Important note

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Below is a list of the journal articles associated with each chapter.

Chapter 2
Bossuyt BTA, Janssen CR. 2004. Long-term acclimation to copper of *Pseudokirchneriella subcapitata* (Korshikov) Hindak: changes on tolerance and physiology. Aquatic Toxicology 68, 61-74.

Chapter 3
Bossuyt BTA, Janssen CR. 2003. Acclimation of *Daphnia magna* to environmentally realistic copper concentrations. Comparative Biochemistry and Physiology Part C 136, 253-264.

Bossuyt BTA, Janssen CR. 2004. Influence of multi-generation acclimation to copper on tolerance, energy reserves and homeostasis of *Daphnia magna* Straus. Environmental Toxicology and Chemistry 23.

Chapter 4
Bossuyt BTA, Janssen CR. (2004). Multi-generation acclimation of *Daphnia magna* Straus to different bioavailable copper concentrations. Archives of Environmental contamination and Toxicology (submitted January 2004).

Chapter 5
Bossuyt BTA, Janssen CR. (2004) Copper regulation and homeostasis of *Daphnia magna* and *Pseudokirchneriella subcapitata*: influence of acclimation. Environmental Pollution (submitted February 2004).

Chapter 6
Bossuyt BTA, Janssen CR (2004). Copper toxicity to different field-collected cladoceran species: intra- and inter-species variation. Environmental Pollution (submitted April 2004).

Bossuyt BTA, Muyssen BTA, Janssen CR. (2004). Relevance of generic and site-specific species sensitivity distributions in the current risk assessment procedures for copper and zinc. Environmental Toxicology and Chemistry (accepted May 2004).

Chapter 7
Bossuyt BTA, De Schamphelaere KAC, Janssen CR. (2004). Using the biotic ligand model for predicting the acute sensitivity of cladoceran dominated communities to copper in natural surface waters. Environmental Science and Technology (submitted January 2004).

UNIVERSITEIT GENT

FACULTEIT LANDBOUWKUNDIGE
EN TOEGEPASTE BIOLOGISCHE
WETENSCHAPPEN

Academiejaar 2003-2004

ACCLIMATION OF FRESHWATER ORGANISMS TO COPPER: EFFECTS ON HOMEOSTASIS AND TOLERANCE

ACCLIMATISATIE VAN ZOETWATERORGANISMEN AAN KOPER: EFFECTEN OP HOMEOSTASIS EN TOLERANTIE

door

ir. Bart T.A. BOSSUYT

Thesis submitted in fulfilment of the requirements for the degree of
Doctor (Ph.D.) in Applied Biological Sciences

Proefschrift voorgedragen tot het bekomen van de graad van
Doctor in de Toegepaste Biologische Wetenschappen

op gezag van
Rector: **Prof. dr. apr. A. DE LEENHEER**

Decaan:
Prof. dr. ir. H. VAN LANGENHOVE

Promotor:
Prof. dr. C. JANSSEN

ISBN 90-5989-018-3

Gold is for the mistress – silver for the maid –
Copper for the craftsman, cunning at his trade.

Author unknown

De promotor, The promoter,

Prof. Dr. Colin Janssen

De auteur, The author

Bart Bossuyt

Dit weten we.
Alle dingen zijn verbonden
als het bloed
dat een familie bijeenhoudt…

Wat de aarde overkomt,
overkomt de zonen en dochters van de aarde.
De mens heeft het levensweb niet geweven;
hij is daarin niet meer dan één draad.
Alles wat hij het web aandoet,
doet hij zichzelf aan.

Ted Perry
Geïnspireerd door Opperhoofd Seattle

This we know.
All things are connected
like the blood
which unites one family…

Whatever befalls the Earth
befalls the sons of the Earth.
Man did not weave the web of life
he is merely strand in it.
Whatever he does to the web,
he does to himself.

Ted Perry
Inspired by Chief Seattle

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ONDERZOEKSGROEP
MILIEUTOXICOLOGIE

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Na ruim 1500 dagen, zullen mijn troeteldiertjes het voortaan zonder mijn gezelschap moeten stellen. Ik denk dat zij mij zullen missen, maar waarschijnlijk nog meer de tientallen liters

lekkere algen die ik dagelijks op hun menu plaatste. En over mijn bezorgdheid voor de goede kweekomstandigheden zullen ze ook niet mogen klagen. Alleen jammer dat tienduizenden watervlooien zijn gesneuveld op het veld van de wetenschap. Bovendien denk ik dat de resultaten van dit onderzoek wel de enkele grammen koper verrechtvaardigen die ik gebruikt heb in de loop van het onderzoek.

Nazareth, 11 februari 2004

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List of abbreviations

AF	Application factor
AFA	Active fulvic acid
AHA	Aldrich humic acid
ANOVA	analysis of variance
ASTM	American Society for Testing and Materials
BAF	Bioaccumulation factor
BCF	Bioconcentration factor
BLM	Biotic ligand model
CEA	Cellular energy allocation
DOC	Dissolved organic carbon
DW	Dry weight
ε_{350}	Absorption coefficient at 350 nm
E_a	Energy available
E_c	Energy consumption
EC50	Effect concentration resulting in 50 % effect
EC10	Effect concentration resulting in 10 % effect
EDTA	Ethylenediaminetetraacetic acid
EQC	Environmental quality criteria
ERA	Environmental risk assessment
E_rC_{50}	Effect concentration resulting in 50 % growth rate inhibition (algae)
EU	European Union
F_x	generation x
HC5	Hazardous concentration that protects 5 % of the organisms
HC50	Hazardous concentration that protects 50 % of the organisms
ISO	International Organization for Standardization
LC50	Lethal concentration for 50 % of the tested organisms
LOEC	Lowest observed effect concentration
l_x	Age-specific survival
MEC	Measured environmental concentration
MOPS	3-N morpholino-propane-sulfonic acid
m_x	Age-specific reproduction
N	Cell density (algae)

ND	Not determined
NOEC	No observed effect concentration
NRA	No risk area
OCEE	Optimal concentration range for essential elements
OECD	Organization for Economic Cooperation and Development
p.a.	Pro analysis
PEC	Predicted environmental concentration
PNEC	Predicted no effect concentration
R_0	Net reproduction
R^2	Determination coefficient
RF	Resistance factor
r_m	intrinsic rate of natural increase
RS	Relative sensitivity (compared to *Daphnia magna*)
RQ	Risk quotient
SD	Standard deviation
SSD	Species sensitivity distribution
SWAD	Surface water database
TGD	Technical guidance document
US EPA	United States Environmental Protection Agency
UV	Ultra violet
WER	Water effect ratio
WHAM	Windermere humic aqueous model
WQC	Water quality criteria

Introduction

This doctoral thesis is situated in the field of aquatic toxicology, *i.e.* the study of the effects of natural and anthropogenic substances on the structure and functioning of freshwater and marine ecosystems (Boudou and Ribeyre, 1989). More specifically, this thesis focuses on the ecotoxicology of metals in freshwater ecosystems. This implies an interdisciplinary approach by using analytical chemistry, environmental toxicology and ecology in order to evaluate the hazard and risks of existing and new chemicals on the function of the receiving ecosystem.

Contrary to the numerous man-made organic chemicals, metals are naturally occurring substances and life has evolved in the presence of these elements. Some of these, the essential metals (like copper), have become incorporated into metabolic processes crucial to survival, growth and reproduction of organisms (Marx, 1987; Linder, 1991; Keen *et al.*, 1993; O'Halloran, 1993). Each species has for each essential element an optimal concentration range (OCEE) in which it can satisfy its metabolic requirements and develop and perform in an optimal way (Van Assche *et al.*, 1997; Hopkin, 1989). This OCEE is linked to the natural (bioavailable) concentration of the essential element in the species' natural habitat and by the species' homeostatic capacity that allows it to regulate its internal essential element concentration. However, when the external concentration of the essential element becomes too low or too high, this homeostatic regulation will fail and deficiency or toxicity may occur, respectively.

Although copper is a very potent toxicant, it has unique properties that justify its use in many wide-spread applications such as electric wires, roof sheeting, water pipes, biocides and soil fertilizers (Landner and Lindeström, 1999). The wide-spread use in these numerous applications and the natural occurrence indicates that organisms are continuously exposed to sub-lethal and lethal copper concentrations.

Risks of copper are being managed through the establishment of environmental quality criteria or by environmental risk assessments (ERA). Recently, it has been recognised that for metals standard procedures for deriving these criteria may not be appropriate to accurately assess their true impact on the ecological quality of aquatic ecosystems (Bergman and Dorward-King, 1997; Janssen *et al.*, 2000). Several possible reasons have been put forward:

(1) ecological relevance of the test media; (2) acclimation/adaptation of species to environmental copper concentrations; (3) community differences in the field; (4) total/dissolved copper concentrations.

First, water quality criteria are mostly derived, using various extrapolation methods, from laboratory toxicity data. As such, the quality and the relevance of the data are crucial to derive ecological meaningful standards. However, the ecological relevance of the laboratory ecotoxicity data is in most cases not considered. These data are usually established with standard assays performed under unrealistic and very different test conditions than the natural environment (low hardness, no dissolved organic carbon, inadequately described or deficient test media…) and with only a few standard species.

Indeed, for waterfleas, it has been demonstrated that many international prescribed standard artificial culture and test media are inadequate to meet the organisms requirements of essential elements including essential metals (Keating *et al.*, 1989; Elendt and Bias, 1990; Muyssen and Janssen, 2001b). Hence, the knowledge of the consequences of long-term exposure to these sub-optimal conditions on the organism's performance in standard toxicity assays is of major importance.

The second reason is the fact that copper is a naturally occurring element with varying background concentrations in different types of aquatic systems and this has, to date, not been considered in most ERA procedures. Depending on the copper background concentration, biological communities in these different systems may have differentially acclimated/adapted to the natural presence of copper resulting in varying species/community sensitivity. Indeed, organisms in the field are exposed to sub-lethal copper concentrations (*i.e.* background or ambient copper concentrations). This long-term (generations) exposure to these concentrations can lead to changes in their copper tolerance and shifts in their OCEE. To allow for the possible incorporation of such data into a regulatory framework, an evaluation of the natural differences in species/community sensitivities due to natural variation in copper exposure is required.

Several studies have demonstrated that acclimation to high or extremely high (not environmentally relevant) metal concentrations can be induced in the laboratory (LeBlanc, 1982; Stubblefield *et al.*, 1999; Muyssen and Janssen, 2001b, Muyssen *et al.*, 2002).

However, no data are available on the acclimation effects and the possible shift in OCEE of organisms exposed to environmentally relevant copper concentrations.

Thirdly, extrapolation of laboratory ecotoxicity data to realistic field responses is based on assessment factors or (theoretical) modelling considerations. Aquatic communities exist of a great amount of organisms. As different ecological systems can have different natural background metal concentrations, the resident aquatic communities can have different sensitivities (cf. above) towards metals and may thus respond differently than standard laboratory species. Additionally, the various resident species can react differently to the ambient copper concentration occurring in the ecosystem.

Finally, current water quality criteria and ERA of metals are based on total or dissolved metal concentrations. However, there is extensive evidence that neither total nor dissolved aqueous concentrations of a metal are good predictors of its bioavailability and hence its eventual impact on an ecosystem's structure and functioning (Bergman and Dorward-King, 1997). To fully evaluate the bioavailability and toxicity of metals, assessment of the physico-chemical characteristics of the aquatic system and the background metal concentration on the one hand, and the sensitivity of the biological assemblages on the other hand, are required.

To include bioavailability in the ERA, models have been developed to predict metal toxicity to aquatic organisms, among others for copper and *D. magna* (*i.e.* the biotic ligand model: BLM; Paquin *et al.*, 2002; De Schamphelaere and Janssen, 2002). As this model is only developed for a limited number of standard laboratory species, species-specific differences (not only between laboratory species but also among field-collected species originating from different aquatic systems) in metal sensitivity are not accounted for.

It is obvious that there are a number of scientific problems which are currently not investigated. Reviewing the laboratory to field extrapolation process we can state that (1) optimal concentration ranges of copper for (standard) organisms have not been established and hence concentrations resulting in deficiency have not been assessed; (2) an evaluation is required of the natural differences in species and/or community sensitivities due to the natural variation in metal background exposure and the relevance of this acclimation (or adaptation) should be considered in the current ERA procedures; (3) the ecological relevance of standard organisms compared to the resident cladoceran species of aquatic systems is unknown; and

(4) the predictability of the acute copper sensitivity of various cladoceran species by the BLM has not been investigated.

The main goal of the current study consisted of two parts. In the first part, we investigated the influence of long-term acclimation of freshwater organisms (green algae and waterfleas) to environmentally relevant copper concentrations (ranging from deficient to toxic) on their copper tolerance, homeostasis, population dynamics and energy budgets and the consequences for ERA procedures were determined. In contrast to these laboratory studies, the second part focussed on field-collected cladoceran species/communities sampled in several aquatic systems. The copper sensitivity of these various species/communities was investigated and related to the water characteristics (ambient copper concentration) and to their ecology. This thesis consists of eight chapters covering the following subjects:

1. a general introduction to the conceptual framework of the study,
2. long-term acclimation of *Pseudokirchneriella subcapitata* to copper,
3. influence of multi-generation acclimation of *Daphnia magna* to copper,
4. multi-generation acclimation to different bioavailable copper concentrations,
5. copper accumulation and homeostasis in *P. subcapitata* and *D. magna*,
6. copper toxicity to field-collected cladoceran species: intra- and interspecies sensitivity, species sensitivity distributions and community sensitivity,
7. the use of the biotic ligand model to predict the acute copper sensitivity in field-collected cladoceran species, and
8. general conclusions and research perspectives.

