Q_{50} was calculated to be 4.857. Rearrangement of Equation 5.5 for the calculation of Q_{10} yielded a Q_{10} of 5.342.

Using these model parameters, the model's accuracy clearly improved. The 72-h E_rC50_{Nidiss} and 72-h E_rC10_{Nidiss} were predicted by an average error of 1.3 (prediction error range = 1.0-1.6) and 1.3 (1.0-1.7) in the univariate test series used for model development (data point obtained at pH 6.01 included) and 1.3 (1.0-1.8) and 1.6 (1.0-2.2) in the bivariate pH-magnesium test series, respectively. To account for the observed lower sensitivity of the algae in both validation test series, Q_{50} and Q_{10} were optimized based on the results from the tests conducted in OECD medium (Q_{50} = 4.340, Q_{10} = 5.153). Compared to the BLM-type model developed in this study (which was also calibrated to account for the observed sensitivity shift), the new model also yielded more accurate predictions in the natural and synthetic test waters used for model validation, as demonstrated by an average prediction error of 1.2 (1.0-1.6) and 1.4 (1.0-2.1) for the 72-h E_rC50_{Nidiss} and 72-h E_rC10_{Nidiss}, respectively.

Log-linear pH-toxicity relationships have previously been demonstrated to provide a solid basis for modeling copper and zinc toxicity to algae (De Schamphelaere et al., 2003, 2005a; De Schamphelaere and Janssen, 2006). Most interestingly, they have also been successfully used for modeling the effect of pH on long-term nickel toxicity to rainbow trout and chronic nickel toxicity to *D. magna* (chapter 2 and 4). This observation suggests that pH affects chronic (or long-term) nickel toxicity – at least to a certain extent – in a similar way in different aquatic organisms. The similarity between the bioavailability models developed for different groups of organisms may also benefit their wider application in regulatory exercises.

5.5. Conclusion

Based on the results of univariate experiments, a bioavailability model was developed in which the protective effects of Ca^{2+}, Mg^{2+} and H^{+} on nickel toxicity to *P. subcapitata* were modeled as BLM-type single-site competition effects. The model's performance was acceptable in both synthetic and natural waters. However, the fact that the relationship between Ni^{2+} toxicity and H^{+} activity was not linear over the entire tested pH range indicates that the effect of pH can probably not be attributed to H^{+} competition for a single site alone.

The model's accuracy was further improved when the effect of pH was modeled based on the empirical linear relationship between 72-h $E_rC50_{pNi2+}{}^{*}$ and pH, according to the method described in chapter 2. This method was originally developed for modeling long-term nickel toxicity to rainbow trout. It was also successfully applied in chapter 4 for modeling chronic nickel toxicity to *D. magna*. Although the mechanisms by which pH affects nickel toxicity are not yet completely understood, the results of this study strongly suggest that pH affects chronic (or long-term) nickel toxicity – at least to a certain extent – in a similar way in different aquatic organisms. The similarity between the bioavailability models developed for predicting chronic (or long-term) nickel toxicity to fish, daphnids and algae may benefit their wider application in regulatory exercises.

Chapter 6

Comparison of nickel toxicity to cladocerans in soft versus hard surface waters

Redrafted from

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Chapter 6

Comparison of nickel toxicity to cladocerans in soft versus hard surface waters

Abstract. The aims of the present study were to investigate (i) whether cladocerans living in soft water (operationally defined water hardness < 10 mg $CaCO_3$/L) are intrinsically more sensitive to nickel than cladocerans living in hard water (operationally defined water hardness > 25 mg $CaCO_3$/L), and (ii) whether a single bioavailability model can be used to predict the protective effect of water hardness on the toxicity of nickel to cladocerans in both soft and hard water. To address these research questions, acute and chronic bioassays were conducted with ten different cladoceran species collected in soft and hard water lakes in Sweden. Soft water organisms were tested in a 'soft' and a 'moderately hard' test water (nominal water hardness = 6.25 and 16.3 mg $CaCO_3$/L, respectively). Hard water organisms were tested in a 'moderately hard' and a 'hard' test water (nominal water hardness = 16.3 and 43.4 mg $CaCO_3$/L, respectively). The results of the toxicity tests in the 'moderately hard' test water revealed no significant differences between the intrinsic sensitivity of soft and hard water organisms. Modeling exercises indicated that water hardness significantly reduced nickel toxicity to both the soft and the hard water organisms tested. Although predictions of chronic nickel toxicity were sufficiently accurate using the same log K_{CaBL} and log K_{MgBL} (i.e. the model parameters describing the protective effect of water hardness) for all organisms under consideration, predictions of acute nickel toxicity were significantly more accurate when separate log K_{CaBL} and log K_{MgBL} values were used for the soft and the hard water organisms tested. This is due to the fact that the relative decrease of acute nickel toxicity to soft water organisms in 'moderately hard' compared to 'soft' test water was significantly higher than for hard water organisms in 'hard' compared to 'moderately hard' test water.

6.1. Introduction

The importance of considering water hardness in the risk assessment of metals in freshwater is obvious as it protects freshwater biota against the toxicity of cationic metals (De Schamphelaere and Janssen, 2002; Heijerick et al., 2002b; Niyogi and Wood, 2004), including nickel (Chapman et al., 1980; Meyer et al., 1999; Pyle et al., 2002a; Hoang et al., 2004; Keithly et al., 2004). This protective effect can be considered the result of competition between Ca^{2+} and/or Mg^{2+} and the free metal ion (Me^{2+}) for binding to transport sites and/or

sites of toxic action (commonly termed 'biotic ligand' (BL), Di Toro et al., 2001) at the organism-water interface. Stability constants representing the strength of binding of each of these cations to the BL (log K_{CaBL}, log K_{MgBL} and log K_{MeBL}) are used in biotic ligand models (BLMs) to predict the relationship between water hardness and toxicity of metals (e.g., Di Toro et al., 2001; De Schamphelaere and Janssen, 2002), including nickel (Wu et al., 2003; Keithly et al., 2004; chapter 2, 3 and 4).

For fish (*Pimephales promelas* and *Oncorhynchus mykiss*), relations between water hardness and nickel toxicity have been established within an overall hardness range of 20 to 305 mg $CaCO_3$/L (Meyer et al., 1999; Pyle et al., 2002a; Hoang et al., 2004; chapter 2). For crustaceans (*Ceriodaphnia dubia* and *Daphnia magna*), investigated water hardness levels ranged from 42 to 476 mg $CaCO_3$/L (Chapman et al., 1980; Keithly et al., 2004; chapter 3 and 4).

All crustacean nickel bioavailability models developed so far (Wu et al., 2003; Keithly et al., 2004; chapter 3 and 4) are based on experiments with *D. magna* and *C. dubia*. These models have a lower water hardness boundary between 42 and 50 mg $CaCO_3$/L. Cladocerans are known to be one of the organism groups that are most sensitive to metals (Brix et al., 2001; Von Der Ohe and Liess, 2004), including nickel (Keithly et al., 2004). An important question therefore is whether or not bioavailability models based on hardness-toxicity relationships for water hardness levels $\geq$ 42 mg $CaCO_3$/L can be used to predict the effect of water hardness on nickel toxicity at lower hardness levels.

Based on acute copper toxicity experiments with fathead minnows, Van Genderen et al. (2005) demonstrated that an extrapolation of the acute copper BLM of Santore et al. (2001) below water hardness levels of 50 mg $CaCO_3$/L may result in underestimation of copper toxicity. For nickel however, the applicability of the existing bioavailability models below their lower hardness boundary has not been evaluated so far.

In Europe, nickel is considered a priority substance under the Water Framework Directive (European Commission, 2000), implying that environmental quality standards will be developed and endorsed in all member states of the European Union. About 30% of the European waters have water hardness levels < 42 mg $CaCO_3$/L. Large regions in geographic areas such as Scandinavia, Scotland and Northern Portugal are characterized by the

occurrence of surface waters with hardness levels far below 42 mg $CaCO_3$/L (Salminen et al., 2005). Hence, in order to correctly evaluate the risk of nickel in these waters and areas, information is needed on how low water hardness levels affect nickel toxicity.

Nickel is known to be an ionoregulatory toxicant to *D. magna*, by impairing unidirectional Mg^{2+} uptake, which results in a net decrease of whole body Mg^{2+} (Pane et al., 2003b). Several studies have suggested that the interaction between Ni^{2+} and Mg^{2+} is the result of competition for uptake at Mg^{2+} transport channels (e.g., Snavely et al., 1991; Pane et al., 2006a,b). The existence of a shared uptake pathway could explain why magnesium protects against nickel toxicity in freshwater organisms. The protective effect of calcium however may be primarily related to its stabilizing effect on membrane permeability (see previous chapters).

Reviewing mechanisms of acute copper and silver toxicity, two ionoregulatory toxicants disrupting sodium homeostasis, Grosell et al. (2002) argued that a species' sensitivity towards exposure to copper and silver is determined by its sodium uptake rate (in the absence of toxicants) and by its sensitivity to loss of sodium from its body fluids. Similarly, the nickel sensitivity of an organism could be dependent on its magnesium uptake rate, the affinity of its transport channels for magnesium or its sensitivity to loss of magnesium. Since water hardness has already been demonstrated to affect the affinity of organisms not only for uptake of magnesium (Snavely et al., 1991, *Salmonella typhimurium*) but also for calcium (Neufeld and Cameron, 1993, crustaceans), it may be expected that organisms living in waters with different hardness levels have different intrinsic nickel sensitivities and/or do not experience a similar protective effect of water hardness.

Therefore, the two major research questions of this study were (i) whether organisms living in soft water are intrinsically more sensitive to nickel than organisms living in hard water, and (ii) whether a single bioavailability model can be used to predict the protective effect of water hardness on the acute and chronic toxicity of nickel to organisms in both soft and hard water. Soft waters were operationally defined as waters with water hardness levels below the 5th percentile of the water hardness distribution in European surface waters (i.e., < 10 mg $CaCO_3$/L, Salminen et al., 2005). Waters with water hardness levels > 25 mg $CaCO_3$/L were operationally defined as hard waters throughout this study.

To address both research questions simultaneously, we determined the acute and chronic toxicity of nickel to (i) cladocerans collected in soft surface waters and tested in a 'soft' and a 'moderately hard' test solution, and (ii) cladocerans collected in hard surface waters and tested in a 'moderately hard' and a 'hard' test solution. Nominal water hardness of these test solutions was 6.25, 16.3 and 43.4 mg $CaCO_3$/L, respectively.

6.2. Materials and methods

6.2.1. Sampling of water and organisms

First, the geographical distribution of water hardness throughout Sweden (data source: Swedish Agricultural University, http://info1.ma.slu.se) was visualized using a Geographic Information System (GIS, Arcview 3.7a, ESRI Inc., 1996). Next, regions with water hardness < 10 mg $CaCO_3$/L (arbitrarily termed soft water regions) and > 25 mg $CaCO_3$/L (arbitrarily termed hard water regions) were delineated. Regions within a 10-mile radius (~ 16 km) around cities and other urban zones were rejected to avoid confounding effects of anthropogenic activities. A digital land use map including urban zones and cities was therefore downloaded from the GIS data depot (http://data.geocomm.com). Finally, one hard water region and one adjoining soft water region were selected for sampling. These regions were located approximately 200-300 km northwest of Stockholm (geographic area 60°04'49"-61°59'49"N and 15°46'17"-17°14'08"E).

During an exploratory sampling campaign, 13 soft and 13 hard water lakes were investigated. Live zooplankton samples were collected using a plankton sampling net with mesh size 100 µm. Cladoceran species present in the zooplankton samples were identified using the key of Scourfield and Harding (1966). Based on the results of the exploratory sampling campaign, five soft and four hard water lakes were selected for in-depth investigation. Main selection criteria were water hardness, pH (no extremes), species diversity and accessibility of the sites. Anthropogenic influence was very low as evidenced by extremely low trace metal concentrations and NO_3^-, NH_4^+ and PO_4^{3-} concentrations (measured in the field using test kits of Merck, Darmstadt, Germany) being < 10, < 0.2 and < 0.25 mg/L, respectively. Water hardness in the selected soft and hard water lakes varied between 4.68 and 7.16 and between 33.9 and 52.9 mg $CaCO_3$/L, respectively; pH varied between 6.20 and 6.91 in the soft water lakes and between 7.63 and 8.07 in the hard water lakes.

Table 6.1. Geographic coordinates and main physicochemical parameters of the four hard and five soft water lakes of which live zooplankton samples were transported to the laboratory.

Site No.	Geographic coordinates	Name of lake	pH	Ca (mg/L)	Mg (mg/L)	Hardness (mg $CaCO_3$/L)	Na (mg/L)	K (mg/L)	Cl (mg/L)	SO_4 (mg/L)	Alkalinity [b] (mg $CaCO_3$/L)	DOC (mg C/L)
H 10	60°05'45" N 16°18'05" E	- [a]	8.07	15.1	3.65	52.9	3.51	3.02	6.95	6.99	35.4	16.8
H 11	60°05'32" N 15°59'13" E	- [a]	7.91	16.9	1.44	48.2	13.4	9.25	8.29	3.91	72.1	10.3
H 12	60°04'49" N 16°00'37" E	Målsjön	7.71	12.9	2.66	43.2	7.45	2.35	12.1	18.3	24.0	11.6
H 13	60°33'44" N 15°53'30" E	Lintjärnen	7.63	11.3	1.39	33.9	18.4	0.389	27.4	5.40	25.2	7.75
S 14	61°40'25" N 16°25'19" E	- [a]	6.41	1.98	0.538	7.16	1.58	0.167	1.57	1.79	6.86	5.57
S 17	61°42'25" N 15°59'01" E	Abborrtjärnen	6.20	1.94	0.404	6.50	1.81	0.207	2.92	1.40	2.74	12.2
S 18	61°40'18" N 15°52'51" E	Oktjärn	6.91	1.71	0.480	6.24	1.43	0.241	1.20	1.94	6.99	5.15
S 19	61°39'40" N 15°46'17" E	- [a]	6.44	1.26	0.371	4.68	1.19	0.234	1.14	1.10	3.26	6.80
S 21	61°56'54" N 16°11'42" E	Lilla Svartsjön	6.69	1.96	0.535	7.09	1.42	0.078	1.95	1.45	6.02	7.79

Dissolved nickel concentration was below the method detection limit (MDL) of the ICP-OES (i.e. < 1 µg/L) in all lakes. Dissolved concentrations of other trace metals were also very low and can be obtained on request. NO_3, NH_4 and PO_4 concentrations were all < 10, < 0.2 and < 0.25 mg/L, respectively.

[a] Name of lake not indicated on topographic map or in situ.

[b] Alkalinity was calculated from measured inorganic carbon (IC) and pH, using thermodynamic stability constants taken from Stumm and Morgan (1996).

Live zooplankton samples of these nine lakes were transported to the laboratory within 72 hours after collection. At the time of sampling, water temperature was between 19.9 and 26.8 °C. Overall, 19 cladoceran species were successfully transported to the laboratory. They belonged to five different families: Sididae (1 species), Daphniidae (7), Bosminidae (1), Chydoridae (8), and Macrothricidae (2).

For culturing the field-collected species, water was filtered (0.45 µm) at each site and transported to the laboratory. The following physicochemical parameters were analyzed in each lake water: pH (pH-meter P407, Consort, Turnhout, Belgium), dissolved organic and inorganic carbon (DOC and IC) (TOC-5000, Shimadzu, Duisburg, Germany), Ca, Mg, Na, K, Fe, Al, Mn, Ni, Cu, Zn, Pb, Cr, As and Cd (ICP-OES, Perkin Elmer 3300 DV) and Cl, NO_3 and SO_4 (Ion Chromatography, Dionex QIC analyzer, IONPAC AS4A). An overview of the geographic coordinates and the main physicochemical characteristics of the selected lakes is given in Table 6.1.

6.2.2. Culturing of cladoceran populations

We established cultures of all species that were successfully transported to the laboratory. All organisms were cultured in filtered water (0.45 µm) from their lake of origin and were kept at 20 °C and under a light cycle of 12L:12D. The animals were fed ad libitum with an algal mix of *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* in a 3:1 ratio (on a cell number basis). Culture medium was renewed once a week.

6.2.3. Test design

Organisms originating from soft water lakes were tested in a ‘soft’ and a ‘moderately hard’ test medium and organisms originating from hard water lakes were tested in a ‘moderately hard’ and a ‘hard’ test medium. The physicochemical composition of the ‘soft’ and the ‘hard’ test medium reflected the geometric mean of major anion (with the exception of SO_4) and cation (with the exception of sodium, see further) concentrations in the five selected soft and the four selected hard water lakes, respectively. The physicochemical composition of the ‘moderately hard’ test medium was determined as the geometric mean of the composition of the ‘soft’ and ‘hard’ test medium.

Table 6.2. Overview of the physicochemical characteristics (nominal values) of the three synthetic test solutions used for acute and chronic testing.

Test medium	pH	Ca	Mg	Hardness	Na	K	Cl	SO_4	Alkalinity [a]
		(mg/L)		(mg $CaCO_3$/L)	(mg/L)				(mg $CaCO_3$/L)
Soft	7.18	1.75	0.461	6.25	2.61	0.172	1.65	3.98	12.4
Moderately hard	7.18	4.93	0.984	16.3	6.62	0.622	4.40	10.5	12.4
Hard	7.18	13.9	2.10	43.4	16.8	2.25	11.7	28.5	12.4

[a] Alkalinity was calculated from nominal added inorganic carbon (IC) and nominal pH, using thermodynamic stability constants taken from Stumm and Morgan (1996).

To avoid complications related to a combined modification of pH and water hardness, pH was maintained around 7 in all test waters. This was achieved by adding the same amount of $NaHCO_3$ to each test medium. Water hardness of the 'soft', the 'moderately' hard and the 'hard' test medium was 6.25, 16.3 and 43.4 mg $CaCO_3$/L, respectively. Nominal composition of the test media is given in detail in Table 6.2.

6.2.4. Toxicity testing

All tested species are presented in Table 6.3 (see further). Test organisms were only taken from cultures in which organisms had been growing and reproducing well for several months. All tests were performed at 20 °C under a light cycle of 12L:12D.

Acute toxicity tests were performed according to OECD guideline 202 (OECD, 1996) with the exception that juveniles of < 48 hours old (instead of < 24 hours old) were used to initiate tests. Each experiment consisted of a control and five to seven nickel (added as $NiCl_2$) treatments. Each treatment was performed with three replicates (polyethylene cups containing 50 mL of test medium) using five to ten organisms per replicate. The number of 'immobilized' juveniles in each cup was recorded after 24 and 48 hours. Test results were accepted and reported only when mortality in the controls did not exceed 10%.

Chronic tests were initiated with juveniles of < 48 hours old. For each bioassay, a control and five nickel concentrations (added as $NiCl_2$) were prepared. At the start of testing, a single juvenile was transferred to each of the ten replicates per concentration (polyethylene cups containing 50 mL of test medium). Animals were fed daily with an algal mix of *P. subcapitata* and *C. reinhardtii* in a 3:1 ratio (on a cell number basis). Food quantities were dependent on the species tested and varied between 3×10^6 and 12×10^6 cells per individual per day. Every other day, the test medium was renewed, parent mortality noted, and the number of produced juveniles counted. Tests were continued until control organisms had released their third brood. This is in accordance with the survival and reproduction test method of the US EPA for *C. dubia* (US EPA, 2002b). Depending on the species, this occurred between 16 and 21 days after test initiation (indicated in Table 6.3). Test results were accepted and reported only when mortality in the controls did not exceed 20%.

6.2.5. Chemical analyses

Water temperature, oxygen saturation and pH were measured at the start and at the end of testing for acute tests and at each medium renewal for chronic tests. The glass electrode for pH measurements was calibrated with pH 4, pH 7 and pH 10 buffers (Merck, Darmstadt, Germany).

Samples for measurement of total calcium and magnesium and dissolved nickel concentrations (filtration through a 0.45 µm filter, Gelman Sciences, Ann Arbor, MI, USA) were taken at the end of testing for acute tests and once a week for chronic tests. They were acidified (1% v/v) with 0.14 N HNO_3 (Normatom™ ultrapure, VWR, Leuven, Belgium). Calcium and magnesium concentrations were measured using flame atomic absorption spectrometry (F-AAS, SpectrAA100, Varian, Mulgrave, Australia). Nickel was measured using F-AAS for nickel concentrations above 100 µg/L and graphite furnace AAS (GF-AAS, SpectrAA800 with Zeeman background correction, Varian, Mulgrave, Australia) for nickel concentrations below 100 µg/L. Measurement procedures were the same as described in chapter 2.

Inorganic carbon concentrations were measured at the end of testing for acute tests and once a week for chronic tests (TOC-5000, Shimadzu, Duisburg, Germany). Alkalinity was calculated from measured IC concentrations and pH using thermodynamic stability constants from Stumm and Morgan (1996). Means of all nickel, calcium, magnesium and IC measurements were used for data analysis and model development. For Na, K, Cl and SO_4, nominal concentrations were used. Previous studies at our laboratory indicated that measured values of these parameters typically deviate less than 10% from nominal values.

6.2.6. Calculation and statistical comparison of effect concentrations

Acute 48-h EC50 values, chronic LC50 values and their respective 95% confidence intervals were calculated using the trimmed Spearman-Karber method (Hamilton, 1977). Observed ‘immobility’ (acute tests) or mortality (chronic tests) at each measured nickel concentration was used as input for the calculations.

Chronic EC10 and EC50 values (exposure concentrations resulting in 10 and 50% decrease in reproduction, respectively) and their 95% confidence intervals were calculated with a log-logistic model described by De Schamphelaere and Janssen (2004a) using the Levenberg-Marquardt algorithm (Levenberg, 1944; Marquardt, 1963). In this chapter, all EC10, EC50 and LC50 values were based on dissolved nickel measurements. NOEC and LOEC values can be obtained on request.

Acute EC50s, chronic EC50s and chronic LC50s for soft water organisms in 'moderately hard' test medium were statistically compared to those for hard water organisms in the same test medium using the Mann Whitney U test ($p < 0.05$).

6.2.7. Modeling and predicting the effect of water hardness on nickel toxicity

One way to quantitatively evaluate and compare the effects of water hardness on nickel toxicity observed for the different populations is by assuming that the observed effects can be explained by an underlying model, such as the BLM. The choice of a model may result in a biased interpretation, and the potential implications of choosing a particular model will therefore be discussed in the discussion section.

According to the BLM concept, EC50s and LC50s based on Ni^{2+} activity can be predicted for population *i* in test water *j* using the following equation, assuming that the protective effects of calcium and magnesium against nickel toxicity can be represented by competitive unidentate binding to a single BL site, as described by De Schamphelaere and Janssen (2002):

$$E/LC50_{Ni^{2+},i,j,predicted} = E/LC50_{Ni^{2+},0,i}.\left\{1 + K_{CaBL,i}.(Ca^{2+})_j + K_{MgBL,i}.(Mg^{2+})_j\right\} \qquad \text{Eq. 6.1}$$

where $E/LC50_{Ni2+,0,i}$ is the sensitivity parameter (may be interpreted as the $E/LC50_{Ni2+}$ for population *i* in the hypothetical case that no competing cations are present), $(Ca^{2+})_j$ and $(Mg^{2+})_j$ are the chemical activities of the competing cations in test water *j*, and $K_{CaBL,i}$ and $K_{MgBL,i}$ are the stability constants for binding of Ca^{2+} and Mg^{2+} to the BL of population *i*.

The model presented in Equation 6.1 requires the estimation of three model parameters. The sensitivity parameter $E/LC50_{Ni2+,0,i}$ can be estimated for each single population *i*. The parameters describing the protective effect of water hardness, i.e. K_{CaBL} and K_{MgBL}, may also vary among species/populations. However, our limited dataset (only two water hardness levels tested per population) does not allow estimation of these constants for each individual species/population. Furthermore, because calcium and magnesium concentrations were not varied independently of each other in the three test media used in this study, it is not possible to separately optimize K_{CaBL} and K_{MgBL}.

We dealt with these two limitations by estimating K_{CaBL} and K_{MgBL} for groups of tested populations instead of for individual populations (see further) and by assuming a fixed ratio between K_{CaBL} and K_{MgBL} equal to the ratio of these two constants obtained for *D. magna* (acute toxicity: chapter 3; chronic toxicity: chapter 4).

