Uptake of Trace Metals in Aquatic Organisms: a Stable Isotopes Experiment

Dissertation for the academic degree of Doctor of Sciences at the University of Antwerp, to be defended by

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Antwerpen 2009
Uptake of Trace Metals in Aquatic Organisms: a Stable Isotopes Experiment

Opname van Spoormetalen door Aquatische Organismen: een Stabiele Isotopen Experiment

Proefschrift voorgelegd tot het behalen van de graad van Doctor in de Wetenschappen aan de Universiteit Antwerpen te verdedigen door

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Antwerpen, 2009
Front cover illustrations:

1. Water flea *Daphnia magna* ([www.asi-group.com/engineering/ToxicityLab.htm](http://www.asi-group.com/engineering/ToxicityLab.htm))

2. Zebrafish *Danio rerio*
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Acknowledgements

I would like to express my sincere gratitude to my supervisor Prof. Dr. Ronny Blust, who introduced me to the field of aquatic toxicology, for his advice and invaluable support during my studies. Many thanks extend to Prof. Dr. Gudrun De Boeck and Prof. Dr. Lieven Bervoets for their valuable advices. I also would like to thank Karin Van den Bergh, Marcel Selens, Dr. Valentine Mubiana and Nemo Maes for providing technical support and creating friendly working atmosphere.

I want to express my appreciation to the EBT secretary Rosa Hermans for her kindness, warmth and friendly help which always made me feel welcomed in the lab.

My thanks extend to An Hagenaars, Frouke Vermeulen and Niko Celis, with whom I shared the office for the last two years, for creating a light and humorous atmosphere. I would like to thank all colleagues for providing a stimulating and fun environment. I am especially grateful to An Jamers, Judith Voets, Marleen Eyckmans and Tine Vandenbrouck for their friendship.

Last but not least, I would like to thank my family, especially my husband Ivan for his continuous love, encouragement and support during all these years. I would not be able to complete this thesis without you believing in me. I dedicate this thesis to you.
CHAPTER 1

Introduction
All aquatic organisms take up trace metals either from the surrounding aquatic medium or from food, whether or not these metals are essential to metabolism, (Rainbow, 1997, 2002). Thus, metal bioaccumulation is a good integrative indicator of the chemical exposures of organisms in ecosystems. All metals have the potential to cause toxic effects. As a result, metal accumulation is closely linked with toxicity (Vijver et al., 2004; Marsden et al., 2004), although in a complex manner, because metal accumulation is influenced by a variety of factors, such as multiple routes of exposure (diet and solution), chemical composition of the surrounding medium and physiological or biochemical effects on bioavailability (Luoma and Rainbow, 2005). The metal accumulation levels vary widely among metals, organisms, and have different distributions between tissues and organs in the body (Rainbow, 1997). Aquatic organisms living in the same habitat may have very different body concentrations of trace metals, even within closely related species (Luoma and Rainbow, 2005).

To establish a link between metal concentration in ambient water and metal toxicity toward aquatic organisms the Biotic Ligand Model (BLM) has been developed. It is a unifying computational model which predicts the effects of water chemistry on metal bioavailability and toxicity (Paquin et al., 2002). The BLM consists of two sub-models. First, the chemical equilibrium sub-model is used to predict the level of metal accumulation at the site of action (a biotic ligand) depending on water quality. It considers water hardness (i.e. Ca$^{2+}$ and Mg$^{2+}$ ions), pH and dissolved organic carbon as parameters affecting metal accumulation. Then, the toxicity sub-model uses this computed accumulation level to predict a toxicological response. Thus, full understanding of metal accumulation pattern, which depends on metal species, water chemistry characteristics, exposure routes, and species-specific characteristics, is a prerequisite for a successful prediction of the site-specific toxicity of a metal towards aquatic organisms and setting adequate water quality criteria (Luoma and Rainbow, 2005; Rainbow, 2007). Moreover, in real environments aquatic organisms are exposed to trace metals at concentrations far below the toxicity levels. Comprehensive understanding of metal accumulation processes occurring in natural waters in relation to water chemistry
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parameters, including presence of metals in mixtures, is essential for setting adequate water quality criteria. Delineating the effects which \( \text{Ca}^{2+} \) and \( \text{Na}^+ \) ions present in natural waters, pH and interactions among metals have on the uptake of individual elements is a first step in this direction.

1.1. Trace metals and their role in organisms

In leaving organisms metal ions regulate an array of physiological mechanisms. In addition to macro elements (sodium, potassium, calcium and magnesium), which are present in the largest quantities, there will be a range of trace elements. During evolution these trace metals (copper, zinc, iron, cobalt, selenium, manganese and a few others) have become essential for the health of most organisms being components of enzymes and other molecular complexes involved in all aspects of biological function. Nevertheless, in excess essential elements are toxic. They can bind to metal sensitive molecules or form dangerous free radicals. Consequently, there is a fine balance between metal deficiency and surplus and it is vital for organisms to maintain metal homeostasis (Bury et al., 2003).

A metal is considered to be essential when (Reilly, 2004):

1. It is consistently present in all healthy living tissues within a zoological family.
2. Deficiency symptoms are noted with metal depletion or removal, which disappear when the element is supplied again.
3. The deficiency symptoms should be attributed to a distinct physiological or biochemical effect.

The rest of metals occur in organisms only in minute quantities and are called non-essential, since no biological function has yet been identified for them. Sometimes, even a slight increase in their concentration in the body can exert a toxic effect.

1.1.1. Cadmium

Cadmium is a ubiquitous environmental pollutant (Cherian and Goyer, 1989). It is nutritionally non-essential and toxic due to its interaction with the metabolism of at least three essential metals: calcium, zinc, and iron. Cadmium has a profound capacity of
binding to –SH groups, which is higher than that of zinc and thus, has the ability to
displace zinc in a number of zinc-containing enzymes (Wittmann, 1979; Moore and
Ramamoorthy, 1983). In aquatic organisms cadmium interacts with calcium uptake at the
gill surface, which leads to acute hypocalcaemia and eventual death (Verbost et al.,
1989). In fish cadmium accumulates primarily in kidneys, followed by gills, intestine,
liver and muscles. The toxicity involves the renal and skeletal systems and is largely the
consequence of the interactions between cadmium and essential metals, particularly
calcium (Bervoets and Blust, 2003; Raynders et al., 2008). Dissolved cadmium levels in
freshwaters range from 0.3 to 10nmole·L⁻¹; however, due to human activities cadmium
concentrations in polluted areas may exceed 200nmol·L⁻¹ (Bervoets and Blust, 2003;
Voets et al., 2006; Campbell et al., 2008).

1.1.2. Copper

Copper is an essential trace element, which acts as a cofactor for a number of key
proteins and plays an important role in cellular respiration. Diet is a major source of
copper for aquatic organisms. Daily dietary requirements for fish, for example, are in the
range 15-60nmoleCu·kg⁻¹ dry mass (Lanno et al., 1985; Watanabe et al., 1997). However,
elevated cellular copper can cause rapid generation of free radicals in tissues where it is
accumulated due to the redox properties of copper. Branchial uptake also contributes
considerably to copper accumulation through highly regulated transport pathways.
Elevated copper concentrations in water, however, lead to impairment of sodium uptake
in gills, a vital part of freshwater osmoregulation (Grosell and Wood, 2002). In
freshwater systems copper is present at concentrations 8-80nmol·L⁻¹, which is sufficient
to meet 60% of the whole body copper requirements by uptake via gills along (Spry et al.,
1981; Kamunde et al., 2002). In polluted areas dissolved copper concentrations may
exceed 300nmol·L⁻¹ (Bervoets and Blust, 2003; Campbell et al., 2008). Thus, circulating
levels of copper in the plasma must be kept under tight regulation by maintaining a fine
balance between uptake and excretion (Grosell et al., 1997; Rainbow, 2002).
1.1.3. Nickel

Nickel is a naturally occurring element and an anthropogenic contaminant for aquatic and terrestrial environments as it is used in many industrial products. In uncontaminated freshwaters Ni concentrations vary between 10-170nmol·L⁻¹ and can reach up to 2500nmol·L⁻¹ due to human activities (Howells, 1994; Eisler, 1998; Giguere et al., 2005). Several toxic effects of nickel including allergies, carcinogenesis, and cardiovascular and renal disorders have been identified in higher animals including humans (Denkhaus and Salnikow, 2002). In fish nickel acts as a respiratory toxicant affecting arterial oxygen/carbon dioxide tension in gills and causing a respiratory acidosis. Although the details surrounding the mechanism of respiratory toxicity are not thoroughly investigated, the physiological effects observed are presumed to result from impairment of gas exchange between the organism and the water caused by a diffusive limitation at the gill (Pane et al., 2003). Nickel is known to be essential nutrient for microorganisms and aquatic plants, however, the essentiality of nickel to aquatic animals is not established (Muyssen et al., 2004; Chowdhury et al., 2008). Nevertheless, recent study by Chowdhury et al. (2008) indicate that gut and kidney play important roles in nickel homeostasis regulation, a property that is a characteristic of essential metals, by providing uptake, clearance and storage sites in adult rainbow trout.

1.1.4. Lead

Lead is not required for normal physiology in plants or animals and potentially toxic. Lead is a common contaminant in the natural environment that can enter the water column through geologic weathering and volcanic action, or by various anthropogenic activities (World Health Organization, 1995). The principal toxic effects of chronic lead exposure to fish are presumably hematological (Hodson et al., 1978), neurological (Davies et al., 1976) and renal (Patel et al., 2006) impairment. In fish waterborne lead causes the disruption of Na⁺, Cl⁻ and Ca²⁺ regulation during acute exposure, development of black tails and spinal curvature during chronic exposure, and disruption in hemoglobin synthesis (Sippel et al., 1983; Rogers et al., 2003; Rogers et al., 2005). Waterborne lead
concentrations in natural fresh waters are in the range 0.6-1.1nmol·L$^{-1}$ (Fischer and van den Berg, 2001). In polluted areas dissolved lead concentrations vary between 50-400nmol·L$^{-1}$ (Bowles et al., 2006), while lead toxicity has been reported for some freshwater species at Pb concentrations as low as 50nmol·L$^{-1}$ (Grosell et al., 2006).

1.1.5. Zinc

Zinc is an essential element for normal physiological functions. It has vital structural and catalytic importance in more than 300 proteins involved in growth, reproduction and development processes, as well as visual and immune functions (Watanabe et al., 1997). In contrast to copper, it does not form free radicals; however, it becomes toxic at increased waterborne concentrations due to interference with calcium homeostasis leading to hypocalcemia (Spry and Wood, 1985; Hogstrand and Wood, 1996). Levels of zinc in fresh uncontaminated waters are usually low and in the range 30-60nmol·L$^{-1}$ (Voets et al., 2006; Cambell et al., 2008). However, much higher concentrations in a range 2000-3000nmol·L$^{-1}$ and reaching up to 34000nmol·L$^{-1}$ are reported for polluted areas (Bervoets and Blust, 2003; Giguereet et al., 2005).

1.2. Metal bioaccumulation in the cell

Metal bioaccumulation is a complex process. The uptake of dissolved metals by aquatic organisms is considered to be a four-step process (Campbell, 1995):

1. The metal speciation in the surrounding medium determines the transport (diffusion) of metal ions to the cell surface and their adsorption on the cell wall. The metal of interest should be present in a bulk solution in a bioavailable form to be able to interact with a cell wall through the adsorption/desorption processes.
2. Diffusion of the adsorbed metals through a cell wall toward cell membrane.
3. Interaction of metal ions with a cell membrane and their transport through a membrane into cytoplasm (metal internalization).
4. Metal partitioning within a cell and organs of the organism as a result of metal storage in a bioavailable/detoxified forms and elimination processes).
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Metals in natural waters can exist in many different chemical forms, including the free ion (e.g. Cu\(^{2+}\)), dissolved inorganic forms (e.g. hydroxides, carbonates, sulfates), organic complexes (metal bound to dissolved organic matter), and variety of particulate forms bound to clays or incorporated into the matrix of soil particles (Schnoor et al, 1997). The relative distribution of these forms is a function of environmental conditions including pH, hardness, and the presence of organic matter and/or inorganic particulates (Wood et al., 1997). The free metal ion is one of the few metal forms which are transported across the gill membrane. Therefore, anything that reduces the concentration of the free metal ion in water or reduces the number of available binding sites for the free metal on the active site will reduce its toxicity. In general, waterborne metals are most toxic to fish in water that has low hardness, low pH, and low DOC (Playle et al., 1993 a,b). Increase in salinity will shift the equilibrium toward formation of chloride species and decrease the concentration of free metal ions (Blust et al., 1992). Knowing which aqueous species control the rates of trace metal uptake by aquatic biota is essential for modeling their fate and effects in aquatic systems.

To be taken up by aquatic organisms, trace metals and their complexes must first diffuse from the external medium to the surface of the organism (Worms, 2006). According to Hudson (1998), both the bulk solution and cell surface have to be considered when addressing the question which species control the rates of metal uptake by aquatic biota. First, the diffusion of metal species from a bulk solution to the cell surface can be a rate limiting step. Metal complexes are often dynamic, able to dissociate and re-associate (complexation/dissociation) while diffusing to the biological surface. If metal species are sufficiently labile to dissociate within the diffusive boundary layer at the cell surface of the organism, the uptake rate becomes dependent on the bulk concentration of these metals in a solution. Such labile species include complexes with weak inorganic ligands (OH\(^-\), Cl\(^-\), CO\(_3^{2-}\)) and sufficiently abundant organic ligands. For example, the uptake of Fe, Zn, and Cu is controlled by diffusion step when their concentrations in the medium are low and concentrations of competing metals are not high.
The uptake of non-essential metals, in turn, depends on the availability of the essential metal whose transporter is responsible for their uptake. Due to limited space available in plasma membrane, however, slow transport kinetics can become more important in controlling the uptake process than diffusion of the species from a solution to the cell. In many cases, however, trace metal transport across the biological membrane is rate-limiting and the overall process can be simplified to a thermodynamic equilibrium among metal species in the bulk solution and those bound to sensitive sites on the biological surface (Worms et al., 2006). Another rate limiting step is the adsorptive processes on the cell membrane when the metals can be simply adsorbed on cell walls and cell membranes without being transported into the cytoplasm, or undergo a chemical reaction (e.g. enzymatic and non-enzymatic metal reduction) at the cell surface (Price and Morel, 1990). Such cell surface transformations of metals can themselves become limited by diffusion.

With the exception to metals incorporated in cell-surface metalloenzymes, it is metals in the cytoplasm that are either required for or interfere with cellular metabolism (Reinfelder and Fisher, 1998). Various routes have been proposed for the transport of trace metals across the cytoplasmic membrane (Simkiss and Taylor, 1989) including (Fig. 1):

1. Passive diffusion of non-polar lipid soluble metal forms, such as alkyl-metal compounds, and small water soluble solutes.
2. Transport via carrier mediated proteins or ion channels where metals are transported down a concentration gradient within proteins with hydrophilic cores by passive diffusion.
3. Active transport via ion pumps (channels) when a metal ion binds with a membrane protein and is transported across the membrane against a concentration gradient.
4. Endocytosis.
Although most metal species are extremely hydrophilic, a few metal complexes are soluble enough in lipids and able to rapidly diffuse through without energy input (passive diffusion). Passive diffusion is best known for the neutral chloride and hydroxide complexes of Hg\(^{2+}\) and CH\(_3\)Hg\(^{+}\) ions (Bienvenue et al., 1984). Lipophilic organic chelates of several divalent metal ions can also diffuse through cellular membranes, thereby short-circuiting cellular barriers to toxic metal uptake (Phinney and Bruland, 1997).

The majority of trace metals are taken up passively down a concentration gradient. The process is facilitated by carriers (membrane protein transporters) or channels. These carrier proteins, which are imbedded within the cell membrane and have binding sites available from the opposite sides of the membrane and ion channels, which are considered as perforations in the membrane, move metal ions from a higher concentration (outside the cell) to a lower concentration (inside the cell) without utilizing any chemical energy (Simkiss, 1998). Inside the cell the incoming metal is bound to a
Chapter 1

number of metal-binding ligands with increasing metal affinity and is not able to cross the membrane in the opposite direction. Thus, internal metal concentrations can be orders of magnitude higher than those externally, and trace metals may still be taken up passively. Such carrier proteins could either be specific to a particular metal ion or have varied affinities for a range of metals (Simkiss and Taylor, 1989). Three major classes of trace metal transporters have been identified in aquatic organisms (Bury et al., 2003; Worms et al., 2006): P-type ATPases; Divalent Metal Transporter1 (DMT1, Fe\textsuperscript{2+}/H\textsuperscript{+} like symporter) and zinc regulated transporter/ion-regulated transporter (ZRT/IRT). P-type ATPases are involved in both internalization and detoxification or trace metals. DMT1 proteins in addition to Fe\textsuperscript{2+} regulation also participate in the transport of other divalent metals (Cd\textsuperscript{2+}, Co\textsuperscript{2+}, Cu\textsuperscript{2+}, Mn\textsuperscript{2+}, Pb\textsuperscript{2+} and Zn\textsuperscript{2+}). ZRT/IRT proteins have been shown to allow entry of Mn and Cd. Ion channels are aqueous pores through the lipid membrane, funnel-shaped at the entrance and exit. The channels are characterized by a high selectivity towards the radius and charge of the passing hydrated ion. The selectivity of transporting channels also depends on the charge and dimensions of the channel. For example, cations selective channels have a negative charge to attract positively charged ions. Moreover, only small metal species, such as free metal ions, can fit into the channel. Thus, large metal complexes cannot be transported.

Another route of metal uptake from a solution is active transport through ionic pumps, channels or exchangers involving energy input in the form of adenosine triphosphate, or ATP, to move molecules against the concentration gradient. Metals, such as Na, K, Ca and Mg are transported across hydrophobic membranes through ion channels, such as Na\textsuperscript{+}/K\textsuperscript{+} ATPase system (Simkiss and Taylor, 1989). Trace metals may be incorporated into such pumps, particularly if they are of a similar ionic size as the bulk electrolyte metal being transported. Many channels, such as the sodium channel, are gated by a voltage or chemical sensor systems, which block or open the channels in response to a stimulus. Different voltage and receptor-gating systems have been proposed for calcium channel, which selects out Na\textsuperscript{+}, K\textsuperscript{+} and Mg\textsuperscript{2+} ions (Simkiss and Taylor, 1995). Although it is generally assumed that there are specific channels for each essential metal,
there is a potential for any metal ion to enter via other channels according to selecting characteristics of the channel, the size of hydrated dehydrated ion and cost of dehydration. In particular, the size of Ca\(^{2+}\) ion is 0.99 Å, while Cd\(^{2+}\) has an ionic radius of 0.9 Å allowing it to enter the cell via a calcium channel (Rainbow, 1997). Mn\(^{2+}\) and Pb\(^{2+}\) have been also shown to interfere with calcium channels (Simkiss and Taylor, 1995). Similarly, Cu\(^{2+}\) and Ag\(^{+}\) ions interfere with sodium channels, although sodium insensitive pathways have also been identified for copper (Grosell and Wood, 2002). Finally, trace metals can enter the cell by means of endocytosis, which involves the folding of the plasma membrane to form a vesicle with the transported metal inside.

After entering the cell, the subsequent fate of the trace metal depends on the particular physiology of the organism and type of the metal. Depending on whether or not the metal is used for essential metabolic purposes, it can become available to bind to sites where it can play an essential role, be bound to another biomolecule or complexing agent and stored within the cell, be transported to subcellular compartments, excreted, or even gains access to the ‘wrong’ biomolecule and thus exerts a toxic effect (Rainbow, 2002).

Nonessential metals entering the cell may exert a toxic effect due to metal binding to physiologically important sites leading to blocking functional groups of biomolecules (often thiols), displacement of essential metals from their normal sites within biomolecules or resulting in changed conformation (and activity) of biomolecules. To protect the organism against metal toxicity, a variety of subcellular systems have evolved to permit the accumulation, regulation, and immobilization of trace metals (Mason and Jenkins, 1995). These systems include metal binding proteins (such as metallothionein), lysosomes, granules, and membrane-bound vesicles. Nonessential metals associated with these subcellular fractions are normally considered to have been detoxified (Wallace et al., 2003; Wang and Rainbow, 2006). The development of an effective efflux mechanism is another response of an organism to the excess of non-essential metal present in the cell (Campbell et al., 2008). Such complexity of metal
uptake processes is the reason why trace element bioaccumulation is so variable among metals, species, and environments and requires thorough investigation.

1.3. Experimental organisms and their use in aquatic toxicology

1.3.1. Green algae

_Pseudokirchneriella subcapitata_ (Korshikov) Hindak (Fig. 1.2), formerly known as _Selenastrum capricornutum_, is a unicellular species which belongs to the group of green algae, the most diverse group of algae with more than 7000 species growing in a variety of habitats. Algae represent the base of the food chain and, therefore, are essential to most aquatic ecosystems. Changes in their density and composition can affect the chemical and biological quality of the habitat. Algae have been found to be more sensitive than animal species to a variety of potential contaminants, including cadmium, copper, nickel, and zinc and thus, they are frequently used in phytotoxicity tests (Burton et al., 2002). Most of the information available to the scientific community concerning the phytotoxic effects of chemicals and other potential toxicants is based, however, on the results for only a few green algal species, including _Pseudokirchneriella subcapitata_. The use of this alga in various studies started during the 1960s and the 1970s. It is recommended as a standard species for toxicity testing by various scientific and regulatory communities such as OECD, ISO, ASTM (Burton et al., 2002).

![Figure 1.2. Green algae Pseudokirchneriella subcapitata](image)
Introduction

1.3.2. Water flea

Invertebrates occupy a key position as intermediate consumers in the pelagic and the benthic food chains of aquatic ecosystems (Persoone and Janssen, 1993). *Daphnia magna* (water flea) is a freshwater invertebrate crustacean found all over the world (Fig. 1.3). Water fleas inhabit all types of freshwater bodies, from springs to rivers and large lakes. Largest densities of *Daphnia magna* are found in smaller lakes and ponds, mostly without fish. *Daphnia magna* has been extensively used to determine the toxicity of effluents and water samples and has been demonstrated to be sensitive to many environmental contaminants (Adema, 1978). Methods for conducting acute and sublethal (partial or whole life cycle) tests with liquid samples have been well established (ASTM, 1993b; ISO, 1993; US EPA, 1991).

![Image of Daphnia magna](image)

**Figure 1.3.** Water flea *Daphnia magna*

Daphnids are very sensitive organisms and their health is easily affected by the diet and culture medium used. However, once suitable culture conditions have been established, maintenance of the cultures is straightforward. The reasons for the selection of daphnids for the routine use in toxicity testing are both scientific and practical
(Persoone and Janssen, 1993): they are broadly distributed in freshwater bodies, have relatively short life cycle and relatively easy to culture in the laboratory, they are sensitive to a broad range of aquatic contaminants and their small size means that only small volumes of test waters and little bench space are required. Moreover, daphnids are an important link in many aquatic food chains since they graze on primary producers and are food for many fish species.

1.3.3. Zebrafish

The zebrafish, *Danio rerio* (Hamilton) is a freshwater fish inhabiting the Ganges and Brahmaputra river basins in north-eastern India, Bangladesh and Nepal (Fig. 1.4). They are characterized by a small size (<120 mm total length) and a distinctive color pattern based on alternating dark and light horizontal stripes, which may be broken up into blotches or bars. There is a wide range of temperatures within the natural habitat of zebrafish, from as low as 6°C in winter to over 38°C in summer (Spence, 2008).

![Figure 1.4. Zebrafish Danio rerio](image-url)
The zebrafish is one of the most important vertebrate model organisms in genetics, developmental biology, neurophysiology and biomedicine (Rubinstein, 2003; Amsterdam and Hopkins, 2006). It is a small, robust fish, so large numbers can be kept easily and cheaply in the laboratory, where it breeds all year round. Generation time is short, typically 3-4 months, making it suitable for selection experiments. Development is rapid, with precursors to all major organs developing within 36h and larvae displaying food seeking and active avoidance behaviours within five days post fertilisation, i.e. 2-3 days after hatching (Kimmel et al., 1995). Currently, zebrafish is a recommended test organism for juvenile growth, early-life stage toxicity and chronic toxicity tests by a number of regulatory communities (e.g. OECD, USEPA, ISO, ASTM).

1.4. Conceptual framework of the study

Setting of the appropriate site-specific ambient water quality criteria is one of the major tasks in aquatic toxicology. Understanding how metal accumulations processes depend on water chemistry characteristics is an important step in finding the relationship between metals present in water and their toxicity to aquatic organisms.

The central aim of this work was to characterize trace metal uptake processes in green algae Pseudokirchneriella subcapitata, water flea Daphnia magna and zebrafish Danio rerio as a function of water chemistry characteristics, i.e. Ca\(^{2+}\) and Na\(^{+}\) concentrations, pH and metal-metal interactions. The uptake processes were studied during a simultaneous exposure to cadmium, copper, nickel, lead, and zinc at exposure levels corresponding to the concentrations of trace metals found in real environments. Analytical methods were developed for the analysis of trace metals in different matrices and are described in Chapter 2. The stable isotope technique applied in this work allowed to overcome the limitations of the total metal measurement approach employed in the majority of accumulation studies. First, use of stable isotopes made it possible to follow the uptake of each metal individually from a mixture of trace metals. This gives the advantage of direct comparison of effects on the accumulation of different metals since the exposure was done on the same organism. As a result, there was no experimental
uncertainty occurring due to the difference in physiological and exposure conditions when single element exposure studies performed on different individual organisms are compared. Second, a distinction between newly accumulated metals and those originally present in the organism could be made. This is particularly important when the uptake of essential metals (in this case Cu and Zn) is studies. Essential metals are present in the organisms in significant amounts and simple measurements of total metal concentrations in tissues will not reveal their uptake at low exposure concentrations due to high background levels. By using trace metals enriched in one particular low abundant isotope in the exposure experiment, it was possible to follow the concentrations of each isotope in the tissue instead of measuring the total metal concentration. Thus, even a slightest accumulation of the isotope of interest from the exposure solution was detected. Finally, the determination of a primary uptake route was made. Metal uptake in aquatic organisms occurs via both water and dietary routes and it is essential to know which route contributes the most to the metal accumulation in a real environment. To address this issue, green algae and *Daphnia magna* were exposed to trace metals at equal conditions. Then, the metal uptakes from water phase were quantified for algae and *Daphnia* and the metal assimilation efficiencies from ingested algae were determined in *Daphnia magna* (Chapter 3).

As mentioned earlier, water chemistry has a distinct impact on the uptake of trace elements. Nevertheless, there is limiting number of studies investigating the effects from major cations present in ambient waters on the trace metal accumulation. Most of the studies are dealing with single element exposures, which are often performed at toxic levels, or concentrated on the effects from one particular ion, such as Ca-Cd or Na-Cu effects. Additionally, multi-metal interactions are often neglected. In this respect, the present work addresses some of the question marks present in literature by the investigation of the uptake processes of five trace elements as a function of Ca$^{2+}$ and Na$^+$ concentrations, pH and possible inter-metal interactions in water fleas (Chapters 4 and 5) and zebrafish (Chapters 6 and 7). Moreover, the majority of toxicity models developed at present use different fish species as test organisms. Then, the models are simply
recalibrated for other species. However, structural and physiological organizations, and thus sensitivity toward toxicants and toxicological responses, differ dramatically among species. By investigating the uptake processes in the organisms which occupy different niches in a food chain, it was possible to answer the question whether or not the same type of response would be observed in aquatic organisms of different hierarchy at similar exposure conditions and justify the validity such recalibration. Since the uptakes of both essential and non-essential metals have been studied, a complex assessment of changes taking place in two aquatic species exposed to trace metals was performed helping to understand the processes occurring in real environmental conditions.