Chapter 1

General introduction to the conceptual framework of the study

Chapter
1

General introduction to the conceptual framework of the study

1.1. Copper: the facts

1.1.1. The element and its properties

Copper (Cu) is the 29th element, situated between nickel and zinc, with an atomic weight of 63.546 g mol^{-1}. It is placed in the same group (group IB) in the periodic table as silver and gold and is therefore considered as one of the "precious metals" (Landner and Lindeström, 1999). In pure form, copper is a reddish-brown metal, which is relatively soft, but very tough. The density of copper is 8,940 kg m^{-3} at 20°C which ranks it according to Wittmann (1979) into the category of the "heavy" metals. This term is nowadays less frequently used by most environmental scientists due to (a) its inherently negative connotation and (b) the fact that also "lighter" elements (like Be and Sr) were included in this category (Whitton, 1984).

In aerobic freshwater environments, copper mainly occurs in the divalent form, *i.e.* Cu(II), but inside cells it also exists in the monovalent form, *i.e.* Cu(I), which is unstable in oxidizing environments and is rapidly transformed into the Cu(II). Two other oxidation states can occur: metallic copper (Cu(0)) and trivalent copper (Cu(III)). According to the general classification scheme of Turner *et al.* (1981), both Cu(I) and Cu(II) belong to the so-called "borderline metals" with no general preference for either O- and S-donating functional groups (Mason and Jenkins, 1995). According to the Irving-Williams series for complex stability with divalent cations ($Mn^{2+}<Fe^{2+}<Co^{2+}<Ni^{2+}<Cu^{2+}>Zn^{2+}$; Irving and Williams, 1953), copper forms the most stable complexes with both inorganic (ammonia, carbonate, chloride, hydroxide, nitrate and sulphate) and organic complexes (amino acids, amino-sugars, alcohol, urea, etc.) of the 3d-transition metals (Mason and Jenkins, 1995). This also means that copper forms the most stable bonds with functional groups on enzymes and other biomolecules. Hence, copper inherently has the largest toxicity potential of all these metals (Mason and Jenkins, 1995).

Copper was the first metal used by man in any quantity. The earliest workers in copper soon found that it could be easily hammered into sheets and the sheets in turn worked into shapes, which became more complex as their skill increased. The electrical conductivity of copper, which was utilized by Michael Faraday in his epoch-making experiments, remains the key to modern power generation. Indeed, copper has the highest conductivity of any metal other than silver. The ductility of copper, which led to its use for water piping in ancient Egypt, is illustrated by the countless thousands of miles of copper tube in contemporary plumbing and heating systems. The corrosion resistance of copper, which induced the Romans to use it for sheathing the roof of the Pantheon, is today verified by thousands of copper roofs on modern buildings large and small. Other properties that explain the wide-spread use of copper in today's society include its high thermal conductivity, malleability and its good ability to form alloys with other metals (Landner and Lindeström, 1999).

1.1.2. Historical and current use and application (Landner and Lindeström, 1999)

Man recognized very early the special properties of copper, and the use of naturally occurring copper metal is believed to have started as long as 9,000 years ago, in what is now Turkey, as demonstrated by archaeological findings of decorative artefacts from that period. When the technology for mining and smelting carbonate and oxide for copper ores was developed a few thousand years later (Bronze age), there was a considerable rise in copper consumption, often in the form of alloys such as arsenic-copper and bronzes. After the development of new techniques for smelting sulphide ores, there was a further increase in production. It has been estimated that cumulative copper production prior to the establishment of the Roman Empire was more than half a million tonnes. During the Roman period, about 2,000 years ago, production peaked at over 15,000 tonnes per annum, and the cumulative amount used by the Romans has been estimated at about 5 million tonnes. After this period, global copper consumption dropped with temporary peaks during the Sung dynasty of China and after the opening of the Falun mine in Sweden. Large-scale, industrial use of copper did not start until the middle of the 19th century, when the need for copper as material in telegraph cables arose. From that time on, copper mining and production has continued to sharply increase for a large number of applications today.

Nowadays, the most important application for copper metal and copper alloys are: wires and cables for transmission of electricity, the electronic industry, water pipes, heat exchangers, valves, pumps, electrical appliances and motors, cooking utensils and containers, roofing and facing materials on buildings and brake linings. Copper compounds are mainly used in wood preservatives, other biocides and disinfectants, pigments, ship and boat antifouling paints and as nutritive additives to livestock mineral feeds and to fertilizers, in order to prevent copper deficiency (see section 1.2.).

1.1.3. Sources of copper in the environment

1.1.3.1. Occurrence in the earth's crust (Figure 1.1.)

Copper is ranked number 28 among the elements in order of abundance in the earth's crust, with a mean concentration of 50 to 70 mg kg^{-1}. The highest levels are recorded in volcanic, basic rocks, while the lowest concentrations are found in limestone and sandstone. The largest known copper reserves nowadays are located in Chile and the USA, but copper-ores are also found in South Africa, Australia, Canada and Zambia. Known worldwide resources of this metal are estimated at nearly 460×10^6 tonnes of which only about 55×10^6 tonnes (12 %) have been mined throughout history - keeping in mind that nearly all of this 12 % is still in circulation because copper's recycling rate is higher than that of any other engineering metal. Very high concentrations of copper ore deposits exist on all continents of the Earth. In these ores, the copper can occur in sulphide deposits (in the minerals chalcopyrite, bornite, chalcocite and covellite), in carbonate rocks (in the minerals azurite and malachite), in silicate deposits (in the minerals chrysycolla and dioptase) and as pure "native" copper metal.

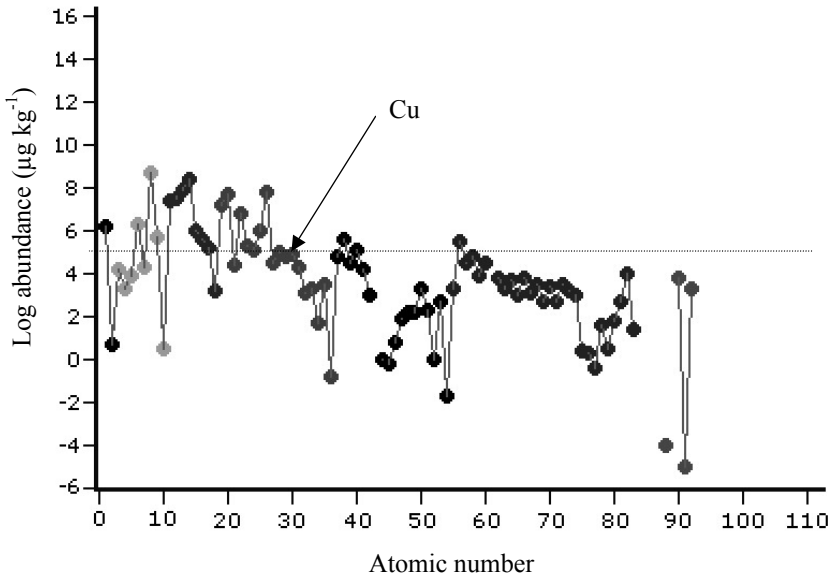

Figure 1.1. Logarithmic abundance of elements in Earth's crust plotted against the atomic number (source: http://www.webelements.com).

1.1.3.2. Natural and anthropogenic sources

All metals present in the environment, except those small quantities that have arrived extra-terrestrially or have been created in nuclear reactions, have been present in and on the Earth since its formation some 4.5 billion years ago (Dalzeil, 1999). According to Landner and Lindeström (1999) 57 % of the copper flux to the atmosphere is natural (*e.g.* volcanic activity, salt spray from oceans, etc.), whereas 43 % is of anthropogenic nature (*e.g.* smelting and refining, mining, etc.). The volcanic eruption of the Pinatubo on 14-15 June 1991, for example, released 600,000 tonnes of copper (Garrett, 2000). Copper fluxes to the oceans are mainly natural (about 80 %, mostly transport from rivers) with only 20 % anthropogenic fluxes (*i.e.* mostly diffuse sources). Rubin (1997) estimated the ocean reservoir for copper to be around 410×10^6 tonnes (with a seawater copper concentration of 0.3×10^{-3} mg kg^{-1}).

Copper fluxes to the freshwater environment highly differ across regions, rivers and water sheds. The combined fluxes of natural sources determine the background concentration of copper in waters (see section 1.1.3.3.), whereas anthropogenic fluxes result in increased (above background) concentrations. It may be assumed that nature has conditioned itself to the fluxes and concentrations of essential trace elements which originate from natural sources and that potential risk stems from anthropogenic sources which cause increases in metal

concentrations above critical levels that may result in adverse effects on ecosystems (Van Tilborg, 2002).

Anthropogenic sources can be classified as point sources or diffuse (non-point) sources (Landner and Lindeström, 1999). Point sources include, among others, mine waste dumps, metal industry, pulp and paper industry, municipal waste water treatment plants, waste dumps and landfills. Diffusive sources include corrosion of copper roofing and copper plumbing systems, street traffic, release of copper from paints, wood preservatives and other pesticides, erosion and leaching from agricultural soils (fertilizers and cattle manure). The relative importance of these sources is geographically very variable.

1.1.3.3. Background copper concentrations

Metals are present naturally throughout the atmospheric, aquatic and terrestrial environments (see section 1.1.3.2.). Background concentrations of metals in the environment are hence above zero and can vary greatly in different media (*e.g.* soils, sediments, water) and are dependent of the geological and environmental conditions. In case of aquatic systems, this is represented in Table 1.1. Background concentrations of copper in surface waters mainly depend on biogeochemical cycling and are, according to Zuurdeeg (1992), between 0.5 and 2.5 µg Cu L^{-1} in Northern Europe. According to an analysis of monitoring data in Europe (data from Belgium, The Netherlands, Sweden, United Kingdom, Germany and Spain; Surface Water Database, SWAD; Heijerick *et al.*, unpublished data) environmental concentrations in Europe range between 0.4 and 11 µg Cu L^{-1} (10^{th} and 90^{th} percentiles, respectively; Figure 1.2). Data from the Banque Nationale des Données sur l'Eau between 1997 and 1999 (n = 4254) showed a mean around 39 µg Cu L^{-1} in the surface waters of France.

These values are not necessarily background concentrations (which are extremely difficult to determine due to wide-spread contamination), but they can be regarded as baseline concentrations because all the samples were taken from locations without known anthropogenic pollution or mineral deposits. The historical background metal concentrations are almost not identifiable. The concentrations nowadays observed in pristine waters should be better termed as environmental/ambient (background) concentration. These concentrations can be considered as "naturally occurring" during the last century.

Table 1.1: Low (10th percentile), average (50th percentile) and high (90th percentile) ambient copper concentration in seven European regions (Heijerick *et al.*, unpublished data).

Region	**Copper concentration (µg Cu L^{-1})**		
	Percentiles		
	10	**50**	**90**
Flanders	2.0	5.4	19.4
Wallon	1.8	2.8	5.4
Germany	2.3	3.5	7.3
Finland	0.2	0.7	4.7
The Netherlands	2.5	3.5	5.4
UK	1.5	3.4	11.2
Sweden	0.4	0.9	2.6

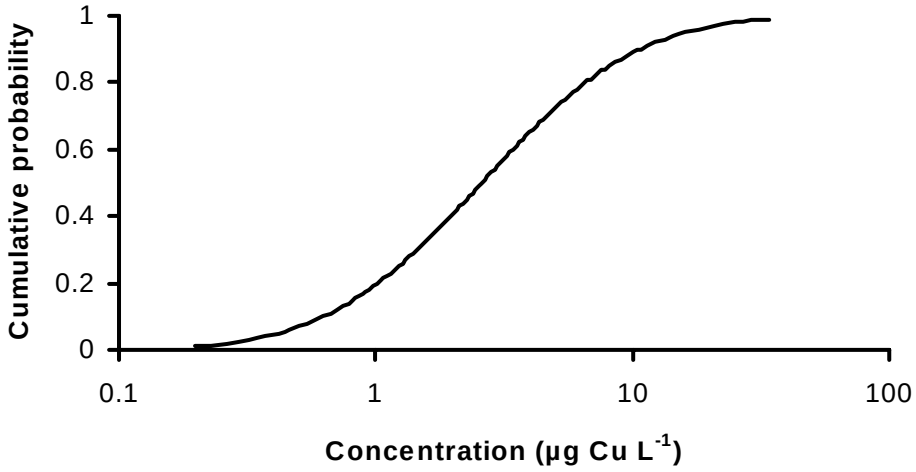

Figure 1.2: Copper concentration distribution (log-normal) for Europe based on the Surface Water Database (SWAD, Heijerick *et al.*, unpublished data).

Local concentrations of metals in the environment can be influenced by human activities. However, unlike synthetic organic chemicals that are introduced into the environment by man, human activities do not introduce additional amounts of metals into the environment. The global bulk concentration of any metal in all media is constant with time. Instead, human activities influence environmental concentrations of metals through enhancing the release of metals and metalloids from the crust and altering their distribution among different

environmental media. In other words, *humans dig out and distribute but do not invent metals and metalloids* (Chapman and Wang, 2000).

1.2. Copper as an essential element: deficiency, homeostasis and toxicity

Several metals (*e.g.* sodium, potassium, magnesium, and calcium) occur in large concentrations in organisms. A second set of metals, termed trace metals, occur at much lower concentrations (normally < 0.01 %). During evolution of life some metals have become essential for normal metabolic functioning. An element is essential when: (1) it is consistently determined to be present in all healthy living tissues within a zoological family, (2) deficiency symptoms are noted with depletion or removal, which disappear when the elements are returned to the tissue, and (3) the deficiency symptoms should be attributed to a distinct biochemical defect (on the molecular level) (Wittmann, 1979). Two separate functions performed by essential metals may be distinguished: (1) metals involved in electron transfer processes (*e.g.* copper, iron, molybdenum), and (2) direct participation of the metal in governing the reaction mechanism (*e.g.* cobalt and zinc). Several metals are essential for various biological functions such as enzymatic and metabolic reactions; a summary is given in Table 1.2 (Leland and Kuwabara, 1985; Depledge and Rainbow, 1990; Goyer, 1996). Note that some metals, such as Fe, Mn, Zn, Cu, Co, and Mo are essential for all living organisms, while the essentiality of other metals, such as Ni, V, I, Cr, and Se, has been established for a limited number of species.

The other metals, for which no biological, nutritional or biochemical function has (yet) been identified, are termed non-essential (*e.g.* cadmium, arsenic, lead, mercury) and can often be highly toxic at certain levels.