Maximum likelihood estimation (MLE) was followed to obtain best-fit values for all model parameters. For large sample sizes, minimizing the sum of squared errors (SSE) is identical to maximizing the likelihood (Kutner et al., 2005):

$$SSE = \sum_{i,j} \left(\log(E/LC50_{i,j,predicted}) - \log(E/LC50_{i,j,observed}) \right)^2 \qquad \text{Eq. 6.2}$$

A first modeling exercise (model 1) assumed $K_{CaBL} = K_{MgBL} = 0$. In other words, it was assumed that effects of water hardness on nickel toxicity are not significant. Fitting of Equation 6.1 was thus reduced to estimating the sensitivity parameter $E/LC50_{Ni2+,0,i}$ for all populations *i*. A second modeling exercise (model 2) consisted of optimizing K_{CaBL} and K_{MgBL} based on the combined toxicity dataset of the soft and the hard water organisms tested. A third modeling exercise (model 3) differed from the second in that optimal values for K_{CaBL} and K_{MgBL} were derived for the soft and the hard water organisms separately.

In this sequence of modeling exercises, model 3 can be considered a 'nested' extension (sensu Kutner et al., 2005) of model 2, which in turn is a 'nested' extension of model 1. Obviously, the SSE for model 3 will be lower than for model 2, since one more parameter is estimated. Similarly, the SSE for model 2 will be lower than for model 1. The performance of model 3 was compared to that of model 2 and the performance of model 2

was compared to that of model 1 using the likelihood ratio test (Kutner et al., 2005). The statistic for the likelihood ratio test, denoted by G^2 (Kutner et al., 2005; Jonker et al., 2005) is calculated using the following equation:

$$G_{1-2}{}^2 = N \times \ln\left(\frac{SSE_{Model1}}{SSE_{Model2}}\right) \text{ and } G_{2-3}{}^2 = N \times \ln\left(\frac{SSE_{Model2}}{SSE_{Model3}}\right) \qquad \text{Eq. 6.3}$$

N is the number of LC50 or EC50 data that were used to fit the model. Large-sample theory states that, when N is large, $G_{1\text{-}2}{}^2$ (or $G_{2\text{-}3}{}^2$) is approximately distributed as χ^2 with one degree of freedom since one more parameter is estimated in model 2 versus model 1 (or model 3 versus model 2). The χ^2 cumulative probability distribution function delivers the probability α for G^2, where α is the probability of validity of the null hypothesis, i.e. that model 1 and model 2 (or model 2 and model 3) have the same predictive capacity. When $\alpha < p = 0.05$, model 2 was considered significantly better than model 1 (or model 3 was considered significantly better than model 2). A significantly better model 2 means that the protective effect of water hardness should be incorporated into the model. A significantly better model 3 indicates that nickel toxicity to the soft and the hard water populations tested should be modeled using separate K_{CaBL} and K_{MgBL} values.

A final modeling exercise (model 4) was conducted to evaluate the predictive capacity of a model that uses the K_{CaBL} and K_{MgBL} values derived for the standard test organism *D. magna* (acute toxicity: chapter 3; chronic toxicity: chapter 4).

In all modeling exercises, the sensitivity parameter $E/LC50_{Ni2+,0,i}$ was optimized for each population *i*. Chemical speciation of nickel and other ions was calculated using WHAM VI software (Tipping, 1998; NERC, 2001) as described in chapter 2. All model parameter values and values for SSE, G^2 and α are reported in Table 6.4 (see further).

6.3. Results

6.3.1. Acute toxicity test results

Acutely, seven hard and four soft water populations were tested successfully. Three cladoceran families were represented among the tested species: Daphniidae (*Ceriodaphnia pulchella, Ceriodaphnia quadrangula, Daphnia longispina, Simocephalus serrulatus* and *Simocephalus vetulus*), Bosminidae (*Bosmina coregoni*) and Chydoridae (*Alona affinis, Camptocercus lilljeborgi, Chydorus ovalis* and *Peracantha truncata*). For *C. quadrangula* both a soft and a hard water population was tested. An overview of all tested populations and their 48-h EC50s is given in Table 6.3.

Overall, the 48-h EC50s of the soft water populations varied between 97.3 and 2200 µg/L in 'soft' test water and between 141 and 2730 µg/L in 'moderately hard' test water. The 48-h EC50s of the hard water populations varied between 401 and 3330 µg/L in 'moderately hard' test water and between 511 and 5540 µg/L in 'hard' test water. Thus, there is an upward shift of the EC50 ranges with increasing water hardness.

Also, for all individual populations, increasing water hardness resulted in decreased toxicity. For the soft water populations, a water hardness increase from 6.25 to 16.3 mg $CaCO_3$/L (nominal values) resulted in a 1.2- to 3.4-fold increase of the 48-h EC50s. For the hard water populations, 48-h EC50s increased by a factor 1.1 to 3.1 with an increase of water hardness from 16.3 to 43.4 mg $CaCO_3$/L (nominal values).

Considering all 48-h EC50s obtained in the 'moderately hard' test water, no significant differences were observed between those obtained with soft and those obtained with hard water organisms (Mann Whitney U test, $p < 0.05$). However, for *C. quadrangula*, the 48-h EC50 for the soft water population was a factor 2.8 lower than the 48-h EC50 for the hard water population in the same test medium. The observed effects of increasing water hardness are visualized in Figure 6.1.

Table 6.3. Toxicity of nickel to all field populations tested. Effect concentrations (48-h EC50s, chronic LC50s, chronic EC10s and EC50s for the endpoint reproduction) are reported as µg dissolved Ni/L. Numbers between brackets represent 95% confidence intervals – if not reported, no reliable confidence intervals could be calculated due to steep concentration response curves.

Site No.	Species	Chronic test duration (d) [a]	Hard				Moderately hard			
			Acute	Chronic			Acute	Chronic		
			48-h EC50	EC10	EC50	LC50	48-h EC50	EC10	EC50	LC50
H 11	*Ceriodaphnia quadrangula*	17	517 *(252-1060)*	33.1 *(-)*	36.2 *(-)*	25.1 *(16.1-39.1)*	401 *(248-647)*	2.5 *(-)*	11.3 *(5.4-23.9)*	18.0 *(12.1-26.8)*
	Daphnia longispina	21	511 *(290-898)*	113 *(-)*	125 *(-)*	> 118 [c]	455 *(322-644)*	14.8 *(3.0-73.3)*	58.6 *(31.4-110)*	48.3 *(32.8-71.1)*
	Alona affinis	16	5540 *(3830-8020)*	- [b]	- [b]	33.4 *(14.0-79.9)*	3000 *(2030-4430)*	- [b]	- [b]	9.9 *(-)*
	Camptocercus lilljeborgi		1080 *(624-1890)*				526 *(371-745)*			
H 12	*Ceriodaphnia pulchella*	17	981 *(709-1360)*	27.6 *(3.5-217)*	31.2 *(-)*	36.0 *(30.3-42.8)*	842 *(625-1130)*	7.0 *(4.0-12.3)*	16.2 *(12.3-21.3)*	22.1 *(14.1-34.7)*
	Chydorus ovalis		4260 *(3200-5650)*				3330 *(2520-4410)*			
H 13	*Simocephalus vetulus*	21	1490 *(963-2290)*	23.3 *(-)*	28.9 *(25.3-33.1)*	38.8 *(-)*	483 *(331-704)*	9.0 *(5.6-14.3)*	11.2 *(-)*	13.5 *(-)*
Site No.	Species	Chronic test duration (d)	Soft				Moderately hard			
			Acute	Chronic			Acute	Chronic		
			48-h EC50	EC10	EC50	LC50	48-h EC50	EC10	EC50	LC50
S 14	*Ceriodaphnia quadrangula*	17	97.3 *(66.7-142)*	3.0 *(2.1-4.2)*	4.4 *(3.3-6.0)*	3.9 *(2.7-5.5)*	141 *(91.9-215)*	21.7 *(-)*	23.4 *(-)*	13.9 *(10.0-19.3)*
	Peracantha truncata	17	2200 *(1780-2720)*	4.9 *(1.7-14.4)*	15.3 *(10.1-23.2)*	21.0 *(15.2-29.0)*	2730 *(2390-3100)*	24.7 *(8.8-69.4)*	47.2 *(31.8-69.9)*	29.3 *(16.7-51.7)*
S 17	*Simocephalus serrulatus*	17	641 *(505-812)*	6.9 *(-)*	7.7 *(-)*	9.4 *(5.6-15.7)*	1430 *(1140-1800)*	45.3 *(-)*	54.2 *(-)*	47.3 *(36.4-61.5)*
S 19	*Bosmina coregoni*		165 *(136-201)*				559 *(421-741)*			

[a] Test duration of chronic tests was dependent on the time needed for control animals to release three broods.

[b] No reproduction observed.

[c] No LC50 could be calculated – 70% survival at highest exposure concentration of 118 µg/L.

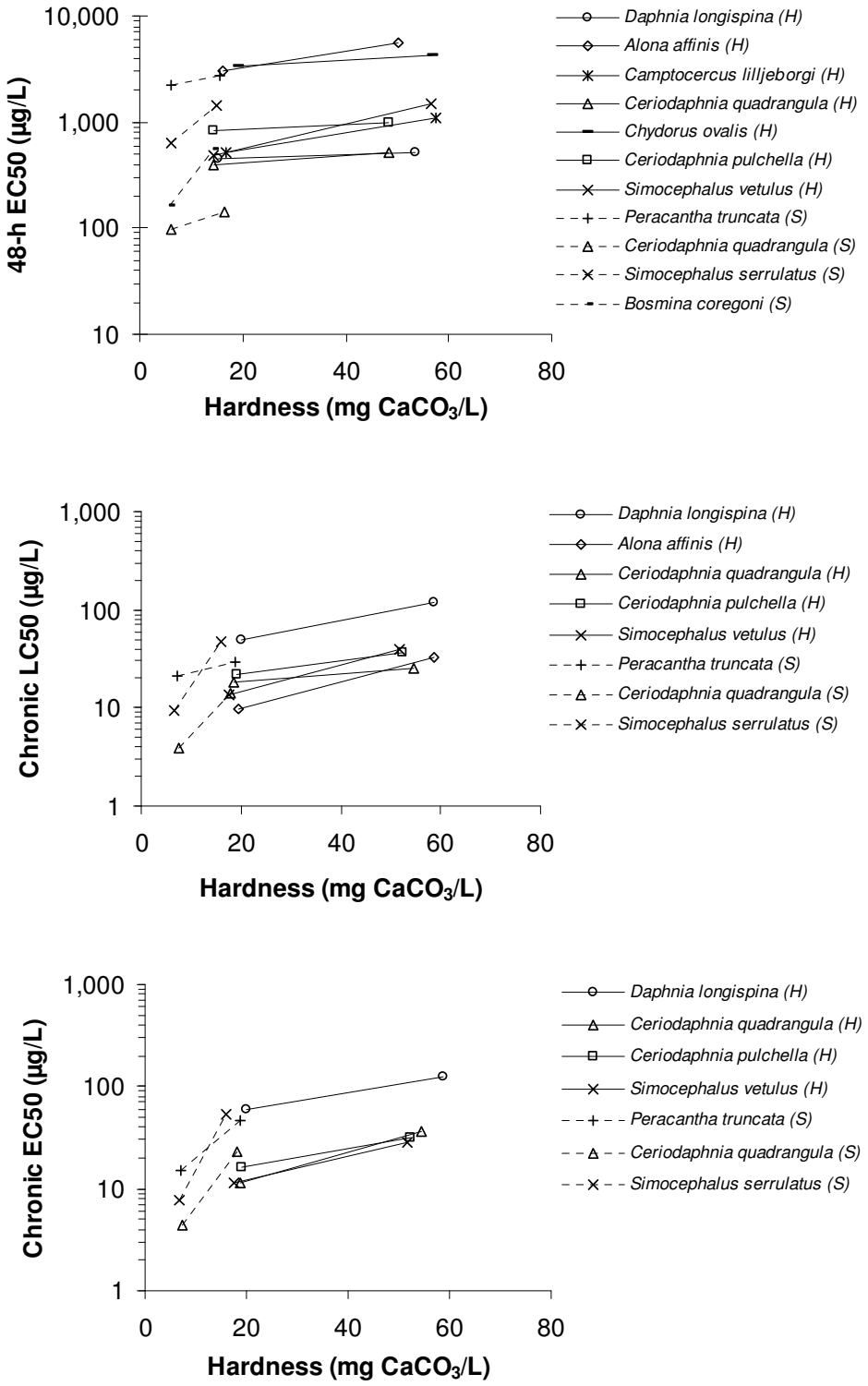

Figure 6.1. Acute (48-h EC50) and chronic (LC50 and EC50) toxicity of nickel as a function of water hardness (mg $CaCO_3$/L) for cladoceran populations originating from soft (S, dashed lines) and hard (H, solid lines) water lakes. Soft water populations were tested at water hardness levels of 6.25 and 16.3 mg $CaCO_3$/L (nominal values); hard water populations were tested at water hardness levels of 16.3 and 43.4 mg $CaCO_3$/L (nominal values). All EC50s and LC50s are plotted against measured water hardness levels.

6.3.2. Chronic toxicity test results

The same populations were used in chronic assays, with the exception of *C. lilljeborgi, C. ovalis* and *B. coregoni*. Test duration, LC50s, EC10s and EC50s are reported in Table 6.3. Overall, the LC50s of the soft water populations varied between 3.9 and 21.0 µg/L in 'soft' test water and between 13.9 and 47.3 µg/L in 'moderately hard' test water. For the hard water populations, LC50s varied between 9.9 and 48.3 µg/L in 'moderately hard' test water and between 25.1 and > 118 µg/L in 'hard' test water. The EC50s of soft water populations varied between 4.4 and 15.3 µg/L in 'soft' test water and between 23.4 and 54.2 µg/L in 'moderately hard' test water. For the hard water populations, EC50s varied between 11.2 and 58.6 µg/L in 'moderately hard' test water and between 28.9 and 125 µg/L in 'hard' test water.

For the soft water populations, an increase of water hardness from 6.25 to 16.3 mg $CaCO_3$/L (nominal values) resulted in a 1.4- to 5.0-fold increase of the LC50s and a 3.1- to 7.1-fold increase of the EC50s. For the hard water populations, toxicity decreased 1.4- to 3.4-fold (LC50s) and 1.9- to 3.2-fold (EC50s) when water hardness increased from 16.3 to 43.4 mg $CaCO_3$/L (nominal values). Thus, water hardness clearly protected all tested species against chronic exposure to nickel.

Overall, the LC50s and EC50s obtained with the soft water organisms in the 'moderately hard' test water did not significantly differ from those obtained with the hard water organisms in the same test water (Mann Whitney U test, $p < 0.05$). Moreover, the soft water population of *C. quadrangula* was observed to be equally sensitive as the hard water population when tested in the same test medium. The observed effects of increasing water hardness are visualized in Figure 6.1.

6.3.3. Biotic ligand model application

The first modeling exercise (model 1) assumed $K_{CaBL} = K_{MgBL} = 0$. Fitting of Equation 6.1 was thus reduced to estimating the sensitivity parameter $E/LC50_{Ni2+,0,i}$ for each population *i*. Average prediction errors were 1.3 (prediction error range = 1.0-1.8), 1.6 (1.1-2.2) and 1.8 (1.3-2.6) for the models based on acute 48-h EC50s, chronic LC50s and chronic EC50s, respectively (Table 6.4).

In the second modeling exercise (model 2), K_{CaBL} and K_{MgBL} were optimized based on the combined toxicity dataset for soft and hard water populations. The acute model (log K_{CaBL} = 3.2, log K_{MgBL} = 2.6) predicted toxicity with an average error of factor 1.2 (1.0-1.7). Optimized log K values for chronic LC50s (log K_{CaBL} = 4.2, log K_{MgBL} = 3.9) and EC50s (log K_{CaBL} = log K_{MgBL} = 4.6) were more than 1 log-unit higher than in the acute model. Chronic LC50s and EC50s were predicted with an average error of factor 1.3 (1.0-1.7) and 1.3 (1.0-1.8), respectively (Table 6.4). Prediction errors were clearly reduced compared to the scenario where no hardness effect was taken into account (model 1). In all cases, the likelihood ratio test indicated a significant improvement of predictions using model 2 compared to model 1 ($\alpha < p = 0.05$, acutely; $\alpha < p = 0.001$, chronically; Table 6.4). This demonstrates the need to incorporate the protective effect of water hardness into the nickel bioavailability model.

In the third modeling exercise (model 3), optimal values for log K_{CaBL} and log K_{MgBL} were determined for soft and hard water populations separately. Log K values for the acute model were log K_{CaBL} = 4.2 and log K_{MgBL} = 3.6 for soft water populations and log K_{CaBL} = 3.2 and log K_{MgBL} = 2.6 for hard water populations. Optimal log K values for the model based on chronic LC50s were log K_{CaBL} = 4.6 and log K_{MgBL} = 4.2 for soft water populations and log K_{CaBL} = 3.9 and log K_{MgBL} = 3.5 for hard water populations. Chronic EC50s were predicted in an optimal manner using log K_{CaBL} = log K_{MgBL} = 4.7 and 4.1 for soft and hard water populations, respectively.

The acute model predicted the observed 48-h EC50s with an average error of factor 1.2 (1.1-1.4). The chronic models predicted LC50s and EC50s with an average error of factor 1.2 (1.1-1.6) and 1.3 (1.0-1.8), respectively (Table 6.4). For both endpoints the optimized log K_{CaBL} and log K_{MgBL} values were higher for the soft water populations than for the hard water populations. Average prediction errors and/or prediction error ranges obtained using separate log K values for soft and hard water populations (model 3) were slightly (acute 48-h LC50s and chronic LC50s) or not (chronic EC50s) smaller than those obtained using a single set of log K values for soft and hard water populations (model 2). The likelihood ratio test indicated that acutely, predictions with model 3 were significantly better than those obtained with model 2 ($\alpha < p = 0.05$). Chronically however, no significant differences were observed ($\alpha > p = 0.05$). The predictive capacity of the retained models (acute: model 3; chronic: model 2) is visualized in Figure 6.2.

Table 6.4. Model parameters (log K_{CaBL}, log K_{MgBL} and $E/LC50_{Ni2+,0,i}$, i.e. the population-specific sensitivity parameter), average prediction errors and prediction error ranges for four different modeling exercises based on acute 48-h EC50s and chronic E/LC50s (see Table 6.3 for chronic test duration for each tested population). Model 1 = no hardness effect assumed; model 2 = log K values determined for soft and hard water populations combined; model 3 = log K values determined for soft and hard water populations separately; model 4 = *Daphnia magna* model as described in chapter 3 (acute) and chapter 4 (chronic). SSE = sum of squared errors (Equation 6.2); G^2 = test statistic for likelihood ratio test (Equation 6.3); α = probability of validity of null hypothesis (if α < p = 0.05, null hypothesis is rejected, i.e., model performance is significantly better than previous model). Log K_{CaBL}, log K_{MgBL} and prediction errors of retained models are printed in bold.

	Acute 48-h EC50				Chronic LC50				Chronic EC50			
Model	1	2	**3** [a]	4	1	**2**	3 [a]	4	1	**2**	3 [a]	4
log K_{CaBL}	-	3.2	**3.2/4.2**	3.10	-	**4.2**	3.9/4.6	3.61	-	**4.6**	4.1/4.7	3.53
log K_{MgBL}	-	2.6	**2.6/3.6**	2.47	-	**3.9**	3.5/4.2	3.22	-	**4.6**	4.1/4.7	3.57
SSE	0.365	0.235	0.144	0.249	0.684	0.219	0.176	0.343	0.961	0.253	0.238	0.581
G^2 [b]	-	9.71 [c]	10.7 [d]	-	-	18.2 [c]	3.44 [d]	-	-	18.7 [c]	0.906 [d]	-
α	-	0.00183 [c]	0.00103 [d]	-	-	0.0000194 [c]	0.0638 [d]	-	-	0.0000156 [c]	0.341 [d]	-
Average prediction error	1.3	1.2	**1.2**	1.2	1.6	**1.3**	1.2	1.3	1.8	**1.3**	1.3	1.5
Prediction error range	1.0-1.8	1.0-1.7	**1.1-1.4**	1.0-1.7	1.1-2.2	**1.0-1.7**	1.1-1.6	1.0-2.0	1.3-2.6	**1.0-1.8**	1.0-1.8	1.1-2.3

Species/population		Population-specific sensitivity parameter $E/LC50_{Ni2+,0,i}$ (µM)										
HARD	*Ceriodaphnia quadrangula*	6.2	4.7	4.7	4.9	0.29	0.060	0.099	0.14	0.27	0.024	0.062	0.14
	Daphnia longispina	6.6	4.8	4.8	5.1	1.0	0.20	0.34	0.49	1.2	0.095	0.25	0.57
	Alona affinis	55	41	41	43	0.24	0.049	0.081	0.12	-	-	-	-
	Camptocercus lilljeborgi	10	7.4	7.4	7.8	-	-	-	-	-	-	-	-
	Ceriodaphnia pulchella	12	9.3	9.3	9.8	0.38	0.080	0.13	0.19	0.30	0.027	0.070	0.16
	Chydorus ovalis	51	36	36	39	-	-	-	-	-	-	-	-
	Simocephalus vetulus	12	8.4	8.4	8.9	0.31	0.068	0.11	0.16	0.24	0.022	0.058	0.13
SOFT	*Ceriodaphnia quadrangula*	1.7	1.5	1.1	1.5	0.11	0.043	0.024	0.077	0.15	0.029	0.024	0.11
	Peracantha truncata	36	32	23	33	0.36	0.15	0.080	0.26	0.39	0.076	0.063	0.28
	Simocephalus serrulatus	14	12	8.9	13	0.30	0.13	0.072	0.23	0.29	0.062	0.052	0.22
	Bosmina coregoni	4.4	4.0	2.9	4.1	-	-	-	-	-	-	-	-

[a] Log K_{CaBL} and log K_{MgBL} values for hard and soft water populations are given before and after the slash, respectively.

[b] N = 22 acute 48-h EC50s, 16 chronic LC50s, 14 chronic LC50s (Equation 6.3).

[c,d] Values for statistical comparison of model 2 with model 1 and model 3 with model 2, respectively.