1.5. References


Introduction


Chapter 1


CHAPTER 2

Determination of Trace Metals in Different Matrices
2.1. Application of an ICP-MS technique for trace metal determination

Inductively coupled plasma-mass spectrometry (ICP-MS) is a powerful tool for the analysis of elements. This technique offers determination of more than 80 elements and provides isotopic information in a simple spectrum with limited spectral overlaps and modest matrix effects (Ray et al., 2004). High sensitivity, low detection limits (DL), wide linear dynamic range, multi-element capability and the potential of coupling to various separation and sample preparation techniques make ICP-MS advantageous over other spectroscopic techniques including flame and graphite furnace atomic absorption spectrophotometry (FAAS and GFAAS) and inductively coupled plasma–atomic emission (ICP-AES) instruments for the determination of elements at trace concentrations (Linge, 2007). The technique has been extensively applied in material science, geological and biological areas for the determination of impurities in semiconductor materials, isotopic composition characterization in the age dating studies, for the trace metal speciation, partitioning, bioavailability, turnover and isotope variations studies (Heumann et al., 1998; Gee and Bruland, 2002; Eimers et al., 2002; Mason and Borja, 2002; Rodriguez-Gonzalez et al., 2005).

ICP-MS instruments, regardless of their specific design, comprise six functional elements: a sample introduction system, an ion source (plasma), an interface region, an ion optics system, a mass analyzer and a detector. Each of these elements exists in many forms and is combined to produce a wide variety of ICP-MS instruments with specific characteristics. A general scheme of an ICP-MS system is presented in Figure 2.1. The sample is introduced to an ICP-MS instrument via a sample introduction system. At present, sample introduction systems for the analysis of gaseous, liquid and solid samples have been developed. Nevertheless, the majority of applications involve analysis of liquids. When the liquid sample enters the sample introduction system, it is converted into a fine aerosol by a nebulizer and a spray chamber in order to be efficiently ionized in the plasma discharge. The formed fine droplets (~5–10μm in diameter) are transported into the sample injector of the plasma torch. As the sample droplet moves through the plasma
formed in the torch, it undergoes a number of physical changes and eventually becomes a positively charged ion. Formed ions are directed into the interface (sampler and skimmer cones) of the mass spectrometer. The role of the interface is to transport the ions efficiently, consistently and with electrical integrity from the plasma, which is at atmospheric pressure (760 Torr), to the mass spectrometer analyzer region, which is at vacuum (approximately $10^{-6}$ Torr). The ion focusing system (ion optics) helps to transport the maximum number of analyte ions from the interface region to the mass separation device, while rejecting as many of the matrix components and nonanalyte-based species as possible. The ions emerging from the ion optics enter the hart of the ICP-MS system, the mass analyzer, where they are separated according to their mass-to-charge ratio. There are basically four kinds of commercially available mass analyzers: quadrupole mass filters, magnetic sector (single or double focusing MS, also referred to as high resolution), collision–reaction cell and time-of-flight analyzers. Finally, the ions reach the detector, which converts them into electrical pulses. The magnitude of the electrical pulses corresponds to the number of analyte ions present in the sample. The pulses are counted by an integrated measurement circuitry.

![Schematics of an ICP-MS system](image_url)

**Figure 2.1.** Schematics of an ICP-MS system (adapted from Thomas, 2004).
Earlier designs employed channel electron multipliers (channeltron) for low ion count rates and Faraday collectors for high-count rates. The modern ICP-MS systems used for ultratrace analysis are equipped with detectors that are based on the active film or discrete dynode electron multipliers. Some of the ICP-MS systems dedicated to a high precision isotope ratio analysis are the double focusing magnetic-sector instruments, where multiple detectors, often referred to as multicollector systems, are used instead of just one detector.

At present quadrupole ICP-MS (Q-ICP-MS) are the most frequently used instruments for a routine elemental analysis as they represent more than 90% of all inductively coupled plasma mass spectrometry systems installed worldwide (Thomas, 2004). Such systems are capable to detect elements in a 0-300 atomic mass units (amu) range with high sensitivity (10-50 million counts per second (cps) per ppm for most metals), relatively low background noise (10-20cps) depending on the sample matrix and a short-term precision (based on several consecutive measurements) of 1-3%. The typical resolution of the quadrupole ICP-MS instruments is 0.7-1 amu with an operational resolving power of ~ 300-400, which is quite satisfactory for most environmental applications. In theory, the resolution of a quadrupole mass filter can be varied between 0.3 to 3.0 amu, although improved resolution is always accompanied by a sacrifice in sensitivity. Presence of isobaric (e.g. $^{58}\text{Ni}$ on $^{58}\text{Fe}$, $^{40}\text{Ar}$ on $^{40}\text{Ca}$) and polyatomic overlaps ($^{40}\text{Ar}^4\text{O}$ on $^{56}\text{Fe}$, $^{38}\text{Ar}^1\text{H}$ on $^{39}\text{K}$, $^{35}\text{Cl}^4\text{O}$ on $^{51}\text{V}$, $^{40}\text{Ar}^{35}\text{Cl}$ on $^{75}\text{As}$, $^{40}\text{Ar}^{40}\text{Ar}$ on $^{80}\text{Se}$) occurring when isotopes of interest have the same mass as another species, have made the Q-ICP-MS technique less adequate for determination of elements (i.e. Fe, K, As, V, Se and Cr) that are prone of spectroscopic interferences (Moldovan et al., 2004; Thomas, 2004). Isobaric interferences are predictable and can be minimized by application of the ‘cool plasma’ technique (Patterson et al., 1999), use of appropriate internal standards and correction equations or selection of a different isotope for the measurement (Taylor, 2001). However, formation of interfering polyatomic species from matrix elements and plasma gases (e.g. $^{40}\text{Ca}^1\text{O}$, $^{40}\text{Ar}^1\text{O}$, $^{40}\text{Ar}^1\text{H}$, $^{36}\text{Cl}^1\text{O}^1\text{H}$, reactive oxides and organic molecular ions) is more problematic (Zoorob et al., 1998).
Approaches used to overcome spectral interferences include application of
collision/dynamic reaction cells and high resolution magnetic sector ICP-MS instruments
(Moldovan et al., 2004). In a first approach the ICP produces an ion beam composed of
analyte ions, matrix component ions, and polyatomic molecules, which is then
transmitted through the cell by the action of a multipole (a quadrupole, hexapole, or
octapole). The collision/reaction cell is usually positioned before the analyzer quadrupole.
An externally supplied gas (hydrogen or helium in a collision cell and more reactive
ammonia or methane in a dynamic reaction cell) selectively reacts with the polyatomic
molecular ions in the ion beam. These reactions effectively remove the molecular ions
from the beam prior to its entrance into the mass analyzer for ion separation without a
significant loss of sensitivity for the analyte species. Formation of secondary
interferences from reaction products can be a problem under some conditions and their
control requires careful selection of operating conditions. Moreover, careful consideration
should be given to the choice of collision gases in multielemental analysis because the
gas may reduce interferences on one element whilst decreasing sensitivity to other
elements being measured (Taylor, 2001; Thomas, 2004).

Magnetic-sector ICP-MS (single and multi collector instruments) are capable of
providing a resolving power of up to 10,000 in high resolution mode. At such operating
conditions a successful separation of any interfering species (e.g. $^{56}\text{Fe}^+$ at 55.935amu and
$^{40}\text{Ag}^{16}\text{O}^+$ at 55.957amu) is achieved. In low resolution mode magnetic sector analyzers
offer high sensitivity of typically 100–200 million counts per second per ppm and
extremely low background noise of 0.1–0.2cps, which make them excellent for the
determination of minute elemental quantities. Besides good detection capability, magnetic
sector instruments are able to quantify elements with excellent precision due to the
characteristically flat topped spectral peaks, resulting in high-precision data. In the low
resolution mode, relative standard deviation (RSD) values of 0.01–0.05% are fairly
common and less than 0.1% RSD values are typical for a high resolution mode, which is
ideal for carrying out high precision isotope ratio work (Vanhaecke et al., 1996). It should
be mentioned, however, that increase in resolution unavoidably leads to the loss in
sensitivity making it challenging to determine elements at 1µg·L⁻¹ level in a high resolution mode (Morton, 2005). Moreover, HR-ICP-MS analysis is time consuming and expensive.

2.2. Application of stable isotopes as tracers in biological systems

The ability of ICP-MS instruments to determine the isotopic composition promoted the application of enriched stable isotopes of elements as tracers for elucidating the fate and metabolic pathways of trace metals and biomolecules in many biological systems including aquatic and terrestrial ecosystems, cell cultures, animals and humans (Crews et al., 2000; Mota et al., 2000; Eimers et al., 2002; Stürup, 2004; Evans et al., 2006). Stable isotope tracer experiments are very similar to radioisotope studies, but can be used in experiments involving living organisms without any radiotoxicological concerns. The approach enables exposure of organisms to several metals simultaneously at environmentally realistic concentrations (Evans et al., 2006, Croteau et al., 2007). Many trace elements have several stable isotopes, thus two or more isotopes can be added to the system concurrently to label different compartments such as food, water and sediment (Smokorowski et al., 1998; Eimers et al., 2001). The method allows one to follow each tracer independently through the entire accumulation-elimination process and simultaneously measure the concentrations of added and naturally occurring (background) elements in the exposure medium and organisms (Evans et al., 2002; Croteau et al., 2004).

The main drawback is that in the case of essential elements a significant amount of the selected isotope, for example ⁶⁵Cu, is often present as part of the naturally occurring element in the biological system under investigation. Hence, a relatively large amount of the enriched stable isotope tracer, compared to radioactive isotopes, must be administered to induce a detectable shift in the tracer abundance in the sample of interest (Stürup et al., 2008).

In this work the stable isotope approach was used for studying trace metal uptake and accumulation in aquatic organisms under different exposure scenarios using a
quadrupole ICP-MS for the quantification of trace metal concentrations. The exposure medium was spiked with trace metals enriched in one low abundant stable isotope of each examined element without addition of regular metals. The exposure concentrations and sampling periods were chosen to ensure that enough isotopes quantities were accumulated by an organism to allow direct measurement of their concentrations rather than determination of isotope ratios in a sample. Precise determination of the isotope ratios is critical for the reliable quantification of the taken up metals. However, the accuracy of the isotope ratio measurements provided by Q-ICP-MS is not satisfactory for such work. By using the alternative approach of the direct measurement of the accumulated isotopes, the lower resolution characteristics of Q-ICP-MS compared to high resolution MC-ICP-MS did not pose a problem since concentrations of tracers in the samples were well above detection limits. The optimization of an ICP-MS method ensured collection of the reliable data.

2.3. Method development for the ICP-MS analysis

Determination of trace metal isotopes was performed using a Varian Expert 700 quadrupole ICP-MS (Fig. 2.2), which is capable to determine middle masses with sensitivity over 100 million cps per ppm in a normal sensitivity mode (comparable with that of MC-ICP-MS operating in a high resolution mode) without compromising oxide and doubly charged interference performance. At the beginning of each run the nebulizer/plasma flow rates and ion optics were tuned to provide maximum sensitivity and to minimize polyatomic spectral interferences by keeping the formation of oxide and double charged species below 1% and 3%, respectively. The method development included optimization of the following parameters:

1. Number of scans per replicate.
2. Dwell time (amount of time spent for measurement of one isotope).
3. Possible isobaric interferences from other elements.
4. Selection of an appropriate internal standard for the instrument drift and matrix effects corrections.
Increasing the number of scans per replicate improves the reproducibility and the accuracy of the measurements, but also significantly increases the analysis time. An increase in dwell time also may improve the quality of the measurements for low abundant isotopes, but requires longer running time. Therefore, a compromise between the quality and time/cost of analysis had to be found. The precision of measurements is determined by % RSD values. In quadrupole ICP-MS instruments the RSD values below 3% are usually considered to be representative of a precise measurement (Thomas, 2004). Increasing the number of scans per replicate to 50 has significantly improved the precision of measurements and decreased RSDs values below 3% (data not shown). However, further increase in the number of scans to 100 did not significantly improve the quality of measurement (Table 2.1).
Table 2.1. Precision of Q-ICP-MS measurements of selected isotopes at 50 and 100 scans per replicate (5 replicates per sample, 10µg·L⁻¹ multielement solution in 1% HNO₃, peak hopping scan mode)

<table>
<thead>
<tr>
<th></th>
<th>Ni60</th>
<th>Ni62</th>
<th>Cu63</th>
<th>Cu65</th>
<th>Pb206</th>
<th>Pb207</th>
<th>Pb208</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 scans</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSD % in sample</td>
<td>1.75</td>
<td>2.75</td>
<td>1.41</td>
<td>1.73</td>
<td>1.90</td>
<td>1.14</td>
<td>1.40</td>
</tr>
<tr>
<td>RSD % inter sample</td>
<td>0.62</td>
<td>0.74</td>
<td>0.24</td>
<td>0.78</td>
<td>1.51</td>
<td>0.36</td>
<td>1.06</td>
</tr>
<tr>
<td>100 scans</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSD % in sample</td>
<td>1.83</td>
<td>2.27</td>
<td>1.79</td>
<td>1.67</td>
<td>1.59</td>
<td>1.67</td>
<td>1.44</td>
</tr>
<tr>
<td>RSD % inter sample</td>
<td>0.77</td>
<td>0.69</td>
<td>0.41</td>
<td>0.41</td>
<td>0.44</td>
<td>0.22</td>
<td>0.45</td>
</tr>
</tbody>
</table>

Thus, 50 scans per replicate were chosen as an optimum value. The typical dwell time used by default in Varian Q-ICP-MS instruments for a multi-element analysis is 20ms per isotope. To obtain more precise measurements, the instrument was allowed to spend more time on the measurement of low abundant isotopes compared to more abundant species. The results showed that such approach improved the precision of isotope measurement within sample replicates, as well as between the samples (Table 2.2).

Polyatomic interferences from other elements present in a sample matrix at high concentrations can result in increased number of counts measured for an isotope of interest and, therefore, overestimated concentration. For example, 27Al⁴⁰Ar⁺ ions formed in plasma or 27Al⁴⁰Ca⁺ ions formed from the sample matrix ions may overlap with a signal from ⁶⁷Zn. Similarly, ²⁴Mg⁴⁰Ar⁺ (²⁴Mg⁴⁰Ca⁺), ²⁶Mg⁴⁰Ar⁺ (²⁶Mg⁴⁰Ca⁺) or ⁶⁶Zn⁴⁰Ar⁺ (⁶⁶Zn⁴⁰Ca⁺) ions may overlap with ⁶⁴Zn, ⁶⁶Zn and ¹⁰⁶Cd signals, respectively. If this is the case, a correction equation should be determined for each interfering element. For this purpose, samples containing 10µg·L⁻¹ of Cd or Zn were spiked with variable concentrations of Mg, Al and Ca (Fig. 2.3). The analysis showed the absence of interfering effects from Mg, Al and Ca on ⁶⁶-⁶⁸Zn or ¹⁰⁶Cd signals at optimized operation conditions.
Table 2.2. Precision of Q-ICP-MS measurements of selected isotopes as a function of dwell time ($N=10$, 50 scans per replicate, 5 replicates per sample, 10µg·L$^{-1}$ multielement solution in 1% HNO$_3$, peak hopping scan mode)

<table>
<thead>
<tr>
<th>Isotope</th>
<th>abundance %</th>
<th>% RSD at 20ms dwell time</th>
<th>Variable dwell time, ms</th>
<th>% RSD at variable dwell time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>in sample</td>
<td>inter sample</td>
<td>in sample</td>
<td>inter sample</td>
</tr>
<tr>
<td>$^{111}$Cd</td>
<td>12.80</td>
<td>2.02</td>
<td>30</td>
<td>1.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.68</td>
<td></td>
<td>0.26</td>
</tr>
<tr>
<td>$^{112}$Cd</td>
<td>24.13</td>
<td>1.92</td>
<td>30</td>
<td>1.36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.58</td>
<td></td>
<td>0.57</td>
</tr>
<tr>
<td>$^{114}$Cd</td>
<td>28.37</td>
<td>2.03</td>
<td>30</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.88</td>
<td></td>
<td>0.74</td>
</tr>
<tr>
<td>$^{116}$Cd</td>
<td>7.49</td>
<td>1.92</td>
<td>30</td>
<td>1.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.63</td>
<td></td>
<td>0.35</td>
</tr>
<tr>
<td>$^{63}$Cu</td>
<td>69.17</td>
<td>1.92</td>
<td>30</td>
<td>1.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.72</td>
<td></td>
<td>0.55</td>
</tr>
<tr>
<td>$^{65}$Cu</td>
<td>30.83</td>
<td>2.20</td>
<td>30</td>
<td>1.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.48</td>
<td></td>
<td>0.63</td>
</tr>
<tr>
<td>$^{60}$Ni</td>
<td>26.22</td>
<td>1.95</td>
<td>30</td>
<td>1.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.29</td>
<td></td>
<td>0.33</td>
</tr>
<tr>
<td>$^{61}$Ni</td>
<td>1.14</td>
<td>2.18</td>
<td>90</td>
<td>1.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.61</td>
<td></td>
<td>0.30</td>
</tr>
<tr>
<td>$^{62}$Ni</td>
<td>3.63</td>
<td>1.81</td>
<td>60</td>
<td>1.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.55</td>
<td></td>
<td>0.58</td>
</tr>
<tr>
<td>$^{206}$Pb</td>
<td>24.10</td>
<td>1.67</td>
<td>30</td>
<td>1.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.44</td>
<td></td>
<td>1.10</td>
</tr>
<tr>
<td>$^{207}$Pb</td>
<td>22.10</td>
<td>1.92</td>
<td>30</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.59</td>
<td></td>
<td>1.16</td>
</tr>
<tr>
<td>$^{208}$Pb</td>
<td>59.40</td>
<td>1.86</td>
<td>30</td>
<td>1.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.49</td>
<td></td>
<td>1.18</td>
</tr>
<tr>
<td>$^{65}$Zn</td>
<td>27.90</td>
<td>2.39</td>
<td>30</td>
<td>1.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.75</td>
<td></td>
<td>0.36</td>
</tr>
<tr>
<td>$^{67}$Zn</td>
<td>4.10</td>
<td>2.01</td>
<td>60</td>
<td>1.59</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.52</td>
<td></td>
<td>0.41</td>
</tr>
<tr>
<td>$^{68}$Zn</td>
<td>18.75</td>
<td>1.92</td>
<td>30</td>
<td>1.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.88</td>
<td></td>
<td>0.36</td>
</tr>
</tbody>
</table>
Addition of internal standards (IS) to calibration standards and samples helps to compensate for instrumental drift during the analysis run and possible matrix effects (Sartoros and Salin, 1999; Morton, 2005). Several criteria must be met to achieve satisfactory internal standardization. The internal standard element must be absent in the
samples or present at insignificantly low concentrations, it must be available in a high-purity form to avoid contamination of analyte elements, have no interference with measured isotopes or matrix elements and be close by mass to a measured isotope. The typical internal standard elements used in ICP-MS analysis include $^6$Li, $^{45}$Sc, $^{72,74}$Ge, $^{89}$Y, $^{113,115}$In, $^{103}$Rh, $^{169}$Tl and $^{232}$Th. Four elements, namely Sc, Ge, Y and In, were evaluated as possible internal standards for some isotopes to cover the mass range from 60 to 111. It should be noted that $^{232}$Th, which is the closest by mass IS to Pb isotopes, was not available. With exception to $^{115}$In, all evaluated internal standards showed good results for the instrumental drift correction (Fig. 2.4). The analysis of digested tissue samples showed presence of Ge in significant concentrations.

![Figure 2.4](image-url)  
*Figure 2.4. Correction for the instrumental drift on $^{111}$Cd signal with internal standards (10µg·L$^{-1}$ Cd solution in 1% HNO$_3$, total analysis time 3h).*

Thus, $^{45}$Sc and $^{89}$Y were chosen for further evaluation as an appropriate IS for all isotopes monitored in this study. The results of the instrumental drift corrections with both internal standards for all isotopes of interest are presented in Fig. 2.5. Increased signal intensity was observed on isotopes with m/z ratio in the range 60-67 when $^{45}$Sc correction was applied. Therefore, $^{89}$Y was chosen as in internal standard for further measurements.
Figure 2.5. Correction for the instrumental drift with Sc and Y as internal standards (10µg·L⁻¹ Cd solution in 1% HNO₃, total analysis time 3h).
Figure 2.5. Continued
2.4. Choice of a trace metal pre-concentration technique

ICP-MS exhibits low tolerance to total salt and organic content of the sample and in complex matrices the chemical interferences can drastically increase the detection limits and reduce the number of isotopes that can actually be used for analytical purposes due to spectral interferences (Patriarca, 1996). The most common matrix elements leading to the formation of interfering molecular ions include chlorine (Cl), phosphorus (P), sulfur (S), carbon (C) and nitrogen (N) (Plantz, 1996). Thus, some sample pretreatment often in a form of a pre-concentration/separation step may be required (Danielson et al., 1982; Wells et al., 1998; Hirata et al., 2001). To accomplish this task a solvent extraction, co-precipitation or solid phase pre-concentration techniques can be used. Solvent extraction is the oldest pre-concentration method, where metal separation is achieved by the addition of a chelating agent to an aqueous sample and extraction of formed metal complexes into an organic phase. Organic compounds with functional groups containing nitrogen (as in amines, amides or nitriles), oxygen (carboxylic, hydroxyl or ether functional groups) or sulphur (thiols, thiocarboxamides) atoms are capable of chelating trace metals (Camel, 2003). Solid phase extraction has received the most attention due to its simplicity, high concentration factor, low sample volume requirements and more environmentally friendly reagents used. The method employs similar principles of metal partitioning between solid and liquid phases. The sample is passed through a column containing a solid sorbent and retained metals are eluted from the solid phase by an appropriate solvent. Alternatively, functional chelating groups may be introduced into the sorbent (Camel, 2003; Çekiç et al., 2004; Lemos et al., 2005). A variety of commercially available sorbents and chelating agents creates multiple possibilities for modifications of existing methods and achieving high metal recoveries at reasonably high flow rates.

In spite of the wide application of the solvent and solid phase extraction methods, some limitations have been noticed. Some metals, such as Ni, Mn or Fe, can form kinetically inert non-extractable complexes with organic ligands present in the sample due to neutralisation prior to analysis resulting in low recoveries (Danielson et al., 1892; Mackey et al., 1997; Ndung’u et al., 2003). Co-precipitation methods may overcome the
limitations of solvent and solid phase pre-concentration techniques due to a different mechanism of extraction. The basic principle involves three steps. First, a trace element reacts with an organic/inorganic compound and forms a solid phase. Then, the major precipitate reacts with other metals to form chelates. Finally, solid particles are separated from the aqueous media and re-dissolved in acid or in an organic solvent, such as isobutyl methyl ketone (Hengwu et al., 1997). In spite of its simplicity, this technique received less attention than solid phase extraction.

There is a diversity of methods available for the determination of trace metals in seawater samples. However, many of them were evaluated using synthetic solutions with concentration of metals 10-100 times higher than in natural samples (Elci et al., 1997; Vasconcelos et al., 1997; Ramesh et al., 2002). As a result, it is not always clear how well the methods perform at low and realistic metal concentrations and the procedures may over or underestimate the metal levels at these low concentrations. The comparative study investigating the extraction efficiency of conventional solvent extraction, solid phase extraction and co-precipitation methods used for pre-concentration of Cd, Cu, Ni Pb and Zn from natural seawater samples showed that all techniques successfully removed interfering matrix ions (Komjarova and Blust, 2006). The traditional liquid-liquid extraction was the most efficient pre-concentration technique for a multi-elemental analysis with low experimental errors (Figure 2.6). However, the method was labour intensive, required large sample volumes, which are not always available, and employed environmentally unsafe solvents. Application of a novel Dowex Optipore V-493 resin instead of the traditionally used Amberlite XAD-4 for a solid phase extraction proved to be a good alternative to solvent extraction. High pre-concentration factors and low sample volume requirements were major advantages of the technique. However, a pre-treatment with UV irradiation was recommended for samples with high organic content. The cobalt co-precipitation method was successfully applied for the fast and accurate determination of Cu and Ni, satisfactory determination of Cd and Zn, but could not be used for Pb at low concentrations. Although high pre-concentration factors can be achieved, the relative large sample volume requirement is a disadvantage of this technique. Therefore, the
selection of the most suitable method depends on several factors, such as the metal of interest, sample volume available and practical operation.

Figure 2.6. Analysis of certified reference material BCR-403 (mean values ± standard deviation, N=3) performed by three extraction techniques.
2.5. Conclusions

The analysis methods were developed for the accurate determination of the trace metals in different matrices using a quadrupole ICP-MS instrument. The methods were applied in the following studies for the quantification of the trace metal uptake occurring in aquatic organisms at environmentally relevant concentrations. At such low exposure concentrations, the stable isotope approach was particularly useful for the characterization of the uptake of the essential metals, such as Cu and Zn, which are initially present in the organisms at high quantities.

2.6. References


Determination of Trace Metals in Different Matrices


Determination of Trace Metals in Different Matrices


CHAPTER 3

Determination of the Simultaneous Uptake of Cd, Cu, Ni, Pb and Zn by the Water Flea *Daphnia magna* from Water and the Green Algae *Pseudokirchneriella subcapitata*
Abstract

Accumulation and toxicological effects of water and dietary born metals in aquatic organisms can be potentially very different. Therefore, it is important to know the relative contribution of these different sources in metal exposure, availability and accumulation. In this chapter the uptake of Cd, Cu, Ni, Pb, and Zn by the green algae *Pseudokirchneriella subcapitata* and the water-flea *Daphnia magna* was studied during simultaneous exposure to the five metals at environmentally realistic concentrations from separate water and dietary routes. Green algae take up Cu faster compared to Cd, Ni, Pb and Zn and the distribution of metals between the external and internal compartments is metal and population growth stage dependent. The metal accumulation reached a steady-state within 24-48 hours for all metals. In daphnids the metal uptake rate constants from water (total metal concentration scale) were highest in case of Cu and lowest in case of Ni. The differences were accentuated when results were expressed on a free metal ion scale. Metal assimilation efficiencies in *Daphnia magna* from the food source varied with metal, ranging from approximately 80% in case of Cd to near 0% in case of Ni. For all five metals studied, water appeared to be the most important route of uptake by *Daphnia magna*.

3.1. Introduction

In aquatic organisms the accumulation and toxicity of trace metals highly depends on water chemistry, exposure conditions and exposure routes. In aquatic environments metals exist in different forms such as free ions, organic complexes or metals adsorbed on or trapped in (suspended) particles and food. Aquatic organisms are exposed to metals via these dissolved and solid phases and their importance for uptake depends on the concentrations and availability of the metals in these different phases and the structural and functional organization of the organisms (Moore and Ramamoorthy, 1984; Rainbow, 2002). Metal concentrations in food and other particles can be
considerably higher than in the dissolved phase, but that does not necessarily imply that the bioavailability and uptake from these sources is also higher (Luoma, 1989). The chemistry of the gill and gut environment is very different and the effects of digestive processes on metal speciation may result in drastic changes in metal availability, which are difficult to predict in advance. Once inside the organism, metals taken in from water and food do not necessarily follow the same internal pathways resulting in different internal tissue distributions, accumulation levels and toxicological effects (Luoma, 1995; Allen, et al., 1995; Roy and Hare, 1999; Chan et al., 2003; Sofyan et al., 2006a). Therefore, a realistic assessment of the effects of metals on aquatic biota has to consider the importance of the different exposure routes for metal uptake, accumulation and toxicity.