Copper is required (as a co-factor) for the functioning of a variety of enzymes (at least 13 in humans) such as superoxide dismutase (a scavenger of toxic oxy-radicals), cytochrome *c*-oxidase (part of the electron transport system in eukaryotic cells), several oxidases (*e.g.* amino oxidase, ascorbate oxidase), mono-oxygenases and di-oxygenases (Cass and Hill, 1980; Lewis, 1993). Copper is also essential for haemocyanin, which is a wide-spread oxygen-carrier in molluscs and arthropods and which is the second most widely distributed pigment in the animal kingdom (Brunori *et al.*, 1979; Cass and Hill, 1980).

Table 1.2: Examples of some essential trace elements and their function (Zumdahl, 1992; Stumm and Morgan, 1996; Butler, 1998; Lane and Morel, 2000).

Element	Biological functions
Cd	Co-factor of carbonic anhydrase when Zn is depleted
Co	Component of vitamin B_{12}
Cr	Co-factor for insulin action, involved in control of cholesterol
Cu	Co-factor of cytochrome, ascorbate oxidase, and plastocyanin; assists in iron storage; involved in production of colour pigments
Fe	Co-factor of cytochrome, catalase, peroxidase, and chelatase; component in haemoglobin and myoglobine
Mn	Co-factor of superoxide dismutase and O_2 evolving enzyme
Mo	Co-factor of nitrogenase; involved in electron transfer processes
Ni	Co-factor of urease and hydrogenase
Se	Co-factor of glutathione peroxidase
Zn	Co-factor of DNA and RNA polymerases, carbonic anhydrase, and alkaline phosphatase

According to Liebig's law of the minimum, each species has for each essential element an optimal concentration range in which it can satisfy its metabolic requirements and develop and perform in an optimal way (Hopkin, 1989). Van Assche *et al.* (1997) termed this range the Optimal Concentration range for Essential Elements (OCEE; Figure 1.3), although the existence of optimal concentrations of essential elements is a fundamental principle in *e.g.* medicine (Wittmann, 1979; Hopkin, 1989). The OCEE is linked with the natural concentration of the essential element in the species' natural habitat. It is further determined by the species' homeostatic capacity that allows it to regulate actively its metabolically required tissue concentrations and maintain optimal levels under varying external concentrations of the essential element. However, when the external concentration of the element becomes too low or too high, homeostatic regulation will not be sufficient and deficiency or toxicity can occur, respectively.

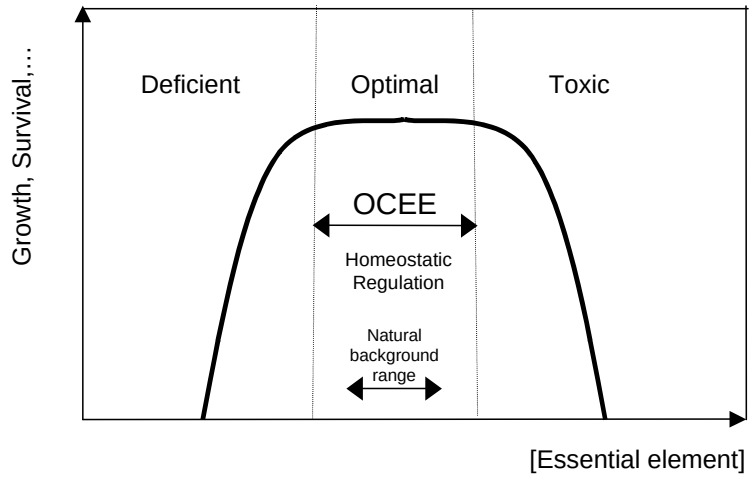

Figure 1.3: The Optimal Concentration range for Essential Elements (OCEE) for a species in a given habitat-type (adapted from Van Assche *et al.*, 1997).

Metal bioaccumulation is an important process in the homeostatic regulation whereby aquatic organisms obtain these essential metals (Adams *et al.*, 2000). Bioaccumulation may be defined as the uptake and net accumulation of a chemical substance by an organism from its environment and/or diet. In the case of aquatic organisms, the ratio of the tissue concentration to the water concentration is termed the bioaccumulation factor (BAF). BAF estimates assume exposure from water or diet or both and are often derived from field data. Bioconcentration factors (BCFs) are used to define the ratio of the tissue concentration to the water concentration and assume water exposure and no dietary exposure. BCFs are typically derived in laboratory experiments and are often used instead of BAFs because the latter are not typically available. Aquatic biota regulate their internal concentrations of essential metals in three ways: active regulation, storage, or a combination of both. As a result of these processes - and more specifically due to active regulation - an inverse relationship exists between water concentrations the of metal and the corresponding BCF. Thus, at low water concentrations, organisms are actively accumulating essential metals (and often non-essential metals via the same uptake mechanisms) to meet their metabolic requirements. At higher water concentrations, organisms with active regulatory mechanisms are able to excrete excess metals or limit uptake (Figure 1.4). An extensive literature review demonstrates that this hypothesis is correct for both essential and non-essential metals for most organisms (Brix and Deforest, 2000).

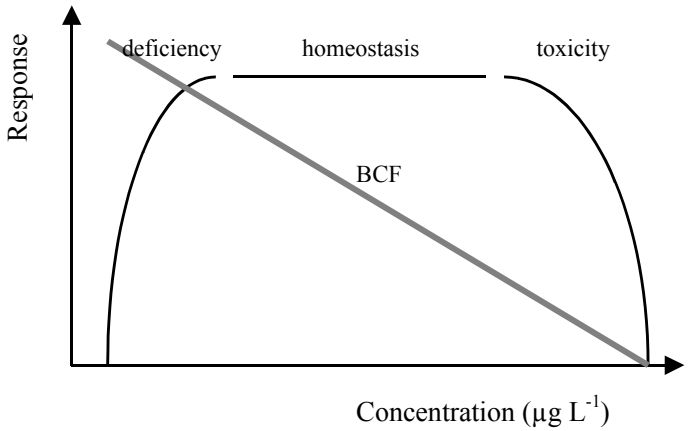

Figure 1.4: Metal regulation showing ranges of deficiency, homeostasis and toxicity (adapted from Adams *et al.*, 2000).

Reports on copper toxicity to aquatic biota are very abundant (Lewis, 1993). Conversely, data on copper deficiency in aquatic organisms are extremely scarce (Stokes and Dreier, 1981). Copper deficiency is observed in plants (agricultural crops, fruit trees, cultivated plants, forest trees and non-cultivated grass species; Landner and Lindeström, 1999). Most of the knowledge concerning copper deficiency and its symptoms in higher animals (mammals and birds) has been acquired through studies with domestic animals from many parts of the world. Naturally occurring copper deficiency among domestic animals is almost exclusively associated with grazing cattle and sheep, whose feed has very low natural copper concentrations (Landner and Lindeström, 1999; McMurray, 1980). Even in humans, copper deficiency (of nutritional origin) has been recognized as an important part of complex nutritional problems in Peru, as an occasional event in premature babies in Western countries, and as a real hazard of over-zealous zinc therapy or of prolonged parental alimentation in children or adults (Danks, 1980; Lewis, 1993).

1.3. Acclimation and adaptation

1.3.1. Effects on metal tolerance

Natural populations in polluted areas are subjected to selective pressures to develop increased resistance (*i.e.* the ability to withstand a toxicant exposure that ultimately results in death) or tolerance (*i.e.* the ability to withstand a toxicant exposure to a given concentration for an indefinite period of time) towards the contaminants present (LaMontagne and McCauley, 2001). This may be achieved either by physiological acclimation or genetic adaptation.

Acclimation and adaptation can be distinguished by the fact that increased tolerance due to adaptation is passed on to subsequent generations, while for acclimation the offspring must also be pre-exposed to acquire it. So, adaptation is a genetic process, beyond the lifespan of the individual and may occur without appreciable metabolic cost (though perhaps with a cost to the population in terms of loss of individuals or genetic diversity; Chapman *et al.*, 1998); Acclimation is a physiological/structural mechanism of gaining increased tolerance within the lifespan of the individual, and may have appreciable metabolic cost. In reviewing the evolution of tolerance to metals in aquatic organisms, it can be concluded that most, but not all, populations in polluted areas do have an increased tolerance (if the published literature accurately represents the situation, *i.e.* negative results having an equal chance of being published) (Klerks and Weis, 1987). If this increased tolerance is caused by acclimation or adaptation is often not reported nor examined. Since metals existed on Earth since its formation of the planet, organisms emerging and evolving on Earth should also be more or less acclimated/adapted to (natural) background levels of metals in their habitats.

Genetic adaptation to metals seems more prevalent in Prokaryotes and Protista than in Metazoa (Klerks and Weis, 1987). This could be expected as micro-organisms have on the average a shorter generation time and gene selection can work faster. Reports on induction of adaptation in the laboratory are therefore restricted to such micro-organisms, *e.g.* with the blue-green alga *Oscillatoria anguistissima* (Ahuja *et al.*, 2001). Another factor hampering induction of adaptation in the laboratory is that genetic adaptation requires sufficient genetic variability which is often lacking in laboratory test organisms (Seitz and Ratte, 1991; Klerks and Levinton, 1993). As a result evidence for metal adaptation comes mainly from field studies with natural populations. In Klerks and Weis (1987) evidence is reviewed on metal adaptation in aquatic organisms, *i.e.* bacteria, fungi, algae, annelids, molluscs, crustaceans, insects and vertebrates. Although definitive evidence for a genetic component in metal tolerance of these aquatic organisms is not always provided, in several studies on different phyla the occurrence of (genetic) adaptation is clearly demonstrated (*e.g.* Bryan and Hummerstone, 1971; Jensen *et al.*, 1974; Foster, 1977; Klerks and Levinton, 1989; Posthuma and Van Straalen, 1993; Postma *et al.*, 1996). Time requirements for adaptation will be species-specific and are generally not mentioned in reports. For the oligochaeta *Limnodrilus hoffmeisteri* increased resistance evolved fairly rapidly, *i.e.* in less than 30 years (Klerks and Levinton, 1989)!

Generally, acclimation to metals is much better documented than the occurrence of adaptation. A major reason for this is that acclimation can be easily induced in the laboratory by pre-exposure to the metal for a duration of only several hours to a few weeks. Metal acclimation has been reported for protozoans (Niederlehner and Cairns, 1992), algae (Kuwabara and Leland, 1986; Wang, 1986; Maeda *et al.*, 1990; Thompson and Couture, 1991; Muyssen and Janssen, 2001a), invertebrates (Saliba and Krzyz, 1976; Stuhlbacher *et al.*, 1992; Pynnönen, 1995; Muyssen and Janssen, 2001b; Muyssen *et al.*, 2002; Muyssen and Janssen, 2002), fish (Dixon and Sprague, 1981; Grosell *et al.*, 1997; Stubblefield *et al.*, 1999; Hollis *et al.*, 1999; Alsop *et al.*, 1999; Grosell *et al.*, 2001), and amphibians (Herkovits and Pérez-Coll, 1995). In all these cases, acclimation was induced by exposure to rather high to extremely high (*i.e.* comparable to those found at contaminated sites) metal concentrations. In general, the change in tolerance is dependent on the acclimation concentration. A theoretical response model is proposed in Figure 1.5. Increased tolerance (response) can be observed with increasing metal concentrations, even at low (natural background) metal concentrations. The upper zone of decreased tolerance may be viewed as a latent lethal effect of the prior exposure alone. In contrast, Chapman (1985) proposed a theoretical model in which this response pattern consists primarily of a zone of increased tolerance bounded on either side by zones of decreased tolerance. The existences of the lower zone of decreased tolerance has been shown in a study of copper-acclimated rainbow trout (Dixon and Sprague, 1981) and probably results from metal uptake insufficient to trigger the induction of detoxification mechanisms.

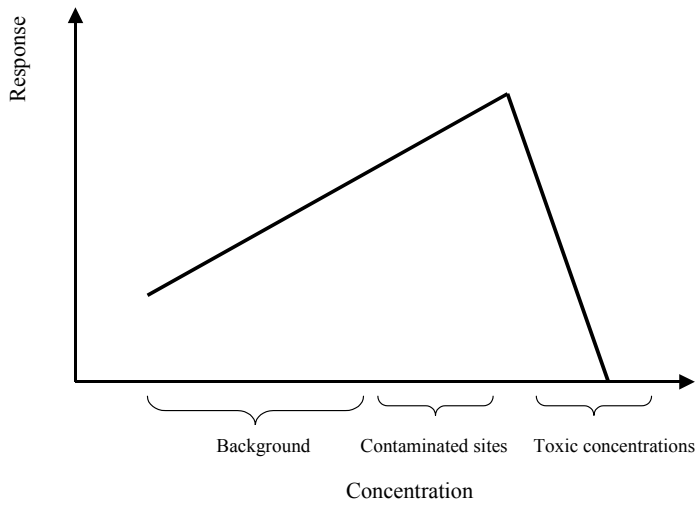

Figure 1.5: Theoretical model of acclimation: effect of metal acclimation on subsequent tolerance to acute metal exposure.

The magnitude of increased tolerance may be metal-specific, and available data suggest that the magnitude of acquired tolerance follows the series Zn > Cu > Cd > Cr (Chapman, 1985). Compared to other toxicants, *e.g.* insecticides (for fish up to a 1000-fold increase in tolerance), acclimation to metals is of much smaller magnitude (Chapman, 1983). Although still substantial, increases in tolerance by a factor up to 7 for daphnids (Stuhlbacher *et al.*, 1992) and up to 13 for fish (Hollis *et al.*, 1999) have been reported for some metals.