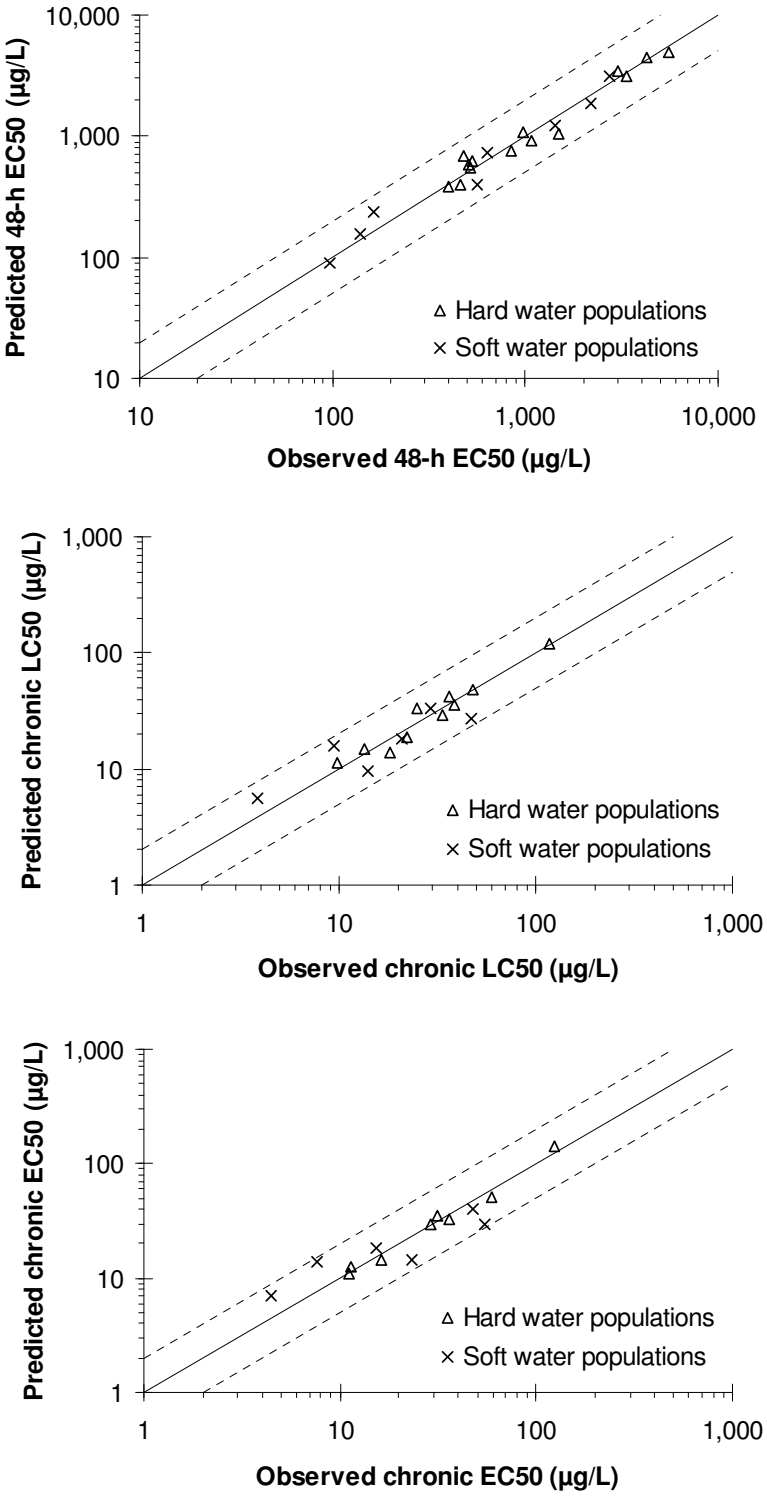

Figure 6.2. Predicted versus observed nickel toxicity (acute: 48-h EC50, chronic: LC50 and EC50). Predictions of acute toxicity were made using log K_{CaBL} and log K_{MgBL} optimized for the soft and the hard water populations separately (model 3). Predictions of chronic toxicity were made using log K_{CaBL} and log K_{MgBL} optimized for the soft and the hard water populations combined (model 2). Model parameters are given in Table 6.4. The solid line indicates a perfect match between predicted and observed E/LC50s; the dashed lines indicate ratios of 0.5 and 2 between observed and predicted E/LC50s.

6.4. Discussion

In this study, we investigated the effect of water hardness on the acute and chronic toxicity of nickel to field-collected cladocerans within a water hardness range of 6.25 to 16.3 mg $CaCO_3$/L (nominal values) for cladocerans collected in soft water and 16.3 to 43.4 mg $CaCO_3$/L for cladocerans collected in hard water. The objectives were to determine (i) whether organisms living in soft water are intrinsically more sensitive to nickel than organisms living in hard water, and (ii) whether a single bioavailability model can be used to predict the protective effect of water hardness on acute and chronic nickel toxicity to organisms in both soft and hard water.

We focused on the effect of water hardness since it has been demonstrated that it is one of the most important factors affecting nickel toxicity. Although several other factors have been demonstrated not to affect nickel toxicity to cladocerans in waters with hardness ≥ 42 mg $CaCO_3$/L (e.g., sodium and potassium, chapter 3), it must be kept in mind that these findings are not yet confirmed in softer waters such as the test waters used in this study.

6.4.1. Modeling the effect of water hardness

Statistical comparison of the predictive capacity of model 2 (hardness effect for soft and hard water populations described using a single set of log K_{CaBL} and log K_{MgBL} values) with the predictive capacity of model 1 (no hardness effect included) revealed that in all cases, model 2 performed significantly better (acutely, $p < 0.05$; chronically, $p < 0.001$). This demonstrates that the protective effect of water hardness on nickel toxicity at low water hardness levels is sufficiently important to be included in nickel bioavailability models and risk assessment exercises.

The explanation for the protective effect of magnesium can be found in the mechanism of nickel toxicity. For *D. magna*, it has been demonstrated that both acute and chronic exposure to nickel result in a decrease of the unidirectional Mg^{2+} uptake rate and the whole body Mg^{2+} concentration (Pane et al., 2003b). This most likely originates from the fact that Ni^{2+} and Mg^{2+}, having similar dehydrated ionic radii (0.066 and 0.069 nm, respectively, Weast, 1973), compete for uptake by the same Mg^{2+} transport systems (Snavely et al., 1991; Pane et al., 2006a,b).

Although less effective than Mg^{2+}, Ca^{2+} has also been identified as a competitive inhibitor of Ni^{2+} uptake by Mg^{2+} transporters (Snavely et al., 1991). However, the protective effect of calcium is expected to be primarily due to its stabilizing effect on membrane permeability (see previous chapters), hereby offering protection against nickel-induced loss of Mg^{2+} from the haemolymph. Further research is needed to elucidate the underlying physiological mechanisms of the protective effect of calcium against nickel toxicity in crustaceans.

To determine whether a single bioavailability model can be used to predict the protective effect of water hardness on acute and chronic nickel toxicity to organisms in both soft and hard water, the predictive capacity of model 3 (log K_{CaBL} and log K_{MgBL} determined for soft and hard water populations separately) was statistically compared to the predictive capacity of model 2 (log K_{CaBL} and log K_{MgBL} determined for soft and hard water populations combined). Only acute nickel toxicity was significantly better predicted using model 3 ($p < 0.05$).

The average prediction error and the prediction error range of the acute model 2 were only slightly lower and smaller than those obtained when no hardness effect was assumed (model 1). Closer inspection of the data indicated that this was due to the fact that prediction errors remained high mainly for soft water populations (e.g., factor 1.7 deviation for *B. coregoni* with both models). Model performance significantly improved when log K_{CaBL} and log K_{MgBL} values were derived for soft and hard water populations separately (model 3).

In model 3, the log K_{CaBL} and log K_{MgBL} values for soft water organisms are about 1 log-unit higher than those for hard water organisms. This is due to the fact that the relative decrease of acute nickel toxicity to soft water organisms in ‘moderately hard’ compared to ‘soft’ test water was significantly higher than for hard water organisms in ‘hard’ compared to ‘moderately hard’ test water. A possible explanation for this could be that soft water organisms have higher affinities for Mg^{2+} and/or Ca^{2+} and thus experience a stronger competitive effect between Mg^{2+} and/or Ca^{2+} and Ni^{2+} at increasing water hardness levels compared to hard water organisms.

Snavely et al. (1991) reported the existence of three different types of magnesium transporters in the prokaryote *Salmonella typhimurium*. CorA is a low-affinity channel, of

which the expression and functioning is not affected by the prevailing external Ca^{2+} or Mg^{2+} concentrations. MgtA and MgtB are high-affinity transport systems, with affinities for Mg^{2+} and Ni^{2+} being two orders of magnitude higher than those of CorA. The expression of MgtA and MgtB is increased at very low Ca^{2+} and/or Mg^{2+} concentrations. On the other hand, Mg^{2+} influx via MgtA and MgtB is decreased with increasing Mg^{2+} concentrations. Both Mg^{2+} and Ca^{2+} inhibited Ni^{2+} uptake via each of these three magnesium transport systems. Obviously, if similar low- and high-affinity transport systems for Mg^{2+} (and Ni^{2+}) exist in cladocerans, this could explain why the relative decrease in acute nickel toxicity with increasing water hardness was significantly higher for soft water cladocerans than for hard water cladocerans.

For calcium, it has already been demonstrated that crustaceans living in soft water have higher affinities for Ca^{2+} than crustaceans living in harder water (Neufeld and Cameron, 1993). Since Ca^{2+} is reported to be a less effective competitive inhibitor of Ni^{2+} uptake via Mg^{2+} transport systems, a higher affinity for Ca^{2+} would only result in a slightly more effective inhibition of Ni^{2+} uptake. Possibly, an increased affinity for Ca^{2+} also positively affects membrane stabilization, hereby increasing the protection against nickel-induced loss of Mg^{2+}. Physiological research with cladocerans is needed to confirm this hypothesis.

It should be noticed that the possible existence of multiple binding sites for Ni^{2+}, Mg^{2+} and/or Ca^{2+} can not be taken into account in a one-binding-site modeling approach. Fitting log K_{CaBL} and log K_{MgBL} assuming only one binding site would mean that we are estimating some kind of 'average' affinity of more than one transport system. This could explain the higher estimates of log K_{CaBL} and log K_{MgBL} for the soft water populations. Borgmann et al. (2005) have demonstrated the usefulness of considering two biotic ligand sites with different affinities for competing cations in explaining bioavailability of copper to *Hyalella azteca*.

Our finding that increasing water hardness does not affect the acute toxicity of nickel to soft and hard water organisms to a similar extent seems to contradict two other studies. First, Erickson et al. (1997) observed that acclimation hardness did not affect the acute toxicity of copper to fathead minnow at two different water hardness levels. However, fish were only acclimated to high water hardness levels (45.8 and 210 mg $CaCO_3$/L), which is an important difference with our study. Furthermore, one could argue that long-term acclimation and/or adaptation of organisms to the water hardness of their natural environment is more likely to result in differences than short-term acclimation in the laboratory. Second, De

Schamphelaere et al. (2007b) found no relation between the protective effect of sodium on the acute toxicity of copper to field cladocerans and the sodium concentration or water hardness of their waters of origin. However, too little data were available to infer general theories on how a different physicochemical environment may result in differing effects of competing cations on the acute toxicity of copper to cladocerans.

Chronically, the predictive capacity of model 3 was not significantly different from the predictive capacity of model 2. As opposed to what was observed for acute nickel toxicity, the difference between the soft and the hard water populations with regard to the effect of increasing water hardness on chronic nickel toxicity was too small to require separate model parameters. This is not due to the fact that fewer populations were tested chronically. A model fitting exercise using only the acute toxicity data for populations that were also tested chronically revealed that the performance of model 3 was still significantly better than the performance of model 2 ($p < 0.05$).

Perhaps the explanation for the difference between the acute and chronic toxicity observations lies in the two main differences between acute and chronic test conditions, i.e. longer exposure duration and presence of food in chronic exposures. First, it has been demonstrated that feeding can protect against metal-induced ionoregulatory disturbance (e.g., Baldisserotto et al., 2004). It has been suggested that this may result in shifts in protective effects of cations on metal toxicity (e.g., De Schamphelaere et al., 2004a). Second, physiological acclimation is more likely to occur during longer exposures. Hogstrand et al. (1995) demonstrated that the affinity of Ca^{2+} transport systems (shared by Zn^{2+}) in rainbow trout gills was modified during long-term exposure to zinc. Further research is needed to investigate how feeding and exposure duration influence the effect of increasing water hardness on chronic nickel toxicity to soft and hard water organisms.

A second important observation with regard to chronic exposure to nickel is that the protective effect of water hardness overall appears to be more important than in acute exposures. As a result, the log K_{CaBL} and log K_{MgBL} values of the chronic models are higher than those of the acute model (Table 6.4). This may be related to the high acute to chronic ratios (48-h EC50/chronic LC50 = 62.4 (4.35-304); 48-h EC50/chronic EC50 = 41.4 (4.10-144); note that both averages and maxima are substantially higher due to extremely high ratios for the chydorids tested). Since chronic nickel toxicity generally occurs at much lower

nickel concentrations, high-affinity sites (as discussed above) might be more involved in chronic than in acute toxicity. This could explain the higher stability constants of both chronic models.

For risk assessment purposes, it was considered interesting to evaluate the predictive capacity of the nickel bioavailability models developed for the standard test organism *D. magna* (chapter 3 and 4) when used for predicting nickel toxicity to the field-collected cladocerans tested in this study (modeling exercise 4). Although the majority of the predictions that had to be made were for water hardness levels below the lower water hardness boundary of both models (i.e., 46 mg $CaCO_3$/L for the acute model; 42 mg $CaCO_3$/L for the chronic models), acute and chronic nickel toxicity to the investigated field cladocerans were fairly well predicted (Table 6.4). However, it must be acknowledged that the *D. magna*-based models were still less accurate than the models specifically developed for the tested field cladocerans. The log K_{CaBL} and log K_{MgBL} values for *D. magna* (Table 6.4, model 4) were lower than those derived for the field cladocerans (Table 6.4, model 2 and 3). Further analysis of the data would be needed to investigate whether the parameter values for the field cladocerans are statistically different from those for *D. magna*.

6.4.2. Intrinsic sensitivity

In order to compare the sensitivity of soft and hard water cladocerans to nickel, both soft and hard water populations were tested in a similar 'moderately hard' test medium (nominal hardness = 16.3 mg $CaCO_3$/L). Acutely nor chronically, significant differences were observed between the E/LC50s for the soft and the hard water populations tested. However, for *C. quadrangula* – the only species of which both a soft and a hard water population was tested – the acute sensitivity of the soft water population was significantly higher than the acute sensitivity of the hard water population. Chronically, no significant sensitivity difference was observed.

The observed factor 2.8 difference in acute sensitivity between both populations of *C. quadrangula* is within the range of inter-population sensitivity differences observed by Muyssen et al. (2005) for zinc (factor 1.2 to 4.6) using a range of field-collected populations of cladoceran species. Among field populations of *D. magna*, Barata et al. (1998) observed a difference in copper sensitivity of up to factor 7. For *S. vetulus*, Bossuyt and Janssen (2005b)

even reported intra-population sensitivity differences of up to factor 2.9 for copper. The lower acute nickel sensitivity of the soft water population of *C. quadrangula* is hence not necessarily related to the low water hardness of its lake of origin.

Comparison of the EC50s and LC50s for species belonging to different cladoceran families clearly reveals the markedly lower acute sensitivity to nickel of most Chydoridae (*A. affinis, C. ovalis,* and *P. truncata*) compared to the other tested families (Daphniidae and Bosminidae). Overall, species of the Chydoridae that were tested by Bossuyt and Janssen (2005b) and Muyssen et al. (2005) also exhibited a relatively low sensitivity to copper and zinc in acute exposures. Why chydorids are – at least acutely – less sensitive to metals than daphnids is still an unresolved research question that certainly deserves further attention.

Comparison of the toxicity data for the hard water organisms in 'hard' test water (nominal hardness = 43.4 mg $CaCO_3$/L) with toxicity data for *C. dubia* (Keithly et al., 2004) in water with comparable water hardness (50 mg $CaCO_3$/L) reveals that both acutely and chronically, all tested field species were less sensitive to nickel than the standard test organism *C. dubia*. *D. magna* exhibits intermediate sensitivity compared to the tested field species, as demonstrated by toxicity data at water hardness levels of 42 to 50 mg $CaCO_3$/L (Chapman et al., 1980; chapter 3 and 4).

Since the sensitivity parameter of bioavailability models (in this study: $E/LC50_{Ni2+,0,i}$) has been previously suggested to represent the intrinsic sensitivity of a species/population (De Schamphelaere et al., 2007b), one may expect that nickel sensitivity differences as observed through comparison of effect data obtained in the 'moderately hard' test water would be reflected in the $E/LC50_{Ni2+,0,i}$ values. This is clearly the case for individual species/populations. As a result, based on the $E/LC50_{Ni2+,0,i}$ values for the retained models (acute: model 3; chronic: model 2), no significant sensitivity difference was revealed between the soft and the hard water populations either.

However, here also, the implications of using a single-site bioavailability model should not be overlooked. A single-site model ignores the possible existence of multiple binding sites with different affinities for both Ni^{2+} and competing cations (Mg^{2+} and Ca^{2+}), as discussed above. If water hardness determines the relative presence of low- and high-affinity binding sites for Ni^{2+} and Mg^{2+} and/or Ca^{2+}, the nickel sensitivity of an organism is expected

to be dependent on the water hardness of its environment. If the soft and/or the hard water cladocerans used in this study experienced a sensitivity shift by exposing them to nickel in the 'moderately hard' test water, to which they were not acclimated before testing, the sensitivity parameter $E/LC50_{Ni2+,0,i}$ would represent the average nickel sensitivity of population *i* at different water hardness levels. This could have masked sensitivity differences between soft and hard water cladocerans.

6.5. Conclusion

It can be concluded from this study that cladocerans living in soft water (water hardness < 10 mg $CaCO_3$/L) are not intrinsically more sensitive to nickel than cladocerans living in hard water (water hardness > 25 mg $CaCO_3$/L). The protective effect of water hardness between 6.25 and 43.4 mg $CaCO_3$/L was demonstrated to be significant and should therefore be incorporated in nickel bioavailability models and risk assessment exercises. Bioavailability models predicting acute nickel toxicity performed significantly better when the distinction was made between soft and hard water organisms. This is due to the fact that the relative decrease of acute nickel toxicity to soft water organisms in 'moderately hard' compared to 'soft' test water was significantly higher than for hard water organisms in 'hard' compared to 'moderately hard' test water. For chronic nickel toxicity, the difference between soft and hard water organisms was not important enough to require separate modeling. Further research is needed to mechanistically explain the protective effect of water hardness on nickel toxicity to soft and hard water organisms.

Chapter 7

Comparison of nickel toxicity to green microalgae in soft versus hard surface waters

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Chapter 7

Comparison of nickel toxicity to green microalgae in soft versus hard surface waters

Abstract. The major research questions addressed in this study were (i) whether green microalgae living in soft water (operationally defined water hardness < 10 mg $CaCO_3$/L) are intrinsically more sensitive to nickel than green microalgae living in hard water (operationally defined water hardness > 25 mg $CaCO_3$/L), and (ii) whether a single bioavailability model can be used for predicting the effect of water hardness on the toxicity of nickel to green microalgae in both soft and hard water. Algal growth inhibition tests were conducted with clones of ten different species collected in soft and hard water lakes in Sweden. Soft water algae were tested in a 'soft' and a 'moderately hard' test medium (nominal water hardness = 6.25 and 16.3 mg $CaCO_3$/L, respectively), whereas hard water algae were tested in a 'moderately hard' and a 'hard' test medium (nominal water hardness = 16.3 and 43.4 mg $CaCO_3$/L, respectively). The results from the growth inhibition tests in the 'moderately hard' test medium revealed no significant sensitivity differences between the soft and the hard water algae used in this study. Increasing water hardness was demonstrated to significantly reduce nickel toxicity to both soft and hard water algae. The parameter values representing the protective effect of water hardness were calculated to be identically the same for the soft and the hard water algae used in this study (log K_{CaBL} = 4.0 and log K_{MgBL} = 5.3). Apparently, a single bioavailability model can be used to predict nickel toxicity as a function of water hardness to green microalgae in soft and hard surface waters.

7.1. Introduction

Water hardness has been demonstrated to be one of the most important factors affecting the toxicity of nickel to freshwater biota. The nickel toxicity mitigating effect of water hardness has been investigated for fish (Meyer et al., 1999; Pyle et al., 2002a; Hoang et al., 2004; chapter 2), crustaceans (Chapman et al., 1980; Kszos et al, 1992; Keithly et al., 2004; chapter 3 and 4) and algae (Issa et al., 1995; chapter 5).

Within the concept of the biotic ligand model (BLM, Di Toro et al., 2001), the protective effect of water hardness on the toxicity of cationic metals is usually considered the result of competition between Ca^{2+} and/or Mg^{2+} and the free metal ion (Me^{2+}) for binding to

transport sites and/or sites of toxic action at the organism-water interface, commonly termed 'biotic ligand' (BL). Stability constants representing the strength of binding between each of these cations and the BL (log K_{CaBL}, log K_{MgBL} and log K_{MeBL}) are used to predict the relationship between water hardness and toxicity (e.g., Di Toro et al., 2001; De Schamphelaere and Janssen, 2002).

For nickel, bioavailability models have been developed for fish (chapter 2), crustaceans (Wu et al., 2003; Keithly et al., 2004; chapter 3 and 4) and algae (chapter 5). The available models have a lower water hardness boundary of 22 mg $CaCO_3$/L for fish, 42-50 mg $CaCO_3$/L for crustaceans and 20 mg $CaCO_3$/L for algae.

In Europe, nickel is considered a priority substance under the Water Framework Directive (European Commission, 2000), implying that environmental quality standards will be developed and endorsed in all member states of the European Union. The environmental quality standard for nickel will be based on the outcome of the European nickel risk assessment, in which the bioavailability models described in chapters 2-5 have been used for determining safe exposure concentrations for the aquatic compartment. Later on, the same models can also be used by the member states to investigate whether their water bodies comply with the imposed quality standard.

In this respect, a major point of concern is that large regions in geographic areas such as Scandinavia, Scotland and Northern Portugal are characterized by the occurrence of surface waters with water hardness levels well below the lower water hardness boundaries of the abovementioned bioavailability models. According to the data included in the FOREGS database (Salminen et al., 2005, http://www.gtk.fi/publ/foregsatlas, Forum of the European Geological Surveys Directors), 22, 32 and 21% of the European surface waters have a water hardness below the lower water hardness boundary of the available nickel bioavailability models for fish, crustaceans and algae, respectively. An important question therefore is whether these models can accurately predict nickel toxicity to organisms living in such waters.

Using the acute copper BLM for fathead minnow (Santore et al., 2001), Van Genderen et al. (2005) observed an underestimation of copper toxicity in waters with hardness levels below the lower water hardness boundary of the model (i.e. 50 mg $CaCO_3$/L). This may

indicate that fathead minnows are more sensitive to copper in these waters than in the waters used for model development. According to Grosell et al. (2002), the sensitivity of freshwater animals to copper and silver (which are two ionoregulatory toxicants disrupting sodium homeostasis) is dependent on the organisms' sodium uptake rate (in the absence of toxicants) and on their sensitivity to loss of sodium from their body fluids.

Similarly, for nickel, which has been demonstrated to be a toxicant affecting magnesium homeostasis (e.g., Pane et al., 2003b), the sensitivity of an organism may be expected to be dependent on its magnesium uptake rate or its sensitivity to magnesium loss. To understand how the water hardness of an organism's environment may influence its sensitivity to nickel, it is necessary to take a closer look at the mechanisms by which water hardness affects nickel toxicity.

The protective effect of magnesium against nickel toxicity is most likely due to competitive inhibition of Ni^{2+} uptake via Mg^{2+} transport systems. Evidence for a shared uptake pathway for Ni^{2+} and Mg^{2+} has been provided for very different groups of organisms (for an overview, see chapter 5). Although there is no direct evidence for Ni^{2+} to be taken up via Mg^{2+} transporters in algae, recent findings of Worms and Wilkinson (2007) indicate that magnesium transporters represent a major pathway for nickel to enter algal cells.

Although Ca^{2+} has also been demonstrated to reduce Ni^{2+} uptake via Mg^{2+} transporters (e.g., Snavely et al., 1991), its protective effect against nickel toxicity may be primarily due to its role in regulating membrane permeability and ion transport. For instance, increased calcium concentrations may protect against nickel-induced magnesium loss and thereby reduce the disturbance of magnesium homeostasis (see chapters 2-5).

As discussed above, the sensitivity of an organism to nickel may be dependent on its magnesium uptake rate or its sensitivity to magnesium loss. From the above mechanisms, it is clear that if the water hardness of an organism's environment would for instance determine the organism's affinity for magnesium and/or calcium, soft and hard water organisms would be clearly different in their sensitivity to nickel.

In this respect, it is important to mention the findings of Snavely et al. (1991) concerning the existence of water hardness-dependent high-affinity nickel transport systems

in the prokaryote *S. typhimurium*. Snavely et al. (1991) investigated the uptake of Ni^{2+} via three different Mg^{2+} transporters: a low-affinity channel (CorA), of which the expression and functioning was not affected by the prevailing Ca^{2+} and Mg^{2+} concentrations, and two high-affinity transport systems (MgtA and MgtB), of which the expression increased at very low Ca^{2+} and/or Mg^{2+} concentrations and through which Mg^{2+} influx decreased with increasing Mg^{2+} concentrations. Both Mg^{2+} and (to a lesser extent) Ca^{2+} inhibited Ni^{2+} uptake via each of these three transport systems. Recently reported results of nickel internalization experiments with the unicellular green alga *Chlamydomonas reinhardtii* suggested that multiple nickel internalization sites that are differently influenced by Ca^{2+} and Mg^{2+} and have different affinities for Ca^{2+}, Mg^{2+} and Ni^{2+} also exist in algae (Worms and Wilkinson, 2007).