Although it is generally found that water is the most important source of metal uptake and toxicity in aquatic organisms (Wang and Fisher, 1996; Rainbow, 2002), a number of studies indicate that food and other particulate sources can be a primary source of metal uptake, especially at environmentally realistic metal concentrations (King et al., 2005; Goulet et al., 2007). The free ion activity and the biotic ligand model, which are currently used to predict and evaluate metal toxicity, are based on water exposure only scenarios (Luoma, 1995; Paquin et al., 2002). However, recent studies indicate that dietary metals are also important sources and may contribute to metal toxicity (Allen et al., 1995; Sofyan et al., 2006a). In these cases, the dietary assimilation of trace metals highly depends on the food concentration, diet type and metals involved. In particular, Cd assimilation efficiency in aquatic insects Sialis velata and Chaoborus punctipennis (Roy and Hare, 1999; Munger and Hare, 2000) or dietary assimilation of Ag in Daphnia magna (Lam and Wang, 2006) increases with decreasing food concentrations and assimilation of Ag, Co, Cs and Mn in Daphnia magna greatly depends on the algal species they graze on (Adam et al., 2002). Other studies demonstrate that water mites, caddisfly larvae and Daphnia magna readily accumulate Cd from a food source, while aqueous uptake is a dominating pathway for Zn accumulation in these organisms (Timmermans et al., 1992; Guan and Wang, 2004). Furthermore, the assimilation of Cd and Zn by barnacles was
found to be closely related to the metal distribution in the algal cytoplasm (Wang and Rainbow, 2000). The toxicological effect of the dietary consumed metals is evident in chronic studies with *Daphnia magna*, where elevated concentrations of Cu and Zn in algae caused reduction in growth and reproduction of organisms (De Schamphelaere et al., 2004a; De Schamphelaere and Janssen, 2004b).

*Daphnia magna* is a widely used standard organism for conducting life-cycle chronic toxicity tests, which are performed in the presence of green algae as food source. Therefore, if dietary metal uptake is an important bioaccumulation pathway the accumulation of metals by algae and transfer to the invertebrates may alter the total effective exposure metal concentration. Information on the accumulation and responses of *Daphnia magna* and other model organisms to metal exposure via water and/or food is limited. For some metals such as Cd or Zn studies with radioactive tracers have been performed to determine uptake and accumulation rates from water and food at ecologically relevant exposure concentrations. For other metals such as Cu, Ni or Pb, no such information is available although these elements are of equal environmental relevance (Paquin et al., 2002).

In the current study we have used stable isotopes of the elements cadmium (Cd), copper (Cu), nickel (Ni), lead (Pb) and zinc (Zn) to determine the importance of water and algae for the uptake of the five metals to the water-flea. The stable isotope tracer technique is a powerful technique to study metal accumulation and elimination processes in biota. In contrast to the radio isotopes approach, where the specific activity of the isotopes inside the organism is usually not known, the analysis of stable isotopes provides information on all isotopes of the same metal present in a given system (Croteau and Luoma, 2005). In addition, the technique can be applied to metals for which no or only short lived radio-tracers are available such as Cu. By labeling various compartments (e.g. water, food or sediment), each bioaccumulation pathway can be followed simultaneously or separately with discrimination among added metals and those originally present in the organism. Stable isotopes can be applied at environmentally relevant concentrations that enable to study the processes under realistic exposure concentrations (Croteau and
Luoma, 2005). In addition, the approach does not require the same safety measures and does not generate the waste disposal problems associated with radioactive tracer studies. In this work we applied the stable isotope tracer technique to investigate the simultaneous uptake of Cd, Cu, Ni, Pb, and Zn by the green algae *Pseudokirchneriella subcapitata* and the water flea *Daphnia magna* from water and the trophic transfer from algae to the water-flea during exposure at environmentally relevant metal concentrations.

### 3.2. Materials and methods

#### 3.2.1. Experimental design

The aim of the experiments was to investigate the simultaneous uptake of five metals under realistic exposure concentrations by the water-flea *Daphnia magna* from water and food. First, green algae were acclimated to medium hard fresh water (OECD guidelines, TG201, 1993) with concentrations of major cations (Na\(^+\), K\(^+\), Ca\(^{2+}\), Mg\(^{2+}\)) adjusted to match the *Daphnia* rearing medium. After acclimation the medium was spiked with stable isotopes of the metals at environmentally relevant concentrations and metal concentrations in algae were followed over seven days. Both intra- and extra-cellular metal concentrations in algal cells were measured. In the second part of the experiment *Daphnia magna* acclimated to medium hard fresh water (OECD guidelines, 202, 1993) were exposed to the same total metal concentrations via water (without food given) or fed labeled algae from the first part of the experiment and metal uptake was followed over 96 hours. By separating the water and food exposure routes, the contribution of each metal source to metal accumulation in *Daphnia magna* was determined. Simultaneous exposure to labeled food and water was not performed in the current experiment due to the absence of multiple low abundant isotopes of Cu and Pb that are required for the labeling of both water and food phases at the same time.
3.2.2. Culture maintenance/acclimation

Green algae *Pseudokirchneriella subcapitata* were cultivated from a stock obtained from the Culture Collection of Algae at the University of Göttingen, Germany. The growth medium used in the experiments was modified OECD medium TG 201 for conducting standard algal growth tests (OECD guidelines 201, 1993) with the following composition: 0.77mM NaHCO₃, 0.28mM NH₄Cl, 2mM CaCl₂·2H₂O, 0.5mM MgSO₄·7H₂O, 0.01mM K₂HPO₄ and 1mM EPPS buffer. The final pH of the medium was 8.0 ± 0.2. The medium was autoclaved in 1L flasks for 20min at 121°C and pre-equilibrated with 1mL of sterile trace metal solution (0.3mM FeCl₂·6H₂O, 3mM H₃BO₃, 2.1mM MnCl₂·4H₂O, 0.0063mM CoCl₂·6H₂O, 0.029mM Na₂MoO₄·2H₂O, 0.27mM Na₂EDTA·2H₂O) for at least 24 hours before inoculating with algae. Reagent grade chemicals purchased from VWR (Leuven, Belgium) and ultra pure water (MilliQ, R>18.2 MΩ) were used for the preparation of the medium. Algal cultures were maintained at 21±1°C and illuminated with a fluorescent light (4500Lx) under a 14h light: 10h dark regime. Compressed air with an in line nylon filter (0.22μm) was used for continuous aeration. To maintain exponential growth of algae, each week an inoculum enough to provide an initial cell density of 2 to 5×10⁴ cells·mL⁻¹ was taken from a pre-culture and transferred to 1L of a sterile medium. The culture was acclimated to the medium for three weeks. The number of cells (and cell volumes) in the culture was monitored on a daily basis using a Beckman Multisizer Z3 Coulter Counter.

The water-fleas used in the experiments originated from a healthy *Daphnia magna* stock reared in our laboratory for several years and originally supplied by the University of Ghent (Belgium). The stock culture was maintained in carbon filtered and aerated tap water under controlled laboratory conditions in 1L polypropylene aquaria. The metal uptake experiments were performed in the standard medium for conducting acute immobilization tests with *Daphnia magna* (OECD guidelines 202, 1993) with the following composition: 2mM CaCl₂·2H₂O, 0.5mM MgSO₄·7H₂O, 0.77mM NaHCO₃ and 0.077mM KCl. The animals were acclimated to the medium for at least 3 generations before starting the experiment. During the rearing period they were fed a mixture of the
green algae *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* in 3:1 ratio. Three times a week the medium was renewed and *Daphnia magna* were fed 400×10⁶ algal cells L⁻¹. The culture was maintained at 21±1°C under a 14h light:10h dark cycle. For all experiments, 15 to 16 day old daphnids were used, which were isolated within one to two days after birth.

### 3.2.3. Metal uptake experiments with *Pseudokirchneriella subcapitata*

To determine metal uptake in algae, the growth medium was spiked with 0.05µM ¹⁰⁶Cd, 0.15µM ⁶⁵Cu, 0.3µM ⁶²Ni, 0.05µM ²⁰⁴Pb and 0.15µM ⁶⁷Zn as nitrate salts (STB Isotope Germany GmbH) and inoculated with 2-5×10⁴ cells·mL⁻¹ of algal culture. The background trace metal concentrations in the medium were less than 0.001µM for Cd, Pb and less than 0.005µM for Cu, Ni and Zn. Since the experiments were conducted in closed flasks and the air for aeration was filtered though a 0.22µm nylon filter, no increase in the metal background concentrations was observed. Free ion metal concentrations and activities in the medium were calculated using Visual MINTEQ 2.4b (Table 3.1). The intra-cellular and extra-cellular metal content of the algae was determined each day according to a procedure adopted from Franklin et al. (2000) and was followed for seven days, which was the time when an algal population growth cycle was completed. Briefly, 50mL of the culture was withdrawn daily from each replicate. Ten mL was used for cell counting and 40mL of the culture was filtered through an acid-washed, pre-weighed 0.45µm cellulose nitrate membrane (NC45, Schleicher & Schüll®) and rinsed with 20mL Milli-Q™ water. The filtrate was acidified to 2% HNO₃ with 69% concentrated nitric acid and analyzed for dissolved metals. Filters with algae were placed in 50mL polypropylene tubes, extracted with 20mL of 0.02M Na₂EDTA for 20 minutes using an orbital shaker and filtered back on the same membrane. The extract was analyzed for adsorbed metals and the filtered algae were washed with Milli-Q™, dried at 60°C and the dry weight of the samples was determined to the nearest 0.100 ± 0.01mg. For the metal measurements, dried algae were digested in 0.5mL of 69% (v/v) HNO₃ in a microwave for 10 minutes together with filters. The samples were diluted with 10mL of
MQ and analyzed for absorbed metals. The data were corrected for metal concentrations in procedural blanks and blank filters.

**Table 3.1.** Total and free-ion metal concentrations and activities in daphnids and algae growth media calculated with the Visual Minteq2.4b speciation model

<table>
<thead>
<tr>
<th>Metal</th>
<th>Total metal, μM</th>
<th>Medium for daphnids</th>
<th>Medium for algae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free ion, μM</td>
<td>Activity, μM</td>
<td>Free ion, μM</td>
</tr>
<tr>
<td>Cd²⁺</td>
<td>0.05</td>
<td>0.036</td>
<td>0.025</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>0.15</td>
<td>0.008</td>
<td>0.006</td>
</tr>
<tr>
<td>Ni²⁺</td>
<td>0.32</td>
<td>0.268</td>
<td>0.183</td>
</tr>
<tr>
<td>Pb²⁺</td>
<td>0.05</td>
<td>0.004</td>
<td>0.003</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>0.15</td>
<td>0.107</td>
<td>0.073</td>
</tr>
</tbody>
</table>

3.2.4. Metal uptake experiments with *Daphnia magna*

All uptake experiments were carried out in 750mL polypropylene containers, each containing 700mL of medium and 25 to 30 daphnids. In the water exposure experiments the medium was spiked with 0.05μM ¹⁰⁶Cd, 0.15μM ⁶⁵Cu, 0.3μM ⁶²Ni, 0.05μM ²⁰⁴Pb and 0.15μM ⁶⁷Zn. The test organisms were not fed during the 96h water exposure. In the food exposure experiment *D. magna* feeding was stopped 24 hours prior to the initiation of the experiment to allow partial depuration of the gut. Complete depuration is not obtained after 24 hours when animals are not fed new algae (Gillis et al., 2005). Then, *Daphnia* were placed in a non-metal spiked medium and fed with green algae *Pseudokirchneriella subcapitata* uniformly labelled with stable isotopes providing a cell density of 400×10⁶ cells·L⁻¹. The medium and food were renewed daily and cell density in the exposure medium was recorded at the beginning and at the end of each renewal. In order to monitor the possible loss of metals from algae to water, three...
replicates of test medium containing only labeled algae and no Daphnia magna were included in the experiment. There were three replicates for each treatment.

Each day water and daphnids were sampled to be analyzed for metals. Ten to fifteen animals were collected from each replicate and rinsed with MQ. In the food experiment the animals were allowed to depurate their gut contents for six hours. Note that depuration without feeding does not provide efficient gut clearance (Gillis et al., 2005). The organisms were dried at 60°C to a constant weight and microwave digested with 69% HNO₃. As well, 20mL of water was filtered through a 0.45μm cellulose nitrate membrane and acidified with 69% HNO₃.

3.2.5. Analytical and computational procedures

All samples were analysed for metals by inductively coupled plasma mass spectrometry (Varian Expert 700 quadrupole ICP-MS, Mulgrave, Australia). The mass spectrometer was calibrated with mixed ICP-MS standards and yttrium as an internal standard. Although the instrument drift was not significant, the machine was recalibrated every 15 to 20 samples. Prior each measurement, the instrument settings were optimised to minimise a formation of interfering oxide and double charged ions, which were kept below 1% and 3% respectively. At least two isotopes were measured for each metal. Non-tracer isotopes, which were not added to the exposure medium (i.e. ¹⁰⁶Cd, ⁶³Cu, ⁶⁰Ni, ²⁰⁸Pb, ⁶⁴Zn), were used for background concentration corrections or determination of the concentrations of metals already present in the organism based on known natural abundances of isotopes. For example, to calculate the background concentration of ¹⁰⁶Cd, ¹¹¹Cd was also monitored and the following equation was applied:

\[
[¹⁰⁶Cd]_{background} = \left[¹¹¹Cd\right]_{sample} \times \left(\frac{abundance^{¹⁰⁶Cd}}{abundance^{¹¹¹Cd}}\right)
\]  

(1)

\[
[¹⁰⁶Cd]_{background} = \left[¹¹¹Cd\right]_{sample} \times \left(\frac{1.21}{12.75}\right)
\]  

(2)
Calculated background isotope concentrations of spiked isotopes were subtracted from measured values to determine the concentration of newly accumulated isotopes.

The algae growth rate was calculated by plotting the natural log of cell number versus time and fitting a Gompertz growth equation to the data:

\[
\frac{2.718\mu}{c}(\text{Lag} - t) + 1
\]

\[\ln(N) = N_0 + C \cdot e^{-e} \quad (3)\]

where \(N_0\) and \(N\) is the initial and current cell number, \(C\) is the difference between initial and final cell number, \(\mu\) is the maximum specific growth rate, \(d^{-1}\), \(\text{Lag}\) is the lag phase (delay before growth, \(d\)) and \(t\) is time, \(d\).

Filtration (\(F\)) and ingestion (\(I\)) rates were estimated on a daily basis from a change in algal cell density in the sample and control containers over 6 to 24 hour depending on the number of daphnids remaining in the vessel, which was sufficient to achieve at least a 25% change in algal density in a test solution.

\[
F = \frac{V \cdot (\ln C_0 - \ln C_t)}{nt} - \frac{\ln C_0 - \ln C'_t}{t} \quad (4)
\]

\[I = F \sqrt{C_0 C_t} \quad (5)\]

where \(V\) is the volume of the test medium (ml), \(C_0\) and \(C_t\) are the initial and final food concentrations (cells·ml\(^{-1}\)) in the exposure aquaria, \(C'_t\) is the final cell concentration in a control aquaria with algae only, \(t\) is the time of exposure (h) and \(n\) is a number of \(Daphnia magna\) per aquarium.
Uptake of Cd, Cu, Ni, Pb and Zn by Daphnia from water and algae

The metal uptake rates from water were estimated using zero and first order accumulation kinetics. In general terms, the accumulation process can be described by a simple differential equation

\[
\frac{dC_{\text{organism}}}{dt} = \text{accumulation rate} = k_u \cdot C_{\text{water}} - k_e \cdot C_0 \quad (6)
\]

where \( C_{\text{organism}} \) is the concentration of the metal in the organism on a dry weight basis (µmol·kg\(^{-1}\)), \( C_0 \) is the initial metal concentration in the organism at \( t=0 \), \( C_{\text{water}} \) is the concentration of the metal in the water phase (µmol·L\(^{-1}\)), \( t \) is the exposure time (d), \( k_u \) is the uptake rate constant (d\(^{-1}\)), \( k_e \) is the elimination rate constant (d\(^{-1}\)). When the elimination process is negligible (\( k_e = 0 \)), the uptake rate becomes directly proportional to the metal concentration in water resulting in a simple linear function:

\[
C_{\text{organism}} = k_u \cdot C_{\text{water}} \cdot t + C_0 \quad (7)
\]

When the elimination term is significant, the integration of Equation 6 has the following solution:

\[
C_{\text{organism}} = C_{\text{water}} \cdot \frac{k_u}{k_e} \cdot \left( 1 - e^{-k_e \cdot t} \right) + C_0 \quad (8)
\]

In case of uptake from food the equation takes the following form:

\[
C_{\text{organism}} = C_{\text{food}} \cdot \frac{IR \cdot AE}{ke_{\text{food}}} \cdot \left( 1 - e^{-ke_{\text{food}} \cdot t} \right) + C_0 \quad (9)
\]
where IR is the ingestion rate, AE is the assimilation efficiency and $IR \cdot AE = k_u \text{food}$. Combination of both models provides a model for the accumulation from both water and food in a form:

$$C_{organism} = C_{water} \frac{k_u \text{water}}{k_e \text{water}} \left( 1 - e^{-ke \text{water} \cdot t} \right) + C_{food} \frac{IR \cdot AE}{ke \text{food}} \left( 1 - e^{-ke \text{food} \cdot t} \right) + C_0 \tag{10}$$

Note, that a term accounting for growth dilution (i.e; growth rate constant ($k_g$)) was not included because growth was not significant in these adult animals over the short exposure period. GraphPad Prism4 and Statistica6 software was used for all curve fittings and statistical analysis.

### 3.3. Results and discussion

#### 3.3.1. Metal accumulation in algae

After 21 days no further changes in three consecutive growth cycles were observed and the algae were considered to be acclimated to the medium. A population growth cycle was completed within 6 to 7 days. The lag period lasted for 1 to 2 days followed by an exponential growth phase (from 2 to 4 days) and from day 5 a steady-state growth phase was achieved. The growth rate was determined by fitting experimental data to a Gompertz growth equation (eq.3). The lag period and the maximum specific growth rate estimated by this equation were equal to $0.95 \pm 0.37d$ and $0.921 \pm 0.096d^{-1}$ respectively. The obtained value of growth rate is comparable with data reported by Bossuyt and Janssen (2004), where under similar conditions a growth rate of $Pseudokirchneriella subcapitata$ in the range of $0.92$ to $0.94d^{-1}$ was observed. During the stable isotope exposure experiments metal concentrations in algae and water were...
measured daily for seven days. The total, adsorbed (external), and absorbed (internal) metals concentrations are presented in Fig. 3.1. The total concentrations of the metals increased very rapidly from day one to day two, the time when the algae were in their exponential growth phase. In the following days of exposure the total concentrations showed some fluctuation but remained more or less constant for four of the five studied metals. In case of Cu, a decrease in total concentration was observed following day two, which stabilised after day four. The maximum internal concentration of about 1800 µmole·kgdw⁻¹ was reached after two days and then decreased to 800-1100µmole·kgdw⁻¹ during the next few days. In contrast to Cu, the other important essential metal Zn did not show this type of regulation and internal Zn concentrations were increasing over the entire exposure period. The distribution of the metals between the external and internal fraction changes as a function of exposure time and was very different among the metals.

During the first four days, higher concentrations of Cd, Ni and Zn were found on the outer surface than inside the cells. In the course of the experiment a decrease in concentrations of externally adsorbed Cd, Ni and Zn was observed accompanied by an increase in internal metal concentrations. Internal Cu and Pb concentrations were always considerably higher than external concentrations. The bioconcentration factors (BCFs) for the specific exposure conditions calculated at day 2 and 7 using nominal metal concentrations in water (L·kgdw⁻¹) were 4545±973 – 4325±257 for Cd, 13336±1281 – 7230±966 for Cu, 3681±308 – 4036±371 for Zn, 2081±118 – 2215±170 for Ni and 2219±445-6135±389 for Pb. The interaction between algae and dissolved metal involves three major steps: metal diffusion to the algal surface, adsorption on binding sites available on the algal surface and intracellular uptake or internalization.
Figure 3.1. Metal accumulation in green algae *Pseudokichneriella subcapitata* in the presence of 0.05µM $^{106}$Cd, 0.15µM $^{65}$Cu, 0.3µM $^{62}$Ni, 0.05µM $^{204}$Pb and 0.15µM $^{67}$Zn (0.77mM Na$^+$, 2mM Ca$^{2+}$, 0.5mM Mg$^{2+}$, pH7, t=21°C, mean ± S.D., N=3).
In general, binding to the cell wall is considered to be a very fast process, while intracellular uptake and in some cases metal diffusion to the biological surface are rate limiting steps (Vercauteren, 2000).

Among the metals, Cu was accumulated by algae in the greatest quantity even though the concentration of free Cu in the medium was only 5.3% of total Cu on a molar basis. Its primary intracellular location indicates faster Cu transfer across cell wall and membranes than that of the other metals. Fast Cu uptake by different algae species compared to other metals has been demonstrated in several studies (Sunda and Huntsman, 1995; Vasconcelos et al., 2001). Our data correspond well with the studies performed by Knauer et al. (1997), where authors found that *Scenedesmus subspicatus* accumulated 35% of Cu intracellularly after only four hours of exposure to Cu and at day five the intracellular Cu accounted for 80% of the total Cu concentration. The algae also exhibited greater ability to accumulate Cu than Zn or Mn whether expressed on a total or free ion basis.

Another study performed by Bossuyt and Janssen (2005) reported the internal Cu concentration in *Pseudokirchneriella subcapitata* in a range from 1200 to 1250μmole·kgdw⁻¹ (79-82μg·gdw⁻¹) after three to six week exposure to 0.19μM Cu, which is comparable with our data. Based on the BCFs and exposure conditions, the following metal accumulation series can be constructed if free metal concentrations are used for calculations: Cd<Zn<Ni<Pb<Cu. However, the order changes to Ni<Pb<Cd<Zn<Cu if total metal concentrations are used. The latter dependence resembles the increase in metal binding capacities found in *Chaetophora elegans* (Andrade et al., 2005). The authors reported an increase in complexation constants in a row Ni(II)<Cd(II)<Pb(II)<Zn(II) in the presence of 0.1M NaNO₃. The short term Cd and Zn accumulation in *Scenedesmus obliquus* at exposure levels 0.02μM and 0.08μM of Cd and Zn respectively demonstrated the percentage of intracellular metals did not exceed 10-12% and the Cd uptake rate was lower (0.093μg·g⁻¹·h⁻¹) than that for Zn (1.154μg·g⁻¹·h⁻¹) (Yu and Wang, 2002). In general the metal sorption capacity is species and metal dependent with Cu being taken up much faster than any other metal in this study.
3.3.2. Metal accumulation in *Daphnia magna*

3.3.2.1. Metal exposure via water

Initial background concentrations of the naturally occurring metals present in the medium did not exceed 0.5nM Cd, 0.01µM Cu, 0.05nM Ni and Pb, and 0.03µM Zn. There were no significant changes in the Cd and Pb background concentrations during the experiment. Airborne contamination in uncovered experimental aquaria led to an increase in the background concentrations of Cu, Ni and Zn. Taking into account the natural abundances of $^{65}$Cu, $^{62}$Ni and $^{67}$Zn, this contamination resulted in a small addition to the amount of spiked isotopes. The concentrations of spiked isotopes increased to 0.157µM $^{65}$Cu, 0.301µM $^{62}$Ni and 0.159µM $^{67}$Zn towards the end of the experiment. Furthermore, we were able to follow the uptake of enriched isotopes and naturally occurring metals separately and thus, make a correction for the change in metal concentration.

The metal accumulations in *Daphnia magna* showing changes in stable isotopes, originally present (naturally occurring) and total metal concentrations in the organisms as function of time are presented in Fig. 3.2. Newly absorbed $^{106}$Cd, $^{62}$Ni and $^{204}$Pb accounted for 95-99% of total metals present in *Daphnia*, while newly absorbed $^{65}$Cu comprised 40-49% and newly absorbed $^{67}$Zn only 5-15% of the total Cu or Zn present in the organisms. The increase in the body concentrations of naturally occurring Cu, Ni and Zn is explained by the inadvertent introduction of airborne metals during the course of the experiment.

The initial and accumulated Cu and Zn concentrations in *Daphnia magna* found in this study correspond well with the literature data. It was shown by Bossuyt and Janssen (2005) that depending on the composition of the medium, the Cu content of *Daphnia magna* varied between 17-25µg·gdw$^{-1}$ (260-380µmole·kgdw$^{-1}$) at a total dissolved Cu concentration of 0.18µM and increased to 35-72µg Cu·gdw$^{-1}$ (535-1100µmole·kgdw$^{-1}$) when the total dissolved Cu concentration was 1.54µM. Studies performed by Muyssen and Janssen (2002) demonstrated that *Daphnia magna* contained 3058-3820µmole Zn·kgdw$^{-1}$ when exposed to 4.6-9.2µM (Zn) and accumulated about
1375µmole Zn·kgdw⁻¹ when exposed to lower 0.045-0.2µM (Zn) concentrations. Even though in our study *Daphnia magna* were cultured in the medium with low Cu and Zn concentration in water (0.05-0.1µM), the metal content of algae fed to the organisms during the rearing period was relatively high (9930µmole Cu·kgdw⁻¹ and 1580µmole Zn·kgdw⁻¹). Thus, the body concentrations of natural Cu and Zn were equal or higher than the concentrations of the accumulated stable isotopes ⁶⁵Cu or ⁶⁷Zn.

The uptake of Cd, Zn, Ni, and Pb by *Daphnia magna* from the water phase could be described by simple linear uptake kinetics without evidence of elimination during the four day exposure period. The uptake rate constants for newly absorbed isotopes presented in Table 3.2 were obtained by taking the slope of the linear regression (eq.7) between the metal concentration in the organisms (µmole·kgdw⁻¹) and the duration of exposure (days) and dividing it by the total concentration of metal in water. In our study the uptake of Cu followed first order accumulation kinetics showing clear evidence of saturation. In order to estimate the uptake and elimination rate constants for Cu presented in Table 3.2, the concentration of metal in the body (µmole·kgdw⁻¹) was plotted as a function of time and the data fitted to a one compartmental accumulation model (eq.8).

Among the five metals the uptake rate constant increased in the following order from Ni<Zn<Cd<Pb<Cu. The trend remained the same when expressed on a free metal ion concentration or activity basis. However, the free metal ion based uptake rates constants for Cu and Pb showed a strong increase because of the more pronounced complexation effect for these two metals compared to the three others. Similar Cd and Zn results were reported by Yu and Wang (2002), Guan and Wang (2006), Tan and Wang (2008), where a radiotracer technique was used. When *Daphnia* were exposed to 0.02µM(Cd) and 0.08µM(Zn) in the medium soft creek water (0.6mM Ca²⁺, 0.13mM Mg²⁺, 0.25mM SO₄²⁻, pH8.2, DOM not reported) uptake rate constants of the same magnitude were reported (1500 and 1100L·kgdw⁻¹·d⁻¹ respectively).
Figure 3.2. Metal uptake of new isotopes in *Daphnia magna* exposed to 0.05µM $^{106}$Cd, 0.15µM $^{65}$Cu, 0.3µM $^{62}$Ni, 0.05µM $^{204}$Pb and 0.15µM $^{67}$Zn via water (0.77mM Na$^+$, 2mM Ca$^{2+}$, 0.5mM Mg$^{2+}$, pH7, t=21°C, mean ± S.D., N=3). Solid lines represent modeled isotope uptakes.
Little is known about Ni and Pb uptake kinetics in *Daphnia magna* and no data on Ni and Pb uptake rates in *Daphnia magna* to compare with could be found in literature. Studies performed by Pane et al. (2004) indicated that internal Ni concentration in *Daphnia* exposed to 0.14–0.34µM(Ni)·L⁻¹ varied between 85–850µmole·kgdw⁻¹.