1.3.2. Mechanisms of metal resistance

Acclimation and adaptation are based on the development of new or the induction, amelioration or expansion of existing detoxification mechanisms. Bruins *et al.* (2000) identified six mechanisms in micro-organisms: metal exclusion, active transport, extracellular sequestration, intracellular sequestration, enzymatic detoxification and bypassing (alternate pathways) sensitive components. Although, the genetic basis of metal resistance mechanisms in higher organisms is less understood, identical or similar mechanisms have been identified. Metal exclusion, for example, has been found in algae and invertebrates, with or without the release of extracellular metabolites (Bryan and Hummerstone, 1971; Foster, 1977; McKnight and Morel, 1979; Fisher and Fabris, 1982). Active metal excretion as a part of active metal regulation is probably restricted to certain essential trace metals (particularly zinc and copper), and perhaps to particular invertebrate taxa (especially decapod crustaceans) (Rainbow and Dallinger, 1993). Intracellular sequestering through protein binding is reported in plants (incl. algae) and animals by means of phytochelatins or metallothioneins, respectively (Grill *et al.*, 1985; Gekeler *et al.*, 1988; Roesijadi, 1992). In addition to function in the regulation of the essential metals, metallothioneins (in animals and bacteria) and phytochelatins (in algae, plants and some fungi) are also involved in the detoxification of both non-essential and essential metals due to their high affinity for metals (Roesijadi, 1992; Ahner and Morel, 1995). When organisms are exposed to elevated levels of metals, these low-molecular-weight proteins and peptides (6.5 kD and 10 kD, respectively) are induced to bind and detoxify excess metals. Contrary to metallothioneins, phytochelatins are no primary gene products. Glutathione or γ-glutamyl-cysteine have been proposed as precursors for these peptides (Grill *et al.*, 1987). Metallothioneins were first reported as cadmium-binding proteins in horse kidney (Margoshes and Valle, 1957; Kagi and Vallee, 1960) and have since been observed in at least 80 species of fish and invertebrates (Roesijadi, 1992). Studies on the structure,

function, and molecular regulation have, besides their detoxification function, established a central role of these molecules in the regulation of the essential metals copper and zinc.

Other detoxification mechanisms include sequestering of metals in cysts, granules or vesicles. These are found either within the cells or outside the cells in a wide variety of organisms (Silverberg *et al.*, 1976; Brown, 1977; George and Pirie, 1979; Lowe and Moore, 1979; Mason *et al.*, 1984; Bardeggia and Alikhan, 1991). Metal-containing cells and cysts as well as granules are often found in association with the digestive tract or circulatory system (Parametrix, 1995). Metals bound to granules in the gut epithelium can be easily eliminated (by exocytosis or degeneration of complete cells) (Noël-Lambot, 1981; Postma *et al.*, 1996). Calcium deposits, both intracellular and extracellular, sequester divalent cations in a number of marine invertebrates (Fowler *et al.*, 1981). Since many of these animals deposit the calcareous compounds in shells which are metabolically inactive or in exoskeletons which are shed during moulting, the organisms are effectively eliminating metabolically active metals (Fowler, 1977; Hall, 1982; Alikhan, 1990; Groenendijk *et al.*, 1999; Giusti *et al.*, 1999).

1.3.3. Consequences of metal acclimation/adaptation

Adaptation to metal exposure may improve fitness of the individual in contaminated habitats, but several potentially deleterious consequences are identified, which (compared to fitness of sensitive populations) determine future performance, and therefore population persistence at contaminated sites. Firstly, maintenance of tolerance mechanisms (as described above) may be physiologically or energetically costly, so that allocation of nutrients or energy to other functions is reduced. Such "cost of tolerance" is frequently suggested as an inevitable consequence of being tolerant (Posthuma and Van Straalen, 1993).

If metabolic costs are increased in response to toxicant exposure (and energy income is unaffected), production processes must be reduced. That is, there should be a trade-off between the capacity to survive toxic stress and the growth rate and reproductive output (Calow, 1991). Wilson (1988) first demonstrated cost of tolerance expressed as a reduced relative growth rate of metal-tolerant clones of the grass *Agrostis capillaris*. Two types of costs can be distinguished (Calow, 1991). The first type includes the costs associated with a facultative (inducible) adaptation (or acclimation), *i.e.* a capacity to "switch on" energy expensive stress-resisting processes at appropriate times. On the other hand, there may be

expenditures associated with the maintenance of a tolerance mechanism evolved by a fixed genetic adaptation: this is a constitutive, inherited characteristic of a tolerant individual, caused by negative genetic correlations between tolerance and other features. Genetic evidence for "cost of tolerance" is provided by the fact that in the absence of metals, tolerant genotypes perform worse than sensitive genotypes (Sibly and Calow, 1989).

Other possible negative consequences of acclimation and/or adaptation include a decrease of genetic variation due to strong directional selection. The results can be directly interpreted as an alteration of the possibilities to respond to future environmental variation (Posthuma and Van Straalen, 1993). Finally, the resistance at a given trophic level may provide a means by which potentially toxic quantities of bioconcentrated chemicals can be passed on to non-resistant consumers (LeBlanc, 1982).

1.4. Current shortcomings in metal risk assessment procedures

This section is based on Janssen *et al.* (2000), and gives the outline of this dissertation.

1.4.1. Background concentrations vs. acclimation/adaptation

The naturally occurring concentrations of metals, *i.e.* the background concentrations (see section 1.1.3.3.), in various environmental systems can vary substantially depending on the geographic area. Variability in composition of the Earth's crust, soil type and geochemical processes, which are to a large extent responsible for this metal background variability, are discussed by Garret (2000).

Regulatory organisations have not taken into account the possible ecological importance and implications of different metal background concentrations. Especially for essential metals, this variability may have important consequences for the derivation of environmental quality criteria. Since organisms are dependent on essential metals for optimal growth and development, all species are acclimated (or adapted) to a range of bioavailable metal background concentrations occurring in their habitat. In analogy with the concept of eco-regions proposed by Baily (1998), in which ecosystems are classified based on soil type, climate and water requirements, it has been suggested to classify parts of ecosystems, habitats or geographic areas in metallo-regions: *i.e.*, areas with different (bioavailable) background

concentrations of one or more essential metals. Based on the results of laboratory acclimation research and ecological theory, the following hypothesis may be proposed: aquatic communities present in areas with different natural background concentrations of essential metals exhibit a different sensitivity towards this metal. In Figure 1.6A this hypothesis is illustrated for single species living at various background concentrations: as the background concentration of the essential metal increases (within natural limits), the sensitivity (to the metal) of the acclimated/adapted organisms decreases. Expanding this hypothesis to all organisms of an aquatic community living in a specific metal background concentration range, two hypothetical cases may be discerned:

- The shift in the communities' sensitivity due to the acclimation/adaptation process is larger than the span of the species' sensitivity distribution resulting in a distinctive sensitivity of each community, *i.e.* no or little overlap of the sensitivity distributions (Figure 1.6B). In this case, different Water Quality Criteria (WQC) or Predicted No Effect Concentrations (PNEC) for environmental systems with different backgrounds should be derived.
- If the species sensitivity distributions largely overlap, *i.e.* acclimation-induced sensitivity shifts did not or only slightly affect the tolerance of most species in the community, the proposed hypothesis should be rejected and may not be useful in regulatory context (Figure 1.6C).

Insufficient laboratory or field research is presently available to support or reject this conceptual framework. Considering that laboratory toxicity data are used for WQC derivation and the establishment of PNEC in environmental risk assessments, these acclimation-induced sensitivity shifts in metallo-regions may affect the (ecological) relevance and effectiveness of these Environmental Quality Criteria (EQC).

The metallo-region concept may be used effectively in different ways. First, a classification may be developed which is based on water or soil types, thus including both the metal background concentration and the factors influencing bioavailability. Secondly, perhaps as an initial step in the improvement of PNEC and WQC derivations of essential metals, appropriate toxicity data should be used and/or developed. Suggested improvements include:

(1) if standard test organisms are used they should be cultured at a (bioavailable) metal background concentration that is representative of the environmental system under consideration (*i.e.* metallo-region or –class);
(2) the dilution medium (and controls) used in toxicity tests should contain the same representative background concentration;
(3) the dilution medium should be a natural medium representative of the system;
(4) test organisms may be collected in a representative natural environment, cultured in natural medium and used in the toxicity assays.

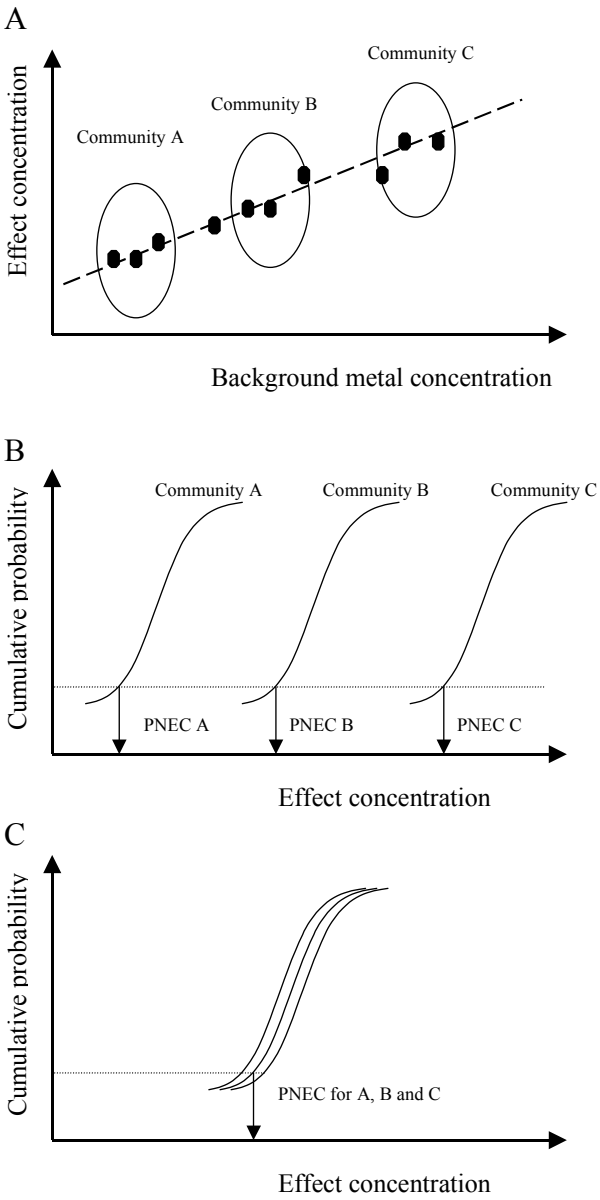

Figure 1.6: Hypothetical relationship between the sensitivity of a single species (A) or different communities (B and C) and the background concentration of an essential metal. (See text for further explanation).

Additionally, many recommended artificial culture and test media do contain no or few essential metals. It has been demonstrated that standard organisms cultured in media with low essential element concentration exhibit an overall decreased fitness (Caffrey and Keating, 1997; Elendt, 1990; Elendt and Bias, 1990; Fort *et al.*, 1998; see section 1.2.). Furthermore, organisms cultured at these low metal concentrations acclimate to these conditions and become more sensitive to stress, including exposure to metals (Muyssen and Janssen, 2001a, b, c, 2002).

New research efforts are required to assess the ecological relevance and regulatory application potential of these suggestions. The main goal of this thesis was therefore to address these knowledge gaps. As such, this study will support future metal risk assessments in general and that of copper more specifically.

1.4.2. Organisms only respond to bioavailable metals

Current WQC and risk assessment procedures for metals are predominantly based on total or dissolved metal concentrations. However, there is extensive evidence that neither total nor dissolved aqueous concentrations of a metal are good predictors of its bioavailability and toxicity (Bergmann and Dorward-King, 1997; Janssen *et al.*, 2000). Metal toxicity towards freshwater organisms has been shown to be highly dependent on a variety of ambient water quality characteristics, *e.g.* pH, hardness and organic matter content. Recent research efforts led to an improved understanding of how water chemistry affects bioavailability, how metals interact with aquatic organisms to exert toxic effects at the organism's site of action, and how toxic effect levels can be predicted. The integration of these approaches has resulted in the development of (mechanistic) toxicity-related bioavailability models commonly referred to as Biotic Ligand Models – BLM (Playle *et al.*, 1992; Di Toro *et al.*, 2001; Paquin *et al.*, 2002; De Schamphelaere and Janssen, 2002; Heijerick *et al.*, 2002a, b; De Schamphelaere *et al.*, 2002; Janssen *et al.*, 2003). It should be clear that when metal essentiality is discussed, the bioavailable concentration should be considered, rather than the total amount of metal present in the medium.

1.5. Essentiality and PNEC derivation

1.5.1. General

One of the ultimate goals of aquatic toxicity testing is to generate effect-based environmental protection levels generally expressed as a Maximum Tolerable Concentration (MTC) or Predicted No Effect Concentrations (PNEC). For new and existing chemicals this assessment of the "safe concentration", is based on the hazard and risk assessment procedures outlined by the OECD and the EU (OECD, 1995; EC, 1996, 2003). Environmental risk managers are often asked to make decisions based on incomplete knowledge and sometimes large uncertainty. To incorporate uncertainty in risk assessment procedures and to reduce the probability of causing harm to the environment, assessment factors (also named uncertainty or safety factor) are applied to the risk estimate. With these factors, one tries to take into account the variability between the effects detected in laboratory assays (at different levels of biological organisation) and those occurring in the natural ecosystem (Persoone and Janssen, 1994; Figure 1.7). Laboratory assays can provide effect concentrations for one or more organisms, which then can be translated to a PNEC. However, in natural systems, organisms are dependent on the background (or ambient) concentrations to which they can acclimate or adapt; essentiality in the case of copper; metal bioavailability and other toxicity modifying factors (temperature, life stage, food concentration, etc.). If all these uncertainties are know for one or more species, the PNEC will be closer to the (possible) observed effect in the field.

Hence, these assessment factors try to incorporate uncertainties in experimental variability (due to biological and experimental variation), variability encountered when extrapolating laboratory effect data to the field, variability caused by predicting chronic toxicity values based on acute toxicity data and variability caused by extrapolation of the effects to higher levels of ecological organisation (Suter *et al.*, 1985; Persoone and Janssen, 1994). Such empirical approaches may have little or no relevance to actual uncertainty, but they greatly reduce the probability of under-estimating risk. Because they are "safe" and provide clear-cut answers, they are generally used. However, their use consequently also greatly increases the possibility of over-estimating risk and may (and often does) lead to unrealistic answers in hazard and risk assessment, *e.g.* values below background metal concentration.