It is clear that an increased importance of high-affinity BL sites at water hardness levels below the lower hardness boundaries of the developed nickel bioavailability models would affect (i) nickel sensitivity of organisms living at these low hardness levels, and (ii) the extent to which water hardness cations protect against the toxicity of nickel to these organisms.

Because of the concern that 'hard-water' bioavailability models may underestimate nickel toxicity to soft water organisms, it was considered necessary to investigate (i) whether organisms living in soft water are intrinsically more sensitive to nickel than organisms living in hard water, and (ii) whether a single bioavailability model can be used to predict the protective effect of water hardness on the toxicity of nickel to organisms in both soft and hard water. To address these research questions, we studied the protective effect of water hardness on the toxicity of nickel to a variety of cladocerans and green microalgae collected in soft and hard water lakes in Sweden. In this study, soft waters were operationally defined as waters with water hardness levels below the 5th percentile of the water hardness distribution in European surface waters (i.e. < 10 mg $CaCO_3$/L, Salminen et al., 2005), while waters with water hardness levels > 25 mg $CaCO_3$/L were operationally defined as hard waters.

The results from the study with the cladocerans have been reported in chapter 6 and demonstrated that (i) soft water cladocerans are not significantly more sensitive to nickel than hard water cladocerans, and (ii) a single bioavailability model can predict the effect of water hardness on the chronic toxicity of nickel to both soft and hard water cladocerans, while for acute nickel toxicity, separate models should be used.

In this study, a similar test design was used as in the study with the cladocerans (chapter 6). Nickel toxicity to soft water algae was investigated in a 'soft' and a 'moderately hard' test solution, whereas nickel toxicity to hard water algae was investigated in a 'moderately hard' and a 'hard' test solution. Nominal water hardness of these test solutions was 6.25, 16.3 and 43.4 mg $CaCO_3$/L, respectively. The algae used in this study originated from the same lakes as the cladocerans used in chapter 6.

7.2. Materials and methods

7.2.1. Lake selection and physicochemical characterization

The sampling area was located approximately 200-300 km northwest of Stockholm (geographic area 60°04'49"-61°59'49"N and 15°46'17"-17°14'08"E) and was selected following the procedure described in chapter 6. Based on the results of an exploratory sampling campaign (see chapter 6), five soft water lakes (labeled S14, S17, S18, S19, S21) and four hard water lakes (labeled H10, H11, H12, H13) were selected for in-depth investigation.

At each site, filtered (0.45 µm) water samples were taken for chemical characterization in the laboratory. The following physicochemical parameters were analyzed: pH (pH-meter P407, Consort, Turnhout, Belgium), dissolved organic and inorganic carbon (DOC and IC) (TOC-5000, Shimadzu, Duisburg, Germany), Ca, Mg, Na, K, Fe, Al, Mn, Ni, Cu, Zn, Pb, Cr, As and Cd (ICP-OES, Perkin Elmer 3300 DV) and Cl^-, NO_3^- and SO_4^{2-} (Ion Chromatography, Dionex QIC analyzer, IONPAC AS4A). Anthropogenic influence on the selected lakes was low, as evidenced by extremely low trace metal concentrations and NO_3^-, NH_4^+ and PO_4^{3-} concentrations < 10, < 0.2 and < 0.25 mg/L, respectively (NH_4^+ and PO_4^{3-} were only measured in the field using Merck test kits).

Water hardness in the selected soft and hard water lakes varied between 4.68 and 7.16 and between 33.9 and 52.9 mg $CaCO_3$/L, respectively. The pH varied between 6.20 and 6.91 in the soft water lakes and between 7.63 and 8.07 in the hard water lakes. For an overview of the geographic coordinates and the main physicochemical characteristics of the selected lakes, see Table 6.1 in chapter 6.

7.2.2. Sampling, isolation, culturing, selection and identification of green microalgae

Phytoplankton was collected in each lake using a plankton sampling net with mesh size 10 µm. At the time of sampling, water temperature was between 19.9 and 26.8 °C. All samples were transported to the laboratory within 72 hours. During transport, the samples were kept in the dark at 15 °C. In the laboratory, the samples were stored in a cooling chamber at 15 °C under a low-intensity (ca. 30 µmol photons/m^2/s) light cycle of 8L:16D.

The phytoplankton samples were used for inoculation of agar plates. The agar plates were incubated for several days (up to a week) under continuous light at 25 °C. Based on the algal growth on the agar plates, 61 isolates of green microalgae were obtained through micromanipulation with Pasteur pipettes. Isolates were transferred to WC medium (Guillard and Lorenzen, 1972). $Na_2SiO_3.9H_2O$ was not added to the medium since silicates are of no importance to green algae.

It was attempted to establish sustainable cultures of as many isolates as possible. Cultures were renewed every three weeks. Erlenmeyer flasks containing 50 mL of WC medium were inoculated and placed on a shaking device under continuous light at 25 °C. After six to nine days (depending on the species), algae were in the late exponential growth phase and were transferred to a cooling chamber were they were stored at 15 °C under a low-intensity (see above) light cycle of 12L:12D.

For inoculation of agar plates, isolation and culturing, the water hardness of the growth medium was adjusted to 6.25 mg $CaCO_3$/L for the soft water strains and 43.4 mg $CaCO_3$/L for the hard water strains. These values were based on the geometric mean of the calcium and magnesium concentrations measured in the five soft and the four hard water lakes, respectively. The calcium to magnesium ratio was maintained at the same level as in the soft and hard water lakes (molar ratio = 2.3 and 4.0, respectively).

Out of all strains that yielded homogenous cultures which were easy to handle, five soft and five hard water strains were randomly selected for testing. Genus and/or species were identified using keys and descriptions from Hindák (1984, 1988, 1990), John et al. (2002) and Wehr and Sheath (2003).

7.2.3. Test design and test media

Algae from the soft water lakes were exposed to nickel in a 'soft' and a 'moderately hard' test medium whereas algae from the hard water lakes were exposed to nickel in a 'moderately hard' and a 'hard' test medium. Test media were based on the OECD medium proposed by the revised test guideline 201 (OECD, 2006). The water hardness of the 'soft', 'moderately hard' and 'hard' test medium was 6.25, 16.3 and 43.4 mg $CaCO_3$/L, respectively. The water hardness of the 'moderately hard' test medium was based on the geometric means of the calcium and magnesium concentrations in the 'soft' and 'hard' test medium.

Care was taken to maintain the desired calcium to magnesium ratios in each test medium (i.e. 2.3, 3.0 and 4.0 in 'soft', 'moderately hard' and 'hard' test medium, respectively). Following Heijerick et al. (2002a), EDTA was replaced by artificial DOC (50 µg/L AHA-DOC, Aldrich Humic Acid, Sigma-Aldrich Chemie, Steinheim, Germany) to prevent undesirable nickel complexation and to keep iron in solution at the same time. Nickel concentration series were prepared by adding $NiCl_2$. Test solutions were allowed to equilibrate for 24 hours at 25 °C prior to being used in the toxicity tests.

7.2.4. Toxicity testing

All growth inhibition tests were performed according to the revised OECD test guideline 201 (OECD, 2006). All tested species are presented in Table 7.1 (see further). Microscopic inspection before testing did not reveal any contamination in the selected cultures. Dissolved nickel concentrations in the culture medium were always below 3 µg/L, i.e. the method detection limit (MDL) of the graphite furnace atomic absorption spectrometer used in this study (GF-AAS, SpectrAA800 with Zeeman background correction, Varian, Mulgrave, Australia).

Prior to testing, the algae were allowed to grow in hardness-adjusted WC medium (see above) in Erlenmeyer flasks. The flasks were placed on a shaking device under continuous light (240 µmol photons/m^2/s) at 25 °C. Growth was followed daily. After five to eight days (depending on the species), the algal cultures were in the exponential growth phase and were ready for use as inoculum for the tests.

Growth inhibition tests were performed in 100 mL Erlenmeyer flasks containing 50 mL of test medium. Three replicates were used for the control treatment as well as for each of the six to seven nickel treatments. For each tested species, an appropriate initial cell density was determined (varying between 3000 and 5000 coenobia/mL for coenobial algae and between 15000 and 150000 cells/mL for single-celled algae). After inoculation, all test flasks were incubated at 25 °C on a light table (24 hours light, 120 µmol photons/m^2/s) and were manually shaken twice daily.

After 48 and 72 hours, light absorbance was measured at λ = 662 nm (i.e. one of the absorption maxima of chlorophyll a) using a Jenway 6300 Spectrophotometer (Dunmow, Essex, UK). To relate light absorbance measurements to cell densities, a conversion factor was determined for each tested species. To this end, light absorbance and cell density were determined daily in three additional control replicates. Cell densities were determined by performing cell counts using a Sedgewick Rafter counting cell. When necessary, algal suspensions were diluted to cell densities appropriate for counting.

7.2.5. Chemical analyses

The pH of each test solution was measured at the start and at the end of testing. The glass electrode was calibrated with pH 4, pH 7 and pH 10 buffers (Merck, Darmstadt, Germany). A maximum difference of 0.3 pH units was allowed between the lowest and the highest measurement made in a single test.

Samples for analysis of total calcium and magnesium and dissolved nickel (filtration through a 0.45 µm filter, Gelman Sciences, Ann Arbor, MI, USA) were taken at the start and at the end of testing and were acidified (1% v/v) with 0.14 N HNO_3 (Normatom™ ultrapure, VWR, Leuven, Belgium). Calcium and magnesium concentrations were measured using flame atomic absorption spectrometry (F-AAS, SpectrAA100, Varian, Mulgrave, Australia). Nickel was analyzed using F-AAS for nickel concentrations > 100 µg/L, and GF-AAS for nickel concentrations < 100 µg/L. Measurement procedures were the same as described in chapter 2.

Inorganic carbon concentrations were also measured at the start and at the end of testing. Alkalinity was calculated from measured IC concentrations and pH using

thermodynamic stability constants from Stumm and Morgan (1996). Dissolved organic carbon concentrations were not measured since De Schamphelaere et al. (2006) demonstrated that background DOC does not significantly contribute to nickel complexation in synthetic test solutions. Means of all pH, nickel, calcium, magnesium and IC measurements were used for data analysis and model development. For Na, K, Cl and SO_4, nominal concentrations were used. Previous studies at our laboratory indicated that measured values of these parameters typically deviate less than 10% from nominal values.

7.2.6. Calculation and statistical comparison of effect concentrations

All effect concentrations (72-h NOE_rC, LOE_rC, E_rC10 and E_rC50) were calculated using growth rate as an endpoint. In this study, the average specific growth rate μ (d^{-1}) in each replicate over a 72 hour exposure period was calculated as the slope of the linear relationship between ln N_x and t_x, with N_x representing the algal cell density at day x (t_x). Three data points were used to determine the average specific growth rate: (t_0,N_0), (t_2,N_2) and (t_3,N_3), representing the initial cell density and the cell densities at day 2 and 3, respectively. For each replicate at each nickel concentration, N_x was calculated using the species-specific conversion factor relating light absorbance measurements to cell densities (see above).

NOE_rCs and LOE_rCs were determined using the Mann Whitney U test ($p < 0.05$). E_rC10s, E_rC50s and their 95% confidence intervals were calculated with a log-logistic model described by De Schamphelaere and Janssen (2004a) using the Levenberg-Marquardt algorithm (Levenberg, 1944; Marquardt, 1963). All effect concentrations were calculated based on measured dissolved nickel concentrations.

The E_rC50s for the soft water strains obtained in 'moderately hard' test medium were statistically compared to those for the hard water strains obtained in the same test medium using the Mann Whitney U test ($p < 0.05$).

7.2.7. Modeling and predicting the effect of water hardness on nickel toxicity

As in our previous study with field-collected cladocerans (chapter 6), we assumed that the observed effects of water hardness can be explained by an underlying model, such as the BLM. The usefulness of the BLM for predicting nickel toxicity to algae as a function of

calcium and magnesium concentrations has been demonstrated in chapter 5, based on results from tests with *Pseudokirchneriella subcapitata*. According to the BLM concept, E_rC50s based on Ni^{2+} activity can be predicted for strain *i* in test water *j* using the following equation, assuming that the protective effects of calcium and magnesium against nickel toxicity can be represented by competitive unidentate binding to a single BL site, as described by De Schamphelaere and Janssen (2002):

$$E_rC50_{Ni^{2+},i,j,predicted} = E_rC50_{Ni^{2+},0,i}.\left\{1 + K_{CaBL,i}.(Ca^{2+})_j + K_{MgBL,i}.(Mg^{2+})_j\right\} \quad \text{Eq. 7.1}$$

where $E_rC50_{Ni2+,0,i}$ is the sensitivity parameter (may be interpreted as the E_rC50_{Ni2+} for strain *i* in the hypothetical case that no competing cations are present), $(Ca^{2+})_j$ and $(Mg^{2+})_j$ are the chemical activities of the competing cations in test water *j*, and $K_{CaBL,i}$ and $K_{MgBL,i}$ are the stability constants for binding of Ca^{2+} and Mg^{2+} to the BL of strain *i*.

The model presented in Equation 7.1 requires the estimation of three model parameters. The sensitivity parameter $E_rC50_{Ni2+,0,i}$ can be estimated for each single strain *i*. The parameters describing the protective effect of water hardness, i.e. K_{CaBL} and K_{MgBL}, may also vary among the algal strains tested. However, our limited dataset (only two water hardness levels tested per strain) does not allow estimation of these constants for each individual strain used in this study. Furthermore, because calcium and magnesium concentrations were not varied independently of each other in the three test media used in this study, it is not possible to separately optimize K_{CaBL} and K_{MgBL}.

We dealt with these two limitations by estimating K_{CaBL} and K_{MgBL} for groups of tested strains instead of for individual strains (see further) and by assuming a fixed ratio between K_{CaBL} and K_{MgBL} equal to the ratio between these two constants obtained for *P. subcapitata* (chapter 5). Maximum likelihood estimation (MLE) was followed to obtain best-fit values for all model parameters (see chapter 6).

A first modeling exercise (model 1) assumed $K_{CaBL} = K_{MgBL} = 0$. In other words, it was assumed that effects of water hardness on nickel toxicity are not significant. Fitting of Equation 7.1 was thus reduced to estimating the sensitivity parameter $E_rC50_{Ni2+,0,i}$ for all strains *i*. A second modeling exercise (model 2) consisted of optimizing K_{CaBL} and K_{MgBL}

based on the combined toxicity dataset of the soft and the hard water strains tested. A third modeling exercise (model 3) differed from the second in that optimal values for K_{CaBL} and K_{MgBL} were derived for the soft and the hard water strains separately.

Statistical comparison of the accuracy of model 2 to that of model 1 and the accuracy of model 3 to that of model 2 was performed using the likelihood ratio test (Kutner et al., 2005; Jonker et al., 2005) as described in chapter 6. A significantly better model 2 means that the protective effect of water hardness on nickel toxicity is significant and should be incorporated into the model. A significantly better model 3 means that nickel toxicity to the soft and the hard water strains tested should be modeled using separate K_{CaBL} and K_{MgBL} values.

A final modeling exercise (model 4) was conducted to evaluate the predictive capacity of a model that uses the K_{CaBL} and K_{MgBL} values derived for the standard test organism *P. subcapitata* (chapter 5).

In all modeling exercises, the sensitivity parameter $E_rC50_{Ni2+,0,i}$ was optimized for each population *i*. Chemical speciation of nickel and other ions was calculated using WHAM VI software (Tipping, 1998; NERC, 2001) as described in chapter 2. All model parameter values and values for SSE, G^2 and α are reported in Table 7.2 (see further). Details on the calculation of SSE (sum of squared errors), G^2 (statistic for the likelihood ratio test) and α (probability of validity of the null hypothesis) are given in the materials and methods section of chapter 6.

7.3. Results

7.3.1. Toxicity test results

Five soft and five hard water strains were successfully used in algal growth inhibition tests. The five soft water strains were identified to be *Chlamydomonas* sp.*, Ankistrodesmus falcatus, Scenedesmus accuminatus, Chlorella* sp. and *Desmodesmus spinosus*. The five hard water strains were identified as *Desmodesmus* sp.*, Pediastrum duplex, Pseudokirchneriella* sp.*, Coelastrum microporum* and *Spermatozopsis exsultans*. For *Desmodesmus* sp. and *C. microporum*, two tests were conducted with an interval of about four months between the first

and the second test. An overview of all tested species and the observed effect concentrations (72-h NOE_rCs, 72-h LOE_rCs, 72-h E_rC10s and 72-h E_rC50s) is given in Table 7.1.

The 72-h E_rC50s for the repeated tests with *Desmodesmus* sp. and *C. microporum* indicated that the sensitivity of the algae shifted upward by a factor of 1.7 and downward by a factor of 2.0 between the first and the second test, respectively.

Overall, the 72-h E_rC50s for the soft water strains varied between 58.8 and 826 µg/L in 'soft' test water and between 147 and 1430 µg/L in 'moderately hard' test water. The 72-h E_rC50s for the hard water strains varied between 40.7 and 280 µg/L in 'moderately hard' test water and between 97.2 and 559 µg/L in 'hard' test water. Thus, the E_rC50 ranges shifted upward with increasing water hardness.

Also, increasing water hardness resulted in decreased toxicity for all individual species tested. For the soft water strains, an increase of water hardness from 6.25 to 16.3 mg $CaCO_3$/L (nominal values) resulted in a 1.7- to 2.5-fold increase of the 72-h E_rC50s. For the hard water strains, 72-h E_rC50s increased by a factor of 1.7 to 2.4 with an increase of water hardness from 16.3 to 43.4 mg $CaCO_3$/L (nominal values). The protective effect of water hardness against nickel toxicity is also clear from the other effect concentrations given in Table 7.1 (72-h NOE_rCs, 72-h LOE_rCs and 72-h E_rC10s).

Statistical comparison (Mann Whitney U test, $p < 0.05$) of the 72-h E_rC50s obtained in the 'moderately hard' test water did not reveal any significant differences between the 72-h E_rC50s for the soft water strains and those for the hard water strains. The observed effects of increasing water hardness are visualized in Figure 7.1.

7.3.2. Biotic ligand model application

The first modeling exercise (model 1) assumed $K_{CaBL} = K_{MgBL} = 0$. Fitting of Equation 7.1 was thus reduced to estimating the sensitivity parameter $E_rC50_{Ni2+,0,i}$ for each strain *i*. Because the sensitivity of the *Desmodesmus* sp. strain and the *C. microporum* strain slightly shifted during the four months between the first and the second test, the sensitivity parameters for the algal populations in the first and the second test were optimized separately (this was done in all four modeling exercises).

Table 7.1. Toxicity of nickel to all field-collected algae tested. Effect concentrations (72-h NOE_rCs, 72-h LOE_rCs, 72-h E_rC10s and 72-h E_rC50s) are reported as µg dissolved Ni/L. Numbers between brackets represent 95% confidence intervals. Soft water algae were tested in 'soft' and 'moderately hard' test medium whereas hard water algae were tested in 'moderately hard' and 'hard' test medium. See text for details. The physicochemical characteristics of the sampling sites are reported in chapter 6, Table 6.1.

Site number	Species		Hard				Moderately hard			
			NOE_rC[b]	LOE_rC	E_rC10	E_rC50	NOE_rC[b]	LOE_rC	E_rC10	E_rC50
H10	*Desmodesmus* sp.	Test 1[a]	43.3	91.1	98.0 (58.3-165)	517 (430-621)	43.7	92.1	86.8 (64.4-117)	236 (210-266)
		Test 2[a]	96.2	168	67.7 (38.3-120)	333 (293-379)	29.1	46.9	67.7 (38.3-120)	140 (109-178)
	Pediastrum duplex		39.5	77.2	16.4 (4.7-56.8)[c]	97.2 (57.4-165)	23.5	38.3	32.2 (4.5-232)	40.7 (21.0-78.9)
H11	*Pseudokirchneriella* sp.		13.8	25.9	12.9 (7.2-23.0)	140 (110-179)[d]	3.5	7.5	4.9 (2.5-9.8)	68.9 (56.4-84.2)
H12	*Coelastrum microporum*	Test 1[a]	< 140	140	54.3 (25.3-116)[c]	243 (178-333)	< 84.0	84.0	38.6 (9.2-163)[c]	139 (75.3-257)
		Test 2[a]	< 40.2	40.2	100 (79.1-126)	559 (514-608)	< 31.2	31.2	66.7 (50.5-88.0)	280 (253-309)
	Spermatozopsis exsultans		< 41.9	41.9	28.6 (18.5-44.4)[c]	263 (222-311)	< 22.9	22.9	17.1 (11.3-25.8)[c]	118 (99.9-139)
Site number	Species		Soft				Moderately hard			
			NOE_rC[b]	LOE_rC	E_rC10	E_rC50	NOE_rC[b]	LOE_rC	E_rC10	E_rC50
S14	*Chlamydomonas* sp.		8.3	16.0	26.4 (16.9-31.6)	58.8 (49.5-64.9)	27.5	42.2	45.0 (35.1-54.8)	147 (130-165)
	Ankistrodesmus falcatus		24.6	42.9	18.3 (5.8-58.2)	237 (168-335)	43.0	105	43.6 (19.5-97.4)	394 (306-507)
S18	*Scenedesmus accuminatus*		< 6.2	6.2	7.0 (4.0-12.1)	83.3 (69.4-99.9)	12.3	25.4	18.5 (10.8-31.9)	189 (160-224)
	Chlorella sp.		98.2	168	64.7 (25.9-162)	826 (601-1130)	< 97.7	97.7	90.5 (48.8-168)[c]	1430 (1150-1780)
S21	*Desmodesmus spinosus*		< 6.9	6.9	5.5 (3.1-9.8)[c]	88.1 (71.7-108)	22.5	41.7	28.9 (19.7-42.3)	171 (149-196)

[a] Two growth inhibition tests were conducted with the same strain.

[b] In some cases, the 72-h NOEC was higher than the 72-h ErC10. This is due to partial overlap of the growth rate ranges at the lower exposure concentrations with the growth rate range in the control while average growth rates gradually decreased.

[c] 72-h ErC10 extrapolated below lowest test concentration.

[d] 72-h ErC50 extrapolated above highest test concentration.

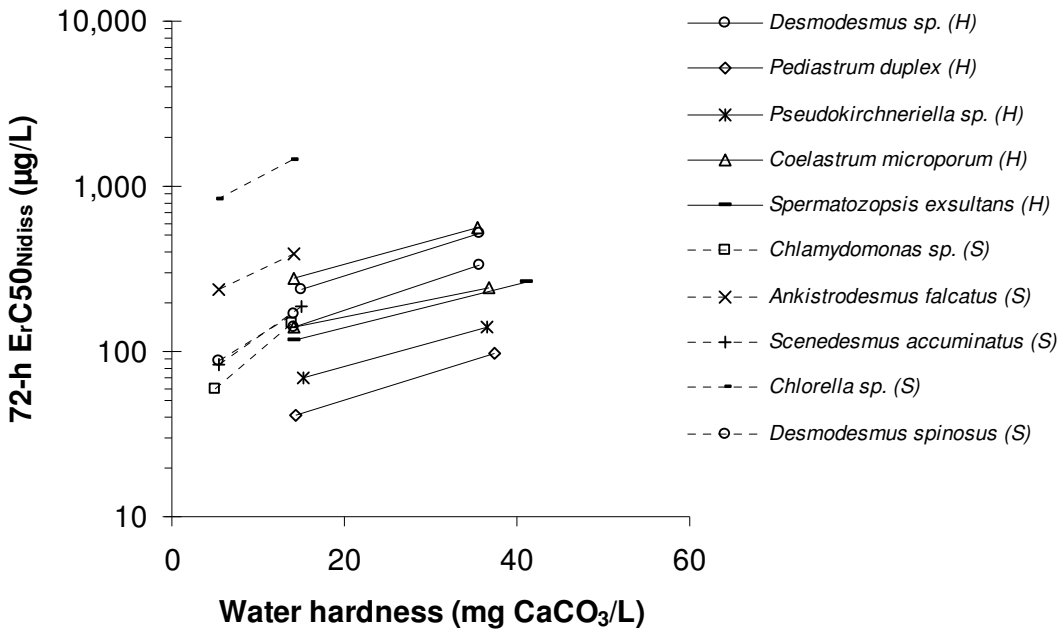

Figure 7.1. Observed 72-h E_rC50 (µg dissolved Ni/L) as a function of water hardness (mg $CaCO_3$/L) for algae originating from soft (S, dashed lines) and hard (H, solid lines) water lakes. Algae from soft water lakes were tested at water hardness levels of 6.25 and 16.3 mg $CaCO_3$/L (nominal concentrations); algae from hard water lakes were tested at water hardness levels of 16.3 and 43.4 mg $CaCO_3$/L (nominal concentrations). All 72-h E_rC50s are plotted against measured water hardness levels.