**Table 3.2.** Cd, Cu, Ni, Pb, and Zn uptake rate constants (total metal concentration scale) and assimilation efficiencies (IR=0.17076 kgdw(algae)·kgdw(daphia)⁻¹·d⁻¹) in *Daphnia magna* exposed to metal isotopes mixtures via water or algae (N=3, mean ± SE)

<table>
<thead>
<tr>
<th>Metal</th>
<th>$k_u$ water L.kgdw⁻¹d⁻¹</th>
<th>$k_e$ water L.kgdw⁻¹d⁻¹</th>
<th>$R^2$</th>
<th>$R^2$</th>
<th>$AE$ food kgdw.kgdw⁻¹d⁻¹</th>
<th>$k_e$ food kgdw.kgdw⁻¹d⁻¹</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{106}$Cd</td>
<td>1341 ± 116</td>
<td>-</td>
<td>0.98</td>
<td>0.81 ± 0.23</td>
<td>0.65 ± 0.23</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td>$^{65}$Cu</td>
<td>3745 ± 1529</td>
<td>0.76 ± 0.36</td>
<td>0.96</td>
<td>0.61 ± 0.07</td>
<td>1.43 ± 0.18</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>$^{62}$Ni</td>
<td>875 ± 85</td>
<td>-</td>
<td>0.97</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>$^{204}$Pb</td>
<td>1770 ± 251</td>
<td>-</td>
<td>0.94</td>
<td>0.06 ± 0.09</td>
<td>1.60 ± 2.58</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>$^{67}$Zn</td>
<td>1228 ± 74</td>
<td>-</td>
<td>0.99</td>
<td>0.32 ± 0.02</td>
<td>0.32 ± 0.04</td>
<td>0.99</td>
<td></td>
</tr>
</tbody>
</table>

Cu and Zn are essential elements, which are present in organisms at significantly higher concentrations compared to other metals. In contrast to Cu and Zn, lower quantities of Ni are required by organisms, while Cd and Pb are toxic even at low body concentrations. In general, aquatic organisms such as algae and crustaceans are able to regulate the accumulation of essential metals by maintaining constant metal concentrations through excreting the metals at rates comparable to uptake rates. Studies showed that *Daphnia magna* are able to regulate total concentrations of Cu and Zn in the body in response to elevating metal concentrations in the medium, although it was not
known whether these regulations were due to a reduction of metal uptake, or enhanced excretion (Muysen and Janssen, 2002; Bossuyt and Janssen, 2005). The results obtained in the current study show regulation of Cu accumulation but no indication of regulation of Zn accumulation of either the Zn isotope spike or the total Zn concentration within the concentration range and short exposure period.

3.3.2.2. Metal exposure via food

Measured nominal concentrations of metals in the algae used during the four day exposure were $468 \pm 24\mu\text{mole}\cdot\text{kg}_{\text{dw}}^{-1}$ of $^{106}\text{Cd}$, $1452 \pm 77\mu\text{mole}\cdot\text{kg}_{\text{dw}}^{-1}$ of $^{65}\text{Cu}$, $395 \pm 33\mu\text{mole}\cdot\text{kg}_{\text{dw}}^{-1}$ of $^{62}\text{Ni}$, $2326 \pm 167\mu\text{mole}\cdot\text{kg}_{\text{dw}}^{-1}$ of $^{204}\text{Pb}$ and $673 \pm 79\mu\text{mole}\cdot\text{kg}_{\text{dw}}^{-1}$ of $^{67}\text{Zn}$. The ingestion and filtration rates in control daphnids (eq.s 4 and 5) and those exposed to contaminated food were nearly constant over the entire experiment and were in the range $1.14 \times 10^5$ to $1.88 \times 10^5 \pm 0.3 \times 10^5 \text{cell}\cdot\text{daph}\cdot\text{h}^{-1}$ and 0.506 to 0.52 $\pm 0.12 \text{mL}\cdot\text{daph}\cdot\text{h}^{-1}$, respectively. A control experiment with labelled algae placed in OECD water showed very limited metal release from algal cells to the medium and therefore no isotope uptake from the water phase occurred or was minimal. Accumulation of $^{106}\text{Cd}$, $^{65}\text{Cu}$ and $^{67}\text{Zn}$ was greatest in the first 24 hours (Fig. 3.3). No further accumulation of $^{204}\text{Pb}$ was observed after this time. In case of $^{65}\text{Ni}$ the uptake was near to zero or at least very low and not quantifiable in terms of the assimilation efficiency. The assimilation efficiencies were obtained by fitting the accumulation results to a one compartmental accumulation function (eq.9) with ingestion rate (IR) included in the equation as a fixed value (i.e. $0.17076\text{kg}_{\text{dw}}(\text{algae})\cdot\text{kg}_{\text{dw}}(\text{daphnia})^{-1}\cdot\text{d}^{-1}$). The obtained elimination rate constant ($k_e$) was used to estimate the possible loss of metal from the body during the 6h gut depuration period. If the results indicated that this loss was of any significance then a correction was made to the original data on the basis of the elimination rate constant obtained and the results were fitted again to equation 9. The metal assimilation efficiencies and elimination rate constants estimated by fitting the experimental data to the metal accumulation from food function are presented in Table 3.2.
Figure 3.3. Metal uptake of new isotopes in *Daphnia magna* fed with 400×10^6 cells L⁻¹·d⁻¹ of labeled algae *Pseudokichneriella subcapitata* (0.77mM Na⁺, 2mM Ca²⁺, 0.5mM Mg²⁺, pH7, t=21°C, mean ± S.D., N=3). Solid lines represent modeled isotope uptakes.
During the first days of exposure, elimination of naturally occurring Cd, Cu, and Zn was observed, which occurred at higher rates than the uptake of the newly accumulated metal isotopes. As a result, the total Cu and Zn concentrations decreased during first the 24 hours and then remained constant. The elimination of naturally occurring Cd had less impact on the total metal concentration since not much Cd was originally present in the organisms. The fast elimination of a fraction of the originally present Cd, Cu and Zn most likely reflects the further depuration of previously ingested metals from the gut which were not removed during the 24 hours starvation period prior to the start of the dietary exposure experiment. It has indeed been reported that starvation does not result in a complete depuration of the gut (Gillis et al., 2005). The results showed relatively high assimilation efficiencies and elimination rate constants for Cd (AE: 0.81±0.23; ke 0.65±0.23), Cu (AE: 0.61±0.07; ke 1.43±0.18) and Zn (AE: 0.32±0.02; ke 0.32±0.04). In contrast the assimilation efficiency obtained in the case of Pb (AE 0.06±0.09; ke 1.60±2.58)-was much lower and the high ke value resulted in a steady-state situation reached within 24 hours after the start of the exposure. In case of Ni, accumulation from the dietary source was negligible and the assimilation efficiency near zero. Note that the ke values were obtained over a short accumulation period and only reflect elimination from the rapidly exchangeable pool and are therefore higher than values obtained in more chronic exposure and elimination studies reflecting elimination from the slower exchangeable pools.

Studies on the relative importance of water and dietary metal uptake by Daphnia magna are only available for a limited number of metals including Cd and Zn. Yu and Wang (2002) showed that the AE of Cd and Zn by D. magna is dependent on algal diet and concentration and ranged from 30-77% for Cd and 7-66% for Zn with the lowest values observed at the highest food concentrations. Guan and Wang (2004) investigated the dietary assimilation of Cd and Zn by Daphnia magna from two types of green algae (Scenedesmus obliquus and Chlamydomonas reinhardtii) exposed to metals for 3 days at different concentrations. When algae were loaded with trace metals at ambient concentrations similar to that used in the present study (0.009-0.046µM(Cd) and 0.031-
0.154µM(Zn)), the Zn assimilation efficiency did not exceed 5-7% from both types of algae. Assimilation efficiency of Cd was higher and depended on the type of algae and ranged from 10 to 36% when Scenedesmus obliquus were used as a food source and from 44 to 51% when Daphnia magna were fed Chlamydomonas reinhardtii. In another study water and dietary Cd uptake by different clones of Daphnia magna using Chlorella vulgaris as a food source was also investigated (Barata et al., 2002). Although the Cd uptake from water varied a lot between the clones, it was always 2-3 folds higher than that from food. The authors indicated that water was the major source of Cd uptake in Daphnia magna when total Cd concentration in ambient medium was 0.09µM. No literature was found on the uptake of Ni or Pb by Daphnia magna from a food source.

Several studies have demonstrated the decrease in Daphnia feeding rates when the organisms were fed Cd or Cu enriched algae. The signs of the digestive system poisoning were noticed of the Daphnia in the forms of decreased enzymatic activity in the gut and altered feeding mechanism (Taylor et al., 1998; Sofyan et al., 2006 b). Our results indicate that at environmentally realistic exposure concentrations, the feeding rates are not affected by Cd or Cu and the assimilation efficiencies are quite high.

To estimate the relative importance of water and food under the given exposure scenarios equation 10 was used to calculate the relative contribution of both sources for the five metals studied. This was done under the assumption that metal uptake from both sources is additive (Goulet et al., 2007). The results shown in Table 3.3 represent the relative contribution of water and food after one day and four days of exposure and show that for all five metals waterborne exposure is the main source of metal uptake. D. magna accumulates the largest portion of dietary metals at the first day of exposure followed by a decrease in the uptake of metals from algae. Waterborne metals, in turn, continue to accumulate in the body of D. magna as the time of exposure progresses. It should be noted that among the five studied metals Cd has the largest AE from the dietary source. Nevertheless, the Cd accumulation from a food source becomes a dominant route of uptake only at low external free Cd ion concentrations (<1nM Cd$^{2+}$), as demonstrated by Goulet et al. (2007), while water exposure became the most important route at higher
exposure concentrations (>10nM Cd\(^{2+}\)). Although the exposure conditions were not exactly the same, the results of our simulation presented in Table 3.3 are in reasonable agreement. Our results indicate that the dietary route contributes 42% after one day of exposure and 26% after four days of exposure of the newly accumulated Cd in the body under our experimental conditions. Similar calculations performed based on the data presented by Goulet et al. (2007) resulted in an estimated value of 52% as chronic Cd contribution from the food source under a similar Cd concentration in water, but somewhat different exposure conditions. Therefore, as shown by our and literature results, it is important to include the dietary uptake component in the current BLM models, at least for such toxic element as Cd, when low exposure concentrations are considered.

### Table 3.3. Relative importance of Cd, Cu, Ni, Pb, and Zn uptake via water and algae by *Daphnia magna* after 1 and 4 days of exposure based on the fraction of bioaccumulated metals from water and dietary sources (eq.10).

<table>
<thead>
<tr>
<th>Metal</th>
<th>Water/Algae 1 day exposure</th>
<th>Water/Algae 4 day exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{106})Cd</td>
<td>0.58/0.42</td>
<td>0.74/0.26</td>
</tr>
<tr>
<td>(^{65})Cu</td>
<td>0.83/0.17</td>
<td>0.87/0.13</td>
</tr>
<tr>
<td>(^{62})Ni</td>
<td>1.00/0.00</td>
<td>1.00/0.00</td>
</tr>
<tr>
<td>(^{204})Pb</td>
<td>0.88/0.12</td>
<td>0.96/0.04</td>
</tr>
<tr>
<td>(^{67})Zn</td>
<td>0.85/0.15</td>
<td>0.90/0.10</td>
</tr>
</tbody>
</table>

### 3.4. Conclusions

The present study illustrates that the stable isotope technique is a powerful tool to investigate the uptake and accumulation kinetics in aquatic biota. It allowed us to follow simultaneously the uptake of Cd, Cu, Ni, Pb, and Zn in algae and daphnids. The approach
Uptake of Cd, Cu, Ni, Pb and Zn by Daphnia from water and algae provides information on the behaviour of both naturally present and spiked isotopes of the same element and the technique is especially valuable for metals for which no or only short-lived radio-isotopes exist, such as Cu. Since the data for the different metals are obtained on the same multi-metal exposed organisms, the results are directly comparable among the metals, which is another important advantage of the approach. The results show the important differences in metal uptake kinetics and assimilation efficiencies that exist among the different metals for both algae and daphnids. Aqueous metal exposure was found to be the dominant uptake pathway at environmentally relevant concentrations with short term exposures.

3.5. References

Adam, C., Gardnier-Laplace, J., Baudin, J.P., 2002. Bioaccumulation of $^{110}$Ag, $^{60}$Co, $^{137}$Cs and $^{54}$Mn by the freshwater crustacean Daphnia magna from dietary sources (Scenedesmus obliquus and Cyclotella meneghiana). Water Air Soil Pollut. 136, 125-146.


CHAPTER 4

Effect of Na, Ca and pH on the Simultaneous Uptake of Cd, Cu, Ni, Pb and Zn in the Water Flea *Daphnia magna*
Abstract

The present chapter discusses the effects of Na⁺, Ca²⁺ and pH present in the exposure medium on the kinetics of Cd, Cu, Ni, Pb, and Zn uptake in Daphnia magna. The Cu uptake was not significantly affected by variations in chemical composition of the test medium. Calcium had a suppressing effect on the uptake of Cd, Ni, Pb and Zn. Specifically, Cd and Ni uptake rate constants were linearly decreasing with increase in calcium concentrations from 0.1 to 2.5mM. The uptake of Zn and Pb was significantly suppressed only at 2.5mM Ca. The effect of sodium was less clear. There was no effect from varying sodium concentrations on the Ni uptake rate constants. Cd and Pb showed an increase in the uptake rate constants at elevated sodium concentrations (2-8mM Na⁺ for Cd and 8mM Na⁺ for Pb). A bell-shaped response on increasing Na⁺ concentrations was observed for Zn with a maximum value of uptake rate constant at the middle value (2mM Na⁺). Variation in pH of the medium affected Cd, Ni and Zn uptake processes. When Daphnia were exposed to acidic conditions (pH6), the Cd and Ni uptake rate constants were the highest, while similarly low values were observed at neutral and basic conditions. In contrast, the uptake rates of Zn were linearly increasing with increasing pH of the medium.

4.1. Introduction

The accumulation of metals in aquatic organisms is known to be highly dependent on water chemistry characteristics. Concentrations of major naturally occurring cations (Ca²⁺, Mg²⁺, Na⁺), presence of dissolved organic matter and pH are important factors affecting trace metals accumulations in aquatic organisms. It is well documented for a number of aquatic organisms that hard waters have a protective effect against Cd toxicity as a result of competitive interactions between Cd²⁺ and Ca²⁺ ions for binding sites. Recently, Pb and Zn have been added to the list of metals interfering with Ca²⁺ transporting system (Niyogi and Wood, 2004; Rainbow, 1997). Sodium, in turn, has been shown to affect the acute Cu toxicity in some freshwater species (De Schamphelaere
Effect of Na, Ca and pH on uptake of Cd, Cu, Ni, Pb and Zn in Daphnia

and Janssen, 2002; Borgmann et al., 2005; De Schamphelaere et al., 2007). Another important factor affecting metal accumulation is pH since it is directly linked to the speciation of such metals as Cu and Pb. Acidic conditions increase the portion of free ions, while basic solutions promote carbonate precipitation. Moreover, exposure of aquatic biota to ambient waters with either low or high pH values induces the disturbance of internal ion balance in an organism as a result of H⁺/OH⁻ titration of basic or acidic groups within an ion transporting system (Simkiss and Taylor, 1995). It was found that water acidification decreases Cd, Cu and Zn uptake by aquatic biota and increases bioavailability of Pb (Campbell and Stokes, 1985; Schubauer-Berigan et al., 1993; Yu and Wang, 2002). Despite of a general consensus on pH effects on metal toxicity, some contradictory results were reported by Belanger and Cherry (1990) and Heijerick et al., (2002) showing increasing Zn toxicity with decreasing pH.

Water flea Daphnia magna is a standard organism for toxicity testing, which has a finely balanced ion homeostasis. This leads to high sensitivity of Daphnia to any metabolism disruptions caused by water acidification or presence of ion regulatory toxicants. Nevertheless, number of studies investigating the effects of water chemistry on metal bioaccumulation in Daphnia magna at environmentally relevant concentrations is rather limited. This study investigates the individual effects of Ca²⁺, Na⁺ and pH on the simultaneous uptake of Cd, Cu, Ni, Pb, and Zn in water flea Daphnia magna at realistic levels using a stable isotope technique. From experimental data the individual metal uptake rate constants were derived and compared at each examined condition.

4.2. Materials and methods

4.2.1 Culturing conditions

The water flea Daphnia magna was cultured under controlled laboratory conditions in 1L polypropylene aquaria. Seven experiments were set up to investigate the effects of major cations on metal uptake by Daphnia. The culturing media were prepared by addition of varying amounts of CaCl₂·2H₂O or NaCl according to Table 4.1 to a soft aerated OECD medium of following composition: 0.1mM CaCl₂·2H₂O, 0.5mM
MgSO₄·7H₂O and 0.077mM KCl (ISO-6341-1982, OECD guidelines 202, 2002). The pH of the medium was controlled by adding non-complexing Good’s biological buffers (2mM MES for pH6, 1mM MOPS for pH7 and 2mM EPPS for pH8) and bringing the pH to a desired value with addition of NaOH. During a rearing period *Daphnia* were fed a mixture of the green algae *Pseudokirchneriella subcapitata* and *Chlaidiomonas reinhardtii* in 3:1 ratio. Three times a week the media were renewed and *Daphnia* were fed 400×10⁶ algal cells·L⁻¹. The cultures were maintained at 21±1°C under a 14h light: 10h dark cycle. The 15 to 16 day old daphnids of the fourth generation were used for the experiments.

### 4.2.2. Experimental procedure

All chemicals were purchased from VWR int., (Leuven, Belgium) and were reagent grade. Stable isotopes were obtained from STB Isotope GmbH, Germany. Ultra pure water (MilliQ, R>18.2MΩ) was used for the preparation of the medium. All uptake experiments were carried out in 750mL polypropylene containers. The exposure media were spiked with 0.025µM ¹⁰⁶Cd, 0.05µM ⁶⁵Cu, 0.1µM ⁶⁴Ni, 0.025µM ²⁰⁴Pb, and 0.1µM ⁶⁷Zn. No food was provided to the organisms during a test period. At 4, 21, 32 and 48 hours both *Daphnia* and water were sampled to be analyzed for total metal concentrations. Ten to fifteen animals were collected from each of three replicates and rinsed with MilliQ water. *Daphnia* were dried at 60°C to a constant weight and digested with 69% HNO₃ in a microwave. The 20mL of water sample was filtered through 0.45µm cellulose nitrate membrane (NC45, Schleicher & Schüll), acidified with 69% HNO₃ and kept in a freezer until the analysis.
Table 4.1. Composition of the test media and experimental condition (constant parameters: 0.5mM Mg\(^{2+}\), 0.078mM K\(^+\)). Conditions with significant changes in metal speciation are highlighted in italics. IS is ionic strength.

<table>
<thead>
<tr>
<th>Set</th>
<th>Concentrations of the varying ions</th>
<th>Concentrations of free metal ions, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cad(^{2+})  Cu(^{2+})  Ni(^{2+})  Pb(^{2+})  Zn(^{2+})</td>
</tr>
<tr>
<td>Ca 0.1mM, Na 2mM, pH7, IS 4.2mM</td>
<td>20.4 39.1 95.0 18.2 93.6</td>
<td></td>
</tr>
<tr>
<td>Ca 0.5mM, Na 2mM, pH7, IS 5.4mM</td>
<td>19.7 39.4 95.4 18.2 94.0</td>
<td></td>
</tr>
<tr>
<td>Ca 2.5mM, Na 2mM, pH7, IS 11.1mM</td>
<td>16.9 40.4 96.7 17.8 94.8</td>
<td></td>
</tr>
<tr>
<td>Na 0.5mM, Ca 0.5mM, pH7, IS 3.9mM</td>
<td>21.4 39.0 95.1 18.5 93.8</td>
<td></td>
</tr>
<tr>
<td>Na 2.0mM, Ca 0.5mM, pH7, IS 5.4mM</td>
<td>19.7 39.4 95.4 18.2 94.0</td>
<td></td>
</tr>
<tr>
<td>Na 8.0mM, Ca 0.5mM, pH7, IS 11.3mM</td>
<td>15.5 40.2 96.2 17.1 94.1</td>
<td></td>
</tr>
<tr>
<td>pH6, Na 2.0mM Ca 0.5mM, IS 5.4mM</td>
<td>19.7 46.4 95.5 20.6 94.5</td>
<td></td>
</tr>
<tr>
<td>pH7, Na 2.0mM Ca 0.5mM, IS 5.4mM</td>
<td>19.7 39.4 95.4 18.2 94.0</td>
<td></td>
</tr>
<tr>
<td>pH8, Na 2.0mM Ca 0.5mM, IS 5.4mM</td>
<td>19.6 14.7 94.7 8.3 82.7</td>
<td></td>
</tr>
</tbody>
</table>

4.2.3. Analytical and computational procedures

All samples were analysed for metals by inductively coupled plasma mass spectrometry (Varian Expert 700 quadrupole ICP-MS, Mulgrave, Australia). The calibration standards were prepared from a 1000mg L\(^{-1}\) ICP multi-element standard.
solution IV (Merk) by dilution with 1% (v/v) nitric acid. Yttrium was added to all standards and samples as an internal standard. Although the instrument drift was not significant, the instrument was recalibrated every 15-20 samples. At least two isotopes were measured for each metal. Non-tracer isotopes, which were not added to the exposure medium (i.e. $^{111}$Cd, $^{65}$Cu, $^{60}$Ni, $^{64}$Zn, $^{208}$Pb), were used for background concentration corrections or determination of the concentrations of metals already present in the organism based on known natural abundances of isotopes. For example, to calculate the background concentration of $^{106}$Cd, $^{111}$Cd was also monitored and the following equation was applied:

$$
[^{106}\text{Cd}]_{\text{background}} = [^{111}\text{Cd}]_{\text{sample}} \times \left( \frac{\text{abundance}^{106}\text{Cd}}{\text{abundance}^{111}\text{Cd}} \right)
$$

(1)

$$
[^{106}\text{Cd}]_{\text{background}} = [^{111}\text{Cd}]_{\text{sample}} \times \left( \frac{1.21}{12.75} \right)
$$

(2)

Calculated background isotope concentrations of spiked isotopes were subtracted from measured values.

The experimental data were fitted with either linear or exponential kinetics using GraphPad Prism 4 software:

$$
C_{\text{organism}} = k_u \cdot C_{\text{water}} \cdot t + C_0
$$

(3)

$$
C_{\text{organism}} = C_{\text{water}} \cdot \left( \frac{k_u}{k_e} \right) \left( 1 - e^{-k_e t} \right) + C_0
$$

(4)

where $C_{\text{organism}}$ is the concentration of the metal in the organism on a dry weight basis ($\mu$mol·kg$^{-1}$), $C_0$ is the initial metal concentration in the organism at $t=0$, $C_{\text{water}}$ is the concentration of the metal in the water phase ($\mu$mol·L$^{-1}$), $t$ is the exposure time (d),
Effect of Na, Ca and pH on uptake of Cd, Cu, Ni, Pb and Zn in Daphnia

$k_u$ is the uptake rate constant (d$^{-1}$), $k_e$ is the elimination rate constant (d$^{-1}$). An ANOVA test was used for the statistical analysis of the data.

4.3. Results

In the present study the concentrations of Na$^+$ and Ca$^{2+}$ in the test medium were increasing up to 25 fold and pH ranged from 6 to 8. Therefore, prior acclimatization to the test medium was critical in order to avoid a possible shock from a sudden change in water composition. For evaluating metal accumulation in *Daphnia magna* the organisms of fourth generation grown in the test medium were used and, therefore, considered to be completely acclimatized to the test conditions. Thus, any changes in metal uptake processes were expected to be induced only by the differences in water environment. The time dependent profiles of whole body metal content were constructed for each metal separately (Fig. 4.1) and fitted with a linear or exponential function. The uptake rate constants were then calculated from the experimental data. When the data were fitted with linear functions, the $k_u$ values were obtained by dividing the slopes of corresponding curves by total dissolved isotope concentrations. In cases where exponential functions were fitting better than linear ones, $k_u$ values were calculated by multiplying the slope of the curve by $k_e$ constant estimated from the predicting equation and dividing the result by a total measured dissolved isotope concentration.

The effects of varying sodium concentrations on metal uptake rate constants are presented in Fig. 4.2. The examined Na$^+$ concentrations were ranging from 0.5mM to 8mM covering a physiologically optimum concentration (2mM). A bell-shaped response was noticed for Cd, Cu and Zn with the maximum uptake rate constants at the middle Na$^+$ concentrations, although statistically there was no difference in Cu $k_u$ values. In contrast, Pb uptake rate was doubled in the medium with the highest sodium content compared to soft and medium Na$^+$ conditions. Statistically, there was no effect of varying sodium concentrations on Ni uptake.
Figure 4.1. The effects of Ca\(^{2+}\), Na\(^+\) and pH on Zn uptake in *Daphnia magna* exposed to 0.1µM \(^{67}\)Zn (21°C, 0.5mM Mg\(^{2+}\), 0.078mM K\(^+\)).
Figure 4.2. The effects of sodium on Cd, Cu, Ni, Pb, and Zn uptake rate constants in *Daphnia magna* exposed to 0.025µM $^{109}$Cd, 0.05µM $^{65}$Cu, 0.1µM $^{62}$Ni, 0.025µM $^{204}$Pb and 0.1µM $^{67}$Zn (21°C, 0.5mM Ca$^{2+}$, 0.5mM Mg$^{2+}$, 0.078mM K$^+$, pH7). Statistically different treatments for each metal are denoted by *, **.
The effects of increasing Ca$^{2+}$ concentrations on metal uptake rate constants are presented in Fig. 4.3. Applied calcium concentrations were 0.1mM, 0.5mM and 2.5mM representing typical values in the soft, medium and hard waters. It appeared that hard water had a ‘protective effect’ toward all studied metals except Cu, for which no statistical difference in the uptake rate constants was observed. Nevertheless, a similar pattern of decreasing Cu uptake with increasing Ca$^{2+}$ content in the medium could be noticed. There were significant linear decreases in Cd ($p<0.0001$, $R^2=0.92$) and Ni ($p<0.0001$, $R^2=0.95$) uptake rate constants with increasing calcium concentrations resulting in four and eight times lower values respectively when moving from the lowest to the highest Ca concentrations. Zn and Pb showed similar to each other patterns: there was no difference in the uptake rate constants at 0.1 and 0.5mM ambient Ca$^{2+}$ concentrations, while presence of 2.5mM Ca$^{2+}$ decreased Zn and Pb $k_u$ values 3 and 2 times respectively.

Fig. 4.4 presents the effects of changing pH on metal uptake rate constants. The medium pH was changing from acidic (pH6) to basic conditions (pH8) covering a physiologically feasible range. Cu and Pb uptake processes were not affected by the changes in the medium pH, however, there was a suppression Cd and Ni uptake rates at neutral and basic conditions. In contrast, Zn uptake rate constants were linearly increasing with increasing pH ($p<0.0001$, $R^2=0.93$).

### 4.4. Discussion

Physiological mechanism of metal toxicity has been studied most extensively in fish. It was found that waterborne metals interact predominantly with gills surface and act in three major directions. Monovalent metals (Ag$^{+}$ and Cu$^{+}$) affect Na transport in gills, divalent metals (e.g. Cd$^{2+}$ and Zn$^{2+}$) disrupt Ca metabolism due to increased competition for binding sites, while other metals (e.g. Pb$^{2+}$, Hg$^{2+}$) cross the gills and act centrally. It should be noted that Cu is most likely reduced from its predominant specie Cu$^{2+}$ to monovalent Cu$^+$ before being transported across biological membranes (Paquin et. al., 2002). Although the mechanism of metal toxicity in *Daphnia* has been less investigated,
it is reasonable to expect similar interactions from toxic metals as those discovered for fish. In this respect, the following discussion is divided in parts considering the metals with similar modes of action together.