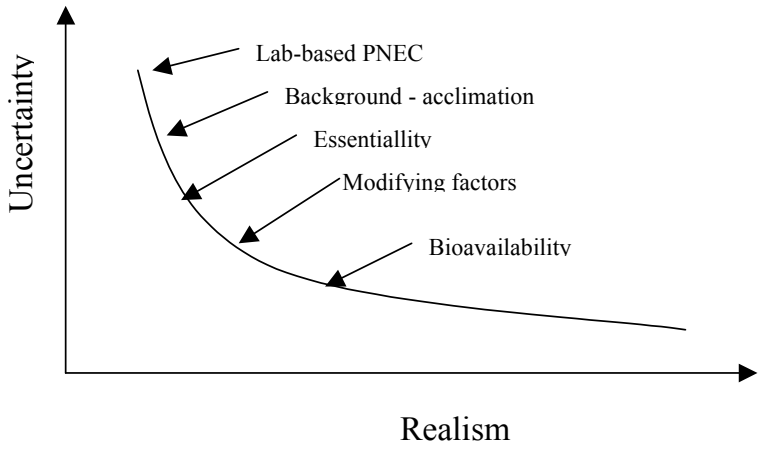

Figure 1.7: Uncertainty decreases and realism (*i.e.* situation-specific knowledge) improves as laboratory data are adjusted extrinsically for factors influencing hazard and risk (adapted from Chapman and Wang, 2000).

The implications of the natural occurrence of metals in the environment for environmental risk assessment procedures have not been well appreciated and adaptation/acclimation to these metal concentrations is therefore often ignored. Specifically, environmental concern related to metals should not be triggered by their presence in the environment, as for some synthetic organic chemicals, but rather by any changes in the level of their natural presence. Both their ambient and background (if determinable) or baseline concentrations need to be determined. The focus should not only be on the absolute value of the ambient concentration, but also on the difference between the ambient and the background concentration. In addition, since the background concentration of a metal varies greatly in different areas, background or baseline concentrations should be measured, where possible, site-specifically. A global, national or regional mean of background concentrations is often useless in an environmental risk assessment (Chapman and Wang, 2000).

1.5.2. Current methods for risk assessment

The method that is often used to provide an assessment of risk is based on the ratio of a predicted or measured environmental concentration (PEC or MEC) and a predicted no effect concentration (PNEC), often referred to as a risk (or hazard) quotient (RQ). This is thus the ratio of the exposure and the effects assessment. The PNEC is derived from some ecotoxicological endpoints (typically survival, reproduction, or growth) that are measured using individuals and can be based on two different approaches as described below.

The Technical Guidance Document (TGD, 1996) used in association with existing and new substances legislation within the European Union (EC, 1996) provides specific recommendations on the size of assessment factors to be applied under different circumstances to derive PNECs for aquatic ecosystems. When only short-term toxicity data are available, an assessment factor of 1000 will be applied on the lowest L(E)C50 of the relevant available toxicity data, irrespective of whether or not the species tested is a standard test organism. A lower assessment factor will be applied on the lowest no observed effect concentration (NOEC) derived in long-term tests with a relevant test organism: 100 in the case there is one NOEC (either fish or *Daphnia*) available, 50 if there are two long-term NOECs available from species representing two trophic levels (fish and/or *Daphnia* and/or algae). A similar set of factors is used in the U.S. by the U.S. Environmental Protection Agency (USEPA) Office of Pollution Prevention and Toxics (OPPT) to set "concern levels" (*i.e.* the level of chemical exposure in the environment at or above which significant risks to aquatic organisms are likely) (Zeeman and Gilford, 1993; Zeeman, 1995).

In response to criticism of the assessment factor approach used in deriving PNECs, an alternative approach has been advocated that attempts to approximate a community species sensitivity distribution (SSD) by incorporating observations from ecotoxicological experiments on a variety of species (see section 1.5.3.; Stephan *et al.*, 1985; Aldenberg and Slob, 1991; Wagner and Løkke, 1991). The resulting SSD enables the estimation of the concentration at which effects on a large fraction of the tested species will not occur. The SSD-approach assumes that the combination of all tested species is representative of aquatic ecosystems and a statistical distribution function is fitted through the data, resulting in the HC5, which is considered to be the hazardous concentration that protects 95 % of the species in a (hypothetical) ecosystem. The TGD proposes the log-logistic distribution (Aldenberg and Slob, 1993), but since this does not always result in statistically significant fit, there is an ongoing debate between academia, regulators and industry on using the best possible fitting distribution function to derive the HC5 (EC, 2003). In the EU regulatory context, the PNEC is subsequently calculated as the HC5 divided by an assessment factor between 1 and 5 depending on, among others, data quality, extremely sensitive species, outcomes of mesocosm studies, etc. However, next to the distribution fitting issue, the size of this assessment factor is currently also a matter of very intensive debate, which is often more inspired by policy than by science.

The resulting PNEC value is then compared to the P(M)EC to get an estimate of risk. An often-cited criticism of the RQ approach is that it does not provide a quantitative probability of risk. In principle, an advantage of the SSD approach is that statistical techniques can be used to combine the uncertainty represented by the frequency distributions of effects and exposure concentrations to provide a statement of probability of harm to the selected group of species. However, since the species used for input into the sensitivity distributions generally are not derived from any known community, the ecological interpretation of the resulting risk is not obvious (see section 1.5.3.).

Van Assche *et al.* (1997) proposed the "No Risk Area" (NRA) concept as a basis for PNEC determination for essential elements. The NRA is determined by the inner envelope of the overlapping OCEE curves of the species, belonging to a given habitat-type. Within the NRA, none of the species is subjected to deficiency or toxicity stress. Using this approach, the EQC can then be presented as a concentration range or concentration window, *i.e.* the NRA. The NRA's upper (toxicity) boundary being determined by the biological species with the lowest toxicity value, the deficiency boundary would then be determined by the species with the highest deficiency value. Recognising that the use of an "EQC window" may pose a problem for applying this concept in a regulatory framework, Van Assche *et al.* (1997) suggested that the PNEC could also be set at the NRA's median or upper boundary, thus protecting all organisms in that environment from both toxicity and deficiency.

Whatever approach is used, it is clear that, when recognising the essentiality and acclimation processes associated with essential metals, different types of habitat or environment may have different EQCs. Currently, the scientific or field evidence for the further development of these concepts and their incorporation in regulatory systems is not available.

1.5.3. Species sensitivity distributions (SSDs)

When these previously mentioned techniques for deriving the PNEC are applied to the essential elements, several conceptual problems and inconsistencies with biological/ecological reality arise. The "assessment factor" approach often leads to PNECs well below the essential element's natural concentration range and would therefore be situated at concentrations that are deficient for (some) organisms in a given ecosystem. As the statistical extrapolation model(s) approach uses statistical (*e.g.* log-logistic) distribution based on ecotoxicity data and

does not consider possible adverse effects due to deficiency, resulting PNECs also may be at the lower end (or beyond) the homeostasis range of some organisms.

The SSD-approach thus uses all available information on effects (in contrast to the assessment factor approach) and has been advocated as a more objective and scientific procedure. However, many researchers forget the major assumptions associated with the theory on which the SSD-approach is based. These main assumptions (behind the theory and application) are described in Forbes and Calow (2000). From their literature review they recognized some commonly occurring problems with the SSD-approach.

- Effects data are mainly taken from the literature and not from the target community/ecosystem of interest.
- The effects represented in a single distribution were often based upon a variety of endpoints for different species.
- A variety of distributions was employed.
- Confidence limits around the effects threshold could not be defined, were not defined, or if defined were specified somewhat arbitrarily.
- Application factors were used to convert acute effects endpoints to chronic values prior to input into the distribution.

An in-depth data collection exercise has recently been performed by Van Sprang *et al.* (EURAS, Zwijnaarde, Belgium, unpublished data). This resulted in high-quality chronic toxicity data of copper for 21 species (3 unicellular green algae, 1 higher plant, 5 crustaceans, 1 snail, 3 insects and 8 fish). Figure 1.8 presents the corresponding SSD. The data used to derive the SSD were obtained from toxicity assays using test media with highly variable physico-chemical characteristics (*e.g.* pH from 5.5 to 9.0, hardness from 8 to 500 mg $CaCO_3$ L^{-1}, DOC from background level in laboratory reconstituted waters to 20 mg L^{-1} in natural waters), which may have added to the high variability of NOEC values within one species (up to a factor 100). Due to this high variability the derived PNEC may be under-protective in one type of surface water, whereas it may be over-protective in another.

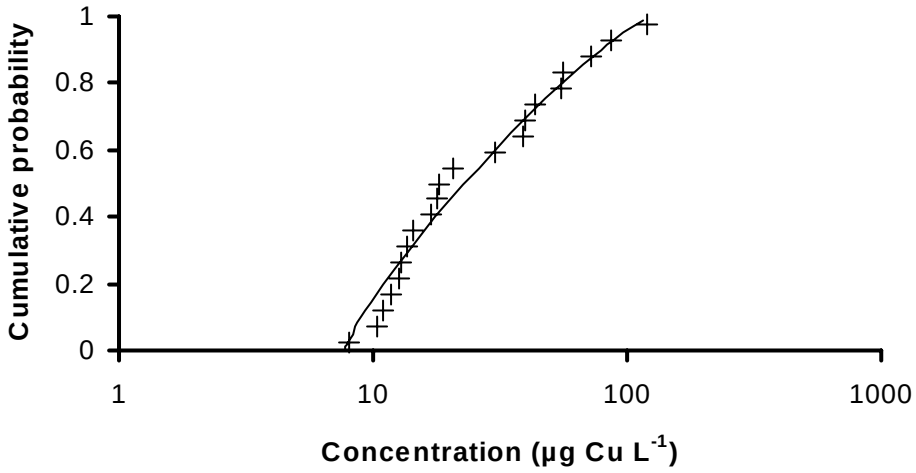

Figure 1.8: Species sensitivity distribution of copper in freshwater (Van Sprang *et al.*, unpublished data) represented by the geometric NOEC-means of 21 different species. The solid line represents the best fitting beta-distribution function. The HC5 is 8.3 µg Cu L^{-1}.

1.6. Introduction to the test organisms

1.6.1. Daphnids

1.6.1.1. Systematic classification and morphology

Daphnids are members of the Phylum of the Arthropoda, Classis of the Branchiopoda. The diversity of crustaceans requires the use of a greater hierarchy of taxa in their classification than usually necessary for other animal groups. Recent molecular evidence suggests that the Cladocera is a monophyletic group (Crease and Taylor, 1998). The suborder of Cladocera is now divided into 11 families, about 80 genera and roughly 440 species (of which about 100 occur in Western Europe; Leentvaar, 1978), although taxonomic revision in all families is ongoing so that the numbers of genera and species are likely to increase. According to Flößner (2000) the taxonomic classification of *Daphnia magna* is as follows:

Phylum:	Arthropoda
Subphylum:	Crustacea
Classis:	Branchiopoda
Subclassis:	Diplostraca
Ordo:	Cladocera
Familia:	Daphniidae
Subfamilia:	Daphniinae
Genus:	*Daphnia*
Subgenus :	*Ctenodaphnia*
Species:	*Daphnia (C.) magna* Straus (1820)

The majority of the waterfleas inhabits freshwater environments. The family of Daphniidae contains 4 widely distributed genera, of which the genus *Daphnia* comprises around 50 species (Brooks, 1957). Of the family of Daphniidae, the genus *Daphnia* is the most ecologically diverse and has a cosmopolitan distribution (Belk, 1982). *D. magna* Straus belongs to the subgenus of the *Ctenodaphnia*, together with *D. similis* and *D. atkinsoni*. *D. magna* Straus is easily distinguished from the other species by the deeply sinuate posterior margin of the post-abdomen and the ridges of the head which run parallel to the mid-dorsal line (Brooks, 1957).

Most cladoceran species range in size between 0.2 and 3.0 mm long (Brooks, 1959; Pennak, 1988) and are characterised by their distinct head and their carapace which covers the thoracic and abdominal regions of the body. The carapace forms a single valve which is closed longitudinal along the dorsal midline.

The general characteristics of the daphnid's morphology are illustrated in Figure 1.9. Daphniidae have an oval, laterally compressed and indistinctly segmented body. The head is rounded anteriorly and possesses one central sessile compound eye. The head is ventrally oriented into an uncinate rostrum and carries five pairs of appendages. The first (unsegemented) pair, the antennules, arise from the ventral margin of the head, carry the olfactory setae and display sexual dimorphism. The second (segmented) pair, the antennae, are inserted laterally and constitute the main locomotor organ for swimming. The last three appendages are situated near the junction of the head and body and form the different mouth parts (mandibules, maxillules, labrum and labium).

The body ends in a posterior abdomen which is flexed forward and carries a claw-like furca. The chitinous transparent carapace, which is reticulated and thickened at the ventral margins, encloses five pairs of thoracic appendages. These (segmented) trunk limbs carry numerous setae and are used for filter feeding. The different limbs are responsible for the generation of a water current in the ventral groove and for the filtering of the suspended particles from the water. The first two pairs of appendages serve as prehensile limbs, while the 3rd and 4th constitute the main filtering apparatus. The fifth pair has no filter plate and serves to clean some of the setae of the 4th limb pair.