Model 1 predicted nickel toxicity by an average error of factor 1.4 (prediction error range = 1.3-1.6) (Table 7.2). As expected, nickel toxicity was systematically underestimated at lower water hardness levels and overestimated at higher water hardness levels.

In the second modeling exercise (model 2), K_{CaBL} and K_{MgBL} were optimized based on the combined toxicity dataset for the soft and the hard water strains tested. The optimal values for log K_{CaBL} and log K_{MgBL} were 4.0 and 5.3, respectively. Using this model, nickel toxicity was predicted by an average error of factor 1.1 (prediction error range = 1.0-1.1) (Figure 7.2, lower panel). Prediction errors were remarkably reduced compared to model 1 (no hardness effect assumed). The likelihood ratio test indicated that the increased accuracy of model 2 was statistically significant ($\alpha < p = 0.05$; Table 7.2). This demonstrates the need to incorporate the protective effect of water hardness into the nickel bioavailability model.

In the third modeling exercise (model 3), optimal values for K_{CaBL} and K_{MgBL} were determined for the soft and the hard water strains separately. Interestingly, the optimized

parameter values for model 3 were exactly the same as for model 2. Consequently, the accuracy of model 3 did not improve compared to model 2 (α = 1; Table 7.2).

Table 7.2. Model parameters (log K_{CaBL}, log K_{MgBL} and the sensitivity parameter $E_rC50_{Ni2+,0,i}$), average prediction errors and prediction error ranges for four different modeling exercises based on 72-h E_rC50s. Model 1 = no hardness effect assumed; model 2 = log K values determined for soft and hard water strains combined; model 3 = log K values determined for soft and hard water strains separately; model 4 = *Pseudokirchneriella subcapitata* model as described in chapter 5. SSE = sum of squared errors (chapter 6, Equation 6.2); G^2 = test statistic for likelihood ratio test (chapter 6, Equation 6.3); α = probability of validity of null hypothesis (if α < p = 0.05, null hypothesis is rejected, i.e. model performance is significantly better than previous model). Log K_{CaBL}, log K_{MgBL} and prediction errors of the retained model are printed in bold.

Model	1	**2**	3	4
log K_{CaBL}	-	**4.0**	4.0	2.0
log K_{MgBL}	-	**5.3**	5.3	3.3
SSE	0.574	0.0191	0.0191	0.491
G^2	-	6.81[a,b]	0[a,c]	-
A	-	0.00909[b]	1[c]	-
Average prediction error	1.4	**1.1**	1.1	1.4
Prediction error range	1.3-1.6	**1.0-1.1**	1.0-1.1	1.3-1.5

Species	Sensitivity parameter $E_rC50_{Ni2+,0,i}$ (µM)			
Hard				
Desmodesmus sp.	4.62	0.455	0.455	4.21
	2.87	0.279	0.279	2.61
Pediastrum duplex	0.833	0.0786	0.0786	0.756
Pseudokirchneriella sp.	1.29	0.121	0.121	1.17
Coelastrum microporum	2.43	0.231	0.231	2.21
	5.26	0.510	0.510	4.79
Spermatozopsis exsultans	2.33	0.220	0.220	2.11
Soft				
Chlamydomonas sp.	1.28	0.247	0.247	1.22
Ankistrodesmus falcatus	4.16	0.778	0.778	3.98
Scenedesmus accuminatus	1.71	0.316	0.316	1.63
Chlorella sp.	14.9	2.80	2.80	14.2
Desmodesmus spinosus	1.67	0.312	0.312	1.60

[a] N = 24 (chapter 6, Equation 6.3).

[b,c] Values for statistical comparison of model 2 with model 1 and model 3 with model 2, respectively.

Finally, in a fourth modeling exercise (model 4), it was investigated how accurate nickel toxicity could be predicted as a function of water hardness when using the log K_{CaBL} and log K_{MgBL} from the nickel bioavailability model developed for the standard test species *P. subcapitata* (log K_{CaBL} = 2.0 and log K_{MgBL} = 3.3, chapter 5). Model 4 predicted nickel toxicity with an average error of factor 1.4 (prediction error range = 1.3-1.5) (Figure 7.2, upper panel). Similar to model 1 (no hardness effect assumed), nickel toxicity was systematically underestimated at lower water hardness levels and overestimated at higher water hardness levels.

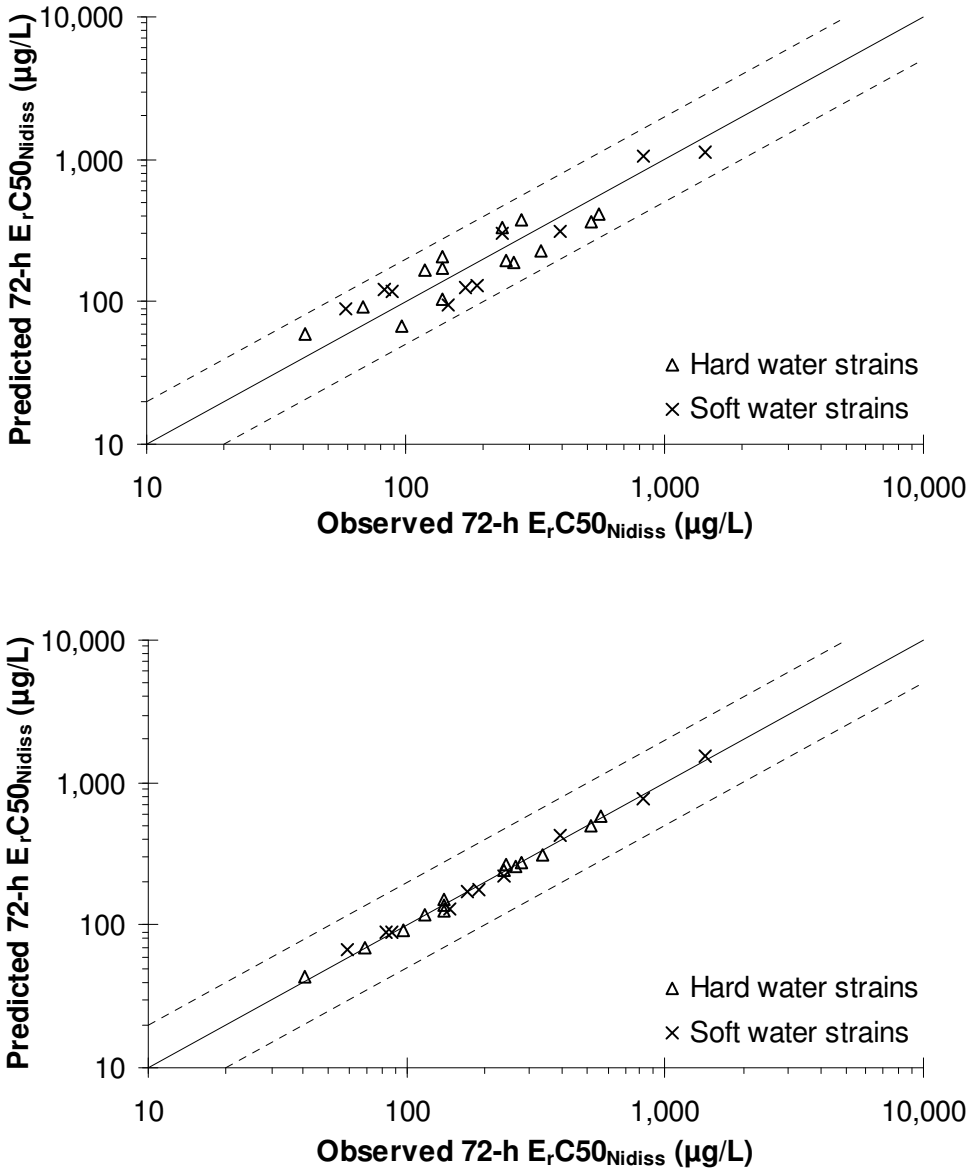

Figure 7.2. Predicted versus observed 72-h E_rC50s (µg dissolved Ni/L). Upper panel: predictions made using the log K_{CaBL} and log K_{MgBL} of the model developed for the standard test species *Pseudokirchneriella subcapitata* (model 4) (see chapter 5); lower panel: predictions made using log K_{CaBL} and log K_{MgBL} values optimized for the soft and the hard water strains combined (model 2). Model parameters are given in Table 7.2. The solid line indicates a perfect match between predicted and observed E_rC50s; the dashed lines indicate ratios of 0.5 and 2 between observed and predicted E_rC50s.

7.4. Discussion

In this study, we investigated the effect of water hardness on the toxicity of nickel to field-collected microalgae within a water hardness range of 6.25 to 16.3 mg $CaCO_3$/L (nominal values) for algae collected in soft water and 16.3 to 43.4 mg $CaCO_3$/L for algae collected in hard water. The objectives of this study were to determine (i) whether algae living in soft water are intrinsically more sensitive to nickel than algae living in hard water, and (ii) whether a single bioavailability model can be used to predict the protective effect of water hardness on the toxicity of nickel to algae in both soft and hard water.

We focused on the effect of water hardness since this water chemistry parameter has been demonstrated to be the most important factor affecting nickel toxicity to algae (chapter 5). However, it must be kept in mind that the effects of other factors – such as pH and DOC (chapter 5) – which have been demonstrated to affect nickel toxicity to algae in hard waters remain to be studied in soft waters.

The inhibitory effect of nickel on algal growth has been observed for a variety of green microalgae using cell counts, biomass measurements, measurements of optical density or light absorbance, (e.g., λ = 750 nm: Issa et al., 1995; Lustigman et al., 1995; Fargašová, 2001; λ = 650 nm: Wong and Chang, 1991), concentrations of photosynthetic pigments (chlorophyll a, chlorophyll b, total chlorophyll, carotenoids) and measurements of photosynthetic parameters (O_2 evolution and carbon fixation) (for an overview, see chapter 1). Fargašová et al. (1999) observed that the EC50s based on chlorophyll a content were very similar to growth rate-based EC50s. Therefore we decided to measure light absorbance at one of the absorption maxima of chlorophyll a (best results obtained at λ = 662 nm). Light absorbance measurements were then related to cell counts to evaluate the effect of nickel on algal growth rate, as prescribed by the revised OECD test guideline 201 (OECD, 2006).

Although nickel toxicity has been studied using a variety of algal species (see chapter 1), comparison of the effect data reported in the available studies is hampered by the use of different test media, different exposure durations and different endpoints. Only two of the available studies investigated the effect of water hardness on nickel toxicity to algae. First, Issa et al. (1995) reported a decrease of nickel toxicity to *Kirchneriella lunaris* at 5.0 mM Ca^{2+} compared to a control (0 mM Ca^{2+}). However, 5.0 mM Ca^{2+} is outside the ecologically

relevant range of calcium concentrations in surface waters. Second, in chapter 5, the effects of calcium and magnesium on nickel toxicity to *P. subcapitata* were extensively studied within a concentration range of (roughly) 0.12 to 3.0 mM. Both calcium and magnesium were observed to ameliorate nickel toxicity, with magnesium offering by far the most effective protection. The protective effects of calcium and magnesium were successfully modeled as BLM-type competition effects represented by log K_{CaBL} and log K_{MgBL}, i.e. the stability constants for binding between calcium and magnesium and the BL on the algal cell surface.

7.4.1. Modeling the effect of water hardness

Statistical comparison of the predictive capacity of model 2 (hardness effect for soft and hard water strains described using a single set of log K_{CaBL} and log K_{MgBL} values) with the predictive capacity of model 1 (no hardness effect included) revealed that model 2 performed significantly better ($p < 0.05$). This demonstrates that the protective effect of water hardness on nickel toxicity at low water hardness levels is sufficiently important to be included in nickel bioavailability models for use in regulatory exercises (e.g., risk assessment, water quality criteria setting). This observation coincides with the results from our study with field-collected cladocerans (chapter 6).

At this point in time, there are only a few studies available reporting on the effects of calcium and/or magnesium on nickel uptake by and/or nickel toxicity to microalgae. Mehta et al. (2000) observed a decrease of nickel adsorption and uptake by *Chlorella vulgaris* with increasing calcium and magnesium concentrations. The study of Issa et al. (1995) demonstrated that both nickel uptake by and toxicity to *K. lunaris* were lower at 5.0 mM Ca^{2+} compared to a control without Ca^{2+}. The observed reduction of nickel toxicity was rather small. In chapter 5, it was demonstrated that both calcium and magnesium protect against nickel toxicity in *P. subcapitata*. However, magnesium appeared to be far more effective than calcium in reducing nickel toxicity. Recent Ni^{2+} internalization experiments conducted by Worms and Wilkinson (2007) indicated that Ni^{2+} internalization fluxes in *C. reinhardtii* decreased with increasing Ca^{2+} and Mg^{2+} concentrations. Here also, the effect of Ca^{2+} was observed to be much smaller than that of Mg^{2+} (only a 3-fold decrease of nickel internalization fluxes upon a 1000-fold increase in calcium concentration).

Based on the results of their Ni^{2+} internalization experiments, Worms and Wilkinson (2007) calculated equal affinity constants for the interaction of Mg^{2+} and Ni^{2+} with the Ni^{2+} uptake site (log K_{MgBL} = log K_{NiBL} = 5.1). This is consistent with magnesium transporters being a major pathway for nickel to enter algal cells. The idea of a common uptake pathway for Ni^{2+} and Mg^{2+} is supported by the fact that Ni^{2+} and Mg^{2+} have similar dehydrated ionic radii (Weast, 1973). Evidence for Ni^{2+} uptake via Mg^{2+} transport systems has been provided for very different types of organisms (for an overview, see chapter 5). Moreover, identification of Mg^{2+} transport proteins in several species suggests at least some degree of conservation throughout evolution, which may explain why nickel-magnesium interactions are so frequently reported in scientific literature. Most remarkably, the log K_{MgBL} reported by Worms and Wilkinson (2007) is very similar to the one obtained for the field-collected microalgae used in this study (log K_{MgBL} = 5.3).

The direct inhibitory effect of Ca^{2+} on Ni^{2+} uptake is much smaller than for Mg^{2+}. This has been demonstrated by several authors. For Ca^{2+}, Worms and Wilkinson (2007) calculated an affinity constant for the interaction with the Ni^{2+} internalization site (log K_{CaBL}) of 3.0. This constant was much smaller than that obtained for Mg^{2+}. This is in agreement with the observations made by Joho et al. (1991) for the yeast *Saccharomyces cerevisiae* and the findings of Snavely et al. (1991) that Ca^{2+} also competes with Ni^{2+} for uptake through all identified Mg^{2+} transporters in *Salmonella typhimurium*, although in a much less effective way than Mg^{2+}.

The results of the Ni^{2+} internalization experiments of Worms and Wilkinson (2007) indicated that the effect of calcium could not be explained by a simple competitive equilibrium with the Ni^{2+} transport sites. In the previous chapters, it was also suggested that mechanisms other than direct competition may be involved in determining the protective effect of calcium on nickel toxicity. Calcium may for instance protect indirectly against nickel toxicity through its function in the regulation of membrane stabilization. Abdel-Basset and Issa (1994) demonstrated that calcium induces membrane stabilization in algae under stressful conditions. Possibly, increased calcium concentrations hereby protect against nickel-induced loss of magnesium (see previous chapters). In this respect, Worms and Wilkinson (2007) suggested that calcium may reduce Ni^{2+} efflux as well, which would counteract the protective effects of calcium mentioned above and may explain the relatively low log K_{CaBL}.

It is noteworthy that the difference between the (chronic or long-term) nickel toxicity reducing capacities of calcium and magnesium is much larger in algae than in cladocerans and fish (*P. subcapitata*: log K_{CaBL} = 2.0, log K_{MgBL} = 3.3, chapter 5; *D. magna*: log K_{CaBL} = 3.5, log K_{MgBL} = 3.6, chapter 4; rainbow trout: log K_{CaBL} = log K_{MgBL} = 3.6, chapter 2). The relatively higher toxicity mitigating potency of magnesium in algae was also observed for zinc (Heijerick et al., 2002a) and may be due to the general protection of increasing magnesium concentrations against the formation of non-functional metal-chlorophyll through substitution of Mg^{2+} in the porphyrin ring (Küpper et al., 2006). Also, the relatively higher protective effect of calcium in multicellular organisms may be attributed to an increased protection against nickel-induced magnesium loss through the effect of calcium on the tightening of paracellular junctions (tight junctions in vertebrates and septate junctions in invertebrates).

To determine whether a single bioavailability model can be used to predict the protective effect of water hardness on the toxicity of nickel to algae in both soft and hard water, the predictive capacity of model 3 (log K_{CaBL} and log K_{MgBL} determined for soft and hard water strains separately) was statistically compared to the predictive capacity of model 2 (log K_{CaBL} and log K_{MgBL} determined for soft and hard water strains combined). There was no significant difference between model 2 and model 3. This was due to the fact that the log K_{CaBL} and log K_{MgBL} values for the soft water algae were identical to those for the hard water algae.

For nickel toxicity to field-collected cladocerans from soft and hard water lakes, modeling exercise 3 yielded higher log K_{CaBL} and log K_{MgBL} values for the soft water cladocerans than for the hard water cladocerans tested (see chapter 6). For predicting acute nickel toxicity, model 3 was demonstrated to be significantly more accurate than model 2, whereas for predicting chronic nickel toxicity, no significant differences were observed between the accuracy of model 2 and model 3. Further research would be needed to explain why higher log K values were obtained for the soft water cladocerans. Possibly, this may be the result of an increased affinity of soft water cladocerans for Ca^{2+} and/or Mg^{2+}. This may be due to a relatively higher occurrence of high-affinity Mg^{2+} transporters in the soft water cladocerans (cf. the findings of Snavely et al. (1991) for *S. typhimurium*). However, hardness-dependent high-affinity Mg^{2+} transporters have not been identified in cladocerans yet.

Recent findings of Worms and Wilkinson (2007) suggest that multiple Ni^{2+} internalization sites also exist in algae. These sites would have different affinities for Ni^{2+}, Ca^{2+} and Mg^{2+} and would be differently influenced by concentrations of Ca^{2+} and Mg^{2+}. The existence of multiple binding sites is theoretically ignored by the single-site modeling approach of the BLM. Consequently, the estimated log K_{CaBL} and log K_{MgBL} values may actually represent the average affinity of all present BL sites for Ca^{2+} and Mg^{2+}. The fact that the optimal log K_{CaBL} and log K_{MgBL} values were exactly the same for the soft and the hard water algae used in this study implies that, if multiple nickel internalization sites with different affinities for Ni^{2+} and competing cations exist in algae, their relative abundance is not significantly influenced by water hardness within the water hardness range investigated in this study.

Although a multiple binding site approach may not be necessary for accurately predicting nickel toxicity to microalgae as a function of water hardness, it may prove useful for increasing the overall accuracy of predictions of nickel toxicity when other toxicity affecting water chemistry parameters are considered (e.g., pH, chapter 5). Indications for the existence of multiple metal binding sites have also been reported for other metals, such as zinc (e.g., Hassler and Wilkinson, 2003) and copper (e.g., Borgmann et al., 2005). The latter authors even demonstrated that copper toxicity to *Hyalella azteca* was more accurately predicted by a two-binding-site model than by a typical single-site BLM and suggested that a multiple binding site approach was also consistent with copper toxicity data reported for other organisms.

Because of the overall indications that the protective effect of calcium on nickel toxicity to algae is much less important than that of magnesium, it could be argued that only a log K_{MgBL} should be used for representing the effect of water hardness in the model. When fitting a log K_{MgBL} alone, the same conclusions could be drawn from the statistical comparison of the predictive capacity of model 3 to that of model 2. The log K_{MgBL} calculated for the soft water organisms was equal to that calculated for the hard water organisms (log K_{MgBL} = 5.5) and was only slightly higher than that obtained in the abovementioned modeling exercises (log K_{MgBL} = 5.3).

For risk assessment purposes, it was considered interesting to evaluate the predictive capacity of the nickel bioavailability model developed for the standard test alga *P.*

subcapitata (chapter 5) when used for predicting nickel toxicity to the field-collected algae tested in this study (model 4). Although two third of the predictions were for water hardness levels below the lower water hardness boundary of this model (i.e. 19.9 mg $CaCO_3$/L), all predictions deviated less than a factor 2 from observed toxicity (Table 7.2). However, because of the lower log K_{CaBL} and log K_{MgBL} values, predictions made with the *P. subcapitata*-based model were less accurate than those made by the model specifically developed for the field-collected algae (model 2). Similar observations were made when using the *Daphnia magna*-based nickel bioavailability models developed in chapter 3 and 4 for predicting nickel toxicity to field-collected cladocerans (chapter 6). The log K_{CaBL} and log K_{MgBL} values for *P. subcapitata* were lower than those derived for the field-collected algae. Further analysis of the available data would be needed to investigate whether the parameter values for the field-collected algae are significantly different from those for *P. subcapitata*.

7.4.2. Intrinsic sensitivity

In order to compare the sensitivity of soft and hard water algae to nickel, both soft and hard water strains were tested in the same 'moderately hard' test medium (nominal water hardness = 16.3 mg $CaCO_3$/L). Statistical comparison of the 72-h E_rC50s obtained in this 'moderately hard' test medium revealed no significant sensitivity differences between the soft and the hard water strains tested. In chapter 6, similar observations were reported for both acute and chronic nickel toxicity to cladocerans originating from soft and hard water lakes.

The sensitivity of the *Desmodesmus* sp. strain and the *C. microporum* strain, which were tested twice within a period of four months, was observed to have shifted slightly (factor 1.7 and 2.0, respectively) during this period. Intraclonal metal sensitivity shifts have been reported previously for algae (e.g., Janssen and Heijerick, 2003; chapter 5) and other organisms, such as cladocerans (e.g., Soares et al., 1992; Baird and Barata, 1997, 1998; chapter 4). Unfortunately, some within-clone variation will always remain uncontrollable. Therefore, the sensitivity parameters for the algal populations in the first and the second test were optimized separately in all modeling exercises.

Comparison of the toxicity data obtained in 'moderately hard' test medium (nominal water hardness = 16.3 mg $CaCO_3$/L) with toxicity data obtained for *P. subcapitata* (chapter 5) in the same medium with comparable water hardness (19.9-20.1 mg $CaCO_3$/L) demonstrated

that *P. subcapitata* exhibits an intermediate to high sensitivity to nickel compared to the tested field species (for only two strains the 72-h E_rC50s were lower than the lowest 72-h E_rC50 obtained for *P. subcapitata* at the abovementioned water hardness – i.e. < 93.7 µg/L, see chapter 5).

When taking account of the fact that (i) water hardness in the *P. subcapitata* tests was slightly higher than in the tests in 'moderately hard' test medium conducted in the present study, and (ii) the calcium to magnesium ratio in the basic test medium used in chapter 5 was lower than the calcium to magnesium ratio in the 'moderately hard' test medium used in the present study, it may be concluded that *P. subcapitata* is rather highly sensitive to nickel. Although nickel toxicity has been investigated using a variety of green microalgae (see chapter 1), comparison with nickel toxicity data obtained with other algal species is even more difficult because of numerous differences between test conditions and test duration and because the composition of the used test media has not always been reported.

Since the sensitivity parameter of bioavailability models (in this study: $E_rC50_{Ni2+,0,i}$) has previously been suggested to represent the intrinsic sensitivity of a species/population (De Schamphelaere et al., 2007b), the interspecies sensitivity differences observed through comparison of the effect data reported in Table 7.1 are also reflected in the $E_rC50_{Ni2+,0,i}$ values reported in Table 7.2. Consequently, statistical comparison of the $E_rC50_{Ni2+,0,i}$ values for the retained model (model 2) did not reveal a significant sensitivity difference between the soft and the hard water strains either.