Figure 4.3. The effects of calcium on Cd, Cu, Ni, Pb, and Zn uptake rate constants in *Daphnia magna* exposed to 0.025μM $^{106}$Cd, 0.05μM $^{65}$Cu, 0.1μM $^{62}$Ni, 0.025μM $^{204}$Pb and 0.1μM $^{67}$Zn (21°C, 2mM Na⁺, 0.5mM Mg²⁺, 0.078mM K⁺, pH7). Statistically different treatments for each metal are denoted by *, **.
4.4.1. Effects of Ca$^{2+}$, Na$^+$, and pH on Cd, Ni and Zn uptake

Sufficient calcium levels in ambient water are extremely important for the healthy development and general fitness of *Daphnia*. In particular, the organisms have an increased calcium demand during a moulting stage and, therefore, are very sensitive to any calcium deficiency. A complete post-moult calcification can be reached only at Ca$^{2+} > 0.13$mM (Alstad et al., 1999). Furthermore, the age specific egg production is strongly reduced at calcium levels below 0.25mM, while a threshold for survival is in a range 2.5-12.5µM (Hessen et al., 2000). On the other hand, calcium in hard waters has a protective effect on organisms during metal exposure due to competition between calcium and other metals for the binding sides. This positive effect of calcium extends, however, to a certain threshold value. For example, a linear reduction of acute Zn toxicity toward *Daphnia magna* was observed with increasing Ca concentrations in the range of 0.25-3mM, while the effect disappears at higher calcium concentrations (Heijerick et al., 2002). Further increase in calcium concentrations (more than 6.5mM) causes an adverse effect on reproduction rate (Cowgill and Milazzo, 1990). Keeping all these factors in mind, we have narrowed the tested calcium concentrations to a range from 0.1 to 2.5mM, which would represent extreme, but still physiologically reasonable conditions.

The evidences of Cd and Zn impacts on Ca metabolism in *Daphnia* have been documented in several studies. The toxicity tests indicated the decrease in Cd, Ni and Zn toxicity with increase in water hardness (Barata et al., 1998; Yim et al., 2006; Heijerick et al., 2003, 2005; Deleebeeck et al., 2008). The investigations on the exact mechanism of Cd action revealed dramatic changes in the structure of gut diverticula (a pair of sacs that curve backward from the midgut and terminate behind the animal’s eye) of *Daphnia magna* following Cd exposure (Griffiths, 1980). These structural changes were accompanied by the appearance of Ca-rich granules in intracellular channels located in diverticula. It was suggested that Cd exposure impairs Ca metabolism and eventually gut diverticula morphology. The hypothesis was further supported by studies of Munger et al. (1999), who investigated the internal distribution of Cd in *Ceriodaphnia dubia* exposed to
Effect of Na, Ca and pH on uptake of Cd, Cu, Ni, Pb and Zn in Daphnia magna exposed to 0.025µM $^{106}$Cd, 0.05µM $^{65}$Cu, 0.1µM $^{62}$Ni, 0.025µM $^{204}$Pb and 0.1µM $^{67}$Zn (21°C, 2mM Na$^+$, 0.5mM Ca$^{2+}$, 0.5mM Mg$^{2+}$, 0.078mM K$^+$, pH7). Statistically different treatments for each metal are denoted by *, **.

Figure 4.4. The effect of pH on Cd, Cu, Ni, Pb, and Zn uptake rate constants in Daphnia magna exposed to 0.025µM $^{106}$Cd, 0.05µM $^{65}$Cu, 0.1µM $^{62}$Ni, 0.025µM $^{204}$Pb and 0.1µM $^{67}$Zn (21°C, 2mM Na$^+$, 0.5mM Ca$^{2+}$, 0.5mM Mg$^{2+}$, 0.078mM K$^+$, pH7). Statistically different treatments for each metal are denoted by *, **.

both water- and foodborn Cd. The results also revealed strong Cd accumulation in the midgut and especially in the gut diverticula.
The adverse effect of chronic waterborn Zn exposure on Ca metabolism in *Daphnia magna* has been reported by Muyszen et al. (2006). However, the restoration of Ca uptake process was observed in two weeks.

In the present study Cd (Ni, Zn)-Ca competition effects were also observed. The highest Cd and Ni uptake rate constants were determined at the lowest calcium concentration followed by their significant decrease with rising calcium concentrations. A suppression of Zn uptake process was evident only at 2.5mM Ca$^{2+}$. A sharp decrease in Cd and Zn uptake rate constants observed at 0.5mM (middle) to 2.5mM (high) Ca indicates that there is a certain limit of calcium level below which no competition between Ca$^{2+}$ and Zn$^{2+}$ ions for binding sides occurs. Ca$^{2+}$ channels are characterized by a multiple metal occupancy and are able to transfer $10^6$ ions per second (Simkiss and Taylor, 1995). Therefore, competition between calcium and other ions becomes significant only when the total metal concentration (Ca$^{2+}$ plus trace metals) is reaching a certain value. When the trace metal concentration in the medium is low, the competitive effect will be observed only at high calcium levels. Conversely, high trace metal concentrations will cause channel blocking at much lower calcium concentrations. Taking into account possible interactions among ions within the channel, the contribution of other metals to the process should not be disregarded.

The effect of increasing sodium concentrations on Cd, Ni and Zn uptake was less clear. The uptake of Ni was not affected by changes in Na$^+$ concentrations, which is consistent with findings by Deleebeeck et al. (2008). Maximum Cd and Zn uptake rates observed at the middle sodium concentration may reflect physiological changes in ion regulation processes occurring in *Daphnia*. It is well documented that sodium uptake is accompanied by NH$_4^+$ or H$^+$ effluxes (Paquin et al., 2002). Low external sodium concentrations could result in increased energy requirements for the active Na$^+$ transport against the concentration gradient need to maintain a proper internal ionic balance and compensate for NH$_4^+/H^+$ losses. The opposite effect would be observed in the medium with high sodium levels, where lower ionic concentration gradient would lead to a decrease in active Na$^+$ uptake resulting in decrease in NH$_4^+$ or H$^+$ effluxes and
Effect of Na, Ca and pH on uptake of Cd, Cu, Ni, Pb and Zn in Daphnia

accumulation of ammonium. All these factors are able to provoke an adverse effect on the general fitness of the organism. This hypothesis is supported by the findings of Arner and Koivisto (1993), who observed higher fitness as well as higher growth and reproduction in Daphnia magna acclimated to salinity of 4ppt compared to daphnids originating from the same clone, but acclimated to salinity levels of 0 and 8ppt.

The effect of pH on Cd, Ni and Zn uptakes varied among the metals. The inverse relationship between pH and Cd/Ni uptake rate constants indicates a similar mechanism involved in the uptake process of both metals. The opposite effect of increasing uptake rates with increasing pH was observed for Zn. Speciation calculations (Visual Minteq 2.4b) showed that changes in pH from 6 to 8 caused less than 1% decrease in Cd$^{2+}$ and Ni$^{2+}$ free ion concentrations, which does not explain the dramatic change in uptake rates. A possible competition between protons and Cd$^{2+}$ ions proposed in earlier studies should not be expected at slightly acidic conditions since Cd binds stronger to a biotic ligand (Daphnia magna) than do protons: according to Playle et al. (1993) the log $K$ value for Cd-BL is higher than that for H-BL (8.6 and 6.7, respectively). At this point it is not clear which mechanism is responsible for a higher uptake at slightly acidic conditions and further investigations are necessary. In contrast to Cd and Ni, concentration of Zn$^{2+}$ as a free ion decreased approximately for 12% when pH of the medium was raised to 8. Nevertheless, Zn uptake rate constant was the highest at pH8. Moreover, a sharp increase in Zn uptake rate constants occurred between pH6 and pH7, where Zn$^{2+}$ free ion concentrations were similar. Thus, changes in free ion metal concentrations do not correlate with the pattern of Zn uptake rates changes. It was proposed by Santore et al. (2002) that in slightly acidic conditions Zn is displaced by protons from the biotic ligand, which decreases the uptake rate. Therefore, higher Zn uptake can be expected at neutral (pH7) and alkaline (pH8) conditions due to a reduction in H$^+$ concentrations. The gill-H$^+$ binding constant in rainbow trout was found to be higher than that of gill-Zn (log $K$ equals to 6.7 and 5.5 respectively). Comparable Zn-BL log $K$ value (5.3) was determined for Daphnia magna by Hijericket et al., (2002), which indicates that similar processes might occur. Although the authors could not determine H-BL binding constant, their
results showed an increase in 48h-EC$_{50}$ total Zn concentrations with increasing pH from 6 to 8.

**4.4.2. Effects of Ca$^{2+}$, Na$^+$ and pH on Cu uptake**

In contrast to Cd, Ni, and Zn, the increases in Ca$^{2+}$, Na$^+$ and H$^+$ concentrations in the medium had little effect on the accumulation of Cu. It is has been shown for several species that acute toxicity of Cu is a result of competition between Cu$^{2+}$ and Na$^+$ for a single binding site (an apical Na channel) leading to a disruption of Na$^+$ transport and inhibition of active sodium uptake (Paquin et al., 2002). Therefore, increasing external sodium concentrations were expected to compete with Cu ions and decrease Cu uptake rates. It should be noted, however, that Cu is an essential element meaning that a certain quantity of the metal is required to meet essential metabolic needs (Rainbow, 2002). Thus, another physiological process can be developed for Cu uptake in cultures acclimated to specific environmental conditions, such as high ambient sodium concentration, where Cu entrance via Na$^+$ channel is blocked by sodium. In 2005, Borgman et al. suggested that *Hyalella azteca* and in *Daphnia magna* have two coexisting Cu uptake pathways, one of which is Na sensitive and the other one is not. Similar observation was reported by Grosell and Wood (2002), who demonstrated the presence of both sodium sensitive and sodium insensitive components in Cu uptake in fish with the later being increasingly important at elevated external Na concentrations.

It was not unexpected that there was no dependence of Cu uptake on external calcium concentrations since there is no evidence of calcium being directly involved in Cu metabolism. Although there was a slight decrease in Cu uptake rate constants with increasing ambient Ca concentrations, statistical treatment showed that there was no difference in the results (ANOVA, $p=0.475$). De Schamphelaere and Janssen (2002) showed that increasing Ca$^{2+}$ activities in the exposure medium mitigate acute Cu toxicity to *Daphnia magna*. However, the obtained results were most likely due to the effect of calcium on sodium metabolism, which then indirectly affected Cu uptake. The experiments were performed at 0.077mM ambient Na$^+$ on organisms which were not
acclimated to such low sodium levels. In this respect, Glover and Wood (2005) showed that active sodium uptake was severely inhibited by 0.1mM calcium gluconate at 0.05-1mM external sodium concentration. Similar to our results were reported by Naddy et al. (2002), who demonstrated that Cu toxicity in Daphnia was a function of Mg^{2+} rather than Ca^{2+} concentration. Similarly, De Schamphelaere and Janssen (2004) did not observe significant Ca^{2+}, Mg^{2+} or combined competition effects on chronic Cu toxicity toward Daphnia magna. Another study also demonstrated no variations in acute Cu toxicity to Ceriodaphnia dubia with varying hardness (Hyne et al., 2005). Gensemer et al. (2002) also evaluated the Cu toxicity to Ceriodaphnia dubia in very hard waters and found that it varied inconsistently as a function of hardness.

Alike calcium, the changes in pH had little effect on total dissolved Cu uptake rate constants. Several studies have reported mitigating effect of decreasing water acidity on both acute and chronic free ion (Cu^{2+}) Cu toxicity to Daphnia magna (De Schamphelaere and Janssen, 2002, 2004; Hyne et al., 2005), while no effect of changing pH on total or dissolved Cu toxicity was reported by Andrew et al. (1977). Moreover, Long et al. (2004) found that pH in the range 5.5-8.5 did not influence acute Cu toxicity in soft waters with hardness 7-20mg-L^{-1} as CaCO_{3} (in our study the pH experiment was conducted at hardness 50mg-L^{-1} as CaCO_{3}). It should be noted that increase in pH causes significant changes in Cu speciation. At acidic conditions Cu^{2+} ions are major Cu specie, while formation of CuOH^{+} ions dominates at pH8. It has been proposed by Schamphelaere and Janssen (2002) that CuOH^{+} ions are also bioavialable to a biotic ligand and explained an increase in EC_{50} with increasing acidity by co-toxicity of Cu hydroxide ions rather then proton competition. The calculated binding constants for two Cu species were close to each other and higher than that for H^{+} (LogK_{Cu^{2+}}^{BL} = 8.02, LogK_{CuOH^{+}}^{BL}= 7.45 and LogK_{H^{+}}^{BL}= 5.4). Thus, decreased toxicity was likely due to less quantity of Cu^{2+} available for binding and increased amount of cationic Cu hydroxide ions bonded to the cell membrane surface.
4.4.3. Effects of Ca$^{2+}$, Na$^{+}$ and pH on Pb uptake

The accumulation of Pb was significantly suppressed by high calcium and sodium concentrations. Statistically, there was no effect of pH on Pb accumulation process, although the uptake rate constant was a little higher at pH8. Several studies have reported the disruption of calcium and sodium metabolism in aquatic organisms as a result of Pb exposure (Rogers et al., 2003, 2005; Grosell et al., 2006a; Patel et al., 2006). Borgmann et al. (2005) demonstrated mitigation of Pb toxicity at elevated ambient Ca concentrations. Investigations on the mechanism of branchial Pb uptake in rainbow trout revealed that Pb$^{2+}$ is taken up via a voltage-independent calcium channel similar to the entry of Ca$^{2+}$ ions (Rogers and Wood, 2004) and thus, able to cause a disruption of Ca metabolism. The effect of lead accumulation on sodium balance appeared to have a biphasic nature (Grosell et al., 2006b). Exposure of *L. stagnalis* to low Pb concentrations (∼0.02-0.06µM) resulted in elevated whole-body Na$^{+}$ concentrations reflecting increased Na$^{+}$/H$^{+}$ exchange rate to compensate for Pb-induced acidosis, while higher Pb concentrations had a direct negative effect on sodium transport proteins. Thus increased Pb uptake at high ambient Na$^{+}$ may be explained by increased sodium uptake rates promoting Pb entrance to the cell.

The lack of pH effect on Pb uptake does not correlate with changes in Pb speciation. Concentrations of Pb$^{2+}$ free ions decrease with increasing pH of the medium and at pH8 Pb$^{2+}$ comprised only 33.4% of total dissolved lead at the studied conditions. Similar finding were reported by Grosell et al. (2006a), who observed increased Pb toxicity at lower and higher pH values compared to neutral conditions during a chronic exposure of fathead minnow to water-born Pb. The obtained results can be explained by reduced proton competition, which begin to influence Pb uptake in waters with high pH.

4.5. Conclusions

The ambient water characteristics, i.e. Na$^{+}$, Ca$^{2+}$ and pH clearly influenced the bioaccumulation of Cd, Ni, Pb and Zn in *Daphnia magna*. There was no effect of either water parameter on Cu uptake process possibly due to a combination of low Cu
concentration and high homeostatic regulation of total body concentration in *Daphnia*. A clear suppression of Cd, Ni, Pb and Zn uptake processes was observed at calcium concentration 2.5mM, which is in agreement with a protective effect of calcium reported in other studies. The effect of sodium was less clear and had a bell-shaped response for Cd and Zn with a maximum uptake rate at the middle sodium concentration. An increased Pb uptake was determined at the highest sodium level, while no effect was observed for Cu and Ni. The effect of pH was metal specific ranging from no effect on Cu and Pb uptake process to a positive linear relationship between Zn uptake rate constants and pH. An increase in pH values from 6 to 7 resulted in a dramatic drop in Cd and Ni uptake rates; however, further increase in pH did not have any effect on uptake of those metals. The obtained results demonstrated that Na⁺, Ca²⁺ and H⁺ cations have a significant impact on metal accumulation process during a simultaneous exposure to several metals even at low exposure concentrations.

### 4.6. References


Effect of Na, Ca and pH on uptake of Cd, Cu, Ni, Pb and Zn in Daphnia


CHAPTER 5

Multi-metal Interactions between Cd, Cu, Ni, Pb and Zn in the Water Flea *Daphnia magna*
Abstract

Metal interaction effects were investigated in *Daphnia magna* exposed to a metal mixture containing 0.0125-0.2µM $^{106}$Cd, 0.025-0.25µM $^{65}$Cu, 0.025-0.25µM $^{204}$Pb, 0.1-1.25µM $^{62}$Ni and 0.1-1.25µM $^{67}$Zn. Cd and Cu exhibited a suppressing effect on the uptake rates of other metals present in the mixture with exception to Pb at all studied concentrations. The effect was already pronounced at low Cd and Cu concentrations and reached a maximum at the higher concentrations. Ni and Zn showed weaker interactions with Cd and between each other, while having no effect on Cu and Pb uptake. There was a high degree of correlation between Cd, Ni and Zn uptake rates indicating that these metals share in part common uptake or interaction pathways. The uptake of Pb was marked by a high initial rate, but the uptake process reached saturation within 24-48 hours. Cd applied at a concentration of 0.2µM was the only metal which stimulated Pb uptake. Addition of 0.25µM Pb to the medium, in turn, increased Cu uptake. Current work illustrates that metal interactions are significant and occur at low environmentally realistic concentration affecting bioavailability of both toxic and essential metals.

5.1. Introduction

Natural waters are frequently contaminated by trace metals as a result of human activities. In such conditions aquatic organisms are often exposed to a mixture of metals rather than a single element (More and Ramamoorthy, 1984). Some of these trace metals, such as Cu and Zn, play an important role in cellular metabolism and their body concentrations can be regulated by the organisms. Others, such as Ag, Cd and Pb, are toxic even at low concentrations and tend to accumulate in the body (Rainbow, 1997, 2002). Essential and non-essential metals may, however, share common uptake routes and interact with each other affecting uptake, bioaccumulation and toxicity. The results of such interactions are highly variable ranging from antagonism to synergism depending on the metal, its external concentration and exposure scenario, length of exposure, studied species and examined organs (Amiard-Triquet and Amiard, 1998; Norwood et al., 2003).
Evidence exists that in crustaceans Cd and Zn are taken up by the same transport pathway, however the type of interaction is inconsistent being both positive/negative or showing no interaction and species dependent (Rainbow, 2000). For example, Zn additions reduced Cd toxicity in the amphipod Corophium volutator (Bat et al., 1998), while increased toxicity was observed in the water flea Daphnia magna and the shrimp Callianassa australiensis during exposure to Cd-Zn mixtures compared to individual metals (Biesinger et al., 1986; Negilski et al., 1981). Synergetic effects of metals interactions in Cu-Cd and Cu-Pb mixtures have also been reported for fish (Pelgrom et al., 1995; Tao et al., 1999).

At present, the BLM models, which aim to predict dissolved metal toxicity to aquatic organisms as a function of external metal concentration and water chemistry characteristics, are used for setting site specific water quality criteria. However, these models have been developed for single metals and do not take into account possible metal interactions. Nevertheless, numerous studies indicate the necessity of such consideration and incorporation of metal interactions is an important future task (Paquin et al., 2002).

The present study is aimed to investigate the interactive effects of Cd, Cu, Ni, Pb, and Zn in Daphnia magna, a standard organism for toxicity testing, during simultaneous exposure to these metals at low concentrations. The number of studies investigating the effect of metal interactions on trace metal uptake and toxicity in Daphnia magna is limited. Contradictory results on toxicity of Cd-Zn mixtures were reported in several studies. In particular, Shaw et al. (2006) found no interactions between Cd and Zn in Daphnia magna, although increasing Cd concentrations decreased the toxicity of Cd-Zn mixtures in other Daphnia species (D. ambigua, D. pulex and C. dubia). Similarly, Jak et al., (1996) examined the effects of simultaneous exposure to As, Cd, Cr, Cu, Hg, Ni, Pb and Zn on population density of Daphnia magna and observed no interactions among metals. In contrast, Canizares-Villanueva et al. (2000), observed an antagonistic effect of Zn on Cd toxicity in Daphnia magna. Another study performed by Barata et al. (2002) showed increased Cd tolerance of three Daphnia magna clones induced by exposure to elevated Zn concentrations. In this work we have studied the effects of metal interactions
during a multi-metal exposure at environmentally relevant concentrations on metal uptake rates using a stable isotope technique that allows direct measurement of newly accumulated metal by ICP-MS. The choice of exposure concentrations was justified by current Flemish water quality criteria set to 1µg·L$^{-1}$ Cd, 50µg·L$^{-1}$ Cu, 30µg·L$^{-1}$ Ni, 50µg·L$^{-1}$ Pb and 200µg·L$^{-1}$ Zn (Flemish Government, 2000) and concentrations of these metals in natural water systems in Flanders (Belgium), which vary between 0.1-12.2µg·L$^{-1}$ Cd, 1-30.1µg·L$^{-1}$ Cu, 2.7-19.8µg·L$^{-1}$ Ni, 0.1-1µg·L$^{-1}$ Pb and 47-2168µg·L$^{-1}$ Zn (Bervoets et al., 2003; Reynders et al., 2008).

5.2. Materials and methods

5.2.1. Culturing conditions

The water flea *Daphnia magna* was cultured under controlled laboratory conditions in 1L polypropylene aquaria. The soft aerated freshwater was used as a rearing medium and had the following composition: 0.5mM CaCl$_2$·2H$_2$O, 0.5mM MgSO$_4$·7H$_2$O, 2mM NaCl and 0.077mM KCl, pH 7. The pH of the medium was controlled by adding a non-complexing Good’s biological buffer (1mM MOPS brought to pH 7 with addition of NaOH) (de Schamphelaere et al., 2004). During a rearing period *Daphnia* were fed a mixture of the green algae *Pseudokirchneriella subcapitata* and *Chlamidomonas reinhardtii* in a 3:1 ratio. Three times a week the medium was renewed and daphnids were fed $400 \times 10^6$ algal cells·L$^{-1}$. The culture was maintained at 21±1°C under a 14h light: 10h dark cycle. Daphnids, 15-16 days old, from fourth generation were used for the experiments.

5.2.2. Experimental procedure

All chemicals were purchased from VWR Int., (Leuven, Belgium) and were reagent grade. Stable isotopes were obtained from STB Isotope GmbH, Germany. Ultra pure water (MilliQ, R>18.2MΩ) was used for the preparation of the medium. All uptake experiments were carried out in 750mL polypropylene containers. The exposure medium was spiked with increasing amounts of $^{106}$Cd, $^{65}$Cu, $^{62}$Ni, $^{204}$Pb, and $^{67}$Zn according to
Table 5.1. No food was provided to the organisms during a test period. At 0, 24, 48, 72 and 96 both *Daphnia* and water samples were collected to be analyzed for metals. Ten to fifteen animals were collected from each of three replicates and rinsed with MQ. The daphnids were dried at 60°C to a constant weight and digested with 69% HNO₃ in a microwave. The 20mL water sample was filtered through a 0.45μm membrane (NC45, Schleicher & Schüll), acidified to 2% HNO₃ with concentrated (69%) nitric acid and kept in a freezer until the analysis.

**Table 5.1.** Exposure concentrations of Cd, Cu, Ni, Pb, and Zn used in the study.
Concentrations of major cations were kept constant at all conditions (2mM Na⁺; 0.078mM K⁺; 0.5mM Ca²⁺; 0.5mM Mg²⁺; pH7, ionic strength 0.0084M).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Metal</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>All metals at minimum level</em></td>
<td>0.0125μM Cd, 0.025μM Cu, 0.1μM Ni, 0.025μM Pb and 0.1μM Zn</td>
</tr>
<tr>
<td>Cd 0.05</td>
<td>0.05μM Cd, 0.025μM Cu, 0.1μM Ni, 0.025μM Pb and 0.1μM Zn</td>
</tr>
<tr>
<td>Cd 0.2</td>
<td>0.02μM Cd, 0.025μM Cu, 0.1μM Ni, 0.025μM Pb and 0.1μM Zn</td>
</tr>
<tr>
<td>Cu 0.05</td>
<td>0.0125μM Cd, 0.05μM Cu, 0.1μM Ni, 0.025μM Pb and 0.1μM Zn</td>
</tr>
<tr>
<td>Cu 0.25</td>
<td>0.0125μM Cd, 0.25μM Cu, 0.1μM Ni, 0.025μM Pb and 0.1μM Zn</td>
</tr>
<tr>
<td>Ni 0.25</td>
<td>0.0125μM Cd, 0.025μM Cu, 0.25μM Ni, 0.025μM Pb and 0.1μM Zn</td>
</tr>
<tr>
<td>Ni 1.25</td>
<td>0.0125μM Cd, 0.025μM Cu, 1.25μM Ni, 0.025μM Pb and 0.1μM Zn</td>
</tr>
<tr>
<td>Pb 0.1</td>
<td>0.0125μM Cd, 0.025μM Cu, 0.1μM Ni, 0.1μM Pb and 0.1μM Zn</td>
</tr>
<tr>
<td>Pb 0.25</td>
<td>0.0125μM Cd, 0.025μM Cu, 0.1μM Ni, 0.25μM Pb and 0.1μM Zn</td>
</tr>
<tr>
<td>Zn 0.25</td>
<td>0.0125μM Cd, 0.025μM Cu, 0.1μM Ni, 0.025μM Pb and 0.25μM Zn</td>
</tr>
<tr>
<td>Zn 1.25</td>
<td>0.0125μM Cd, 0.025μM Cu, 0.1μM Ni, 0.025μM Pb and 1.25μM Zn</td>
</tr>
</tbody>
</table>
5.2.3. Analytical and computational procedures

All samples were analysed for metals by inductively coupled plasma mass spectrometry (Varian Expert 700 quadrupole ICP-MS, Mulgrave, Australia). The metal analysis procedure was the same as described in Chapters 3 and 4.

The experimental data were fitted to either linear or hyperbolic function using GraphPad Prism 4 software to estimate the uptake rates. If a simple linear function was fitting the data the best, then the uptake rate was equal to the slope of the corresponding line. In cases where experimental data could be better described by a hyperbolic function of the form

\[ C_{org} = \frac{a \cdot t}{b + t} \]

where \( C_{org} \) is a metal concentration inside the organism (µmole-gwd\(^{-1}\)) and \( t \) is time (h), then the initial uptake rate was calculated as \( a/b \). An ANOVA test was used to determine differences in the uptake rates. Since we used three separate incubations for each condition as replicates representing repeated measurements, it was possible to construct individual uptake curves for each replicate. Then the derived uptake rates were introduced to ANOVA table as replicate values for each compared condition.

5.3. Results and discussion

In the present study \textit{Daphnia magna} were exposed to a mixture of \(^{106}\text{Cd}, \ 65\text{Cu}, \ 62\text{Ni}, \ 204\text{Pb}, \ \text{and} \ 67\text{Zn} \) stable isotopes in different concentrations. The background concentrations of naturally occurring metals were below 0.0002µM for Cd, 0.002µM for Pb, 0.005µM for Ni and Cu and below 0.06µM for Zn. In total, five different exposure conditions were set up with three replicates for each experiment. Each set was composed in such a way that one of the metals was added in increasing amounts, while
concentrations of the other four elements were fixed at the lowest exposure level. The body concentrations of $^{106}$Cd, $^{65}$Cu, $^{62}$Ni, $^{204}$Pb, and $^{67}$Zn were monitored over a 48 hour time period to determine uptake rates. There was no change in total body concentrations of metals originally present in the organisms. Thus for simplicity, in the following discussion we will refer to $^{106}$Cd, $^{65}$Cu, $^{62}$Ni, $^{204}$Pb, and $^{67}$Zn and their uptake as Cd, Cu, Ni, Pb and Zn. In the majority of cases the bioaccumulation of Cd, Cu, Ni and Zn could be described by simple linear kinetics as illustrated in Fig. 5.1 for Cd-Zn. From the experimental data the uptake rates were calculated for each metal separately. The accumulation of dissolved Pb followed a hyperbolic type of kinetics reaching saturation within 24 hours (Fig. 5.2a). Therefore, in case of Pb reference is made to the initial uptake rate. When Zn was added as an interacting element, the uptake rates could not be determined due to rapid saturation and large variations in Pb body concentrations. Nevertheless, it is clearly seen that Zn addition promoted initial Pb uptake (Fig. 5.2b). There was also a special case of a strong Cd effect on Cu uptake that resulted in a subsequent Cu elimination at Cd concentrations above 0.0125µM (Fig. 5.3). In this case no accurate quantification of Cu uptake rates over the entire exposure period could be made. Thus the data points taken at 96h were excluded from analysis and a hyperbolic function fitted to the rest of the data.