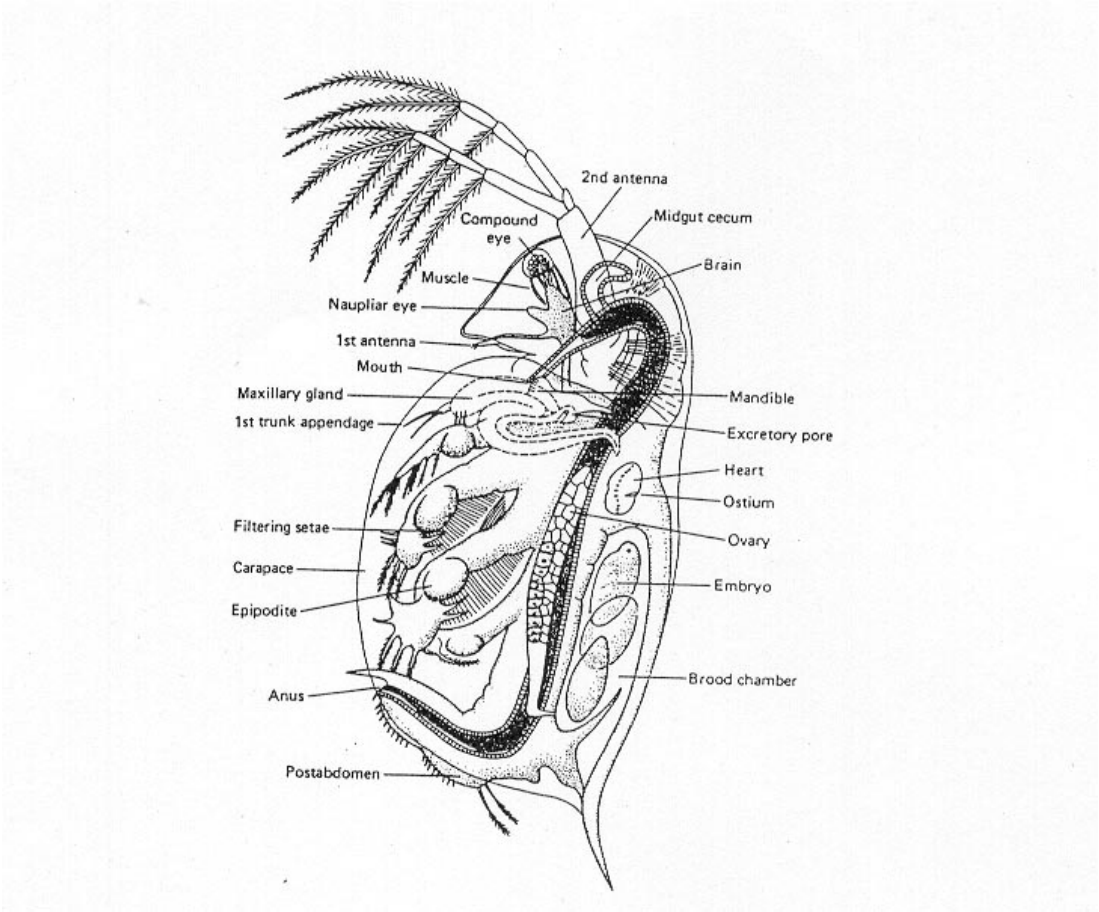

Figure 1.9: A lateral view of a female of *Daphnia pulex* (closely related to *D. magna*), seen through the transparent carapace (from Barnes, 1987).

Another visual characteristic of a daphnid is the brood pouch or the embryonic chamber where eggs are laid and develop. This is situated at the dorsal side between the carapace and the body wall. Oviducts open into this dorsal chamber inside the carapace. Development in most cladoceran species is direct, and the young are released from the brood chamber by the ventral flexion of the post-abdomen of the female. When the young leave the brood chamber,

the skeleton is moulted and a new batch of eggs is released into the new brood chamber. The average brood size of *D. magna* is 14 to 65 juveniles.

Rather than a true abdomen, the genus *Daphnia* has a large post-abdomen which structure is one of the main determinants in the taxonomy of the species. This posterior end of the body carries four pairs of abdominal processes and two long setae. The largest of these processes, the anterior abdominal process, covers the open posterior part of the embryonic chamber in adult females to prevent the eggs from falling out. At the ventral side two claws are positioned with, at the concave side, setae, which are used to clean the thoracic appendages.

1.6.1.2. General ecological aspects

Cladocerans are, with exception of 3 marine genera (*Podon*, *Evadne* and *Pleopsis*), mainly freshwater inhabitants and are found all over the world. They are not present or scarce in fast flowing brooklets, rivers and wells, in ground water and in very salty inland waters. Most of the species are found in ponds and ditches or in the weedy margins of lakes and rivers (Brooks, 1959; Pennak, 1988). More specifically, species of the genus *Daphnia* have a cosmopolitan distribution (Taub, 1982) and are found in both the northern and southern hemisphere. In the European region, *D. magna* is considered as a southern species as it is not encountered in Ireland, Iceland, Greenland or the north and west of Great Britain (Hrbáček, 1987). More globally, *D. magna* is found all over Eurasia, from England and North Africa (in saline lakes in Algeria and Ethiopia) to China and Manchuria (Hebert, 1978; Hrbácek, 1987). On the American continent, it occurs in a large area of northern and western North America, but is absent in Alaska and the south east of the North American region (Brooks, 1957). *D. magna* is a typical inhabitant of polluted small water bodies, mostly without fish. The species is absent in lakes and acid swamps and peats. Remarkable is that this euplanktonic species is usually very transparent, but in our region with shallow and turbid waters they are less transparent and smaller.

Daphnia population densities are high during late spring and summer. Population densities increase until early summer after which they decrease and the species becomes rare or even absent. A second density peak occurs in autumn. During winter and early spring daphnids are scarce or completely absent. It has been shown that natural populations of *D. magna* (Hebert, 1974; Hebert and Ward, 1976; Young, 1979; Korpelainen, 1986; Carvalho, 1987; Carvalho

and Crisp, 1987; Yampolsky and Kalabushkin, 1991), and other *Daphnia* species (*e.g. D. pulex*: Loaring and Hebert, 1981; *D. longispina*: King and Miracle, 1995), consist of a (limited) number of ecologically differentiated clones which may react differently to environmental changes and thus exhibit seasonal changes in relative density (Hebert, 1982).

The genus of *Daphnia* is one of the main consumers of primary producers in many freshwater ecosystems, and consequently, these waterfleas play an important role in the food web as food for both invertebrates and vertebrates (Hebert, 1978). Nannoplanktonic algae are the main food source for daphnids (Lampert, 1987). Bacteria are an important complementary food source (Hadas *et al.*, 1982), just like protozoa (Porter *et al.*, 1979), detritus (Tóth *et al.*, 1987) and some blue-green algae (Holm *et al.*, 1983). As *D. magna* is a large zooplankton species (a mature female can reach a length of 5 to 6 mm) it is mainly encountered in habitats where visually hunting vertebrate predators are rare or absent.

D. magna is a filter-feeding organism, who's feeding apparatus consists of setae-rich thoracic appendages. McMahon and Rigler (1965) found that *D. magna* could efficiently filter particles with sizes ranging from 0.9 to 18,000 μm^3. Compared to other zooplankton species, *D. magna* has a large and energy expensive feeding apparatus. In general, the feeding process is inefficient at low and high algal concentrations. At low food concentrations, too much water has to be filtered in order to collect sufficient particles, while at high food concentrations, more food is collected than can be ingested and food is rejected. According to Richman and Dodson (1983) *D. magna* therefore is a species which ideally inhabits waters with high food concentrations without extremely low or high concentrations.

Another characteristic of *D. magna* is its daily rhythm of vertical migration which is induced by the light intensity (Ringelberg, 1987). During day time, the organisms stay in the hypolimnion to feed on non-algal food, while during night they forage on algae in the epilimnion (Dini *et al.*, 1987). This strategy is likely to be a predator-avoidance behaviour.

The life cycle of the *D. magna* largely depends on the environmental conditions (Green, 1956; Hebert, 1978). *D. magna* reproduces by cyclical parthenogenesis, which involves shifting from a parthenogenic reproduction phase (asexual reproduction) to a phase of gamegonesis (sexual reproduction). Asexual reproduction occurs during favourable conditions: populations entirely consisting out of females produce diploid parthenogenic eggs, which develop into

clones of these females. Under favourable laboratory conditions (*e.g.* 20°C, light/dark cycle of 12 h and abundant food) eggs develop in the embryonic chamber in around 3 days after which neonates are released as free-swimming miniature adults. After 7 to 10 days, during which 5 to 6 moults occur, the organisms release their first brood. From that point onwards after approximately every 3 days, one brood is released followed by a moult shortly thereafter.

The neonate size of the first broods is usually smaller than that of the subsequent broods (Enserink *et al.*, 1990). Moreover, this parameter is ultimately linked to future survival and reproduction of the organism (Tessier *et al.*, 1983; Lampert, 1993). Due to a trade-off mechanism between the egg size and the clutch size, organism are capable to regulate the offspring size (*i.e.* increase in offspring size with increasing food limitation) and therefore control the fitness of the future generations by decreasing the clutch size (Tessier and Consolatti, 1989; Glazier, 1992). Not only the food availability influences the number of eggs produced, but also abiotic factors such as light (Buikema, 1973), temperature (Goss and Bunting, 1983) and dissolved oxygen (Green, 1956). Changes in the offspring number due to these factors have been reported by Hebert (1978) and are possible caused by influencing the food uptake of the organism.

Under unfavourable environmental conditions, *Daphnia* populations reproduce sexually. Initially females start to produce parthenogenic males, usually in single-sexed broods. The sex is determined shortly before the eggs are deposited in the embryonic chamber (Zaffagnini, 1987). Once the males are released, the females start to produce two haploid eggs (Green, 1956) which after fertilisation develop into diapausing eggs and eventually become covered by an ephippium. These ephippia are able to withstand unfavourable conditions such as freezing, drying and passage through the gut of a fish (Hebert, 1978; Mellors, 1975). Under favourable conditions, the ephippia hatch and the eggs develop into parthenogenic females (Hebert, 1978; Zaffagnini, 1987; Van de Vel, 1992; Cotou, 1993; Dos Santos, 1997).

1.6.1.3. The use of daphnids in ecotoxicology

Although several invertebrate species have been used for aquatic toxicity testing, the taxon which has been used most frequently over the last three decades are daphnids (Persoone and Janssen, 1994). At present, the *Daphnia* toxicity assays (both acute and chronic) are one of the aquatic toxicity assays with invertebrates which are officially endorsed by most international

organisations such as the EU, US EPA and OECD. The main reasons why these organisms have been used so frequently are of a practical, economical and scientific nature (Buikema *et al.*, 1980; Baudo, 1987).

- The organisms are broadly distributed in freshwater ecosystems throughout a wide range of habitats.
- The organisms play an important role in many aquatic food chains as they are among the most important groups of organisms grazing on primary producers and are food themselves for many vertebrate and invertebrate predators.
- The organisms are one of the most sensitive species to a wide range of environmental toxicants.
- The organisms are relatively easy to culture under controlled laboratory conditions.
- Due to their small size, they can be manipulated much more easily compared to fish. Additionally, the laboratory bench space required is much smaller.
- Due to their parthenogenic reproduction, the neonates are genetically identical to their mothers which eliminates possible genetic variability towards the toxicant sensitivity.
- Due to their relatively short life cycle, it is much easier to perform chronic toxicity studies compared to fish chronic toxicity testing.
- At present, a large data base is available on the sensitivity of the organism compared to other species.

Both *D. magna* and *D. pulex* are the most commonly used invertebrates in both acute and chronic aquatic toxicity studies. In the USA, however, *Ceriodaphnia* species are the main invertebrate species used in short-term chronic toxicity studies (Horning and Weber, 1985; Versteeg *et al.*, 1997), mainly because it is a more ecologically relevant test species (than *D. magna*) in the USA. In acute toxicity testing, neonates (< 24 h) are exposed to increasing toxicant concentrations for 24 and 48 h, after which the concentration is determined causing 50 % of mortality or immobility (*i.e.* the 24-h or 48-h LC50 or EC50, respectively). In chronic toxicity studies with *D. magna*, the highest concentration which is causing no statistical significant effect (No Observed Effect Concentration or NOEC), the lowest concentration causing a statistically significant effect (Lowest Observed Effect Concentration or LOEC) or the 21-d EC50 on reproduction is determined. Acute toxicity testing with daphnids have been used extensively to study the adverse effects to chemicals – individually or in mixtures

(Enserink *et al.*, 1991) – effluents, leachates from solid wastes, sediments and surface waters (Buikema *et al.*, 1980; Baudo, 1987).

Although in all toxicity test procedures for daphnids and algae a high degree of standardisation is pursued, almost every governmental, regulatory or standardisation organisation has its own specific procedures. Additionally, little attention has been given to the description of the culturing and feeding of the test organisms during chronic tests (Persoone and Janssen, 1994). Well standardized culturing conditions not only provide healthy test organisms but also standardize the nutritional and metabolic status of the organism. Especially for *D. magna*, several studies have indicated that the maternal nutritional status largely influences the sensitivity of the offspring (Baird *et al.*, 1989; Enserink *et al.*, 1990; Naylor *et al.*, 1992). Also the genetic component influencing the difference in sensitivity has been studied (Baird *et al.*, 1990; 1991; Bradley *et al.*, 1993; Barata *et al.*, 1998, 1999). In general, larger genotype-dependent differences have been observed between the results of acute toxicity tests while in chronic tests smaller differences where noted (Baird *et al.*, 1990; 1991). Most of the recommended culture and test media (reconstituted freshwater) for daphnids described in the standard procedures (Clesceri *et al.*, 1998; ASTM, 1993, ISO, 1993; OECD, 1984; USEPA, 1993) are composed of only a few salts and do not contain essential trace elements like copper. Although no results were presented on a changed toxicant sensitivity of organisms cultured in these media, Elendt and Bias (1990) justly questioned their use and optimised culturing conditions by developing new medium, rich in trace elements. On the other hand, natural waters (well, pond, tap water) are frequently used to maintain stock cultures of experimental animals. As the composition of these waters is often not specified and may fluctuate over time, the influence of these conditions on test results remain unknown.