7.5. Conclusion

The results from this study indicate that green microalgae living in soft surface waters (operationally defined water hardness < 10 mg $CaCO_3$/L) are equally sensitive to nickel as green microalgae living in hard surface waters (operationally defined water hardness > 25 mg $CaCO_3$/L). The protective effect of water hardness between 6.25 and 43.4 mg $CaCO_3$/L was demonstrated to be significant and was therefore incorporated in the algal nickel bioavailability model. The parameter values representing the protective effect of water hardness were calculated to be identically the same for the soft and the hard water algae used in this study ($\log K_{CaBL} = 4.0$ and $\log K_{MgBL} = 5.3$). Apparently, a single bioavailability model can be used to predict nickel toxicity as a function of water hardness to green microalgae in

soft and hard surface waters. The predictive capacity of the *P. subcapitata*-based model developed in chapter 5 was acceptable. However, because of the lower log K_{CaBL} en log K_{MgBL} values used by this model, nickel toxicity to the field-collected algae used in the present study was slightly overestimated at higher water hardness levels and slightly underestimated at lower water hardness levels.

Chapter 8

General conclusions and future research perspectives

Chapter 8

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Since water chemistry affects metal bioavailability and toxicity, it is generally accepted that metals require special attention in regulatory actions with regard to the aquatic environment. In countries all over the world (e.g., USA, New Zealand and Australia, Canada) as well as in the European Union, policy makers have provided or are providing the possibility to take into account bioavailability in both risk assessment procedures and procedures for the determination of environmental quality standards for metals. However, to do so, tools have to be developed that are capable of predicting metal bioavailability and toxicity as a function of the physicochemical composition of the environmental compartment under consideration.

The biotic ligand model (BLM) concept, originally developed by Di Toro et al. (2001) and further explored by many researchers (reviewed by Paquin et al., 2002; Niyogi and Wood, 2004) presents an interesting concept that allows the development of bioavailability models that can be used in regulatory exercises concerning metals. However, for nickel, the toxicity data that were available at the start of the present study were not sufficiently detailed and/or not suitable for the development of bioavailability models. Not surprisingly, preliminary BLM-type bioavailability models for nickel developed by Wu et al. (2003) and Keithly et al. (2004) revealed serious drawbacks which would inevitably result in inaccurate predictions (see chapter 1). The overall objectives of this study were therefore to generate new and more comprehensive data on nickel bioavailability to freshwater organisms and to develop, based on these new data, bioavailability models that can be used for regulatory purposes.

In the first part of this study, it was investigated how calcium, magnesium and pH individually affect the toxicity of nickel to three standard test species belonging to three different trophic levels: a unicellular green alga (*Pseudokirchneriella subcapitata*), an aquatic invertebrate (*Daphnia magna*) and a fish (*Oncorhynchus mykiss*, rainbow trout). This study has been the first to investigate the toxicity mitigating effects of calcium and magnesium separately. Sodium was not considered after it was observed not to affect acute nickel toxicity to *D. magna* (chapter 3). Sodium was not expected to alter nickel toxicity since there are no known indications for sodium to be involved in the mechanism of nickel uptake and/or

toxicity. Since chronic toxicity data are recommended for regulatory purposes, the present study mainly focused on the generation of chronic (or long-term) toxicity data.

Both calcium and magnesium were observed to reduce nickel toxicity to all three standard test species. For acute nickel toxicity to *D. magna* (chapter 3), the toxicity mitigating capacity of magnesium was clearly less than that of calcium. However, chronic nickel toxicity to *D. magna* (chapter 4) and long-term nickel toxicity to rainbow trout (chapter 2) were reduced to a similar extent by both hardness cations. For nickel toxicity to *P. subcapitata* (chapter 5), the protective effect of magnesium was even much more important than that of calcium. In all cases, linear relationships (at least within ecologically relevant water hardness ranges) were obtained between the $E/LC50_{Ni2+}$ (i.e. the EC50 or LC50 expressed as free nickel ion activity) and Ca^{2+} and Mg^{2+} activity. These linear relationships allowed modeling the observed effects as BLM-type competition effects.

Although this study is the first to demonstrate the importance of magnesium as nickel toxicity mitigating factor in three of the most commonly used standard test species, nickel-magnesium interactions have been frequently reported in scientific literature (for an overview, see chapter 5). Evidence for Ni^{2+} uptake via Mg^{2+} transport systems has been provided for members of very different groups of organisms and supports the idea that the observed protective effect is the result of competition between Ni^{2+} and Mg^{2+} for a common uptake pathway. Although Mg^{2+} transporters have not yet been identified in fish, aquatic invertebrates or unicellular green algae, recent physiological observations made by Pane et al. (*D. magna*, 2003b; rainbow trout, 2006a,b) and Worms and Wilkinson (*Chlamydomonas reinhardtii*, 2007a) are consistent with Ni^{2+} uptake via Mg^{2+} transport channels in these organisms.

The toxicity mitigating capacity of magnesium on chronic nickel toxicity to *D. magna* was observed to be more important than on acute nickel toxicity. For other metals, such as copper and zinc, it has also been observed that the protective effect of the cation most closely related to the mechanism of toxicity (i.e. sodium for copper and calcium for zinc) becomes increasingly important with increasing exposure duration (see chapter 4). These observations are hence in line with the conclusion that magnesium plays a central role in the mechanism of nickel toxicity. Remarkably, the toxicity mitigating potency of magnesium was much higher in *P. subcapitata* than in the other test species used in this study. This was also observed for

zinc and may be due to the general protection of increasing magnesium concentrations against the formation of non-functional metal-chlorophyll molecules through substitution of Mg^{2+} in the porphyrin ring (see chapter 7).

Like for magnesium, the protective effect of calcium could be modeled as a BLM-type competition effect for all test species used in this study. However, at this point in time, it is not clear whether the observed toxicity mitigating effect of calcium can be considered as a competition-only effect. Based on findings of Joho et al. (1991) and Snavely et al. (1991), part of the observed amelioration of nickel toxicity may be attributed to competition between Ca^{2+} and Ni^{2+} for uptake via Mg^{2+} transport systems. However, the extent to which Ca^{2+} inhibits Ni^{2+} uptake via Mg^{2+} uptake pathways has been reported to be smaller than that of Mg^{2+} itself. Since calcium was observed to be equally protective as magnesium against chronic nickel toxicity to *D. magna* (chapter 4) and long-term nickel toxicity to rainbow trout (chapter 2) and even more protective than magnesium against acute nickel toxicity to *D. magna* (chapter 3), mechanisms other than direct competition for a common uptake pathway are assumed to be involved. For instance, calcium may protect indirectly against nickel toxicity through its role in the regulation of physiological functions such as membrane permeability and ion transport. However, further research is needed to confirm this and to reveal the exact mechanism by which calcium affects nickel toxicity.

The effect of pH on nickel toxicity appeared to be the most complicated effect investigated in this study. Overall, toxicity of the free nickel ion was observed to increase with increasing pH. However, for all three test species, the obtained relationships between the $E/LC50_{Ni2+}$ and H^+ activity were not linear over the entire pH range tested. Apparently, at elevated pH levels, less free metal ion is needed to cause a certain toxic effect. More or less similar nonlinear relationships have also been reported for other metals (see chapters 2 to 5). Such relationships indicate that the effect of pH on metal toxicity can not be attributed to proton competition for a single unidentate site alone.

Several explanations have been given for this in literature (see chapters 2 to 5). For instance, the possibility exists that metal species other than the free metal ion contribute to metal toxicity. For nickel, it may be assumed that $NiHCO_3^+$ and $NiCO_3$, which increase in abundance with increasing pH, are responsible for the higher than expected toxicity at elevated pH levels. Possible explanations for the apparent toxicity of these metal complexes

would be that they dissociate in the physicochemically different gill (*D. magna*, rainbow trout) or cell (algae) microenvironment and/or facilitate Ni^{2+} uptake through Mg^{2+} transport systems such as electro-neutral Mg^{2+}/HCO_3^- symporters (Günther et al., 1986). Unfortunately, the physiology of Mg^{2+} transporters remains to be identified in the test species used in this study. Overall, further research would be needed to unravel the exact mechanisms underlying the higher than expected toxicity at elevated pH (and/or alkalinity) levels.

Interestingly, the effect of pH on chronic nickel toxicity to *D. magna* was observed to be more important than on acute nickel toxicity. Possibly, dietary toxicity is responsible for this observation. In chronic toxicity tests with *D. magna*, live algae are added as food source for the test organisms. Since both metal adsorption to and metal absorption in algal cells generally increase with increasing pH (see chapter 5), exposure to nickel via the diet could be expected to increase with increasing pH. However, it remains to be investigated whether or not dietborne exposure actually contributes to chronic nickel toxicity in *D. magna*. So far, adverse effects of dietborne nickel exposure have only been observed in fish (see chapter 1). It is also highly recommended to investigate which implications dietary toxicity could have for bioavailability modeling.

Because of its limited effect on acute nickel toxicity to *D. magna*, the effect of pH was not incorporated in the acute nickel bioavailability model for *D. magna* (chapter 3). The effect of pH on chronic or long-term nickel toxicity to rainbow trout, *D. magna* and *P. subcapitata* could however not be ignored. Based on the results obtained for rainbow trout, a new modeling approach was developed in which the effect of pH was modeled based on an empirical linear relationship between pH and $E/LC50_{pNi2+}^{*}$ (i.e. the negative logarithm of the $E/LC50_{Ni2+}$ corrected for the presence of calcium and magnesium) and was superimposed on the BLM-type effects of calcium and magnesium (chapter 2). This modeling approach was later adopted for *D. magna* (chapter 4) and *P. subcapitata* (chapter 5). For *P. subcapitata*, a comparison with the original BLM approach (H^+ competition) revealed that the effect of pH was more satisfactorily described when the new approach was followed, resulting in increased accuracy of model predictions.

Since nickel did not significantly affect growth of juvenile rainbow trout, the nickel bioavailability model for rainbow trout was developed based on mortality data (chapter 2). For the development of the acute model for *D. magna*, 48-h EC50s (representing the

concentrations causing 50% immobilization and/or mortality) were used (chapter 3). The chronic model for *D. magna* was based on 21-d EC50s for the endpoint reproduction, since reproduction was observed to be a more sensitive endpoint than survival (chapter 4). Finally, for *P. subcapitata*, the model was developed using growth rate-based 72-h EC50s (chapter 5).

Once all model parameters had been derived for each of the test species under consideration, it was evaluated whether the models were capable of accurately predicting nickel toxicity in natural waters with varying physicochemical composition. In these validation exercises, the models' predictions were compared to toxicity test results obtained in nickel-spiked European surface waters. Except for a few specific cases, all effect concentrations under consideration were predicted within a factor 2 deviation from observed effect concentrations. Overall, it could be concluded that the bioavailability models developed in this study are useful tools for incorporating bioavailability in regulatory exercises.

Nevertheless, there is still some room for improvement. The models presented in this study may be further refined provided that several research topics be addressed in the future. Most of these topics have already been mentioned in the former paragraphs. Other topics of interest may be the possible existence of interactive effects in natural waters and the possibility that dissolved organic carbon (DOC) affects toxicity beyond its effect on nickel speciation (see further).

In chapter 5, it was investigated whether there are interactions between the effects of magnesium and pH, the two most important factors affecting nickel toxicity to *P. subcapitata*. Although some interaction was observed, it was not included in the model at this stage of model development because (i) the observed interaction was very limited, (ii) interactive effects are not sufficiently investigated and understood to allow them to be incorporated in bioavailability models, and (iii) the predictive capacity of the developed model was already very good without taking possible interactive effects into account. Although interactive effects may be subtle and not very important, from a purely physiological point of view, it may be worthwhile to investigate them more closely, since this could lead to new insights in the underlying mechanisms of metal toxicity in natural waters.

The results from the toxicity tests in nickel-spiked surface waters were also used for evaluating the toxicity mitigating effect of DOC. Speciation calculations were conducted

using WHAM VI software and optimized parameters for nickel binding to DOC (Van Laer et al., 2006). In all cases, the $E/LC50_{Nidiss}$ (i.e. the EC50s or LC50s expressed as dissolved nickel concentrations) were positively correlated to DOC content. Overall, the R^2 was higher for test organisms which were adversely affected by lower nickel concentrations. The highest R^2 was obtained for chronic nickel toxicity to *D. magna*, which was observed to be the most sensitive of the three standard test species used in this study. These observations suggest that at higher nickel concentrations, such as those resulting in acute toxicity to *D. magna* and long-term toxicity to rainbow trout, the toxicity mitigating effect of DOC becomes less important since the maximum Ni^{2+} complexation capacity of organic matter is being reached. Although there are no direct indications for DOC to affect nickel toxicity beyond its effect on speciation, further research is needed to exclude this as a possible source of unexplained variation in toxicity in certain surface waters.

Finally, it was investigated whether the models developed for predicting long-term nickel toxicity to rainbow trout (chapter 2) and acute nickel toxicity to *D. magna* (chapter 3) could accurately predict literature toxicity data for similar organisms. In chapter 2, the nickel bioavailability model for rainbow trout, calibrated to account for sensitivity differences between species, life stages and/or exposure durations, was successfully applied to literature data on the acute toxicity of nickel to larval, juvenile and subadult fathead minnow (*P. promelas*). In chapter 3, the acute nickel bioavailability model for *D. magna* was demonstrated to be able to accurately predict earlier published 48-h EC50s for *D. magna* and *C. dubia*, provided that the model was calibrated to account for interclonal or interspecies sensitivity differences.

The predictive capacity of the acute bioavailability model for *D. magna* (chapter 2) was also demonstrated to be better than that of the preliminary BLMs proposed by Wu et al. (2003) and Keithly et al. (2004). These models were based on the dataset of Meyer et al. (1999) on the effect of water hardness on gill nickel accumulation and toxicity to fathead minnow. Although this dataset only justifies the derivation of a K_{NiBL} and a K_{CaBL}, Wu et al. (2003) and Keithly et al. (2004) also incorporated a K_{NaBL} and a K_{HBL} into their models. A K_{MgBL} was not included. A comparison of the predictive capacity of these models to that of the model developed in the present study demonstrated that (i) there is no need to incorporate a K_{NaBL}, (ii) it is important to recognize the protective effect of magnesium, and (iii) the incorporation of a K_{HBL} does not adequately describe the effect of pH.

Although the bioavailability models described in chapters 2 to 5 can be readily used for regulatory purposes, their use is theoretically limited to aquatic systems of which the physicochemical composition falls within the water chemistry ranges for which the models were developed and validated. At the time this study was started, a major point of discussion concerned the use of bioavailability models for predicting metal toxicity in waters with hardness levels below the models' lower hardness boundaries. For nickel, the bioavailability models for fish (chapter 2), crustaceans (chapter 3 and 4) and algae (chapter 5) have a lower water hardness boundary of 22, 42 and 20 mg $CaCO_3$/L, respectively. However, surface waters in large geographic areas of Europe (e.g., Scandinavia, Scotland, Northern Portugal) are characterized by even lower hardness levels (Salminen et al., 2005). Therefore, it was considered necessary to investigate whether organisms living in these waters are affected by nickel in a similar way as organisms living in harder waters and whether possible differences would have implications for modeling. This was the main subject of the second part of this study (chapter 6 and 7).

The major research objectives of chapter 6 and 7 were to investigate (i) whether organisms living in 'soft' water (operationally defined water hardness < 10 mg $CaCO_3$/L) are intrinsically more sensitive to nickel than those living in 'hard' water (operationally defined water hardness > 25 mg $CaCO_3$/L), and (ii) whether a single bioavailability model can be used to predict the protective effect of water hardness on the toxicity of nickel in both soft and hard water. To investigate this, toxicity tests were conducted with cladocerans (acute and chronic, chapter 6) and green microalgae (chronic, chapter 7) that were collected in soft and hard water lakes in Sweden. Organisms collected in soft surface waters were tested in a 'soft' and a 'moderately hard' test solution while organisms collected in hard surface waters were tested in a 'moderately hard' and a 'hard' test solution. Nominal water hardness of these test solutions was 6.25, 16.3 and 43.4 mg $CaCO_3$/L, respectively.

For chronic nickel toxicity to cladocerans and algae, the following conclusions could be drawn from the results of the abovementioned experiments: (i) there are no significant nickel sensitivity differences between soft and hard water organisms, (ii) water hardness significantly protects both soft and hard water organisms against chronic nickel toxicity, and (iii) a single bioavailability model can be used to predict the protective effect of water hardness on chronic nickel toxicity to both soft and hard water organisms. Similar conclusions could be drawn for acute nickel toxicity to cladocerans, except that the protective effect of

water hardness was observed to be more accurately predicted when separate model parameters were used (K_{CaBL} and K_{MgBL}) for soft and hard water cladocerans. The higher K_{CaBL} and K_{MgBL} values for the soft water cladocerans may indicate that the protective effect of water hardness is larger in soft than in hard water cladocerans. Further research is needed to explain why the protective effect of water hardness on acute nickel toxicity to soft and hard water organisms was significantly different, while for chronic toxicity, this was not the case.

From a physiological point of view, it would be interesting to further investigate (i) whether there exist water-hardness dependent high affinity transport systems for Mg^{2+} (and/or Ca^{2+}) and Ni^{2+} in fish, aquatic invertebrates and algae (cfr. the findings of Snavely et al. (1991) for *Salmonella typhimurium*), (ii) whether the presence of such high affinity transporters changes within the ecologically relevant water hardness range, (iii) whether the presence of such transport systems affects Ni^{2+} toxicity, and (iv) whether multiple site models are required for accurately describing Ni^{2+} toxicity within a broad range of ecologically relevant water hardness levels. Similar research with other metals is also recommended.

This is the first study that investigated possible differences in metal toxicity to soft and hard water organisms. For nickel, answers have been provided that may simplify decision-making at the regulatory level whenever this issue is raised. Moreover, the bioavailability models that have been developed based on the results of toxicity testing with field-collected organisms may be further used for regulatory purposes.

Overall, the present study generated a large amount of toxicity data for 23 freshwater species belonging to three different trophic levels, hereby substantially increasing the nickel toxicity dataset for the aquatic compartment. Most of these data were taken up in the effects assessment of the European nickel risk assessment, the outcome of which will be used for determining the European water quality standard for nickel. The models developed in this study are also used in the European nickel risk assessment for correcting species sensitivity distributions for bioavailability, hereby reducing the uncertainty around the derivation of safe concentrations for the aquatic compartment. Later on, the models can also be used by the member states to evaluate whether their water bodies comply with the pan-European water quality standard for nickel. Since the concept of metal bioavailability is increasingly introduced in procedures for metal risk assessment and water quality criteria setting all around the world, the results of this study will definitely serve many countries in the future.

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Summary

During the past decades, risk assessments and water quality standards for metals have been predominantly based on total or dissolved metal concentrations. However, total or even dissolved metal concentrations have been demonstrated not to be good predictors of potential harm to aquatic ecosystems. Indeed, the same total or dissolved metal concentration does not result in the same degree of toxic effect under all conditions. This is mainly due to the fact that metal toxicity is substantially influenced by water chemistry.

Several authorities all around the world (e.g., USA, New Zealand and Australia, Canada, the European Union) have therefore provided the possibility to take into account the effect of water chemistry in risk assessment procedures and procedures for the derivation of water quality standards for metals, or are currently developing new strategies in which special attention is given to this topic. However, to allow taking into account metal bioavailability, tools are to be developed that are capable of predicting metal bioavailability and toxicity as a function of the physicochemical composition of the aquatic compartment under consideration. Without such tools, the proposed water quality criteria may be under- or over-protective, neither of which are desirable, since the former would result in harm to the aquatic environment, whereas the latter would entail unnecessary high costs for environmental sanitation and emission reduction measures.

At the time this study was started, the effect of water chemistry on the toxicity of nickel to freshwater organisms had scarcely been studied (see chapter 1). The available bioavailability data were not sufficiently detailed and/or not suitable for the development of bioavailability models. Not surprisingly, preliminary bioavailability models that were developed based on the available data revealed serious drawbacks which would inevitably result in inaccurate predictions (see chapter 1). The overall objectives of this study were therefore to generate new and more comprehensive data on nickel bioavailability to freshwater organisms and to develop, based on these new data, bioavailability models that can be used for regulatory purposes.

In the first part of this study, the effect of water chemistry on nickel toxicity was investigated using standard test species belonging to three different trophic levels: a unicellular green alga (*Pseudokirchneriella subcapitata*), an aquatic invertebrate (*Daphnia*

magna) and a fish (*Oncorhynchus mykiss*, rainbow trout). The main focus was on the generation of chronic (or long-term) toxicity data, since these are recommended for regulatory purposes. However, for *D. magna*, both an acute and a chronic nickel bioavailability study were performed.

For all three test species, the individual effects of calcium, magnesium and pH on nickel toxicity were studied in synthetic test solutions using a univariate experimental design. In the study on acute nickel toxicity to *D. magna* (chapter 3), sodium was also considered as a possible toxicity mitigating factor. Based on the results of these experiments, a bioavailability model was developed for each test species. To evaluate whether the developed models are capable of accurately predicting nickel toxicity in surface waters with varying physicochemical composition, the models' predictions were compared to toxicity test results obtained in nickel-spiked European surface waters. These test results were also used for evaluating the effect of dissolved organic carbon (DOC) on nickel toxicity. The following paragraphs give an overview of the main results obtained in the first part of this study.

In chapter 2, the effects of calcium, magnesium and pH on the toxicity of nickel to juvenile rainbow trout (*O. mykiss*) were examined during 17- to 26-day exposures to nickel in 15 different synthetic test solutions. Calcium and magnesium concentrations in these test solutions were varied between 4 and 110 mg/L and between 3 and 72 mg/L, respectively. The effect of pH was studied within a pH range of 5.5 to 8.5. The 17-d $LC50_{Nidiss}$ (representing the dissolved nickel concentrations resulting in 50% mortality during an exposure period of 17 days) obtained in the synthetic test solutions ranged from 496 to 2910 µg/L. Overall, increasing chemical activities of Ca^{2+}, Mg^{2+} and H^+ were observed to result in increased 17-d $LC50_{Ni2+}$ (representing the 17-d LC50 expressed as Ni^{2+} activity). The relative importance of the observed effects was pH > Mg ≈ Ca.

Since nickel was not observed to significantly affect growth of juvenile rainbow trout, model development was based on mortality data. The effects of calcium and magnesium were modeled according to single-site competition between Ca^{2+}, Mg^{2+} and Ni^{2+} for binding to sensitive sites on the fish gill (i.e. according to the original biotic ligand model (BLM) approach). The model parameters representing these effects were $\log K_{CaBL} = \log K_{MgBL} = 3.6$. However, since the relationship between the 17-d $LC50_{Ni2+}$ and H^+ activity was not linear over the entire tested pH range, the effect of pH could not be attributed to proton competition

for a single unidentate site alone. Therefore, a new modeling approach was developed in which the slope of the empirical linear relationship between 17-d $LC50_{pNi2+}$ (= – log 17-d $LC50_{Ni2+}$) and pH was used as the basis for modeling the effect of pH. The effect was then superimposed on the effects of calcium and magnesium, assuming that the log K_{CaBL} and log K_{MgBL} are independent of pH.

The 17-d $LC50_{Nidiss}$ obtained in a series of nickel-spiked European surface waters with varying physicochemical composition ranged from 640 to 4140 µg/L. For most of these waters, the model predicted nickel toxicity within a factor 2 deviation from observed toxicity. Based on the observation that only 8 to 20% of the total dissolved nickel was organically complexed at the 17-d LC50 levels, it could be concluded that organic complexation only plays a minor role in determining nickel toxicity to juvenile rainbow trout. Finally, the developed model, calibrated to account for sensitivity differences between species, life stages and/or exposure durations, was also able to accurately predict 96-h LC50s reported in literature for larval, juvenile and subadult fathead minnow (*Pimephales promelas*).

In chapter 3, a nickel bioavailability model was developed for predicting acute nickel toxicity to *D. magna*. To that end, the individual effects of calcium, magnesium, sodium and pH were studied in 48-h immobilization assays in synthetic test waters. The effects of calcium, magnesium and sodium were studied within concentration ranges of 9 to 181 mg/L, 6 to 111 mg/L and 2 to 322 mg/L, respectively, whereas the effect of pH was studied within a pH range of 5.7 to 8.1. The observed 48-h $EC50_{Nidiss}$ ranged from 1820 to 5500 µg/L. Sodium did not significantly affect acute nickel toxicity to *D. magna*. This could be expected, since there are no known indications for sodium to be involved in the mechanism of nickel uptake and/or toxicity. Calcium and magnesium were both observed to protect against acute nickel toxicity. Expressed as Ni^{2+} activity, nickel toxicity was not affected in the pH range of 5.7 to 7.5. However, a further increase of pH up to 8.1 resulted in increased nickel toxicity. The relative importance of the observed effects was Ca > Mg > pH.