5.3.1. Effects of Cd and Cu

The effect of Cd on metal uptake by Daphnia exposed to 0.0125µM, 0.05µM and 0.2µM Cd is presented in Fig. 5.4. The uptake rates of Cd were increasing from 0.49 to 2.44µM·kgdw$^{-1}$·h$^{-1}$ as a linear function of the total dissolved Cd concentration (slope $=15.36\pm1.30$ L·kgdw$^{-1}$·h$^{-1}$, $R^2=0.95$, N=9, p<0.05, runs test for deviation from linearity p>0.05). During the exposure Pb was accumulated at the highest rate compared to other metals. There was an increase of the Pb initial uptake rate at the highest (0.2µM) Cd concentration when it reached 52.2µM·kgdw$^{-1}$·h$^{-1}$. In contrast, Cu, Ni and Zn uptakes were suppressed by Cd.
Figure 5.1. Effect of increasing Cd concentrations on Zn uptake in *Daphnia magna* (mean±SD, n=3, pH 7, 2mM Na⁺, 0.078mM K⁺, 0.5mM Ca²⁺ and 0.5mM Mg²⁺, t=21°C).

The effect was the strongest when dissolved Cd concentration increased from 0.0125 to 0.2µM resulting in 2.5, 2.0 and 2.8 fold decrease in Cu, Ni and Zn uptake rates, respectively.

Addition of Cu to the metal mixture also had a strong suppressing effect on the uptake of Cd, Ni and Zn (Fig. 5.5). Cd and Zn uptake rates were continuously decreasing as more Cu was added to the exposure solution reaching a maximum of 1.6 and 1.8 fold decrease for Cd and Zn, respectively. Ni uptake rates, in turn, were affected the most when the Cu concentration in the medium increased from the lowest (0.025µM) to the middle (0.05µM) exposure concentration and then remained the same for the middle and the highest (0.25µM) Cu concentration. In total, there was a 1.3 time decrease in Ni uptake rates. Accumulation of Pb was not affected by changes in the amount of Cu present in the water. The uptake rates of Cu itself were hyperbolically increasing as a function of Cu concentration in the medium.
Figure 5.2. Effect of increasing Cd and Zn concentrations on Pb uptake in *Daphnia magna* (mean±SD, n=3, pH7, 2mM Na⁺, 0.078mM K⁺, 0.5mM Ca²⁺ and 0.5mM Mg²⁺, t=21°C).
5.3.2. Effects of Ni, Pb and Zn

In contrast to Cd and Cu, additions of Ni, Pb or Zn to the medium had little or no effect on the uptake rates of other elements (Figs 5.6-5.8). Increasing amounts of Ni in the medium resulted in the increase in Ni uptake rates directly proportionally to the dissolved Ni concentration (slope $=4.99\pm0.01\text{L kg}^{-1}\text{kgdw}^{-1}\text{h}^{-1}$, $R^2=0.99$, $N=9$, $p<0.05$ runs test for deviation from linearity $p>0.05$). A suppression of Cd accumulation was observed when the Ni concentration in the medium increased from 0.1 to 0.25µM. After that, no statistically significant change in Cd uptake rate was observed. There was also a slight suppression of Zn uptake rates in the same range of Ni concentrations, although the decrease was not enough to be statistically significant. Interestingly, the uptake of Cu and Pb was not affected by Ni additions.
Figure 5.4. Effect of increasing Cd concentrations on metal uptake rates in *Daphnia magna* exposed to varying Cd concentrations and 0.025µM Cu, 0.1µM Ni, 0.025µM Pb, 0.01µM Zn. Uptake rates shearing the same number of asterisks (*) are statistically the same.
Similarly, there was no effect from Pb additions on the Cd, Ni and Zn uptake rates. However, the highest Pb concentration (0.25µM) stimulated Cu accumulation resulting in a 1.4 time increase in the Cu uptake rate. The initial uptake rates of Pb were proportional to its concentration in the medium (slope =1013±36.64 L·kgdw⁻¹·h⁻¹, $R^2$=0.99, N=9 p<0.05, runs test for deviation from linearity p>0.05). In contrast to Pb, elevating Zn concentrations had a negative impact on Cd and Ni uptake. Zn additions affected Cd uptake stronger than did Cu or Ni. Although there was no statistical difference in the Cd uptake rates at the middle (0.25µM) and highest (1.25µM) Zn concentrations, addition of 1.25µM Zn decreased Cd uptake rates two times compared to
Figure 5.6. Effect of increasing Ni concentrations on metal uptake rates in *Daphnia magna* exposed to varying Ni concentrations and 0.0125µM Cd, 0.025µM Cu, 0.025µM Pb, 0.01µM Zn. Uptake rates shearing the same number of asterisks (*) are statistically the same.

0.1µM Zn. Similarly to Cd, Ni uptake rates were also decreasing, while Cu accumulation remained constant at any Zn concentration. The Zn uptake rates were linearly related to the dissolved Zn concentration (slope =13.77±0.55L⋅kgdw^{-1}⋅h^{-1}, R^2=0.99, N=9, p<0.05), runs test for deviation from linearity, p>0.05). It was not possible to quantify the Pb uptake rates in this case since Pb uptake stopped after a very short exposure period.
5.3.3. Metal correlations

The experimental data showed significant interactions among metals. Therefore, a correlation analysis was performed in order to identify possible similarities in uptake pathways of the different metals. To do so, for each possible metal-metal combination a set of data points with constant concentrations of the corresponding metals in the solution was pulled out from all experimental data. For example, to find a possible correlation between Zn - Cd uptake rates, the data from Cu, Ni and Pb experiments were combined. Then, the Pearson correlation coefficient was calculated for each pair of metals. From all combinations the uptake rates of the following metals was shown to be significantly correlated: Zn-Cd \( (r=0.8335, p=0.0198, n=7) \), Zn-Cu \( (r=0.9290, p=0.0025, n=7) \), Zn-Ni \( (r=0.9226, p=0.0031) \) and Cd-Ni \( (r=0.8926, p=0.0069) \). For all four cases linear regressions fitted the best and deviation from linearity was not significant (runs test).
**Figure 5.8.** Effect of increasing Pb concentrations on metal uptake rates in *Daphnia magna* exposed to varying Pb concentrations and 0.0125µM Cd, 0.025µM Cu, 0.1µM Ni, 0.1µM Zn. Uptake rates sharing the same number of asterisks (*) are statistically the same.

The slopes were as follows: Zn-Cd slope = 0.23±0.07, \( p \) (runs test) = 0.8, Zn-Cu slope = 3.58±0.64, \( p = 0.33 \), Zn-Ni slope = 0.21±0.04, \( p = 0.87 \), Cd-Ni slope = 0.83±0.19, \( p = 0.8 \) (Figs 5.9-5.10). There was no correlation between uptake rates of Pb and any other studied metal.
Figure 5.9. Correlations between Zn and Cd, Cu, Ni uptake rates in *Daphnia magna* exposed to the mixture of metal.

Figure 5.10. Correlation between Cd and Ni uptake rates in *Daphnia magna* exposed to the mixtures of metal.
5.4. Discussion

Three major conclusions can be drawn from the experimental results. First, there were significant and mostly negative (inhibitory) interactions among the uptake of the five metals during a simultaneous exposure to a mixture of the metals. Cd and Cu decreased the uptake rates of the other metals present in the mixture with exception to Pb, while Ni and Zn affected the uptake process of each other, as well as that of Cd. Second, the suppressive effects were already evident at low concentrations and when more of one specific metal was added to the mixture, there was no additional decrease in the uptake rates of the other metals in the majority of cases. Thirdly, Pb exhibited accumulation and interaction patterns which were very different from that of Cd, Cu, Ni or Zn. The Pb uptake was saturated within 24 hours. In spite of the appearance of a plateau in the time dependent uptake curve, the total body concentrations of Pb (on the µM·gdw⁻¹ scale) were 2 to 45 times higher than accumulated levels of Cd, Cu, Ni or Zn at similar exposure concentrations. Moreover, Cd and Zn showed positive Cd-Pb and Zn-Pb interactive effects resulting in stimulation of Pb uptake. It should be noted, however, that exposure concentrations used in this study are likely to be below the acute toxicity limits. Therefore, the toxicity responses may differ from the metal uptake results reported in this study.

The observed inhibitory effects of both Cd and Cu on the uptake of other metals present in the mixture are quite surprising since Cd and Cu are known to enter the cell via different pathways and have different modes of toxic action. Cd as well as Ni and Zn interact actively with calcium channels, whereas Cu uptake interferes with sodium (Paquin et al., 2002). The similarities between Cd, Cu, Ni and Zn interaction effects with all being negative bring the idea of a similar interaction mechanism, which cannot be explained exclusively by a direct competition for the binding sites on the cell membrane. Correlation analysis revealed that Cd, Ni and Zn uptake rates were closely related to each other (Fig. 5.9). Similar slopes of Zn-Cd and Zn-Ni regression lines (0.23 and 0.21 respectively) suggest that these metals share common uptake sites and the competitive
inhibition of uptake is a possible explanation for the interactive effect. Divalent Metal (Ion) Transporter 1 (DMT1), which transports Fe$^{2+}$, may be one of such common uptake sites (Bury et al., 2003). It has been demonstrated by Garrick et al. (2006) that DMT1 has affinity to several metals, which decreases in the following order: Mn$^{2+}$>Cd$^{2+}$>Fe$^{2+}$>Pb$^{2+}$>Co$^{2+}$>Ni$^{2+}$>Zn with unknown position for Cu. The Zn (Cd) induced reduction of Cd (Zn) toxicity has been reported in several studies for different species, although the exact mechanism is not clearly understood (Canizares-Villanueva et al., 2000; Barata et al., 2002; Odendaal et al., 2004; Shaw et al., 2006). Early short term studies by Nugegoda and Rainbow (1995) showed a reduction in the uptake rate of radioactively labelled Zn in *Palaemon elegans* when the organisms were simultaneously exposed to 20µg·L$^{-1}$ Zn and 20µg·L$^{-1}$Cd compared to a single metal exposure, while the opposite trend was observed for Cd. More recently Goto and Wallace (2007) performed a set of experiments intended to elucidate the mechanism of Cd and Zn interactions. The deposit-feeding polychaete *Capitella capitata* was exposed to a mixture of Cd and Zn (1-50µg L$^{-1}$ Cd and 3-86µg L$^{-1}$ Zn) in different combinations and metal accumulation/elimination processes were followed on the whole body and sub-cellular levels. The uptake of both Cd and Zn was suppressed when *C. capitata* were exposed to elevated Cd or Zn concentrations. Subcellular studies indicated that Cd-Zn interactions significantly influenced the distribution of these metals among a variety of subcellular constituents. The authors concluded that Cd-Zn interactions reduced partitioning of both metals to heat stable proteins (HSP) and indicated that metal binding proteins, such as metallothioneins, were important sites of competition. Similarly, Ni induced alteration of HSP–metal partitioning may be a reason of reduction in Cd uptake rate.

There was also a significant correlation between Zn and Cu uptake rates. The slope of the regression line was, however, much steeper (3.58±0.64) compared to Cd and Ni indicating a different interaction mechanism involved. Since additions of Zn did not affect Cu accumulation while in a vice versa scenario smaller additions of Cu continuously decreased Zn uptake rates, it is likely that Cu is governing the interaction effect by interfering with Zn uptake and/or metabolism.
Multi-metal interactions between Cd, Cu, Ni, Pb and Zn in Daphnia

As it was noticed before, reduction in the uptake rates was more pronounced at low concentration range, after which no additional suppression was observed. This finding indicates that metal interactions are more likely to occur within certain concentration ranges when the binding sites are not completely occupied or there is no excess of one of the metals present in the medium. Similar results of interactions occurring only at lower exposure levels were reported in prawn larvae (Devineau and Amiard-Triquet, 1985), when the interactive effects of Zn on Cd uptake were observed at 1.15 -1.9µM Zn and disappeared at higher Zn concentration (4.2µM). Another study by Pelgrom et al. (1995) showed that Cu-Cd interaction in tilapia fish were more pronounced when the organisms were exposed to metals at environmentally realistic concentrations (0.3µM Cu and 0.04µM Cd). Similarly, a reduced Zn uptake in polychaete C. capitata was observed only when the organisms were co-exposed to a low (0.009 µM) Cd concentration (Goto et al., 2007).

Special attention should be directed to the uptake and bioaccumulation of Pb as it was rather distinctive from all other studied metals. Pb uptake was marked by a high initial rate and rapid saturation, sometimes accompanied by a decrease in the total body Pb burdens. Fast Pb accumulation at low exposure levels was also observed in another crustacean Hyalella azteca (MacLean et al., 1996). In this organism the body concentrations of Pb reached 99% of the maximum accumulation level in 5-6 days during exposure to 0.25-0.5µM Pb. The authors also pointed out that Pb uptake kinetics differed from that of Cd, Cu or Zn for the same species. The conclusion was made on the basis of matching Pb elimination rate constants obtained from separate uptake and elimination experiments, whereas other studies demonstrated large differences in Cd, Cu and Zn elimination rate constants estimated from the uptake and depuration studies (Borgman, 1995).

In the present study the uptake rates of Pb were not affected by Cu, Ni or Zn, while enhanced Pb accumulation was observed in the presence of 0.2µM Cd. The highest Pb concentration of 0.25µM, in turn, facilitated Cu uptake. An analogous stimulating
effect of Cu and Pb uptake in fish was reported by Tao et al. (1999). Both Pb and Cu facilitated each other’s uptake during a simultaneous exposure. The effect, however, was observed only when Cu concentrations exceeded 0.8µM and Pb concentrations were above 0.5µM.

The observed differences in metal accumulation may be attributed to a specific ion regulatory toxic action of Pb, which is highly capable of disrupting both Na⁺ and Ca²⁺ homeostasis (Rogers et al., 2003). Studies showed that Pb causes both competitive and non-competitive inhibition of Na⁺ and Cl⁻ influx in rainbow trout (Rogers et al., 2003, 2005). In freshwater pulmonate snails Pb disrupts Na⁺ homeostasis in a complicated pattern suggesting two physiological modes of action depending on the Pb exposure concentration (Grosell et al., 2006). Inhibition of the Ca²⁺-ATPase activity by Pb has been reported in mussels (Viarengo et al., 1993). Direct competition with Ca²⁺ for a binding site in a transport channel as well as a non-competitive reduction of Ca²⁺-ATPase activity was reported for rainbow trout (Rogers et al., 2004). The difference between Pb and other metals extends further to the body level. Ojo and Wood (2007) investigated metal uptake by the gastro-intestinal tract in rainbow trout. Studies demonstrated that area-specific Cd, Cu, and Zn uptake rates were the highest in the anterior intestine and approximately equal in the mid and posterior intestinal segments. However, Pb area-specific and whole organism transport rates were greatest in the mid intestine. Thus, Pb induced disruptions of ion homeostasis and metal absorption processes might be a possible explanation of stimulated Pb uptake in the presence of Cd, as well as the increase in Cu uptake rates provoked by presence of Pb at its highest studied concentration.

5.5. Conclusion

The present study provides evidences of metal-metal interactions that occur in *Daphnia magna* at environmentally relevant concentrations. During a simultaneous exposure to five metals Cd and Cu exhibited the strongest suppressing effects on the uptake of other metals present in the medium at concentrations as low as 0.05µM. Since
with exceptions to Pb the interactions were mostly negative, it is therefore unlikely that contamination of surface waters by one of the studied metals would lead to increased toxicity of other metals present in the environment on the basis of the effects observed at the level of uptake. However, the observation that the uptake of the essential elements Cu and Zn is also already influenced by the presence of other metals at these low concentrations may create problems related to the uptake and homeostatic regulation of the essential metals at these low exposure levels.

5.6. References


Multi-metal interactions between Cd, Cu, Ni, Pb and Zn in Daphnia


CHAPTER 6

Effect of Na, Ca and pH on the Simultaneous Uptake of Cd, Cu, Ni, Pb and Zn in the Zebrafish *Danio rerio*
Abstract

Previous studies with water flea *Daphnia magna* presented in chapter 4 revealed that concentrations of major cations in the medium play an important role in metal accumulation process during a simultaneous exposure to several metals even at low exposure concentrations. Similar experiments performed with zebrafish *Danio rerio* showed that variations in Ca$^{2+}$, Na$^+$ and H$^+$ concentrations in the medium affected the uptake of trace metals by zebrafish showing larger variations in Cd, Cu and Pb uptake rates in fish gills compared to the whole body with the opposite trend observed for Ni. Moreover, a high level of Zn homeostatic regulation marked by a fast elimination of the metal from both gills and body of zebrafish was observed. Addition of Ca$^{2+}$ to the medium had a profound suppressing effect on Cd accumulation, while the highest Cu, Pb and the lowest Ni uptake rates were observed at the middle Ca concentration (0.5mM). Increase in ambient Na concentrations decreased Cd and increased Ni uptake rates in gills. Both low and high Na concentrations in water had a suppressing effect on Pb uptake rates in gills and the total body. The increase in pH of the medium led to the promotion of Cd and Ni uptakes in both gills and the whole body. Cu and Pb, in turn, exhibited higher accumulation at pH7 compared to pH6 and pH8, which is explained by changes in Cu and Pb speciation at acidic and alkaline pH conditions.

6.1. Introduction

Water chemistry characteristics such as calcium and sodium concentration, pH, or dissolved organic matter are known to influence the toxicity of trace metals toward aquatic organisms (Niyogi and Wood, 2004). In fish, naturally occurring waterborne cations (Ca$^{2+}$, Mg$^{2+}$, Na$^+$ and H$^+$) and trace metals interact with each on gills surfaces and other exchange structures resulting in competitive/non-competitive inhibition of the uptake and accumulation processes, which modifies metal toxicity (Paquin et al., 2002). Studies showed that increases in Ca$^{2+}$, pH or Na$^+$ concentrations in water mitigate Cu
Effect of Na, Ca and pH on the uptake of Cd, Cu, Ni, Pb and Zn in Danio

toxicity and protect against Cu accumulation in gills (Playle et al., 1992, 1993; Erickson et al., 1996), but increase Pb toxicity (McDonald et al., 2002). Cd and Zn, which are believed to share a common uptake route (Bentley, 1992; Glynn, 2001), exhibited decreased toxicity at elevated Ca\(^{2+}\) and H\(^+\) concentrations (Hollis et al., 1999; Niyogi and Wood, 2004). In contrast to Cd, Cu, Pb and Zn, which disrupt ionregulatory processes, Ni is known as a respiratory toxicant in fish. The toxicity data for Ni are far less extensive than for other metals because Ni was considered as a metal of minor environmental concern due to its low acute toxicity.

At present, metal toxicity in water of a certain chemical composition is predicted on the basis of gill-metal burdens in the organism of interest causing 50% mortality (LA\(_{50}\)) with acute 96h+ toxicity in relation to the dissolved metal concentration. In fish the LA\(_{50}\) values are derived from the metal-gill binding affinity (log K) and capacity (B\(_{\text{max}}\)) constants, which in practice are determined during a short term (2-3h or 24h) exposure to increasing range of waterborne metal concentrations in ion-poor soft water and measuring gill metal concentration at the end of exposure. The challenge is to link a short-term metal accumulation in fish gills with 96h+ toxicity data. Alternatively, De Schamphelaere and Janssen (2002) proposed to use the toxicity data to determine metal binding constants (log K) in crustaceans assuming that the mortality occurs at the critical levels of metal binding to the action site. The stability constants for competing ions (i.e. Ca\(^{2+}\), Mg\(^{2+}\), and Na\(^+\)) are calculated based on the assumption of a linear relationship between the metal concentration exerting a toxic effect and the concentration of individual competing cations (both expressed as free ion activity). Finally, determined metal binding constants are used to calculate metal burdens on an organism at the site of toxic action as a function of dissolved metal concentration in any given water chemistry. However, any factor influencing the binding constants would have a major implication for the BLM approach regardless of the method of their determination.

To accurately predict trace metal toxicity in different waters, it is important to understand how changes in water chemistry change metal uptake, accumulation and toxicity at exposure levels similar to that in real environment. The transporters and
mechanisms involved in metal uptake under low exposure concentrations are not necessarily the same as those involved at the exposure concentrations used in acute toxicity tests. Consequently, the effects of the different environmental factors may also differ from those observed under non-realistic acute scenarios. Moreover, it has been shown that different types of responses and effects will occur at short and extended exposure periods indicating that mechanisms of toxicity under acute and chronic scenarios may be rather different. The objectives of this work were to determine how changes in concentrations of major cations (Ca$^{2+}$, Na$^+$, and H$^+$) in water affect the simultaneous uptake of Cd, Cu, Ni, Pb, and Zn at environmentally realistic levels.

6.2. Materials and methods

6.2.1. Test organisms

Adult *Danio rerio* were obtained from Aquaria Antwerp (Belgium) and reared in 20L glass tanks containing 25-30 fish per aquarium. Fish were held at their optimum temperature of 25 ± 1 °C (OECD guideline 203, 1992) under a 12h light/12h dark regime and fed with commercial granulated tropical fish food (TetraPrima granular) on a 1% of fish weight daily ration. The aquaria were equipped with trickling filters, which also served as aerators, and the water was checked daily for NH$_4^+$, NO$_2^-$ and NO$_3^-$. If the concentration of any of these ions exceeded 5, 2 or 200µM respectively, the medium was partially renewed. Aquarium media were made up from a base soft fresh water (0.1mM CaCl$_2$·2H$_2$O, 0.5mM MgSO$_4$·7H$_2$O and 0.077mM KCl dissolved in deionized water with R>18.2MΩ) by means of adding varying amounts of CaCl$_2$·2H$_2$O or NaCl (analytical grade, Fluka) according to Table 6.1 and non-complexing biological buffers (2mM MES for pH6.3, 1mM MOPS for pH7.2 and 2mM EPPS for pH8.0, brought to the desired pH with 1M NaOH) and aerated for 48 hours. Fish were acclimatized to the test waters for 20 days. Feeding was stopped 24 hours prior to the start of experiments and no food was provided during the exposure period.
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Table 6.1. Composition of the test media and experimental condition (constant parameters: 0.5mM Mg$^{2+}$, 0.078mM K$^{+}$). Conditions with significant changes in metal speciation are highlighted in italics. IS- ionic strength.

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<th>Concentrations of free metal ions, nM</th>
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6.2.2. Experimental procedure

The experiments were conducted in 5L polypropylene aquaria filled with freshly prepared test media. Each exposure solution was spiked with 0.025µM $^{106}$Cd, 0.05µM $^{65}$Cu, 0.1µM $^{62}$Ni, 0.025µM $^{204}$Pb, and 0.1µM $^{67}$Zn (STB Isotope GmbH, Germany) 24
hours prior to the experiments to allow trace metals to reach equilibrium with the test water. Fish were sampled from the acclimation tanks and randomly transferred to experimental containers with five fish per aquarium and four aquaria per condition. At 17, 22, 40 and 48h one fish was randomly sampled from each test aquarium, allowed to swim in fresh test water without metal additions for 10 to 15 minutes to wash out any metals weakly bounded to the surface, blotted dry and killed by spinal dissection. Then, gills were carefully dissected from fish. Both gills and the rest of the body were dried to a constant weight at 60°C, weighed and digested with concentrated nitric acid/hydrogen peroxide mixture (Ultrapure, Merck) in a microwave. In parallel, water samples were collected for metal measurements. The samples were filtered through a 0.45 μm membrane (NC45, Schleicher & Schüll), acidified with 69% HNO₃ to achieve a final acid concentration of 1% and kept in a freezer until analysis. All samples were analysed for metals by inductively coupled plasma mass spectrometry (Varian Expert 700 quadrupole ICP-MS, Mulgrave, Australia). The calibration standards were prepared from a 1000mg L⁻¹ ICP multi-element standard solution IV (Merck) by dilution with 1% (v/v) nitric acid. Yttrium was added to all standards and samples as an internal standard. The machine was recalibrated every 15 to 20 samples to account for possible machine drift. The calculation procedure, chemical speciation modelling and statistical methods used are identical to those described in Chapter 3.

6.3. Results

In this study separate measurements of metal concentrations in gills and the rest of the body were performed. Gills, body (meaning a whole fish without gills) and whole body metal concentrations were expressed on µM·kgdw(tissue)⁻¹ basis, the uptake data were fitted to either linear or hyperbolic functions and metal uptake rates constants were calculated as described in Chapter 3. Due to the small contribution of the gill tissue, there was no statistical difference in metal uptake rates measured for the body without and with gills within each treatment. For that reason, only gills and whole body metal uptake rate constants are reported. The average background concentrations of naturally occurring
metals in zebrafish did not vary significantly during the exposure period and were equal to (µM·kgdw(tissue)⁻¹): 0.9±0.6(gills) and 0.9±0.5(whole body) for Cd; 86.2±16.3(gills) and 118.5±31.4(whole body) for Cu; 4.1±2.5(gills) and 15.1±4.3(whole body) for Ni; 5.4±1.1(gills) and 4.5±0.9(whole body) for Pb; 3010±369(gills) and 5147±1068(whole body) for Zn. The contribution of the newly accumulated metals to the total metal burdens varied from being significant (in case of Cd, Ni and Pb) to comprising a small fraction (in case of essential Cu and Zn). The maximum concentrations of the enriched isotopes taken up at different conditions were in the range (µM·kgdw(tissue)⁻¹) 4.1-12.3(gills) and 0.7-2.3(whole body) for Cd; 15.4-35.9(gills) and 4.2-17.6(whole body) for Cu; 0.8-2.2(gills) and 1.3-3.8(whole body) for Ni; 7.6-41.7(gills) and 2.9-5.3(whole body) for Pb; 31.4-46.5(gills) and 39.6-50.7(whole body) for Zn.

6.3.1. Cd uptake

The Cd uptake by zebrafish occurred via gills rather than via skin or gastrointestinal tract since metals concentrations increased rapidly in gills after exposure (Fig. 6.1). The gills uptake rate constants (ku) calculated on the total dissolved metal concentration scale were much higher than those of the whole body at all exposure conditions. Increase in ambient calcium concentrations strongly suppressed Cd uptake by gills. The ku dropped from 10.3 to 1.6 L·kgdw(tissue)⁻¹·h⁻¹ when Ca concentrations increased from 0.1 to 2.5mM. However, there was only a slight decrease in the whole body uptake rate constants when ambient calcium concentration increased from 0.1 to 0.5 mM with no further effect observed. Sodium had a similar, although less pronounced, effect on Cd uptake. Increasing sodium concentrations in the medium from 0.5 to 8mM resulted in a double decreased in Cd (gills) ku from 8.6 to 4.4L·kgdw(tissue)⁻¹·h⁻¹, while no statistical difference in the whole body Cd uptake constants was observed (ANOVA, p=0.237). In contrast, increasing the pH of the medium linearly increased both gills and whole body Cd levels. When the ku values were expressed on the free metal ion activity scale, the same trends were observed at all examined conditions.
6.3.2. Cu uptake

Gills and total body Cu uptake rate constants followed similar patterns at all exposure conditions with $k_u$ of gills being much higher than that of the whole body (Fig. 6.2). Both low (0.1mM) and high (2.5mM) calcium concentrations in the medium resulted in a three to five fold suppression of Cu uptake respectively compared to the middle (0.5mM) conditions. The increase in sodium concentrations linearly decreased Cu uptake by gills, although the change was not statistically significant. The whole body Cu uptake rate constant was higher at 2mM sodium compared to 0.5mM and 8mM sodium. A similar effect on the Cu uptake had a change in the pH of the medium. The highest uptake rate constants were observed at neutral conditions (pH7) in both gills and the total body, while lower $k_u$ values were recorded at pH6 and pH8. When the changes in the free metal ion concentrations were taken into account, uptake rate constants expressed on the free metal ion scale were the lower at pH6 compared to pH7 and pH8.