In general, *D. magna* is one of the more sensitive aquatic test organisms. Studies on the relative sensitivity of this species compared to other commonly used organisms have indicated that chronic *Daphnia* toxicity results correlate well with those of long-term fish toxicity tests (Maki, 1979; LeBlanc, 1984). Environmental testing programmes applying a battery of test organisms, usually select *Daphnia* as the representative organism for zooplankton species (Mayer and Allesieck, 1986). The most commonly endpoints in *Daphnia* toxicity tests are immobility and reproduction (Canton and Adema, 1978; Lee *et al.*, 1986; Dillon *et al.*, 1990). Next to these “conventional” endpoints, several authors have used other sensitive endpoints

such as time to first brood, average brood size and the length of the neonates in studies with *D. magna* (Day and Kashik, 1987; Cowgill and Milazzi, 1991; Fernandez-Casalderrey *et al.*, 1993). Although all these reproductive endpoints are relatively sensitive, from an ecological point of view more attention should be paid to effects at the population level, rather than at the individual level (Moriarty, 1988).

Due to its relatively short life cycle (around 60 days at 20°C – Lampert, 1987), *D. magna* is a very attractive species to perform life cycle studies or even multi-generation studies (Daniels and Allan, 1981; Caffrey and Keating, 1997; Muyssen and Janssen, 2001b). Several authors have advocated the use of cohort life-table studies to study the effect of toxic exposure on the population dynamics of the organism (Winner and Farrell, 1976; Van Leeuwen, 1985 a, b; Janssen, 1992; Fernandez-Casalderrey *et al.*, 1993).

Although, to date, most ecotoxicological studies have been carried out with *D. magna* and *D. pulex*, bioassays with other *Daphnia* species, such as *D. ambigua* (Winner and Farrell, 1976; Hanazato, 1991), *D. parvula* (Winner and Farrell, 1976), *D. carinata* (Chandini, 1988; Hickey, 1989), *D. catawba* (Jones *et al.*, 1991), *D. cucullata* (Canton and Adema, 1978), *D. galeata mendotae* (Day and Kaushik, 1987; Stephenson *et al.*, 1991), *D. hyalina* (De Mott *et al.*, 1991), *D. longispina* (Crossland and Hillaby, 1985), *D. obtusa* (Coniglio and Baudo, 1989), *D. pulicaria* (Bridgham, 1988), *D. similis* (Hosokawa *et al.*, 1991), and *D. spinulata* (Alberdi *et al.*, 1990), have also been reported.

1.6.2. *Pseudokirchneriella subcapitata*

1.6.2.1. Systematic classification and morphology

Pseudokirchneriella subcapitata (Korshikov) Hindak was formerly known as *Selenastrum capricornutum* Printz or *Raphidocelis subcapitata* Korshikov (Nygaard *et al.*, 1986). The species *Selenastrum capricornutum* was first described by Printz (1914) and re-described by Korsikov (1953). The strain used in this study was originally isolated by Skulberg in 1959 in River Nitelva, Akershus, Norway and designated as NIVA-CHL 1 strain. In 1981, an uni-algal strain culture of NIVA-CHL 1 was deposited in the Culture Collection of Algae and Protozoa (CCAP), Cambridge, England and thereafter re-designated with a strain number CCAP 278/4. The intention was to make this algal species more easily available to

laboratories that want to use it for bioassay purposes. Indeed, as emphasized by Komarek and Marvan (1979), the uniform and stable designation of experimental strains should be an obvious requirement in bioassays to facilitate comparison of results. Differences in experimental results due to strain differences in algae were observed by Lhotsky (1970) and George (1974). The taxonomic classification of the test organism following Korshikov (1990) is as follows:

Phylum:	Chlorophyta
Classis:	Chlorophyceae
Ordo:	Chlorococcales
Familia:	Chlorellaceae
Subfamilia:	Ankistrodesmoidae
Genus:	*Pseudokirchneriella*
Species:	*Pseudokirchneriella subcapitata*

The Chlorophyceae or green algae have chlorophyll *a* and *b*, and form starch within the chloroplast, usually in association with a pyrenoid. The Chlorophyceae thus differ from the rest of the eucaryotic algae (with the exception of the Charophyta) in forming the storage product in the chloroplast instead of the cytoplasm (Lee, 1980). The Chlorophyceae are primarily freshwater organisms; only 10 % of the algae are marine versus 90 % freshwater organisms (Smith, 1955, as cited by Lee, 1980). The freshwater species have a cosmopolitan distribution with few species endemic in a certain area (Lee, 1980).

The morphology and reproduction of *P. subcapitata* (NIVA-CHL 1; Figure 1.10) as described below have been summarized from Nygaard *et al.* (1986):
Cells in cultures are solitary except during cell division, occurring occasionally confluent to form few-celled clusters enveloped by a delicate and colourless mucilage of varying thickness. Cells are shaped like a helicoidally twisted spindle tapering slightly into blunt apices, heteropolar, although isopolar cells seem to occur. Cells in the vegetative phase are usually semi-circularly curved, while the twisting in old cultures could be one and a half turns. The chloroplast is parietal and devoid of pyrenoid. Reproduction is by division of the mother cell into 2, 4 or 8 autospores.

Dimensions of cells growing exponentially at 20 °C: curvature ranges from 154 - 360° of arc, the diameter from 4.8 to 10.8 μm, width ranging from 1.6 - 4.4 μm and depth/width ratio from 1.7 - 4.1. The cell ends of the spirally twisted algae often seem to be subcapitate. This optical illusion is confined to the cells with their apices turned in a direction sub-parallel to the longitudinal axis of the spiral.

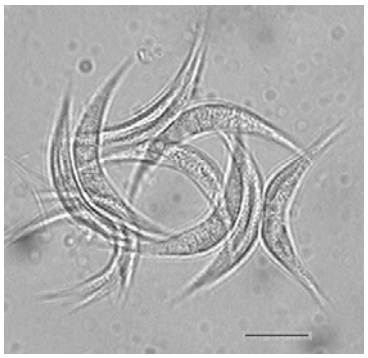

Figure 1.10: Morphological appearance of *Pseudokirchneriella subcapitata* (5 – 7 μm).

In the study of Nygaard *et al.* (1986), it was found that the morphology of *P. subcapitata* did not change substantially when exposed to different growth media. When cells where grown on solid media solitary cells were formed and no mucilage was observed. However, when incubated in aerated batch cultures, solitary cells were also formed but were always enveloped by a more or less thick mucous membrane. The authors also found that the number of cells released from a single mother cell grown in a rich medium differs by a factor of 2 from that of cells incubated in a poor medium. In the same study, the effect of temperature on cell morphology was also taken into account. It was observed that with temperature increases from 10 °C to 30 °C, the degree of curvature of cells increased while a minimal decrease in diameter and width was seen. These characteristics qualify this alga to be considered as a suitable test organism in bioassays considering its low variability with changing environmental conditions which Skulberg (1964, 1966) considers as one of its main advantages.

1.6.2.2. General ecological aspects

Definitions of phytoplankton as an ecological community are abundant and varied in literature. Happey-Wood (1988) describes them as the community of microscopic plants,

existing in suspension in aquatic environments, that is liable to passive movements by wind and currents. Green algae will almost invariably be found in all freshwaters, even if present in only small numbers. Factors affecting growth and distribution of algae in naturally occurring phytoplankton communities may be considered to be of three types related to (1) environmental attributes such as water turbulence or nutrient status, (2) attributes inherent to the cells themselves (morphological, physiological, or genetic), and (3) attributes related to other living organisms within the plankton.

When all these processes are considered in conjunction with the growth characteristics, reproductive mechanisms and life cycles, it is possible to identify a series of survival strategies that have evolved among planktonic green algae. In nutrient-poor waters green micro-algae (0.2 – 20 μm), both motile and non-motile cells, are capable of rapid growth to form a major component of the phytoplankton, persistent often throughout the year. Small size may aid nutrient uptake in waters of low nutrient availability and result in low rates of sedimentation minimising loss through sedimentation during thermal stratification. Grazing pressure by filter feeders is minimal in lakes with low productivity. In eutrophic waters, green micro-algae form significant populations at any time during the year. These growth outbursts depend on the presence of a suitable inoculum, either from another algal community within the water body, from existing small planktonic populations or possibly from aerosols and are coupled with rapid growth rates enabling the small green algae to form populations of significant size between the major growth cycles of larger planktonic algae. Green micro-algae are therefore characterised by "opportunistic growth" in eutrophic waters, producing a sequence of population maxima through the year. These population maxima are likely to be greatest in late spring prior to the high grazing pressure from herbivorous zooplankton present in summer.

Although *P. subcapitata* (*i.e. S. capricornutum* NIVA-CHL 1; CCAP 278/4) has originally been isolated from River Nitelva, Akershus, Norway, it was also found and isolated from the Botanical Garden of Copenhagen, Denmark (Nygaard *et al.*, 1986). According to Blaise (1986) this algae is representative of both oligothrophic and eutrophic freshwater aquatic systems. Other localities where this strain can be found are in Central and Northern Europe and the European part of the former USSR (Nygaard *et al.*, 1986).

1.6.2.3. The use of *Pseudokirchneriella subcapitata* in ecotoxicology

Some authors have considered aquatic plants to be less sensitive to chemicals than aquatic animals (*e.g*. Kenaga and Moolenaar, 1979; Blaylock *et al.*, 1985; Versteeg *et al.*, 1999). This is based on several studies where the acute toxicity of fish, daphnids, several vascular plants and green algae to many chemicals was compared. In contrast, for a variety of potential toxicants, including metals other studies have shown aquatic plants to be more sensitive than invertebrates and fish (Meyerhoff *et al.*, 1985; Thomas *et al.*, 1986; Taraldsen and Norberg-King, 1990; Toussaint *et al.*, 1995). It is obvious, based on current scientific evidence, that the comparative sensitivity of animals and plants to chemicals is unpredictable. Considering this observation and the ecological importance of freshwater plants, it is clear that phytotoxicity data are needed if the environmental risk assessment process is to be effective (Lewis, 1993).

The use of *P. subcapitata* in ecotoxicological testing has encompassed a wide range of applications. Since the isolation of this model organism by Skulberg in 1959, several investigators have focussed on areas like, *e.g.* the assessment of biological activity of nitrogen and phosphorus in the aquatic environment (Skulberg, 1964; 1966; 1973; Condit, 1972; Shiroyama *et al.*, 1975; Parker, 1977; Claesson, 1977; Brown and Button, 1979), studies on the stimulatory and inhibitory effects of wastewater effluents (Miller and Maloney, 1971; Greene *et al.*, 1975; Maloney and Miller, 1975; Greene *et al.*, 1976; Joubert, 1980), the study of pesticides (Turbak *et al.*, 1985; Shigeoka *et al.*, 1988; Abou-Waly *et al.*, 1991) and heavy metals (Bartlett *et al.*, 1974; Hendricks, 1978, Christensen and Scherfig, 1979). *P. subcapitata* is probably the most frequently used algal species world-wide (Forsberg and Claesson, 1981) and has been the subject of more than 200 peer-reviewed articles (Leischman *et al.*, 1979; Janssen and Heijerick, 2003), with a list still growing continuously. It has also long been considered as a sensitive indicator of potential toxicants in aqueous media and standardized procedures have been developed to assess water quality (USEPA, 1971; USEPA, 1978). *P. subcapitata* is one of the recommended species for legislative testing of chemicals and wastes (ISO, EEC, OECD, USEPA).

However, standard toxicity assays with algal species need to be applied with care for the toxicity testing of metals (Janssen and Heijerick, 2003) for the purpose of risk assessment. A common problem is the use of different culture media with different (essential) metal

concentrations that may result in different sensitivities of the used algae and may consequently result in erroneous, irrelevant risk estimates for these organisms.

1.7. Conceptual framework of the study

1.7.1. Goals and objectives

In the present chapter a number of potential short-comings in the currently used environmental risk assessment procedures have been addressed. Integration of these issues in improved risk assessment methodologies has not been possible as a number of questions remained unsolved.

The concept of essentiality (deficiency, optimal and toxicity levels), for example, is universally accepted (see section 1.2.). Nevertheless, deficiency and optimal concentrations have been identified for only a limited number of organisms. No measures have been taken to avoid deficiency in laboratory-reared test organisms. The effect of copper deficiency on copper tolerance and other biological parameters in frequently used test organisms was, prior to this study, not known.

Acclimation and adaptation are well studied in natural populations as well as in laboratory studies (see section 1.3. and 1.4.1.). However, none of these acclimation studies have focussed on low (*i.e.* background/ambient) copper concentrations. Information on acclimation to environmentally relevant copper concentrations is thus scarce. Moreover, acclimation periods in most other studies are short and endpoints at the population and physiological level (*e.g.* energy budgets) are rarely considered. Therefore, this study will focus on long-term (several months) acclimation to environmentally relevant copper concentrations (see section 1.1.3.3.) of two standard laboratory species (*D. magna* and *P. subcapitata*).

The commonly used standard laboratory organisms may not always be representative or relevant for the ecosystem under consideration. Information on copper toxicity (acute and chronic) to field-collected aquatic organisms is scarce and, to date, no attempt has been made to evaluate site-specific species sensitivity distributions and to compare these with standard "hypothetical" species sensitivity distributions (see section 1.4.1. and 1.5.3). This thesis will hence provide acute toxicity data of field-collected cladoceran species, discuss possible

acclimation/adaptation in the field and give insights in site-specific species sensitivity distributions and community sensitivity.

In the current environmental risk assessment of metals, and in this case especially copper, metal toxicity is predicted using the biotic ligand model (see section 1.4.2.). Although this model gives excellent prediction for the standard organism *D. magna*, it has not been validated for cladoceran species naturally occurring in the field. This thesis will include the results of the above mentioned issues and try to predict the copper sensitivity of different resident cladoceran species occurring in different aquatic systems.

1.7.2. Overview of this research

This doctoral thesis is made up of six additional chapters followed by a general conclusion. All six chapters are published or submitted for publication as separate research papers. If necessary, the introduction and material and methods section of these papers were modified to avoid superfluous repetition.

In **chapter 2** copper acclimation in *P. subcapitata* is studied with special attention to the changes in physiology (pigment concentration, growth rate). Acclimation is monitored during a three month period using copper tolerance, growth rate, pigment concentrations and internal copper concentrations as endpoints. An optimal copper concentration range for this alga is determined.