Since the overall variation in toxicity within the tested pH range was relatively small, the effect of pH was not incorporated in the acute nickel bioavailability model. The protective effects of calcium and magnesium were modeled as single-site competition effects based on the linear relationships between 48-h $EC50_{Ni2+}$ and Ca^{2+} and Mg^{2+} activity. The log K_{CaBL} and log K_{MgBL} values were 3.10 and 2.47. To assess the applicability of the model to natural

waters, 16 toxicity tests were conducted in nickel-spiked surface waters from 8 different locations in Europe. The 48-h $EC50_{Nidiss}$ obtained in these tests varied between 860 and 6300 µg/L. These 48-h $EC50_{Nidiss}$ were predicted by the model with an error of less than factor 2 in 15 of 16 cases. Speciation calculations demonstrated that at the 48-h EC50 levels, only 5 to 23% of the total dissolved nickel was organically complexed, suggesting that DOC is of minor importance for determining acute nickel toxicity to *D. magna*.

Provided that the model was calibrated to account for interclonal or interspecies sensitivity differences, it was also capable of accurately predicting 48-h EC50s that are reported in literature for *D. magna* and *Ceriodaphnia dubia*. Finally, the predictive capacity of the model was demonstrated to be better than that of two previously proposed models. These models were based on a dataset developed to assess the effect of calcium on gill nickel accumulation and toxicity to fathead minnow. Although this dataset only supports the derivation of a log K_{CaBL} and a log K_{NiBL}, both models also incorporated a log K_{NaBL} and a log K_{HBL}. A log K_{MgBL} was not included. A comparison of the predictive capacity of these models with the predictive capacity of the new model demonstrated that (i) there is no need to incorporate a log K_{NaBL}, (ii) it is of utmost importance to recognize the individual protective effect of magnesium, and (iii) the incorporation of a log K_{HBL} does not adequately describe the effect of pH.

The bioavailability model that was developed for predicting chronic nickel toxicity to *D. magna* is described in chapter 4. The individual effects of calcium, magnesium and pH were studied in 21-d reproduction tests in synthetic test solutions. Calcium and magnesium concentrations in these artificial waters varied between 7 and 88 mg/L and between 6 and 72 mg/L, respectively, whereas the effect of pH was investigated within a pH range of 5.9 to 8.2. Overall, reproduction appeared to be the most sensitive endpoint, with 21-d $EC50_{Nidiss}$ ranging from 24 to 108 µg/L. The protective effects of calcium and magnesium were modeled according to single-site competition with log K_{CaBL} = 3.53 and log K_{MgBL} = 3.57. Because the increase of the 21-d $EC50_{Ni2+}$ with increasing H^+ activity was not linear over the entire pH range tested, the effect of pH could not be appropriately described by single-site competition. Instead, the modeling approach developed in chapter 2 was adopted for modeling the effect of pH. Overall the relative importance of the observed effects was Ca ≈ Mg > pH.

The importance of DOC in protecting *D. magna* against chronic nickel toxicity was evaluated using the results of 21-d reproduction tests conducted in a series of nickel-spiked surface waters. The 21-d $EC50_{Nidiss}$ obtained in these waters varied between 57 and 292 µg/L. At these concentration levels, 26 to 42% of the total dissolved nickel was complexed by DOC, which is substantially more than at acutely toxic levels (see chapter 3). Consequently, organic complexation plays a more important role in determining chronic nickel toxicity to *D. magna* than in determining acute nickel toxicity to this species. This is reflected in a stronger positive correlation between the 21-d $EC50_{Nidiss}$ and DOC concentration (R^2 = 0.84) than between the 48-h $EC50_{Nidiss}$ and DOC concentration (R^2 = 0.33, see chapter 3).

The results of the toxicity tests in the nickel-spiked surface waters were also used for evaluating the applicability of the model to natural waters. The model systematically tended to overestimate nickel toxicity. The average prediction error and the prediction error range for the 21-d $EC50_{Nidiss}$ were 1.9 and 1.4 to 2.6, respectively. However, since the toxicity tests in the nickel-spiked surface waters were conducted about a year earlier than the toxicity tests in the artificial waters of which the results were used for model development, the observed inaccuracy may simply be due to a sensitivity shift of the test organisms during this period. Indeed, when the model was calibrated to account for this possible sensitivity shift, the average prediction error and prediction error range decreased to 1.2 and 1.0 to 1.5, respectively. Some other possible explanations for the inaccuracy of the model are further discussed in chapter 4.

In chapter 5, the individual effects of calcium, magnesium and pH on the chronic toxicity of nickel to the unicellular green alga *P. subcapitata* were studied in a series of 72-h growth inhibition tests in synthetic test solutions. The calcium and magnesium concentrations were varied between 3 and 144 mg/L and between 3 and 115 mg/L, respectively, whereas the effect of pH was studied within a pH range of 6.0 to 7.9. The 72-h E_rC50_{Nidiss} (i.e. growth rate-based 72-h EC50s) obtained in these artificial test waters ranged from 82 to 1120 µg/L. The 72-h E_rC50_{Ni2+} increased with increasing activities of Ca^{2+}, Mg^{2+} and H^+. The relative importance of the observed effects was Mg > pH > Ca.

The 72-h E_rC50_{Nidiss} obtained in the nickel-spiked surface waters used for model validation ranged from 483 to 1630 µg/L. Speciation calculations demonstrated that at the 72-h E_rC50 levels, 13 to 47% of the total dissolved nickel was complexed by DOC. The 72-h

E_rC50_{Nidiss} obtained in the nickel-spiked surface waters were positively correlated to their DOC content (R^2 = 0.64), indicating the importance of DOC as nickel toxicity reducing factor.

In a first modeling exercise, the protective effects of Ca^{2+} and Mg^{2+} as well as H^+ were modeled as single-site competition effects. The model parameters representing these effects were log K_{CaBL} = 2.0, log K_{MgBL} = 3.3 and log K_{HBL} = 6.5. Using this model, the 72-h E_rC50s obtained in the nickel-spiked surface waters were predicted by an average error of factor 1.3, with a prediction error range of 1.0 to 1.9. However, since the relationship between the 72-h E_rC50_{Ni2+} and H^+ activity was not linear over the entire pH range tested, the model's accuracy could be further improved by modeling the effect of pH according to the method described in chapter 2.

Finally, in chapter 5, it was also investigated whether there are interactions between the effects of magnesium and pH, the two most important factors affecting nickel toxicity to *P. subcapitata*. This was investigated using a bivariate experimental design. Although some interaction was observed, it was considered not necessary to be incorporated in the model at this stage of model development. Indeed, the model's predictive capacity was very good without taking possible interactive effects into account. Moreover, interactive effects have not been thoroughly studied so far and are hence not sufficiently understood to allow them to be incorporated in bioavailability models. Although interactive effects may be subtle and not important enough to be taken into account in bioavailability models, from a purely physiological point of view it may be worthwhile to investigate them more closely.

Although this study was not designed for investigating the mechanisms by which calcium, magnesium and pH affect nickel toxicity, it provided interesting indications concerning this subject. For instance, this study has been the first to demonstrate the importance of magnesium as nickel toxicity mitigating factor for three of the most commonly used standard test species. The protective effect of magnesium on nickel toxicity is believed to be due to competition between Ni^{2+} and Mg^{2+} for uptake via a shared uptake pathway. Direct evidence for Ni^{2+} uptake via Mg^{2+} transport systems has been provided for members of very different groups of organisms (see chapter 5). Although this has not yet been demonstrated for the species used in this study, the results of recent physiological research

with fish, aquatic invertebrates and unicellular green algae are consistent with Ni^{2+} uptake via Mg^{2+} transport systems in these organisms.

The toxicity mitigating capacity of magnesium on chronic nickel toxicity to *D. magna* was observed to be more important than on acute nickel toxicity. For other metals, such as copper and zinc, it has also been observed that the protective effect of the cation most closely related to the mechanism of toxicity becomes increasingly important with increasing exposure duration (i.e. sodium for copper and calcium for zinc) (see chapter 4). Further research would be needed to explain these observations. For nickel, the toxicity mitigating potency of magnesium was observed to be the highest in *P. subcapitata*. This was also observed for zinc and may be due to the general protection of increasing magnesium concentrations against the formation of non-functional metal-chlorophyll through substitution of Mg^{2+} in the porphyrin ring (see chapter 7).

The mechanisms by which calcium protects against nickel toxicity are less clear than for magnesium. It has been reported in literature that Ca^{2+} also competes with Ni^{2+} for uptake via Mg^{2+} transport systems, although to a lesser extent than Mg^{2+} itself. However, in this study, calcium has been observed to be equally protective as magnesium against chronic nickel toxicity to *D. magna* (chapter 4) and long-term nickel toxicity to rainbow trout (chapter 2) and even more protective than magnesium against acute nickel toxicity to *D. magna* (chapter 3). Therefore, further research is needed to investigate whether mechanisms other than direct competition are involved. Possibly, calcium also protects indirectly against nickel toxicity through its role in the regulation of physiological functions such as membrane permeability and ion transport.

The effect of pH on nickel toxicity appeared to be the most complicated effect investigated in this study. For all three test species, the relationships between $E/LC50_{Ni2+}$ and H^+ activity were not linear over the entire pH range tested. Apparently, at elevated pH levels, less free metal ion is needed to cause a certain toxic effect. More or less similar nonlinear relationships have also been reported for other metals (see chapters 2 to 5). Such relationships indicate that the effect of pH on metal toxicity can not be attributed to proton competition for a single unidentate site alone. Several explanations have been given for this in literature (see chapters 2 to 5). For nickel, it may be assumed that $NiHCO_3^+$ and $NiCO_3$, which increase in abundance with increasing pH, are responsible for the higher than expected toxicity at

elevated pH levels. Possibly, these metal complexes dissociate in the physicochemically different gill (*D. magna*, rainbow trout) or cell (algae) microenvironment, hereby increasing the availability of Ni^{2+} for uptake and/or facilitating Ni^{2+} uptake through Mg^{2+} transport systems such as electro-neutral Mg^{2+}/HCO_3^- symporters. Further research is needed to identify the exact mechanisms determining nickel toxicity at elevated pH levels.

Finally, for all three test species, $E/LC50_{Nidiss}$ were observed to be positively correlated with DOC concentration. The strongest correlation was observed for chronic nickel toxicity to *D. magna*, immediately followed by chronic nickel toxicity to *P. subcapitata*. At the nickel concentrations chronically affecting these organisms, organically complexed nickel was calculated to be more abundant than at higher nickel concentrations such as those resulting in acute nickel toxicity to *D. magna* and long-term nickel toxicity to rainbow trout. These findings indicate that the decrease of the toxicity mitigating effect of DOC with increasing nickel concentrations is due to the fact that the Ni^{2+} complexation capacity of organic matter is reached.

Although the models developed in chapters 2 to 5 may be further refined when new insights are acquired into the mechanisms by which water chemistry affects nickel toxicity, they already provide useful tools for taking into account nickel bioavailability in current regulatory exercises. However, their use is theoretically limited to aquatic systems with physicochemical compositions corresponding to the water chemistry ranges for which the models were developed and validated. During the course of this study, a new point of discussion arose which was related to the use of bioavailability models for predicting metal toxicity in waters with hardness levels below the models' lower hardness boundary.

For nickel, the bioavailability models for fish (chapter 2), crustaceans (chapter 3 and 4) and algae (chapter 5) have a lower water hardness boundary of 22, 42 and 20 mg $CaCO_3$/L, respectively. However, surface waters in large geographic areas in Europe (e.g., Scandinavia, Scotland, Northern Portugal) are characterized by lower water hardness levels. The major concern was that the available models would underestimate the potential risks of nickel to aquatic life in these softer waters. In the second part of this study, it was investigated more closely whether this concern is scientifically justifiable.

The major research questions addressed in chapter 6 and 7 were (i) whether organisms living in 'soft' water (operationally defined water hardness < 10 mg $CaCO_3$/L) are intrinsically more sensitive to nickel than organisms living in 'hard' water (operationally defined water hardness > 25 mg $CaCO_3$/L), and (ii) whether a single bioavailability model can be used to predict the protective effect of water hardness on the toxicity of nickel in both soft and hard water. To investigate this, toxicity tests were conducted with cladocerans (acute and chronic, chapter 6) and green microalgae (chronic, chapter 7) that were collected in soft and hard water lakes in Sweden. Organisms collected in soft water lakes were tested in a 'soft' and a 'moderately hard' test solution while organisms collected in hard water lakes were tested in a 'moderately hard' and a 'hard' test solution. The nominal water hardness of these test solutions was 6.25, 16.3 and 43.4 mg $CaCO_3$/L, respectively.

Acutely, 7 hard and 4 soft water cladoceran populations were tested successfully. For one species, both a soft and a hard water population were tested. Consequently, toxicity data were obtained for 10 different species. Overall, the 48-h $EC50_{Nidiss}$ varied between 97 and 5540 µg/L. With the exception of 1 soft and 2 hard water populations, the same cladoceran populations were chronically exposed to nickel (7 different species). The $EC50_{Nidiss}$ for the endpoint reproduction ranged from 4 to 125 µg/L. Chronic nickel toxicity to green microalgae was studied using 5 soft and 5 hard water strains (10 different species). Overall, the 72-h E_rC50_{Nidiss} varied between 59 and 1432 µg/L. Based on the results obtained in the 'moderately hard' test solution, no significant nickel sensitivity differences were observed between soft and hard water organisms.

Statistical evaluation of the effect of water hardness demonstrated that water hardness significantly protects against both acute and chronic nickel toxicity in all species tested. Finally, it was investigated whether a single bioavailability model can be used to predict the effect of water hardness on nickel toxicity to both soft and hard water organisms. The results of this study indicate that the effect of water hardness on chronic nickel toxicity to cladocerans and green microalgae could be described using the same model parameters (log K_{CaBL} and log K_{MgBL}) for both soft and hard water organisms. However, the acute toxicity of nickel to cladocerans was observed to be more accurately predicted when separate model parameters are used for soft and hard water populations. Further research would be needed to explain why the protective effect of water hardness on acute nickel toxicity to soft water

cladocerans was significantly higher than the observed effect in hard water cladocerans, while for chronic toxicity, this was not the case.

This is the first study that investigated possible differences in metal toxicity to soft and hard water organisms. For nickel, answers have been provided that may simplify decision-making at the regulatory level whenever this issue is raised. Moreover, the bioavailability models that have been developed based on the results of toxicity testing with field-collected organisms may be further used for regulatory purposes.

Overall, this study generated a large amount of toxicity data for 23 freshwater species belonging to three different trophic levels, hereby substantially enlarging the nickel toxicity dataset for the aquatic compartment. Most of these data were included in the effects assessment of the European nickel risk assessment, the outcome of which will be used for determining the European water quality standard for nickel. Moreover, the models developed in this study can be readily used for normalizing species sensitivity distributions for bioavailability, thus allowing the performance of site-specific risk assessment exercises and the development of site-specific water quality criteria. Since it is expected that the concept of metal bioavailability will be increasingly introduced in risk assessment and water quality standard setting procedures all over the world, the results of this study will hopefully serve many countries in the future.

Samenvatting

Tot op heden zijn waterkwaliteitscriteria en risicoevaluaties voor metalen voornamelijk gebaseerd op totale of opgeloste metaalconcentraties. Totale en zelfs opgeloste metaalconcentraties zijn echter geen goede maatstaven om de potentiële risico's voor aquatische ecosystemen te voorspellen. Eenzelfde totale of opgeloste metaalconcentratie resulteert namelijk niet noodzakelijk in hetzelfde toxische effect onder verschillende omstandigheden. Dit is hoofdzakelijk het gevolg van de invloed van de watersamenstelling op de toxiciteit van metalen.

Verschillende overheden (onder andere die van de USA, Nieuw Zeeland en Australië, Canada en de Europese Unie) hebben daarom recent de mogelijkheid voorzien om hiermee rekening te houden tijdens risicoevaluatie-oefeningen en procedures voor het bepalen van waterkwaliteitscriteria voor metalen, of zijn volop nieuwe strategieën aan het ontwikkelen waarin speciale aandacht wordt gegeven aan dit onderwerp. Om biobeschikbaarheid in rekening te kunnen brengen moeten er echter modellen worden ontwikkeld die dit toelaten. Zonder dergelijke modellen kunnen waterkwaliteitscriteria te conservatief zijn voor bepaalde waters en onvoldoende beschermend voor andere. Te conservatieve waterkwaliteitscriteria kunnen leiden tot onnodig hoge kosten voor milieusanering en emissiereductie, terwijl onvoldoende beschermende waterkwaliteitscriteria kunnen leiden tot zware schade aan aquatische ecosystemen. Het is duidelijk dat geen van beide wenselijk is.

Bij de aanvang van dit onderzoek was de biobeschikbaarheid van nikkel voor zoetwaterorganismen nauwelijks bestudeerd (zie hoofdstuk 1). De beschikbare data waren ofwel niet bruikbaar ofwel onvoldoende gedetailleerd voor het ontwikkelen van biobeschikbaarheidsmodellen. Toch waren er reeds enkele pogingen tot modellering ondernomen. Het is dan ook niet verwonderlijk dat deze modellen bij nader onderzoek zware nadelen bleken te vertonen die kunnen leiden tot onnauwkeurige voorspellingen (zie hoofdstuk 1). De belangrijkste doelstellingen van dit onderzoek waren daarom (i) het genereren van meer gedetailleerde en onthullende data met betrekking tot de biobeschikbaarheid van nikkel voor zoetwaterorganismen, en (ii) het ontwikkelen van biobeschikbaarheidsmodellen die kunnen worden gebruikt voor normstelling en risicoevaluatie.

In het eerste deel van dit onderzoek werd het effect van de fysicochemische samenstelling van water op de toxiciteit van nikkel onderzocht gebruik makende van standaard testsoorten die behoren tot drie verschillende trofische niveaus: een eencellig groenwier (*Pseudokirchneriella subcapitata*), een aquatische invertebraat (*Daphnia magna*) en een vis (*Onchorhynchus mykiss*, regenboogforel). Aangezien het gebruik van chronische data aanbevolen is voor het bepalen van veilige metaalconcentraties voor aquatische ecosystemen was deze studie geconcentreerd op het genereren van chronische data en de ontwikkeling van chronische biobeschikbaarheidsmodellen. Voor *D. magna* werd echter zowel een acuut als een chronisch model ontwikkeld.

Voor elk van de drie standaard testsoorten die in dit onderzoek werden gebruikt werden de effecten van calcium, magnesium en pH op de toxiciteit van nikkel individueel bestudeerd door middel van een univariate experimentele opzet. Het potentiële effect van natrium werd enkel bestudeerd tijdens acute experimenten met *D. magna* (chapter 3). Op basis van de resultaten van de uitgevoerde experimenten werd vervolgens voor elke testsoort een biobeschikbaarheidsmodel ontwikkeld. Teneinde te evalueren of de ontwikkelde modellen ook in staat zijn de toxiciteit van nikkel te voorspellen in natuurlijke waters werden de voorspellingen van de modellen voor een aantal Europese oppervlaktewateren met gekende fysicochemische samenstelling vergeleken met de resultaten van toxiciteitstesten uitgevoerd in deze waters. De resultaten van deze tests werden tevens gebruikt om het effect van opgelost organisch materiaal op de toxiciteit van nikkel te evalueren. In de volgende paragrafen wordt een overzicht gegeven van de belangrijkste resultaten van het eerste deel van dit onderzoek.

In hoofdstuk 2 werden de effecten van calcium, magnesium en pH op de toxiciteit van nikkel voor juveniele regenboogforellen (*O. mykiss*) bestudeerd tijdens 17 tot 26 dagen durende blootstellingen in 15 verschillende artificiële testmedia. De calcium- en magnesiumconcentraties werden respectievelijk gevarieerd van 4 tot 110 mg/L en van 3 tot 72 mg/L. Het effect van pH werd bestudeerd binnen een pH-bereik van 5.5 tot 8.5. De 17-d $LC50_{Nidiss}$ (i.e. de opgeloste nikkelconcentraties die resulteren in 50% mortaliteit gedurende een blootstellingsperiode van 17 dagen) varieerden van 496 tot 2910 µg/L. De 17-d $LC50_{Ni2+}$ (i.e. de 17-d LC50's uitgedrukt als Ni^{2+} activiteit) namen toe bij toenemende activiteiten van Ca^{2+}, Mg^{2+} en H^{+}. Het relatieve belang van de waargenomen effecten was pH > Mg ≈ Ca.

Nikkel had geen significante invloed op de groei van juveniele regenboogforellen. Daarom werd het biobeschikbaarheidsmodel ontwikkeld op basis van mortaliteitsdata. De bescherming die calcium en magnesium bieden tegen nikkeltoxiciteit werd beschouwd als het gevolg van competitie tussen Ca^{2+}, Mg^{2+} en Ni^{2+} voor binding ter hoogte van nikkelgevoelige sites op de kieuwen. De effecten van calcium en magnesium werden bijgevolg gemodelleerd in overeenstemming met het originele BLM concept (BLM = Biotisch Ligand Model). De parameters die de effecten van calcium en magnesium vertegenwoordigen zijn log K_{CaBL} = log K_{MgBL} = 3.6. Het verband tussen de 17-d $LC50_{Ni2+}$ en H^+ activiteit was niet lineair over het volledige onderzochte pH-gebied. Bijgevolg kon het effect van pH niet bevredigend gemodelleerd worden als competitie-effect. Daarom werd een nieuwe methode ontwikkeld die de helling van de empirische lineaire relatie tussen de 17-d $LC50_{pNi2+}$ (= – log 17-d $LC50_{Ni2+}$) en pH gebruikt als basis voor modellering van het pH-effect. Het effect van pH werd beschouwd als onafhankelijk van de effecten van calcium en magnesium en omgekeerd.

De 17-d $LC50_{Nidiss}$ die werden bekomen in een reeks Europese oppervlaktewateren met variabele samenstelling varieerden van 640 tot 4140 µg/L. Voor de meeste van deze waters kon het ontwikkelde model de toxiciteit van nikkel voorspellen binnen een factor twee afwijking van de waargenomen toxiciteit. Gebaseerd op de waarneming dat slechts 8 tot 20% van het opgelost nikkel organisch gecomplexeerd was bij nikkelconcentraties rond de 17-d LC50's kon er geconcludeerd worden dat complexatie van nikkel door organisch materiaal slechts een beperkte invloed heeft op de toxiciteit van nikkel voor regenboogforel. Tenslotte werd aangetoond dat het ontwikkelde model tevens in staat is 96-h EC50's die in de literatuur werden teruggevonden voor Amerikaanse dikkop-elrits accuraat te reproduceren op basis van de gegeven watersamenstelling. Een belangrijke voorwaarde was wel dat het model werd gecalibreerd voor gevoeligheidsverschillen tussen de verschillende vissoorten, levensstadia (larven – juvenielen – subadulten) en blootstellingsperiodes.

In hoofdstuk 3 werd een acuut biobeschikbaarheidsmodel ontwikkeld voor *D. magna*. Daartoe werden de individuele effecten van calcium, magnesium, natrium en pH op de toxiciteit van nikkel bestudeerd in 48 uur durende immobilisatietesten in synthetische testmedia. De effecten van calcium, magnesium en natrium werden bestudeerd binnen een concentratiebereik van respectievelijk 9 tot 181 mg/L, 6 tot 111 mg/L en 2 tot 322 mg/L. Het effect van pH werd bestudeerd binnen een pH-bereik van 5.7 tot 8.1. De 48-h $EC50_{Nidiss}$ varieerden van 1820 tot 5500 µg/L. Natrium had geen significant effect op de toxiciteit van

nikkel. Dit was te verwachten daar er geen interacties tussen natrium en nikkel werden teruggevonden in de literatuur en er geen aanwijzingen zijn voor de betrokkenheid van natrium bij het mechanisme van nikkelopname en/of –toxiciteit. Zowel calcium als magnesium bleken de toxiciteit van nikkel te reduceren. Binnen een pH-bereik van 5.7 tot 7.5 werd geen effect van pH op de toxiciteit van nikkel waargenomen. Bij een verdere toename van de pH tot 8.1 nam de toxiciteit van nikkel echter plots toe. Het relatieve belang van de waargenomen effecten was Ca > Mg > pH.