6.3.3. Ni uptake

In contrast to Cd and Cu, Ni was accumulated in zebrafish in much lower quantities (Fig. 6.3). The whole body uptake rate constants were slightly higher than those of gills at all experimental conditions with the largest differences observed in the calcium exposures. Addition of calcium at 0.1mM and 2.5mM increased whole body $k_u$ 1.5 times compared to the intermediate condition (0.5mM). At the same time, Ni uptake by gills was not significantly affected by changes in calcium concentrations. Sodium, in turn, promoted Ni uptake by gills at the highest exposure concentration (8mM), while no effect of elevating sodium concentrations on the whole body uptake rate constants was observed. The increasing pH of the medium increased Ni uptake in both gills and the whole body. At pH8, gills and the whole body $k_u$ values increased 1.6 to 2.3 times in comparison with neutral or acidic conditions, where no difference in Ni uptake was observed.
6.3.4. Pb uptake

The results for the Pb uptake showed considerably larger (up to eight times) $k_u$ values for the gills than for the whole body under all conditions, indicating that the gills where the main site of uptake (Fig. 6.4). The highest gill uptake rate constants were obtained at the intermediate conditions (2mM Na, or 0.5mM Ca, or pH7), while both lower and higher concentrations of the same cation suppressed Pb uptake. The whole body Pb uptake rate constants slightly increased at the highest calcium concentration of 2.5mM and remained the same at varying sodium concentrations. Both acidic (pH6) and basic (pH8) conditions decreased the whole body Pb uptake. When the changes in the Pb free ion concentrations were taken into account, lower gill and the whole body uptake rate constants were observed at acidic conditions compared to $k_u$ values calculated at pH7 and pH8, which were statistically the same.

6.3.5. Zn uptake

The Zn accumulation in both gills and whole body followed a more complicated pattern compared to the other metals (Fig. 6.5). Between 17 and 22h, there was a sudden 15 to 20 fold increase in Zn accumulation by gills at all examined conditions. Zn tissue concentrations reached up to 46 $\mu$gZn·kgdw(tissue)$^{-1}$ at 22h, then drop to 4-10 $\mu$gZn·kgdw(tissue)$^{-1}$ by 40h and remained at somewhat constant level further on. The whole body Zn accumulation patterns repeated those of gills in a less extreme manner.
Figure 6.1. Effect of Ca, Na and pH on Cd uptake during exposure to 0.025µM $^{106}$Cd, 0.05µM $^{65}$Cu, 0.1µM $^{62}$Ni, 0.025µM $^{204}$Pb and 0.1µM $^{67}$Zn (25°C, 0.5mM Mg$^{2+}$, 0.078mM K$^+$). Statistically different uptake rates are denoted by different low case (gills) and upper case (whole body) letters.
Figure 6.2. Effect of Ca, Na and pH on Cu uptake during exposure to 0.025µM $^{106}$Cd, 0.05µM $^{65}$Cu, 0.1µM $^{62}$Ni, 0.025µM $^{204}$Pb and 0.1µM $^{67}$Zn (25°C, 0.5mM Mg$^{2+}$, 0.078mM K$^+$). Statistically different uptake rates are denoted by different low case (gills) and upper case (whole body) letters.
Figure 6.2. Continued
Effect of Na, Ca and pH on the uptake of Cd, Cu, Ni, Pb and Zn in Danio

Figure 6.3. Effect of Ca, Na and pH on Ni uptake during exposure to 0.025µM $^{106}$Cd, 0.05µM $^{65}$Cu, 0.1µM $^{62}$Ni, 0.025µM $^{204}$Pb and 0.1µM $^{67}$Zn (25°C, 0.5mM Mg$^{2+}$, 0.078mM K$^+$). Statistically different uptake rates are denoted by different low case (gills) and upper case (whole body) letters.
Figure 6.4. Effect of Ca, Na and pH on Pb uptake during exposure to 0.025μM $^{106}$Cd, 0.05μM $^{65}$Cu, 0.1μM $^{62}$Ni, 0.025μM $^{204}$Pb and 0.1μM $^{67}$Zn (25°C, 0.5mM Mg$^{2+}$, 0.078mM K$^+ $). Statistically different uptake rates are denoted by different low case (gills) and upper case (whole body) letters.
The maximum accumulation levels were between 40 and 51 µg Zn·kgdw(tissue)^{-1}, which gradually decreased to 4-12 µg Zn·kgdw(tissue)^{-1} by the end of the exposure. These results indicate that the exposure to the Zn isotope triggers a regulatory process that eliminates the newly accumulated Zn after an initial uptake phase. To see if there was any change in the accumulation pattern due to increasing Ca^{2+}, Na^{+} or H^{+} water concentrations, the Zn concentrations in gills and whole body were compared at 22h (maximum concentration in tissue) and 48h (end of exposure) by ANOVA test. The
analysis showed that gills accumulated more Zn at the end of exposure with increasing pH ($p=0.0034$), while a significant increase in the maximum whole body Zn concentration was observed with rising calcium concentrations ($p=0.0218$).

### 6.4. Discussion

#### 6.4.1. Gills and whole body metal uptake

The fish gills are the primary target for trace metals entry due to their ion- and acid-base balance regulatory functions (Niyogi and Wood, 2003). Nevertheless, a significant metal influx can occur through the skin, fins, and head of a freshwater fish (Wicklund-Glynn, 2001). Freshwater fish may bind divalent metals at gill surfaces in at least two different sites. High affinity-low capacity binding sites are involved when external metal concentrations are in a low environmentally relevant range, while low affinity-high capacity sites become more involved when exposed to higher metal concentrations such as during acute toxicity tests (Niyogi and Wood, 2003). Studies showed that in rainbow trout Cd and Cu high affinity/low capacity gills binding sites exhibit saturation at waterborne Cd and Cu concentrations below 0.25\( \mu \text{g L}^{-1} \) (Taylor et al., 2000; Hollis et al., 1999). Moreover, both Na\(^+\)-sensitive and Na\(^+\)-insensitive components (Grosell and Wood, 2002) are present in high affinity gill Cu-binding sites. Therefore, changing environmental conditions may have different effects on metal accumulation during exposure at low concentrations compared to higher levels.

In this respect, gills are the first organ to look at for any alterations in metal bioaccumulation. Indeed, our studies revealed that variations in Ca\(^{2+}\), Na\(^+\) and H\(^+\) concentrations in the medium caused larger variations in gills metal uptake rates compared to the whole body uptake, where the effects were mitigated. Moreover, Cd, Cu and Pb were accumulated in gills at much higher rates than in the whole body. In contrast, Ni was taken up faster by the whole body than by the gills. This observation is accordance with findings by Pane et al. (2003), who examined Ni distribution in organs of rainbow trout exposed to 11.6mg (Ni)·L\(^{-1}\) for 117h. The authors determined three fold...
higher Ni accumulation in the intestine and in the plasma of the whole fish (on a wet weight basis) than in gills.

It was not possible to determine Zn uptake rates due to its fast elimination from both gills and body of zebrafish. However, observed accumulation patterns indicate higher Zn accumulation in the body than in gills. It has been shown that in yellow perch both gills and intestine are involved in Zn uptake, but only gills play an important role in maintaining Zn homeostasis by reducing the affinity of waterborne Zn transport pathways and increasing the transport rates during a chronic exposure to elevated Zn concentrations (Niyogi et al., 2007). Thus, lower Zn accumulation in gills compared to the whole body
observed in our study at the end of exposure may be a result of such increase in Zn transport rates in gills.

**6.4.2. Effect of Ca**

Addition of Ca\[^{2+}\] to the medium had variable effects on the uptake of metals. Increasing calcium levels suppressed Cd accumulation in both gills and whole fish. Cu and Pb uptake rates were highest at the middle calcium concentration (0.5mM) and the opposite trend was observed for Ni. Our observations of decreased Cd uptake with elevating Ca\[^{2+}\] concentrations are in accordance with competitive model for Cd and Ca uptake at the same uptake sites and internal receptors leading to toxicity. It is known that Cd uptake occurs first via voltage-insensitive Ca\[^{2+}\] channels on the apical membrane and thus competes with Ca binding to uptake sites. Once inside the cell, Cd blocks Ca uptake via competitive inhibition of basolateral high affinity Ca\[^{2+}\]-ATPase (Niyogi and Wood, 2003, 2004). As a result of such competition, both Cd and Ca influence each other’s uptake. Waterborne Ca\[^{2+}\] at concentrations between 0.04 to 2mM has been shown to decrease branchial Cd uptake in rainbow trout, yellow perch and tilapia during both short time (2 to 3h, 48h or 72h) and chronic (30d) exposures to Cd in a concentration range from 0.02 to 0.45µM Cd (Hollis et al., 2000; Niyogi and Wood, 2008). Detailed studies showed that the acute suppression of Cd accumulation in fish gills with increasing Ca levels in water was a result of decreased gills affinity for Cd and reduction in number of binding sites (Hollis et al., 2000; Niyogi and Wood, 2008) suggesting that the Cd binding characteristics of gills are not constant and depend on the chemistry of ambient water.

Cu accumulation in gills and the whole body were suppressed at low (0.1mM) and high (2.5mM) calcium concentrations in the medium. The literature concerning the effect of water hardness on Cu uptake and toxicity is contradictory. No effect of water hardness on Cu accumulation in juvenile rainbow trout was observed during exposure to Cu in a concentration range 0.4 to 6.2µM for 24h (Lauren et al., 1986). Similarly, studies by Playle et al. (1992) showed no difference in Cu accumulation by gills of fathead
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minnows exposed to 0.25µM Cu for 2 to 3h in soft (0.05mM Ca\textsuperscript{2+}) and hard (2 to 4mM Ca\textsuperscript{2+}) waters at pH6.3, although calcium additions reduced Cu accumulation at pH4.8. On the other hand, the protective effect of calcium on Cu toxicity has been shown for Indian major carp (*Labeo rohita*) and juvenile catfish (*Channa punctatus*) (Erickson et al., 1996; Adhikari, 2003). Radioisotope studies performed by Pyle and Wood (2008) revealed that Cu accumulation in gills of rainbow trout and yellow perch exposed to 0.3-0.9µM Cu was highly variable within the first 12h of exposure and stabilized only within the following 12h. After 25h of exposure both species accumulated more Cu in soft (0.11mM Ca\textsuperscript{2+}) than in hard (1.2mM Ca\textsuperscript{2+}) water. The possible explanation of decreased Cu uptake at low calcium concentration is the alteration of physiological processes at extreme conditions. Taylor et al. (2000) hypothesised that calcium may regulate the number and affinity of binding sites on the gill surface. Indeed, genomic studies revealed that acclimation of zebrafish to soft water resulted in 3.5 fold induction in gene expression of epithelium calcium channels after 6 days of exposure to soft water in an attempt to make up for low external calcium concentrations (Craig et al., 2007), which would lead to more binding sites being available for both Ca and interfering metals. In another study Taylor et al. (2002) investigated Cu accumulation in rainbow trout acclimated to hard water (1.0mM Ca) and exposed to 0.26µM \textsuperscript{64}Cu for three hours. The results showed that when the fish was acutely transferred from hard to soft water (0.13mM) for three hours prior to the Cu exposure, the organisms accumulated 3 times more Cu in gills compared to the fish left in hard water, and five times more Cu when the transfer occurred 24h prior to Cu exposure. The results were explained by disturbances in ion regulatory system caused by changes in ambient calcium concentrations.

Increasing ambient calcium concentrations affected Pb uptake in a similar manner as Cu. The highest Pb gill uptake rate constants were determined at the middle calcium concentrations, although the total body Pb uptake was significantly suppressed only at 2.5mM Ca. Similar effects of water hardness on Pb uptake were observed by Grosell et al. (2006) in fathead minnows. The authors reported a significantly higher Pb bioconcentration factor in fish exposed to 0.1µM Pb at 0.5mM test water Ca\textsuperscript{2+} compared
to the exposure in soft (0.14µM Ca\(^{2+}\)) or hard (1.0µM Ca\(^{2+}\)) waters. Decreased Pb uptake at high Ca levels is not surprising since Pb is known to competitively interact with apical voltage-independent Ca\(^{2+}\) channels and was reported to reduce Ca\(^{2+}\) uptake in rainbow trout and carp (Stouthart et al., 1994; Rogers and Wood, 2003). At the same time, increasing waterborne Ca concentrations reduce branchial Pb accumulation in rainbow trout (Rogers and Wood, 2004). Due to a finite number of Ca\(^{2+}\) transport proteins at the gill surface, it should be expected that increasing Ca\(^{2+}\) will out-compete Pb binding to active sites and reduce Pb uptake during exposure at low Pb concentrations. Reduced Pb uptake in soft waters may be a result of increased expression of calcium transporting proteins in gill membranes of zebrafish and facilitated calcium transport in an attempt to maintain ion homeostasis (Craig et al., 2007).

The absence of a calcium effect on Ni accumulation in zebrafish gills is not unexpected since Ni is not an ion regulatory toxicant in fish. No impact of acute Ni exposure on average unidirectional or net fluxes of Na\(^{+}\), Cl\(^{-}\) or Ca\(^{2+}\) was observed in juvenile trout exposed for 48 to 60 hours to 15.6mg (Ni)-L\(^{-1}\) (Pane et al., 2003). Slight decrease in total body Ni uptake rate constant observed in our study at the middle 0.5mM Ca concentration may be an indication of physiological changes taking place in the whole organism.

### 6.4.3. Effect of Na

The observed suppression of Cu accumulation at increased sodium concentrations in water is in accordance with the mechanism of branchial Cu toxicity which involves interference with active sodium uptake via apical Na\(^{+}\) channel and basolateral transport by Na\(^{+}/K^{+}\)-ATPase (Bury et al., 2003).

It has been generally accepted that Cd does not interfere with sodium uptake pathways. Therefore, little attention has been paid to the possible effect of sodium on Cd accumulation in freshwater fish species. Nevertheless, increasing ambient sodium concentrations decreased Cd uptake by gills. A recent study by Zhang and Wang (2007) showed a significant decrease in Cd uptake by both gills and the whole body of
eurhythaline black sea bream (*Acanthopagrus schlegeli*) with increasing salinities (0-35psu). Although the reported decrease in Cd uptake in saline water was mainly attributed to the changes in speciation due to formation of Cd-chloride complexes, our observations indicate that sodium also plays a significant role in Cd uptake and further research should be conducted in that direction to elucidate a possible mechanism of Cd-Na interactions.

There is an evidence of Pb induced disruption of Na⁺ and Cl⁻ balance in rainbow trout reported by Rogers et al. (2005). The authors found that waterborne Pb decreases the maximal Na⁺ influx rate without a change in transport affinity and immediately decreases carbonic anhydrase activity. The time study revealed that exposure to 0.48µM Pb inhibited Na⁺-K⁺-ATPase activity by 24h, while addition of 4.8µM Pb resulted in immediate enzyme inhibition. The analysis of Pb burdens showed higher Pb accumulation in gills at 1.0mM Na⁺ compared to 0.5mM and 10mM external Na during exposure to the maximal Pb concentration (4.8µM), while no changes were recorded at 0.48µM Pb. In spite of a significant reduction in Na⁺ influx rates, the reduction in enzyme activity was weakly correlated with gill Pb accumulation after three and eight hours of exposure; however, the relationship strengthened by 24h. Moreover, the authors found a significant reduction in Na⁺ efflux rates resulting in a net Na loss occurred during a prolonged 24 to 48h exposure to Pb. Therefore, to observe any changes in Pb accumulation at low exposure levels, longer than 24h exposure duration might be necessary. Our studies showed that Pb uptake rate peaked at the middle 2mM Na concentration during a 48h exposure to 0.025µM Pb, which is in agreement with observations by Rogers et al. (2005) reported for 4.8µM Pb.

The number of studies investigating the mechanism of Ni toxicity at different water chemistries is rather limited and to our knowledge none of them have investigated the effect of changing Na concentrations in ambient water. As mentioned earlier, Ni does not cause any changes in unidirectional or net fluxes of Na⁺, Cl⁻, or Ca²⁺ in juvenile trout exposed for 48 to 60h to 15.6mg (Ni)·L⁻¹ (Pane et al., 2002). Therefore, the increase in gills Ni uptake at the highest Na concentration of 8mM was surprising. The fact that no
changes in the whole body Ni accumulation were observed is in agreement with another study by Pane et al. (2004), who stated that Ni toxicity is an exclusively branchial phenomenon of respiratory nature. Our results indicate that there is some degree of Ni-Na interactions in zebrafish, which requires further investigations.

6.4.4. Effect of pH

The results showed increasing Cd and Ni uptake in both gills and the whole body with increasing pH. Cu and Pb, in turn, exhibited highest accumulation at pH7 when the \( k_n \) values were calculated using total dissolved isotope concentrations. Playle et al. (1993) reported reduced Cd accumulation in gills at pH4.8 during a short-term acute Cd exposure due to \( \text{H}^+ / \text{Cd}^{2+} \) (similar to \( \text{Ca}^{2+} / \text{Cd}^{2+} \)) competition on the gill surface. In contrast, recent studies by Niyogi et al. (2008) showed no effect of pH in a broad range (4.5 to 9.5) on Cd accumulation in gills of rainbow trout during a three hour exposure to 0.16µM Cd. It should be noted, however, that those experiments were performed at Cd concentrations close to the saturation level of high affinity-low capacity Cd binding sites. In our studies, we exposed zebrafish to much lower Cd concentration (0.0125µM) and thus, allowed possible \( \text{H}^+ / \text{Cd}^{2+} \) competition to take place.

The literature concerning the effect of pH on Ni toxicity is contradictive. The toxicity studies performed by Deleebeeck et al. (2007) reported a 4.5 decrease in Ni LC\(_{50}\) values determined during a 17d exposure of rainbow trout to Ni with increasing pH in a range of 5.5 to 8.5. In another study, Hoang et al. (2004) observed no effect of pH at low levels of hardness and alkalinity (20mg CaCO\(_3\)·L\(^{-1}\)), but reported a 1.5-fold increase in Ni toxicity over a pH range of 7.3 to 8.5 at higher levels of hardness and alkalinity (100mg CaCO\(_3\)·L\(^{-1}\)). In contrast, Pyle et al. (2002) showed 3-4 times increase in 96h Ni LC\(_{50}\) values determined for fathead minnows with increasing pH in a range 5.5-8.5. The results were explained by a significant decrease in free ion Ni\(^{2+}\) concentration at pH8.5 occurring in the soft water used during toxicity testing. Our speciation calculations showed that changes in pH from 6 to 8 caused less than 1% decrease in Ni\(^{2+}\) free ion concentrations, which does not explain the significant change in the uptake rate. At this point the
underlying mechanism of pH effect on Ni uptake and toxicity remains unclear and requires further investigation.

A similar pattern of higher Cu and Pb accumulation at pH7 compared to pH6 and pH8 suggests similar mechanisms being involved in $\text{H}^+/\text{Cu}^{2+}$ and $\text{H}^+/\text{Pb}^{2+}$ interactions, which can be explained by changes in Cu and lead speciation. Calculations using Visual Minteq2.4b software showed that free ion Cu$^{2+}$ and Pb$^{2+}$ concentrations decrease with increasing pH. At pH6 $\text{H}^+/\text{Cu}^{2+}$ and $\text{H}^+/\text{Pb}^{2+}$ competitions lead to decreased metal uptake. Lower uptake rates observed at pH8 are due to decreased free ion concentration, which is confirmed by similar $k_u$ values obtained when correction for the free ion concentration was made. Similar results were reported by Grosell et al. (2006), who found the absence of a pH effect on Pb bioaccumulation during a chronic 30d exposure of fathead minnow, although both reduced and increased pH (6.7 and 8.1 respectively) substantially increased Pb toxicity compared to the control pH of 7.4. De Schamphelaere and Janssen (2002) proposed that CuOH$^+$ ions are also bioavailable to a biotic ligand and explained an increase in EC$$_{50}$ with increasing acidity by co-toxicity of Cu hydroxide ions rather than proton competition. Since our results showed decreased rather than increased Cu and Pb uptake at pH8, it is possible that hydroxide ions make a noticeable contribution to metal uptake only at metal concentrations close to the acute toxicity levels.

6.5. Conclusions

The study revealed that variations in Ca$^{2+}$, Na$^+$, and H$^+$ concentrations in the medium affected the uptake of trace metals by zebrafish showing larger variations in Cd, Cu and Pb uptake rates in fish gills, the primary target for trace metals entry, compared to the whole body and the opposite trend observed for Ni. The suppression of Cu uptake at different water chemistry parameters and pH dependent increase in Cd and Ni accumulations should be the main points of concern when setting water quality criteria. Moreover, changes in concentrations of major cations in water did not influence the uptake of trace metal in zebrafish and *Daphnia magna* in the same manner due to
differences in structural and functional organizations of the organisms. Therefore, direct recalibration of fish-based biotic ligand models to other species based solely on toxicity data without consideration of the toxicity mechanism is inadequate approach.

6.6. References


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Effect of Na, Ca and pH on the uptake of Cd, Cu, Ni, Pb and Zn in Danio


CHAPTER 7

Multi-metal Interactions between Cd, Cu, Ni, Pb and Zn
Uptake from Water in the Zebrafish Danio rerio
Abstract

In natural aquatic environments trace metals are often present together. Uptake of metals in mixtures may demonstrate a series of interactive effects and modify the toxicity of individual elements. In the current chapter the uptake of essential (Cu, Ni and Zn) and non-essential (Cd and Pb) metals in gills and the body of zebrafish exposed to a mixture of trace elements was assessed. Stepwise additions of trace elements to the exposure medium revealed the presence of both negative and positive interactions occurring at environmentally relevant concentrations. The gills and total body uptake displayed linear trends except in the case of Zn. The Pb uptake process was influenced the most by other metals. The Pb uptake rate was enhanced in the presence of Ni and Zn at concentrations above 0.1µM and suppressed at Cd and Cu concentrations above 0.0125 and 0.4µM, respectively. Additions of Pb, in turn, had an antagonistic effect on Cd uptake and remarkably promoted uptake of Cu at Pb concentrations above 0.025µM. The uptake of Cd was decreased by Cu, Pb and Zn and increased in the presence of Ni at concentrations above 0.1µM. The observed 3.5 time reduction of Cd uptake and doubling of the Ni uptake rate in zebrafish gills in the presence of 0.4µM Cu was surprising since these metals are known to enter the cell via different routes. These results demonstrate that metals interact significantly at the level of uptake under environmentally realistic concentrations and these interactions may be of key significance in understanding and predicting of metal uptake, accumulation and toxicity in multi-metal exposure scenarios.

7.1. Introduction

In natural environments aquatic organisms are often affected by trace metals due to anthropogenic activities. In contaminated environments several metals are often present together at elevated concentrations (Borgmann et al., 2008). Some of these trace metals, such as Cu and Zn, play an important role in cellular metabolism and their body concentrations can be regulated by the organisms. Others, such as Cd and Pb, are toxic
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even at low concentrations and tend to accumulate in the body (Rainbow, 1997, 2002). Present as a mixture in ambient waters trace metals may, however, enter the organisms via common uptake routes and interact with each other affecting uptake, bioaccumulation and toxicity. Uptake of metals in mixtures may demonstrate competitive and non-competitive inhibition and in some cases enhanced uptake (Borgmann et al., 2008). The type of interactions depends on the metals involved, their external concentration, availability and exposure scenario, length of exposure, studied species and examined organs (Amiard-Triquet and Amiard, 1998; Norwood et al., 2003). The existence of metal-metal interactions occurring at low, environmentally relevant concentrations has been reported for different crustacean (Rainbow et al., 2000; Komjarova and Blust, 2008) and fish species (Tao et al., 1999; Birceanu et al., 2008). Thus, it is necessary to consider possible multi-metal interactions, which are currently disregarded by water quality guidelines, when setting site specific water quality criteria (Norwood, 2003). At present, the BLM is the most wide-spread model used for prediction of metal toxicity in aquatic systems (Borgmann et al., 2008). However, it was developed for single metals and, therefore, the problem of modeling metal toxicity in multi-metal environments has not been solved by the BLM and further work is required (Paquin et al., 2002).

The first step in the development of metal toxicity involves the initial binding and internalization of the metals, followed by the internal partitioning of the accumulated metals between metabolically active and detoxified forms (Luoma and Rainbow, 2005). Thus, changes in the uptake process will be the first indicator of metal interactions occurring in the organism. The purpose of this work was to assess how and to what extent the presence of metals in a mixture affects their uptake in zebrafish Danio rerio. The uptakes of Cd, Cu, Ni, Pb, and Zn from a mixture of metals in different proportions were studied using a stable isotope technique that allows direct measurement of newly accumulated metal by ICP-MS. The choice of exposure concentrations was based on available information concerning environmental metal concentrations in more or less polluted waters (e.g. concentrations of these metals in natural water systems in Flanders
(Belgium) vary between 0.1-12.2µg·L⁻¹ Cd, 1-30.1µg·L⁻¹ Cu, 2.7-19.8µg·L⁻¹ Ni, 0.1-
1µg·L⁻¹ Pb and 47-2168µg·L⁻¹ Zn, Bervoets et al., 2003; Reynders et al., 2008).

7.2. Materials and methods

7.2.1. Test organisms

Adult *Danio rerio* were obtained from Aquaria Antwerp and maintained in 20L
glass tanks containing 25-30 fish per aquarium. Fish were held at 25 ± 1°C (OECD
guideline 203, 1992) under a 12h light/12h dark regime and fed with the commercial
granulated tropical fish food TetraPrima granular on a 1% of fish weight daily ration. The
aquaria were equipped with trickling filters, which also served as aerators. The water
was checked daily for NH₄⁺, NO₂⁻ and NO₃⁻. If the concentration of any of these ions
exceeded 5, 2 or 200µM respectively, the medium was partially renewed. The aquarium
medium was prepared by dissolving 0.5mM CaCl₂·2H₂O, 2mM NaCl, 0.5mM
MgSO₄·7H₂O, 0.077mM KCl and 1mM MOPS (to keep the pH at 7-7.2) in deionized
water with R>18.2MΩ and aerating for 48 hours. Fish were acclimatized to the test
waters for at least 20 days. Feeding was stopped 24 hours prior to the start of experiments
and no food was provided during the exposure period.

7.2.2. Experimental procedure

All chemicals used in the experiments were reagent grade. Stable isotopes were
obtained from STB Isotope GmbH, Germany. Ultra pure water (MilliQ, R>18.2MΩ) was
used for the preparation of the medium. The experiments were conducted in 5L
polypropylene aquaria filled with freshly prepared test media. The exposure medium was
spiked with increasing amounts of ¹⁰⁶Cd, ⁶⁵Cu, ⁶²Ni, ²⁰⁴Pb, and ⁶⁷Zn (according to Table
5.1) 24 hours prior to conducting the experiments to allow trace metals to reach
equilibrium with the test water. Acclimated organisms were randomly distributed among
experimental containers with 5 fish per aquarium and four aquaria per condition. At 3, 21,
28 and 46h one fish was sampled randomly from each aquarium, allowed to swim in fresh
test water without metal additions for 10-15 minutes to wash out any metals weakly
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bounded to the surface, blotted dry and killed by spinal dissection. Then, gills were carefully dissected from fish and the other parts were pooled. Both gills and the rest of the body were dried to a constant weight at 60°C, weighed and digested with 69% nitric acid/35% hydrogen peroxide mixture (Ultrapur, Merck) in a microwave. In parallel, water samples were collected for the total dissolved metal concentrations measurements. The samples were filtered through a 0.45μm cellulose nitrate membrane (NC45, Schleicher & Schüll), acidified with 69% HNO₃ to achieve final acid concentration of 1% and kept in a freezer until analysis. Analytical and computational procedures were analogous to those described in Chapter 3.

7.3. Results and discussion

During the course of the experiments the concentrations of metals (added isotopes and naturally occurring metals) were followed separately in the gills and the body of each fish, which allowed both gills and total body metal uptake rates to be quantified. The uptakes of all added metal isotopes with exception to Zn, could be described by simple linear functions at all examined conditions. Thus, the uptake rates were equal to the slope of the linear regression analysis describing the increase in metal concentrations due to the uptake of the stable tracers (further referred to as metals) in tissues as a function of time (Fig. 7.1). In case of Zn accumulation patterns in both gills and the body were more complicated. The Zn uptake by gills was marked by a fast, concentration dependent increase in metal concentrations within 21-28 hours followed by the elimination of the Zn from the tissue (Fig. 7.2A) and the presence of a regulation mechanism was evident on a total body level (Fig. 7.2B). Thus, accurate quantification of Zn uptake rates could not be made since uptake appeared to shut down after an initial uptake phase. However, it was possible to determine the effect of Zn on the uptake rates of other metals. A similar Zn accumulation pattern was observed in a previous study (Komjarova and Blust, submitted) suggesting a high level of homeostatic Zn regulation in zebrafish. The limited accumulation of Zn in fish at higher exposure levels than those used in the current study was reported earlier. For example, a study performed by McGeer et al. (2000) showed
only a slight Zn buildup in the gills and a negligible internal accumulation during a 10 day exposure of rainbow trout to 250µg·L⁻¹ waterborne Zn. In another study by Wepener et al. (2001), a decrease in Zn concentration in gills of *Tilapia sparrmanii* was observed during the first 12 hours of exposure to 1000µgZn·L⁻¹. Although the amounts of accumulated Zn gradually increased within the next 48 hours, the concentrations of Zn in gill tissue of tilapia returned to a control level after 96h.

![Figure 7.1](image_url)

**Figure 7.1.** Determination of Cd uptake rates by gills of zebrafish exposed to a range of 106Cd total dissolved concentrations. (mean±SD, n=4, pH7, 2mM Na⁺, 0.078mM K⁺, 0.5mM Ca²⁺ and 0.5mM Mg²⁺, t=25°C).
Figure 7.2. Zn uptake by zebrafish gills (A) and the whole body (B) exposed to a range of $^{67}$Zn total dissolved concentrations (mean±SD, n=4, pH7, 2mM Na$^+$, 0.078mM K$^+$, 0.5mM Ca$^{2+}$ and 0.5mM Mg$^{2+}$, t=25°C).
7.3.1. Effect of Cd on multi-metal uptake

During the course of the exposure Cd was progressively accumulating in the gills and the body of zebrafish as a function of the Cd concentrations in the medium (Fig. 7.3). The Cu, Ni and Pb uptake rates in zebrafish gills were not affected by changes in ambient Cd concentrations, although some variations in the total body Cu and Ni concentrations were observed. In contrast, the total body Pb uptake rates decreased significantly with increasing amounts of Cd in water.

The absence of a Cd effect on Cu and Ni uptake was expected, since these elements are known to enter fish via different uptake routes: Cd actively interacts with calcium channels, whereas Cu uptake interferes with sodium pathways (Paquin et al., 2002). A study by Hollis et al. (2001) showed that exposure to 0.03µM Cd did not alter Cu burdens in gills, kidney or liver of rainbow trout.

Previous studies performed by Rogers and Wood (2004) with rainbow trout reported a reduction in Pb accumulation in gills of fish exposed to 4.8µM Pb for 48h with increasing Cd concentrations, which was attributed to a competition between Cd and Pb for entry into the cell via Ca²⁺ channels. Authors observed a continuous decrease in branchial Pb accumulation with stepwise additions of Cd to the medium until Cd concentrations reached 0.01µM. After that the Pb uptake rate remained constant in spite of a further increase in Cd concentrations in water. The absence of a Cd effect on branchial Pb uptake in zebrafish observed in our study is not in contrast with the study by Rogers and Wood (2004) since we used Cd concentrations in the range 0.0125-0.2µM and lower Pb exposure concentrations. Moreover, Birceanu et al. (2008) observed only a slight and insignificant decrease in Pb accumulation in gills of rainbow trout acclimated to soft water and exposed for 96h to 0.01µM Pb with stepwise Cd additions up to 0.07µM Cd. After initial absorption, Pb, as well as Cd, enters the blood stream and rapidly accumulates in soft tissues, primarily in kidney (Hollis et al., 2001; Rogers et al., 2003; Patel et al., 2006). The transfer of Cd and Pb into the blood stream and their uptake in soft tissues is mediated by a high-affinity Ca²⁺-ATPase enzyme, which activity is reduced by Cd stronger compared to Pb (Verbost et al., 1987; Rogers and Wood, 2004).
Figure 7.3. Effect of increasing Cd concentrations on metal uptake rates in gills (A) and the whole body (B) of zebrafish exposed to varying Cd concentrations and 0.025μM Cu, 0.1μM Ni, 0.025μM Pb, 0.1μM Zn. (mean±SD, n=4, pH 7, 2mM Na+, 0.078mM K+, 0.5mM Ca$^{2+}$ and 0.5mM Mg$^{2+}$, t=25°C). For each metal, the treatments resulting in statistically different uptake rates are denoted by different low case letters.
Several studies indicated that Cd accumulates in higher concentrations in kidney than in gills during acute and chronic exposures resulting in a synthesis of Cd binding metallothioneins, while the opposite trend is observed for Pb without induction of the Pb-MT complexes (Hollis et al., 2000; Hollis et al., 2001; Huang et al., 2007). In contrast to Cd, Pb was found to be associated mostly with a noncytosolic fraction of the cell (Linde et al., 1999).

7.3.2. Effect of Cu

Increasing concentrations of Cu in water had a significant effect on Cd, Cu, Ni and Pb uptake rates showing similar patterns in both gills and the body (Fig. 7.4). Addition of Cu to the external medium decreased Cd uptake 3.5 and 3 times in gills and the body respectively as Cu concentration reached 0.4µM. In contrast, Ni uptake was promoted in the presence of Cu at concentrations above 0.1µM. Cu affected Pb accumulation in a more complex manner. The Pb uptake process was slowed down at both low (0.025µM) and high (0.4µM) Cu concentrations. The uptake rates of Cu itself were increasing as a function of Cu concentrations in the medium.

The observed inhibitory effect of Cu on Cd accumulation is quite surprising due to the difference in Cu and Cd uptake and toxicity modes, as mentioned earlier. Nevertheless, the presence of Cu-Cd interactions has been reported for different fish species (Pelgrom et al., 1995; Eroglu et al., 2005). The study by Pelgrom et al. (1995) showed that Cu-Cd interactions in tilapia were more pronounced when the organisms were exposed to metals at environmentally realistic concentrations (0.3µM Cu and 0.04µM Cd). A stimulating effect of Cu on Pb uptake and vice versa was reported by Tao et al. (1999) in neon tetras. Studies showed that Pb causes both competitive and non-competitive inhibition of Na⁺ and Cl⁻ influx in rainbow trout (Rogers et al., 2003, 2005). Thus, a direct competition at Na⁺ channels for binding sites may be partially responsible for the reduction in Pb uptake rates at the highest Cu concentration.
Figure 7.4. Effect of increasing Cu concentrations on metal uptake rates in gills (A) and the whole body (B) of zebrafish exposed to varying Cu concentrations and 0.0125µM Cd, 0.1µM Ni, 0.025µM Pb, 0.1µM Zn. (mean±SD, n=4, pH7, 2mM Na⁺, 0.078mM K⁺, 0.5mM Ca²⁺ and 0.5mM Mg²⁺, t=25°C). For each metal, the treatments resulting in statistically different uptake rates are denoted by different low case letters.
The inhibitory effect of Cu on Cd uptake we observed, as well as the promotion of Ni accumulation, implies the existence of other than Na+/Ca2+ channel for these metals. Divalent Metal (Ion) Transporter 1 (DMT1), which transports Fe2+, may be one of such common uptake sites (Bury et al., 2003). It has been demonstrated by Garrick et al. (2006) that DMT1 has affinity to several metals, which decreases in the following order: Mn>?Cd>?Fe>Pb~Co~Ni>Zn with unknown Cu position.

### 7.3.3. Effect of Ni

As expected, Ni accumulation in gills and the body increased as a function of dissolved Ni concentration. Changes in the Ni concentrations in the medium affected Cd and Pb uptake processes and had no effect on Cu uptake (Fig. 7.5). Ni concentrations above 0.1µM decreased Cd uptake rates in gills, while the amounts of Cd accumulated by the whole zebrafish were increasing with rising Ni concentrations in water. A similar pattern, although statistically not significant, was observed for Cu uptake. The Pb uptake rates in both gills and total body greatly increased with Ni additions to water and the largest effect was observed at the lower end of the exposure Ni concentrations. In total, Pb uptake rates doubled as Ni concentrations increased from 0.1µM to 1.6µM.

Ni is a respiratory rather than an ionregulatory toxicant in fish, which causes ultrastructural damage to the respiratory epithelium of the fish gills (Brix et al., 2004; Pane et al., 2003). A protective effect of Ca2+ and Mg2+ ions against Ni toxicity in rainbow trout has been reported by Deleebeeck et al. (2007). Moreover, both Ca and Mg have been demonstrated to be involved in the regulation of membrane permeability (Ebel and Günther, 1980; McWilliams, 1983; Hunn, 1985). After entering a blood stream Ni accumulates significantly only in kidneys, where it interferes with Mg2+ metabolism presumably by a direct competition with Mg for reabsorption pathways (Pane et al., 2005). Based on obtained and literature results, Deleebeeck et al. (2007) postulated that observed protective effect of Mg on Ni toxicity to rainbow trout was partly due to the existence of a shared Ni2+/Mg2+ transport system. Similar protective effects of waterborne Ca2+ and Mg2+ ions against Pb toxicity in rainbow trout were reported in the literature (McDonald et al., 2002; Rogers and Wood, 2004).
**Figure 7.5.** Effect of increasing Ni concentrations on metal uptake rates in gills (A) and the whole body (B) of zebrafish exposed to varying Ni concentrations and 0.0125µM Cd, 0.025µM Cu, 0.025µM Pb, 0.1mM Zn. (mean±SD, n=4, pH7, 2mM Na⁺, 0.078mM K⁺, 0.5mM Ca²⁺ and 0.5mM Mg²⁺, t=25°C). For each metal, the treatments resulting in statistically different uptake rates are denoted by different low case letters.
Thus, increased Pb uptake at elevated Ni concentrations may be partially due the presence of a shared pathway or existence of a multi-metal transporter with affinities for both Ni\(^{2+}\) and Pb\(^{2+}\). At this point, however, the mechanisms underlying Ni-Pb and Ni-Cd interactions remain unclear.

### 7.3.4. Effect of Pb

Pb uptake in gills and body increased with Pb concentrations in water. Additions of increasing amounts of Pb to water had a significant interacting effect on Cd, Cu and Ni uptake in gills, as well as in the body (Fig. 7.6). Cd uptake was initially promoted by addition of 0.1µM Pb to water. However, when more Pb was added (0.4µM), the amounts of accumulated Pb dropped significantly. The effect was more pronounced in the body than in the gills. A similar pattern was observed for Ni with the gills showing the most pronounced effect compared to the whole body. The Cu uptake rates in gills were strongly increasing with elevating ambient Pb concentrations, although in the body the effect was less pronounced.

The observed interaction between Pb and Cd is in agreement with the inhibition effects obtained in the Cd and Pb experiments. Indeed, regression analysis revealed the presence of strong Pb-Cd interaction on the total body level (Pb=f(Cd), slope=2.71±0.95, Pearson \(r =0.785, p=0.04, N=7\)), which was the only interaction observed in present study. Less than additive metal-gill binding was reported in rainbow trout exposed to a mixture of Pb and Cd at environmentally relevant concentrations in soft, moderately acidic waters (Birceanu et al., 2008). However, the ionic disturbances caused by Cd plus Pb were greater than additive, which ultimately may increase the toxicity of Cd–Pb mixtures to fishes. Moreover, the Pb facilitated Cu uptake indicates a disruptive Pb interference with one or more Cu uptake systems with possible involvement of unknown transporter proteins in the accumulation process.
Multi-metal interactions between Cd, Cu, Ni, Pb and Zn uptake in Danio

Figure 7.6. Effect of increasing Pb concentrations on metal uptake rates in gills (A) and the whole body (B) of zebrafish exposed to varying Pb concentrations and 0.0125µM Cd, 0.025µM Cu, 0.1µM Ni, 0.1µM Zn. (mean±SD, n=4, pH7, 2mM Na⁺, 0.078mM K⁺, 0.5mM Ca²⁺ and 0.5mM Mg²⁺, t=25°C). For each metal, the treatments resulting in statistically different uptake rates are denoted by low case letters.
7.3.5. Effect of Zn

The uptake rates of all studied metals were affected by changes in the dissolved Zn concentrations (Fig. 7.7). Cd uptake in the total body and Ni uptake in both the gills and the body of zebrafish significantly decreased at Zn concentrations above 0.1µM. In contrast, positive interactions were observed for Cu and Pb at Zn concentrations above 0.1µM. Increase in ambient Zn concentrations strongly affected Pb uptake by gills resulting in three times higher Pb uptake rates at 1.6µM Zn compared to 0.1µM Zn.

Branchial Zn uptake occurs via epithelial calcium channels, a number of Zn regulated transporting proteins and via an amino acid transporter (Bury et al., 2003; Glover, 2003). Based on the observation of Zn accumulations in the plasma, but not in the gill tissue of rainbow trout, it has been suggested that Zn enters the gill tissue and passes through it into a blood stream (Spry et al., 1988). Wepener et al. (2001) also suggested that Zn is easily replaced in gills by more competitive Cu resulting in Zn traversing the gill tissue and entering a blood stream. In contrast to the gills, Zn uptake via the gastrointestinal tract does not appear to involve Ca uptake pathways (Ojo and Wood, 2008). Several studies have reported the interaction of Zn with the uptake of other metals. For example, a reduction of Cd uptake by increasing Zn concentrations in the range 2-16µM was observed in zebrafish exposed to 0.1µM Cd (Glynn et al., 2001). A reduction in the uptake of both Cd and Zn was reported in the deposit-feeding polychaete Capitella capitata exposed to elevated Cd or Zn concentrations (Goto and Wallace, 2007). Dethloff et al. (1999) found that gill Cu concentrations of rainbow trout increased following the exposure to a metal mixture containing Cu and Zn.
Multi-metal interactions between Cd, Cu, Ni, Pb and Zn uptake in Danio

Figure 7.7. Effect of increasing Zn concentrations on metal uptake rates in gills (A) and the whole body (B) of zebrafish exposed to varying Zn concentrations and 0.0125µM Cd, 0.025µM Cu, 0.1µM Ni, 0.025µM Pb. (mean±SD, n=4, pH7, 2mM Na⁺, 0.078mM K⁺, 0.5mM Ca²⁺ and 0.5mM Mg²⁺, t=25°C). For each metal, the treatments resulting in statistically different uptake rates are denoted by low case letters.
7.4. Conclusions

The stable isotope technique employed in this study revealed the presence of significant interactive effects on metal uptake in multi-metal exposure uptake experiments in the zebrafish *Danio rerio* at low and environmentally relevant exposure concentrations. Among all studied metals, Pb uptake appeared to be the most sensitive to the presence of other metals. Although Cd and Cu suppressed Pb uptake at concentrations above 0.0125µM and 0.4µM, respectively, Ni and Zn enhanced the uptake of Pb at concentrations above 0.1µM. Additions of Pb, in turn, had an antagonistic effect on Cd uptake and remarkably promoted Cu uptake at Pb concentrations above 0.025µM. The uptake of Cd was reduced by Cu, Pb, and Zn. The observed 3.5 time reduction in Cd uptake and doubling of Ni uptake rate in zebrafish gills in the presence of 0.4µM Cu was surprising since these metals are known to enter the cell via different routes. This study has demonstrated the existence of several stimulatory and inhibitory interactions among metals during uptake. The observation that these interactions occur at environmentally relevant concentrations implies that they have to be taken into account when performing metal risk assessments and setting environmental quality criteria.

7.5. References


Multi-metal interactions between Cd, Cu, Ni, Pb and Zn uptake in Danio


Multi-metal interactions between Cd, Cu, Ni, Pb and Zn uptake in Danio


CHAPTER 8

CONCLUSIONS
Conclusions

This thesis focuses on the study of trace metals uptake processes in aquatic organisms *Pseudokirchneriella subcapitata*, *Daphnia magna* and zebrafish *Danio rerio* during a simultaneous exposure to Cd, Cu, Ni, Pb and Zn at environmentally realistic concentrations using a stable isotopes approach. Additionally, the transfer of metals from water to algae and from algae to daphnids has been studied. Special attention is given to the influence of major cations (Ca$^{2+}$, H$^+$, and Na$^+$) present in ambient waters and possible interactions between metals on the uptake of trace elements. Chapter 1 is introductory and provides background information on trace metal uptake and accumulation. Chapter 2 deals with the instrumental technique used and the optimization of the metal isotopes measurements by quadrupole ICP-MS. The original research is described in the following five chapters corresponding to peer-reviewed articles that have been published or submitted to international journals.

Metal uptake by *Daphnia magna* from water and food sources

Aquatic organisms are exposed to metals via dissolved and solid phases. The importance of each metal source for the uptake depends on the concentrations and availability of the metals in these different phases and the structural and functional organization of the organisms. Once inside the organism, metals taken in from water and food do not necessarily follow the same internal pathways resulting in different internal tissue distributions, accumulation levels and toxicological effects. Therefore it is important to know the relative importance of these different sources in metal exposure, availability and accumulation. In this study a stable isotope technique was applied to investigate the uptake of Cd, Cu, Ni, Pb and Zn by the green algae *Pseudokirchneriella subcapitata* and the water-flea *Daphnia magna* during simultaneous exposure to five metals at environmentally realistic concentrations from water and the subsequent transfer of the metals from the algae to the daphnids via a dietary route. Simultaneous multi-metal exposure allowed direct comparison of the results among the metals since the data for the different metals were obtained on the same organisms. The results showed that green
algae take up Cu faster than Cd, Ni, Pb and Zn and the distribution of metals between the external and internal compartments depends on the metal and algae growth stage. The metal uptake in *Daphnia magna* was characterized in terms of uptake rate constants. When metals were taken up by daphnids from the water phase the uptake rate constants expressed on a total metal concentration scale increased in the following order: Ni<Zn<Cd<Pb<Cu. The trend remained the same when expressed on a free metal ion concentration or activity basis. However, the free metal ion based uptake rate constants for Cu and Pb showed a strong increase because of the more pronounced complexation effect for these two metals compared to the three others at neutral or alkaline pH. The accumulation of metals by *Daphnia* from a food source was quantified in terms of assimilation efficiencies, which showed strong differences among metals ranging from about 80% in case of Cd to near 0% in case of Ni. The accumulation of Cd, Cu and Zn by daphnids from algae was accompanied by a fast elimination of a fraction of these metals originally present in the organisms. Overall, water appeared to be the most important route of uptake by *Daphnia magna* compared to food when both algae and *Daphnia* were exposed to metals in the same environment.

**Effect of Ca\(^{2+}\), Na\(^{+}\) and pH on metal uptake in *Daphnia magna***

The accumulation of trace metals in aquatic organisms is known to be highly dependent on concentrations of major naturally occurring cations (Ca\(^{2+}\), H\(^{+}\), Mg\(^{2+}\), Na\(^{+}\)) present in ambient water. It is well documented for a number of aquatic organisms that hard waters have a protective effect against Cd toxicity, while sodium, in turn, has been shown to affect the acute Cu toxicity. Another important factor affecting metal accumulation is pH since it is directly linked to the speciation of such metals as Cu and Pb and may also influence the activity of metal membrane transporters. Nevertheless, the number of studies investigating the influence of water chemistry on metal uptake or accumulation in *Daphnia magna* at environmentally relevant concentrations is rather limited. The results of the present work showed that ambient water characteristics, i.e. Ca\(^{2+}\), Na\(^{+}\), and pH clearly influenced the uptake of Cd, Ni, Pb and Zn in *Daphnia magna*. At the same time there was no effect from increasing concentrations Ca\(^{2+}\), Na\(^{+}\), or pH on
Cu uptake process possibly due to a combination of low Cu concentration and high homeostatic regulation of total body concentration in *Daphnia*. Calcium had a pronounced suppressing effect on the uptake of Cd and Ni and decreased Pb and Zn uptake rates at 2.5mM (Ca). The effect of sodium on metal accumulation was more complex. Increased Cd and Pb uptake rate constants were observed at elevated sodium concentrations. A bell-shaped distribution of uptake rate constants with a maximum at the middle Na$^+$ concentration of 2mM was observed for Zn. Moreover, there was no effect from varying sodium concentrations on the Ni uptake process. Variations in pH of the medium affected Cd, Ni and Zn accumulation and had no effect on Cu and Pb uptake. When *Daphnia* were exposed to acidic conditions (pH6), the Cd and Ni uptake rate constants were the highest, while equally low values were observed at neutral and basic conditions. In contrast, the uptake rates of Zn were linearly increasing with increasing pH of the medium. The obtained results demonstrate that concentrations of major cations in the medium have an important effect on metal uptake and that the effects are metal specific. The results also show that the observed effects on metal uptake at these low ecologically relevant exposure concentrations are not exactly the same as previously reported under considerably higher and often acute toxic metal exposure scenarios.

**Metal interactions in *Daphnia magna***

Natural waters are frequently contaminated by trace metals as a result of human activities. In such conditions aquatic organisms are often exposed to a mixture of metals rather than a single element resulting in possible inter-metal interactions during (and after) the uptake process. Such effects are poorly documented and generally neglected when developing toxicity guidelines. In this respect, the stable isotope technique applied in this study allowed to reveal the presence of significant metal interactions in *Daphnia magna* that occurred at environmentally realistic concentration and affected bioavailability of both toxic and essential metals. Specifically, Cd and Cu exhibited a suppressing effect on the uptake rates of other metals, with exception to Pb, present in the mixture. The effect was already pronounced at low Cd and Cu concentrations and reached a maximum at higher concentrations. Ni and Zn showed weaker interactions with Cd and
between each other, while having no effect on Cu and Pb uptake. There was a high degree of correlation between Cd, Ni and Zn uptake rates indicating that these metals share at least partially common uptake or interaction pathways. Moreover, a significant correlation between Zn and Cu uptake rates suggested that more than one mechanism is involved in the accumulation process. The uptake of Pb was marked by a high initial rate reaching saturation within 24-48 hours. Cd applied at a concentration of 0.2µM was the only metal which affected the Pb uptake process by stimulating it. Pb added to the medium at a concentration of 0.25µM, in turn, increased Cu uptake. Since with exceptions to Pb the interactions were mostly negative, it is possible that contamination of surface waters by one of the studied metals will not increase the toxicity of other metals present in the environment. However, the observation that the uptake of the essential elements Cu and Zn is already influenced by the presence of other metals at such low concentrations may create problems related to the uptake and homeostatic regulation of the essential metals even at low exposure levels.

Effect of Ca\(^{2+}\), Na\(^{+}\) and pH on metal uptake in Danio rerio

To accurately predict trace metal toxicity in different waters, it is important to understand how changes in water chemistry change metal uptake, accumulation and toxicity at exposure levels similar to that in real environments. Previous studies presented in chapter 4 showed that ambient water characteristics, i.e. concentrations of Ca\(^{2+}\), H\(^{+}\), and Na\(^{+}\) cations significantly affected the uptake of Cd, Ni, Pb and Zn in Daphnia magna exposed to trace metals at environmentally relevant concentrations. Therefore, it was worth investigating whether similar effects will be observed in organisms of higher development level, such as zebrafish, at the equivalent exposure conditions. The studies revealed that variations in Ca\(^{2+}\), Na\(^{-}\) and H\(^{+}\) concentrations in the medium affected, although in a different manner compared to Daphnia, the uptake of trace metals by zebrafish showing larger variations in Cd, Cu and Pb uptake rates in fish gills, the primary target for trace metals entry, compared to the whole body with the opposite trend observed for Ni. Moreover, a high level of Zn homeostatic regulation, marked by a fast elimination of the metal from both gills and body of zebrafish was observed. In contrast
to *Daphnia*, where increasing Ca levels suppressed uptake of all metals except Cu, addition of Ca$^{2+}$ to the medium had a profound suppressing effect only on Cd accumulation. The highest Cu, Pb and the lowest Ni uptake rates were observed at the middle Ca concentration (0.5mM). Increase in ambient Na concentrations had no or very little effect on the whole body Cd, Cu and Ni uptake. However, decreased Cd, Cu and increased Ni uptake rates were observed in gills with elevating sodium concentrations which may be related to ionregulatory alterations. Both low and high sodium concentrations in water had a suppressing effect on Pb uptake rates in gills and the total body. The increase in pH of the medium led to the promotion of Cd and Ni uptakes in both gills and the whole body. Cu and Pb, in turn, exhibited higher accumulation at pH7 compared to pH6 and pH8, which is explained by changes in Cu and Pb speciation at acidic and basic pH conditions. Overall, the suppression of Cu uptake at low calcium and pH, and high calcium and sodium concentration in the medium and the pH dependent increase in Cd and Ni accumulations should be the main points of concern when setting water quality criteria. Moreover, changes in concentrations of major cations in water did not influence the uptake of trace metal in zebrafish and *Daphnia magna* in the same manner due to differences in structural and functional organizations of the organisms. Therefore, extrapolation of the impact of environmental conditions on metal uptake and toxicity from one species to another without consideration of the uptake and toxicity mechanisms may produce unrealistic results.

**Metal interactions in *Danio rerio***

Previous studies with *Daphnia magna* (chapter 5) showed the presence of significant inter-metal interactions affecting the uptake of trace metals at low exposure levels. The next question was whether interactive effects among metals also occurred in *Danio* and whether the effects were comparable with those observed in *Daphnia*. The Pb accumulation process was influenced the most by other metals. The Pb uptake rate was enhanced in the presence of Ni and Zn at concentrations above 0.1µM and suppressed at Cd and Cu concentrations above 0.0125µM and 0.4µM respectively. Additions of Pb to the medium had, in turn, an antagonistic effect on Cd uptake and remarkably promoted
Conclusions

uptake of Cu at Pb concentrations above 0.025\(\mu\)M. Taking into account multiple sources and the extent of Pb pollution, the increase in Cu uptake already occurring at low ambient Pb concentrations may result in higher Cu toxicity in polluted areas. The accumulation of another common pollutant, Cd, was reduced by Cu, Pb and Zn and increased in the presence of Ni at concentrations above 0.1\(\mu\)M. The observed 3.5 time reduction of Cd uptake and doubling of Ni accumulation rate in zebrafish gills in the presence of 0.4\(\mu\)M Cu was surprising since these metals are believed to enter the cell via different routes. In contrast to Daphnia, where most of the metal interactions were negative, the observation of positive interactions in zebrafish on metal uptake illustrates the complexity of multi-metal exposure. Given the importance of these effects and the fact that they already occur at environmentally relevant exposure levels, it implies that future risk assessment of metals should consider these effects much more than is currently the case.

Perspectives

This study has explored and applied the possibilities of the stable isotopes technique in combination with ICP-MS to study the uptake of metals in three different aquatic organisms under controlled conditions. The study was performed under relatively low exposure concentrations to create conditions that are environmentally relevant. This is in contrast with many of the existing studies where the uptake and effects of metals have been studied under acutely toxic scenarios using exposure concentrations that organisms never encounter in the real world. The results revealed a number of effects and interactions for the different metals studied that have not been reported earlier or showed to be different from those reported at higher exposure levels. Some of these effects, such as the metal-metal interactions, may have important consequences for the risk assessments of metals, which are currently largely based on single metal approaches. This study is a starting point and has mainly illustrated and confirmed the large potential of the stable isotope technique, which revealed a number of effects and interactions previously not described. Towards the future, the technique has to be further developed employing advanced analytical instrumentation such as high resolution ICP-MS and
applied to more chronic studies, aiming to link exposure, uptake and toxicity in a dynamic manner.