Het effect van pH werd niet opgenomen in het biobeschikbaarheidsmodel aangezien de algemene variatie in toxiciteit binnen het bestudeerde pH-bereik vrij klein was. De beschermende effecten van calcium en magnesium werden gemodelleerd als competitie-effecten. De log K_{CaBL} en log K_{MgBL} werden bepaald op basis van de lineaire relaties tussen de 48-h $EC50_{Ni2+}$ en Ca^{2+} en Mg^{2+} activiteit (log K_{CaBL} = 3.10 en log K_{MgBL} = 2.47). Teneinde de toepasbaarheid van het ontwikkelde model op natuurlijke waters te evalueren werden 16 toxiciteitstesten uitgevoerd in oppervlaktewater bemonsterd op 8 verschillende locaties in Europa. De 48-h $EC50_{Nidiss}$ in deze waters varieerden tussen 860 en 6300 µg/L. In 15 van de 16 gevallen kon het model de waargenomen toxiciteit binnen een factor twee afwijking voorspellen. Speciatieberekeningen toonden aan dat slechts 5 tot 23% van het opgelost nikkel organisch gecomplexeerd was bij deze nikkelconcentraties. Dit suggereert dat opgelost organisch materiaal slechts een beperkte invloed heeft op de acute toxiciteit van nikkel voor *D. magna*.

Na calibratie voor gevoeligheidsverschillen tussen verschillende soorten en/of klonen was het model ook in staat 48-h EC50's te reproduceren die in de literatuur werden gerapporteerd voor *D. magna* en *Ceriodaphnia dubia*. Het nieuwe model was ook duidelijk beter dan twee eerder ontwikkelde modellen. Deze twee modellen waren gebaseerd op data met betrekking tot het effect van calcium op de accumulatie van nikkel op de kieuwen van de Amerikaanse dikkop-elrits en de relatie tussen kieuwaccumulatie en de toxiciteit van nikkel voor deze vis. Hoewel deze data eigenlijk enkel de bepaling van een log K_{CaBL} en een log K_{NiBL} toelaten stelden beide modellen ook een log K_{NaBL} en een log K_{HBL} voor. Een log K_{MgBL} werd daarentegen niet opgenomen in beide modellen. Een grondige vergelijking van deze modellen met het nieuwe model beschreven in hoofdstuk 3 toonde aan dat (i) er geen reden is om een log K_{NaBL} op te nemen in het model, (ii) het van groot belang is het individuele

beschermende effect van magnesium te erkennen, en (iii) de opname van een log K_{HBL} geen accurate voorspelling van het pH-effect garandeert.

De ontwikkeling van een chronisch biobeschikbaarheidsmodel voor *D. magna* werd besproken in hoofdstuk 4. De effecten van calcium, magnesium en pH op de chronische toxiciteit van nikkel voor *D. magna* werden onderzocht gebruik makende van 21 dagen durende reproductietesten in synthetische testmedia. De calcium- en magnesiumconcentraties in deze testmedia varieerden respectievelijk tussen 7 en 88 mg/L en tussen 6 en 72 mg/L. Het effect van pH werd onderzocht binnen een pH-bereik van 5.9 tot 8.2. Over het algemeen bleek reproductie het meest gevoelige eindpunt te zijn. De 21-d $EC50_{Nidiss}$ varieerden tussen 24 en 108 µg/L. De beschermende effecten van calcium en magnesium werden gemodelleerd in overeenstemming met het oorspronkelijke BLM concept met log K_{CaBL} = 3.53 en log K_{MgBL} = 3.57. Aangezien de relatie tussen de 21-d $EC50_{Ni2+}$ en H^+ activiteit niet lineair was over het volledige onderzochte pH-bereik kon het effect van pH niet gemodelleerd worden als competitie-effect. Het pH-effect kon echter wel succesvol gemodelleerd worden volgens de methode beschreven in hoofdstuk 2. Het relatief belang van de waargenomen effecten was Ca $\approx$ Mg > pH.

De 21-d $EC50_{Nidiss}$ die werden waargenomen in een reeks toxiciteitstests in Europese oppervlaktewateren varieerden tussen 57 en 292 µg/L. Bij deze nikkelconcentraties was 26 tot 42% van het opgelost nikkel organisch gecomplexeerd. Dit is een aanzienlijk grotere fractie dan bij acuut toxische nikkelconcentraties (hoofdstuk 3). Blijkbaar speelt organisch materiaal een belangrijkere rol in het bepalen van de chronische toxiciteit van nikkel voor *D. magna* dan in het bepalen van acute toxiciteit voor dit organisme. Het belang van organisch materiaal blijkt ook uit de sterke positieve correlatie tussen de 21-d $EC50_{Nidiss}$ en de concentratie opgelost organisch materiaal (R^2 = 0.84). Voor de 48-h $EC50_{Nidiss}$ was deze correlatie veel zwakker (R^2 = 0.33, zie hoofdstuk 3).

Op basis van de resultaten van bovenvermelde toxiciteitstests werd tevens de toepasbaarheid van het model op natuurlijke waters geëvalueerd. Het model bleek de toxiciteit van nikkel systematisch te overschatten. Aangezien de tests in de natuurlijke waters ongeveer een jaar eerder waren uitgevoerd dan die in de artificiële testmedia waarvan de resultaten werden gebruikt voor de ontwikkeling van het model zou dit eenvoudigweg te wijten kunnen zijn aan een verschuiving in de gevoeligheid van de testorganismen tijdens deze periode.

Wanneer het model gecalibreerd werd voor dit mogelijke gevoeligheidsverschil werden veel accuratere voorspellingen bekomen van de toxiciteit van nikkel in natuurlijke waters. De gemiddelde, minimum en maximum fout op de predicties waren respectievelijk factor 1.2, 1.0 en 1.5. Andere mogelijke verklaringen voor de minder accurate voorspellingen in natuurlijke waters werden uitgebreid besproken in hoofdstuk 4.

In hoofdstuk 5 werden de individuele effecten van calcium, magnesium en pH op de chronische toxiciteit van nikkel voor het eencellige wier *P. subcapitata* onderzocht in een reeks 72 uur durende groei-inhibitie-experimenten in synthetische testmedia. De calcium- en magnesiumconcentraties werden respectievelijk gevarieerd tussen 3 en 144 mg/L en tussen 3 en 115 mg/L. Het effect van pH werd bestudeerd binnen een pH-bereik van 6.0 tot 7.9. De 72-h E_rC50_{Nidiss} (i.e. 72-h EC50's gebaseerd op groeisnelheid) in deze synthetische testmedia varieerden tussen 82 en 1120 µg/L. De 72-h E_rC50_{Ni2+} namen toe met toenemende activiteiten van zowel Ca^{2+}, Mg^{2+} als H^+. Het relatief belang van de waargenomen effecten was Mg > pH > Ca.

In een reeks natuurlijke waters werden 72-h E_rC50_{Nidiss} bekomen van 483 tot 1630 µg/L. Speciatieberekeningen toonden aan dat 13 tot 47% van het opgelost nikkel organisch gecomplexeerd was bij deze nikkelconcentraties. De 72-h E_rC50_{Nidiss} waren positief gecorreleerd aan de concentratie opgelost organisch materiaal (R^2 = 0.64). Dit toont aan dat organisch materiaal een belangrijke rol speelt in het bepalen van de toxiciteit van nikkel voor het geteste wier.

In een eerste model werden de beschermende effecten van Ca^{2+} en Mg^{2+} zowel als van H^+ gemodelleerd als competitie-effecten met log K_{CaBL} = 2.0, log K_{MgBL} = 3.3 en log K_{HBL} = 6.5. Dit model voorspelde de toxiciteit van nikkel in de geteste natuurlijke waters met een gemiddelde fout van factor 1.3 (minimum-maximum = 1.0-1.9). Aangezien de relatie tussen de 72-h E_rC50_{Ni2+} en H^+ activiteit niet lineair was over het volledige onderzochte pH-bereik nam de precisie van het model toe wanneer het pH-effect werd gemodelleerd volgens de methode beschreven in hoofdstuk 2.

In hoofdstuk 5 werd ook onderzocht of er interacties zijn tussen de effecten van pH en magnesium, i.e. de twee factoren die de meeste invloed hebben op de toxiciteit van nikkel voor *P. subcapitata*. Daartoe werd een bivariate experimentele opzet aangewend. Dit

experiment toonde aan dat de interactie tussen beide effecten minimaal is. Vanuit een puur fysiologisch standpunt is het wel aan te raden de interacties verder te onderzoeken, opdat dit zou kunnen leiden tot een beter begrip van de onderliggende mechanismen van nikkeltoxiciteit.

Hoewel dit onderzoek niet als doel had de onderliggende mechanismen van de effecten van calcium, magnesium en pH op de toxiciteit van nikkel bloot te leggen gaven de resultaten hieromtrent toch belangrijke aanwijzigingen. Dit onderzoek is bijvoorbeeld het eerste waarin werd aangetoond dat magnesium een belangrijk beschermend effect heeft op de toxiciteit van nikkel voor drie van de meest frequent gebruikte testsoorten. Het beschermend effect van magnesium is waarschijnlijk te danken aan competitie tussen Ni^{2+} en Mg^{2+} voor opname via een gemeenschappelijk transportsysteem. Hoewel er voor de gebruikte testsoorten nog geen directe bewijzen zijn voor Ni^{2+} opname via Mg^{2+} transportsystemen zijn deze er wel voor verschillende andere soorten behorende to zeer diverse groepen van levende organismen. Tevens zijn de resultaten van recent fysiologisch onderzoek in vissen, aquatische invertebraten en algen consistent met het bestaan van een gemeenschappelijk opnamesysteem voor Ni^{2+} en Mg^{2+}.

Het beschermend effect van magnesium op de chronische toxiciteit van *D. magna* bleek belangrijker te zijn dan voor acute toxiciteit. Voor andere metalen, zoals koper en zink, werd ook waargenomen dat het beschermend effect van het kation dat meest gerelateerd is met het toxiciteitsmechanisme van het metaal belangrijker werd bij toenemende blootstellingsduur (i.e. natrium voor koper en calcium voor zink) (zie hoofdstuk 4). Verder onderzoek is nodig om deze waarnemingen te kunnen verklaren. Het beschermend effect van magnesium was over het algemeen het belangrijkst voor *P. subcapitata*. Dit is in overeenstemming met waarnemingen voor zink en zou te maken kunnen hebben met de algemene bescherming van toenemende magnesiumconcentraties tegen de subsitutie van Mg^{2+} door Ni^{2+} in chlorofylmoleculen (zie hoofdstuk 7).

De onderliggende mechanismen van het beschermend effect van calcium zijn minder duidelijk dan voor magnesium. Ca^{2+} werd ook waargenomen in competitie te treden met Ni^{2+} voor opname via Mg^{2+} transportsystemen. De competitieve inhibitie van Ni^{2+} opname veroorzaakt door Ca^{2+} zou echter veel minder belangrijk zijn dan die veroorzaakt door Mg^{2+}. In deze studie bleek calcium echter even beschermend te zijn als magnesium tegen chronische

toxiciteit van nikkel voor *D. magna* (hoofdstuk 4) en regenboogforel (hoofdstuk 2) en zelfs meer beschermend dan magnesium tegen acute toxiciteit van nikkel voor *D. magna* (hoofdstuk 3). Verder onderzoek is dus noodzakelijk om te weten te komen welke andere mechanismen een invloed hebben op het effect van calcium op nikkeltoxiciteit. Mogelijks biedt calcium ook indirecte bescherming tegen nikkeltoxiciteit door het reguleren van fysiologische functies zoals de permeabiliteit van membranen en transport van ionen.

Het effect van pH was ongetwijfeld het meest complexe effect dat werd bestudeerd in dit onderzoek. De relatie tussen de $E/LC50_{Ni2+}$ en H^+ acitvity kon niet als lineair worden beschouwd over het volledige bestudeerde pH-bereik. Blijkbaar is er bij hogere pH minder vrij nikkel nodig om een bepaald toxisch effect te verkrijgen. Vergelijkbare niet-lineaire effecten werden ook geobserveerd voor andere metalen (zie hoofdstukken 2 tot en met 5). Dergelijke niet-lineaire verbanden tonen aan dat het effect van pH niet alleen kan worden toegeschreven aan protoncompetitie. In de literatuur zijn hiervoor uiteenlopende verklaringen te vinden (zie hoofdstukken 2 tot en met 5). Voor nikkel zou kunnen worden aangenomen dat $NiHCO_3^+$ en $NiCO_3$ bijdragen aan de toxiciteit van nikkel bij hogere pH. Mogelijks dissociëren deze complexen in de onmiddellijke omgeving van de kieuwen (*D. magna*, regenboogforel) of cellen (wieren). Hierdoor zou de beschikbaarheid van Ni^{2+} voor opname verhogen en zou eventueel de opname van Ni^{2+} via neutrale Mg^{2+}/HCO_3^- symporters kunnen worden gestimuleerd. Verder onderzoek naar de onderliggende mechanismen van het pH-effect is noodzakelijk om te kunnen verklaren waarom de toxiciteit van nikkel hoger is dan verwacht bij hoge pH.

Voor elke testsoort bleken de $E/LC50_{Nidiss}$ positief gecorreleerd te zijn aan de concentratie opgelost organisch materiaal in natuurlijke waters. De sterkste correlaties werden bekomen voor de chronische toxiciteit van nikkel voor *D. magna* en *P. subcapitata*. Bij de nikkelconcentraties die chronisch toxisch waren voor deze organismen bleek er relatief meer nikkel organisch gecomplexeerd te zijn dan bij de veel hogere nikkelconcentraties die acuut toxisch waren voor *D. magna* en chronisch toxisch voor regenboogforel. Dit suggereert dat organisch materiaal een minder belangrijke rol speelt bij hogere nikkelconcentraties daar de complexatiecapaciteit van het organisch materiaal voor nikkel is bereikt bij zulke hoge nikkelconcentraties.

Hoewel de modellen die werden ontwikkeld en beschreven in hoofdstukken 2 tot en met 5 nog verder kunnen worden verfijnd wanneer nieuwe inzichten worden verworven met betrekking tot de mechanismen die de toxiciteit van nikkel bepalen worden ze reeds met succes gebruikt voor risicoevaluaties en het bepalen van waterkwaliteitscriteria voor nikkel. Theoretisch gezien is het gebruik van de ontwikkelde modellen echter beperkt tot waters met een fysicochemische samenstelling binnen het bereik waarvoor de modellen werden ontwikkeld. Een belangrijk punt van discussie is bijvoorbeeld het gebruik van de ontwikkelde modellen voor het voorspellen van de toxiciteit van nikkel in oppervlaktewateren met een waterhardheid lager dan de laagste waterhardheid waarvoor de modellen zijn ontwikkeld.

De laagste waterhardheidsgrenzen van de biobeschikbaarheidsmodellen ontwikkeld voor vissen (hoofdstuk 2), aquatische invertebraten (hoofdstuk 3 en 4) en wieren (hoofdstuk 5) zijn respectievelijk 22, 42 en 20 mg $CaCO_3$/L. Binnen Europa zijn bepaalde geografische regio's (zoals Scandinavië, Schotland en Noord-Portugal) echter gekenmerkt door het voorkomen van veel zachtere oppervlaktewateren. Men was vooral bezorgd dat de beschikbare modellen de toxiciteit van nikkel zouden onderschatten in deze zachtwaterecosystemen. In het tweede deel van dit onderzoek werd nagegaan of deze bezorgdheid al dan niet een wetenschappelijke bestaansreden heeft.

De belangrijkste onderzoeksvragen die werden behandeld in hoofdstuk 6 en 7 waren (i) of organismen uit 'zacht' water (waterhardheid < 10 mg $CaCO_3$/L) intrinsiek gevoeliger zijn voor nikkel dan organismen uit 'hard' water (waterhardheid > 25 mg $CaCO_3$/L), en (ii) of één enkel biobeschikbaarheidsmodel gebruikt kan worden om het effect van waterhardheid op de toxiciteit van nikkel te voorspellen voor organismen uit 'zacht' zowel als 'hard' water. Hiertoe werden toxiciteitstests uitgevoerd met cladoceren (acuut zowel als chronisch, hoofdstuk 6) en planktonische groenwieren (chronisch, hoofdstuk 7) die werden gecollecteerd in zacht- en hardwatermeren in Zweden. Organismen uit zachte waters werden getest in een 'zacht' en 'gematigd hard' testmedium terwijl organismen uit harde waters werden getest in een 'gematigd hard' en 'hard' test medium. De waterhardheid van deze drie testmedia was respectievelijk 6.25, 16.3 en 43.4 mg $CaCO_3$/L.

Van de gecollecteerde cladoceren werden zeven hard- en vier zachtwaterpopulaties succesvol acuut getest. Voor één enkele soort werd zowel een zacht- als een hardwaterpopulatie getest. Bijgevolg werden acute toxiciteitsgegevens bekomen voor tien

verschillende soorten. De 48-h $EC50_{Nidiss}$ varieerden tussen 96 en 5540 µg/L. Met uitzondering van één zacht- en twee hardwaterpopulaties werden dezelfde populaties chronisch blootgesteld aan nikkel (data voor zeven verschillende soorten). De $EC50_{Nidiss}$ voor reproductie varieerden van 4 tot 125 µg/L. De chronische toxiciteit van nikkel voor planktonische groenwieren werd bestudeerd voor vijf soorten gecollecteerd in zachte waters en vijf soorten gecollecteerd in harde waters. De 72-h E_rC50_{Nidiss} voor de geteste soorten varieerden tussen 59 en 1432 µg/L. Op basis van de resultaten van de tests in 'gematigd hard' test medium kon er worden geconcludeerd dat er geen significante gevoeligheidsverschillen zijn tussen zacht- en hardwaterorganismen.

Statistische evaluatie toonde aan dat het effect van waterhardheid op zowel de acute als de chronische toxiciteit van nikkel voor de geteste organismen significant was. Tenslotte werd aangetoond dat het effect van waterhardheid op de chronische toxiciteit van nikkel voor organismen in zacht en hard water accuraat voorspeld kan worden met één enkel biobeschikbaarheidsmodel. Het effect van waterhardheid op de acute toxiciteit van nikkel voor cladoceren werd echter significant beter voorspeld wanneer aparte parameters werden gebruikt voor zacht- en hardwaterorganismen. Verder onderzoek is noodzakelijk om deze waarneming te kunnen verklaren.

Dit onderzoek is het eerste waarin werd onderzocht of er verschillen zijn tussen de toxiciteit van een metaal voor zacht- en hardwaterorganismen. De resultaten van dit onderzoek leverden – althans voor nikkel – antwoorden op die het nemen van beslissingen op regulatorisch niveau kunnen vereenvoudigen wanneer dit punt van discussie zich opwerpt.

Dit onderzoek levert een grote hoeveelheid nieuwe toxiciteitsdata voor 23 zoetwatersoorten behorende tot drie verschillende trofische niveaus. Hierdoor werd de beschikbare informatie in verband met de toxiciteit van nikkel voor aquatische organismen aanzienlijk uitgebreid. De meeste data werden opgenomen in de de effectevaluatie van de Europese risicoevaluatie voor nikkel, waarvan de resultaten zullen worden gebruikt voor het bepalen van een pan-Europese waterkwaliteitsnorm voor nikkel. De biobeschikbaarheidsmodellen beschreven in dit proefschrift kunnen worden gebruikt voor het normaliseren van SSDs voor biobeschikbaarheid. Hierdoor wordt het mogelijk plaatsspecifieke waterkwaliteitscriteria te bepalen en risicoevaluaties uit te voeren voor individuele waterlichamen. Aangezien verwacht wordt dat steeds meer landen het concept van

metaalbiobeschikbaarheid in rekening zullen brengen bij het uitvoeren van risicoevaluaties of het bepalen van waterkwaliteitsnormen voor metalen zal het toepassingsgebied van de modellen en resultaten voorgesteld in dit proefschrift ongetwijfeld steeds groter worden.

Curriculum vitae

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Posters (presenting author)

Heijerick, D.G., De Schamphelaere, K.A.C., Van Sprang, P.A., Deleebeeck, N., Janssen, C.R., 2003. Biotic ligand model predicting acute nickel toxicity to *Daphnia magna*: development and validation. SETAC North America 24th Annual Meeting, Austin, Texas, USA, 9-13 November 2003.

Deleebeeck, N.M.E., De Schamphelaere, K.A.C., Heijerick, D.G., Janssen, C.R., 2004. Development and validation of a biotic ligand model predicting nickel toxicity to *Pseudokirchneriella subcapitata*. SETAC Europe 14th Annual Meeting, Prague, Czech Republic, 18-22 April 2004.

Deleebeeck, N.M.E., De Schamphelaere, K.A.C., Heijerick, D.G., Van Sprang, P., Janssen, C.R., 2005. Accounting for bioavailability in the ecological risk assessment of nickel: the use of biotic ligand models. SETAC Europe 15th Annual Meeting, Lille, France, 22-26 May 2005.

Van Sprang, P., Deleebeeck, N., De Schamphelaere, K., Heijerick, D., Janssen, C., 2005. Derivation of ecologically relevant threshold concentrations for nickel in surface waters: an European experience. SETAC North America 26th Annual Meeting, Baltimore, Maryland, USA, 13-17 November 2005.

Deleebeeck, N.M.E., De Schamphelaere, K.A.C., Muyssen, B.T.A., Janssen, C.R., 2006. Nickel toxicity to 'soft' versus 'hard water' organisms: a comparison of sensitivity and bioavailability. SETAC Europe 16th Annual Meeting, The Hague, The Netherlands, 7-11 May 2006.

De Schamphelaere, K., Van Laer, L., Deleebeeck, N., Muyssen, B.T.A., Degryse, F., Smolders, E., Janssen, C.R., 2006. Speciation and toxicity of nickel in natural surface waters. SETAC Europe 16th Annual Meeting, The Hague, The Netherlands, 7-11 May 2006.

Scientific reports

Deleebeeck, N.M.E., De Schamphelaere, K.A.C., Heijerick, D.G., Bossuyt, B.T.A., Janssen, C.R., 2005. Development and validation of biotic ligand models for predicting nickel toxicity to fish, daphnids and algae. Report prepared for the Nickel Producers Environmental Research Association (NiPERA), Durham, North Carolina, USA. Gent University, Laboratory of Environmental Toxicology and Aquatic Ecology, Gent, Belgium.

De Schamphelaere, K.A.C., Van Laer, L., Deleebeeck, N.M.E., Muyssen, B.T.A., Degryse, F., Smolders, E., Janssen, C.R., 2006. Nickel speciation and ecotoxicity in European natural surface waters: development, refinement and validation of bioavailability models. Report prepared for the Nickel Producers Environmental Research Association (NiPERA), Durham, North Carolina, USA. Gent University, Laboratory of Environmental Toxicology and Aquatic Ecology, Gent, Belgium.

Deleebeeck, N.M.E., De Schamphelaere, K.A.C., Muyssen, B.T.A., De Laender, F., Janssen, C.R., 2006. Ni-SOFT. Nickel toxicity to soft versus hard water organisms: a comparison of sensitivity and bioavalability. Report prepared for the Nickel Producers Environmental Research Association (NiPERA), Durham, North Carolina, USA. Gent University, Laboratory of Environmental Toxicology and Aquatic Ecology, Gent, Belgium.

Conferences and workshops

April 1st 2004
Beltox meeting: Role of bioavailability in environmental risk assessment, Liege, Belgium.

April 18th – 22nd 2004
14th Annual Meeting of SETAC Europe, Prague, Czech Republic.

May 22nd – 26th 2005
15th Annual Meeting of SETAC Europe, Lille, France.

September 23rd 2005
Studiedag KULeuven. Zware metalen in het leefmilieu: nieuwe inzichten, nieuwe wetten? Leuven, Belgium.

May 7th – 11th 2006
16th Annual Meeting of SETAC Europe, The Hague, The Netherlands.

Membership of professional communities

Member of the Society of Environmental Toxicology and Chemistry (SETAC) since 2003.