In **chapter 3**, multi-generation acclimation experiments are performed with *D. magna* exposed to different copper acclimation concentrations ranging from environmentally relevant (background) to sub-lethal concentrations. Acute copper tolerance, chronic survival and reproduction, body size, total body concentrations and energy budgets are monitored during 15 months. An optimal copper concentration range, based on these parameters, is established.

In **chapter 4**, a second multi-generation study with *D. magna* is conducted. The influence of similar total copper concentrations with different bioavailable copper activities (as Cu^{2+}) is investigated. Daphnids are exposed to media with different water characteristics. Copper tolerance, energy reserves and accumulation is monitored during a six month period.

In **chapter 5**, copper accumulation and homeostasis in *D. magna* and *P. subcapitata* acclimated to copper as described in the three previous chapters, is discussed. The following issues are addressed: influences of acclimation on total body burdens, internal body concentrations, bioconcentration factors and copper regulation mechanisms. Influence of acclimation on these investigated issues is determined and linked with the results of the previous chapters.

In **chapter 6**, inter- and intra-species copper sensitivity is assessed for 22 different cladoceran species collected from six different aquatic systems. The sensitivity of these field-collected organisms to copper exposure is investigated and compared to that of the *D. magna* laboratory clone. Species sensitivity distributions are constructed and their relevance discussed. Acclimation of the field species to the natural background copper concentration of the ecosystems is investigated. At the same time, the community sensitivity of the different aquatic systems is compared.

In **chapter 7**, the copper sensitivity of the different field-collected cladoceran species is assessed in their natural water of origin and predicted with the use of the biotic ligand model. The community sensitivity and the site-specific species sensitivity distribution of the sampled aquatic systems are also predicted with this model.

In **chapter 8**, general conclusions are drawn and future research needs are formulated.

Chapter 2

Long-term acclimation to copper of *Pseudokirchneriella subcapitata* (Korshikov) Hindak: effect on tolerance and physiology

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Chapter
2

Long-term acclimation of *Pseudokirchneriella subcapitata* (Korshikov) Hindak to different copper concentrations: changes in tolerance and physiology

Abstract - The effect of long-term copper acclimation of the freshwater green algae *Pseudokirchneriella subcapitata* to copper was investigated using different physiological and toxicological endpoints. The algae were exposed to seven - five of which are ecologically relevant for European surface waters - copper concentration ranging from 0.5 to 100 µg Cu L^{-1} during a 3 month period. A standard medium was used as culture and test medium with an addition of 2 mg DOC L^{-1} (replacing EDTA). At certain intervals, experiments were performed to assess algal biomass, growth rate, chlorophyll and carotenoid content, pigment diversity, autotrophic index, intracellular and adsorbed copper, and the sensitivity of the algae to copper. Chronic copper tolerance (mean ± standard deviation) increased significantly from 88 ± 15 to 124 ± 25 µg Cu L^{-1} for *P. subcapitata* acclimated to 0.5 and 100 µg Cu L^{-1}, respectively. Based on the algal biomass, the growth rate, the pigment diversity and the autotrophic index, an optimal concentration range was observed between 1 and 35 µg Cu L^{-1}. Significant decreases in algal biomass, pigment diversity and autotrophic index were observed in algal cultures acclimated to 0.5 µg Cu L^{-1} and 100 µg Cu L^{-1}. Chlorophyll *a* content (mean ± standard deviation) increased from 8.4 ± 3.1 to 28.6 ± 7.5 10^{-14} g $cell^{-1}$ and carotenoid content (mean ± standard deviation) increased from 3.7 ± 0.8 to 7.1 ± 1.2 × 10^{-14} g $cell^{-1}$ for algae exposed to 0.5 and 100 µg Cu L^{-1}, respectively. Intracellular copper increased from 0.099 to 20.6 × 10^{-15} g Cu $cell^{-1}$ and adsorbed copper increased from 0.026 to 1.8 × 10^{-15} g Cu $cell^{-1}$ for algae acclimated for 12 weeks to 0.5 and 100 µg Cu L^{-1}, respectively. This research demonstrates that the use of standard culture media, some of which may be deficient in copper, can result in sub-optimal performance of the organisms, which in turn may affect toxicity test results. Additionally, this work also established an optimal concentration range for copper for this algal species. This phenomenon should be taken in consideration when performing environmental risk assessments of essential elements.

2.1. Introduction

Copper is a trace element essential for all living organisms (Price and Morel, 1994). In plants it participates in photosynthetic electron transport and also plays a role as a co-factor of several oxidizing enzymes. Although copper can act at many different levels in the cell, one of the most important mechanisms of copper action in green plants is believed to be the

inhibition of electron transfer in the chloroplasts (Shioi *et al.*, 1978), the formation of reactive radicals (Flemming and Trevors, 1989; Fernandes and Henrique, 1991), the destruction of the chloroplast membrane (Sandmann and Böger, 1980), inhibition of the formation of photosynthetic pigments and decrease in intracellular K^+ and Na^+ concentrations (DeFilippis, 1979). A lack of copper, on the other hand, may interfere with a number of important functions, such as photosynthesis, respiration, protein synthesis, lignification, auxin regulation, disease resistance and reproduction (Shorocks and Alloway, 1985).

To cope with the dualism of copper's essential functions versus its potential toxicity, all living cells have developed strong capabilities to manage the cellular copper levels. Indeed, intracellular copper routes studies have demonstrated that the intracellular copper regulation is generally controlled by 2 key elements: P-type ATPases that can pump copper across biological membranes in either direction and copper chaperones, small intracellular copper binding proteins that allow safe intracellular transport of copper to copper requiring proteins. It is thereby interesting to know that these copper homeostasis mechanisms, only functional within certain concentration limits, have been found in yeast and bacteria to mammalian cells and are therefore considered as being highly conserved through evolution (Wunderli-Ye and Solioz, 1999; Harrison *et al.*, 2000). Additionally, different organisms have developed species-specific copper management mechanisms resulting in different species-specific internal copper levels and copper susceptibilities. For algae, it was indeed demonstrated that copper regulating mechanisms involve the ability of algae to excrete metal-binding compounds to the surrounding medium inducing a reduction in the metal bioavailability and metal uptake (McKnight and Morel, 1979; Lumsden and Florence, 1983) and the production of intracellular phytochelatin, a metal binding peptide, which can detoxify copper (Silverberg *et al.*, 1976; Gekeler *et al.* 1988; Knauer *et al.*, 1998). However, different algal species appear to differ in their ability to produce such compounds, which is reflected in the large variability in inter-species sensitivity of copper (Takamura *et al.*, 1989). Additionally, intra-species variability in observed copper sensitivity can span several orders of magnitude (Janssen and Heijerick, 2003) and have been, at least partially, attributed to differences in test water chemistry: nutrient supply, pH, hardness, the concentration of organic complexing agents in the water (Knauer *et al.*, 1997; Janssen and Heijerick, 2003).

The exposure history of the algal population community may however also play an important role in determining the response of the algae to a given copper concentration. The importance

of exposure history in ecotoxicological studies with algae has been recognized since Kellner (1955) demonstrated that *Ankistrodesmus braunii* can develop a tolerance to elevated copper and rubidium concentrations. Since then, several studies have assessed the effect of metal-polluted environments on algal sensitivity (Foster, 1982; Whitton, 1984; Kuwabara and Leland, 1986; Hall *et al.*, 1989; Muyssen and Janssen, 2001a). Possible effects of - relatively low - natural (background) copper concentrations on the physiology and tolerance and the optimal concentration range of micro-algae to copper have, however, not been investigated.

Background copper concentrations can vary considerably with geographical area. According to Heijerick and Janssen (2000, updated database 2003, personal communication) the 5th and 95th percentile of the (total) copper concentrations in unpolluted European waters is 0.4 and 16.0 µg Cu L^{-1}, respectively. The 5th and 95th percentile for dissolved copper is 0.6 to 10.9 µg Cu L^{-1} (based on data only from The Netherlands, Germany and UK). Knauer *et al.* (1997) found a total dissolved copper concentration in lake Greifen (Switzerland) ranging between 0.2 to 1.8 µg Cu L^{-1}. In a survey of 11 pristine surface waters in 5 European countries, we found dissolved copper concentrations between 0.1 to 11 µg Cu L^{-1} (Bossuyt *et al.*, unpublished data; Heijerick *et al.*, unpublished data).

As organisms are dependent on essential metals for optimal growth and development, it may be hypothesized that species occurring in ecosystems with different background concentrations are differentially acclimated or adapted. Consequently, as each organism has an optimal concentration range for copper which is dependent on the prevailing copper background, it is suggested that with increasing copper concentration the organism's copper tolerance will increase. To test this hypothesis for algae, we exposed *P. subcapitata* to seven different copper concentrations during three months. At regular time intervals, experiments were performed to assess the effect of copper acclimation on the algal biomass, growth rate, pigment diversity, autotrophic index and their tolerance to copper.

2.2. Materials and methods

2.2.1. Acclimation of the algae

Acclimation experiments were performed with the green algae *P. subcapitata* (Korshikov) Hindak (CCAP 278/4, formerly known as *Selenastrum capricornutum* Printz and

Raphidocelis subcapitata Korshikov) obtained from the Culture Collection of Algae and Protozoa (CCAP; CEH, Ambleside, UK). Preparation of the synthetic freshwater ISO (International Organisation for Standardisation) culture medium and the maintenance of algal cultures during the acclimation period of three months followed the procedures described in ISO protocol 8692 (ISO, 1989). In the medium used for culturing and toxicity testing, ethylenediaminetetraacetic acid disodium salt (EDTA, Sigma Aldrich Chemie, Steinheim, Germany) was replaced by Aldrich humic acid (AHA, Sigma Aldrich Chemie, Steinheim, Germany) at a concentration of 2 mg L^{-1} as dissolved organic carbon (DOC) (see discussion); this medium will be referred to as modified ISO medium. The final pH of this medium was 7.8 ± 0.1. Seven different copper acclimation treatments were simultaneously started: < 0.5 (nominal zero), 1, 5, 12, 35, 60 and 100 µg Cu L^{-1}. To minimise metal contamination, all materials in contact with the culture or test medium were soaked 24 h in 1 % HNO_3 (p.a., VWR International, Leuven, Belgium) and rinsed with deionised water. All chemicals used for the preparation of the medium were reagent grade quality and were purchased from VWR International (Leuven, Belgium). Stock solutions of DOC were prepared by dissolving 5 g of AHA in 2 L of deionised water, equilibrating the solution for 24 h at 4 °C and filtering it through a 0.45 µm filter (Gelman Sciences, Ann Arbor, MI, USA). DOC was measured with a TOC-5000 analyser (Shimadzu, Duisburg, Germany).

During the acclimation period, the algae were cultured in 125 mL erlenmeyer flasks containing 50 mL of sterilised medium. The erlenmeyer flasks were autoclaved for 20 min at 121 °C and 1 kg cm^{-2} (pbi international S.p.A., Milano, Italy). After initiating the cultures (inoculum of 10^4 cells mL^{-1}), these flasks were closed with loose cotton stopper and shaken manually three times a day. The cultures were maintained at 22 ± 1 °C and illuminated continuously at 7250 cd m^{-2}. Each week, an inoculum (10^4 cells mL^{-1}) was taken from these cultures and transferred to fresh sterile medium to maintain exponential growth. Before inoculation, all cultures were visually inspected for contamination using a microscope (Kyowa, Tokyo, Japan) with 10x20 magnification. Cultures were performed in triplicate. Each week, cell densities were measured (Coulter, Model DN, Harpenden, Herts, England). Average weight of the algae was determined in triplicate for all tested concentrations and did not differ significantly between treatments (ANOVA, $p > 0.05$). Algal biomass was calculated using the particle counter data and an average (± standard deviation; $n = 21$) cell dry weight of $1.5 \pm 0.1 \times 10^{-11}$ g $cell^{-1}$.

2.2.2. Growth inhibition experiments

Prior to the acclimation experiments, a growth inhibition test was performed in standard ISO medium. During the acclimation experiment, the sensitivity of the acclimated algae to copper was also evaluated. Growth inhibition tests according to the ISO protocol 8692 (ISO, 1989) were performed every week up to week 4 and from then onwards every two weeks. Copper test concentrations were prepared by diluting a concentrated stock solution of $CuCl_2$ (0.1 g Cu L^{-1}) in modified ISO medium. Each test consisted of six copper concentrations ranging from < 0.5 (nominal zero = modified ISO) to 320 µg Cu L^{-1} with three replicates per concentration. Test vessels were incubated as described for the acclimation cultures. Algal densities were measured after 24, 48 and 72 h as cell numbers (algal cells mL^{-1}) using a particle counter (Coulter Model DN, Harpenden, Herts, England). At the same time, the pH of the medium was measured and noted. Initial cell density in all experiments was 10^4 cells mL^{-1}. A test was considered as valid when pH variation at the end of the test was less than one pH-unit and when the final cell concentration in the control exceeded 1.6×10^5 cells mL^{-1}.

The growth rate μ (day^{-1}) was calculated as follows (ISO, 1989):

$$\mu = \frac{\ln N_t - \ln N_0}{t_n}$$

with N_t the final cell density (cells mL^{-1}), N_0 the initial cell density (cell mL^{-1}) and t_n the time (d) after the initiation of the test.

The 72-h EC_{50} values were calculated based on the percentage inhibition of the growth rate (72-h E_rC_{50}) compared to the control (OECD, 1996a). The inhibition percentages were plotted against the measured copper concentrations and a statistically significant ($p < 0.05$) sigmoidal concentration-effect relationship was fitted through the data points using the software package SigmaPlot® 2000 (SPSS, Chicago, IL, USA). From this relationship, the 72-h E_rC_{50} and the 95 % confidence intervals were derived.

2.2.3. Absorbed and adsorbed metal concentration

The method used to measure the absorbed and adsorbed metal concentration is based on Franklin *et al.* (2000). These measurements were performed every week up to week 4 and from then onwards every two weeks. At the moment an inoculum of the acclimated algae was transferred to a sterile medium, 40 mL of the algal culture was transferred to 50 mL acid-washed Teflon centrifuge tubes. Samples were centrifuged (Centra-8, IEC, USA) at 500 g for 20 min and 20 °C. 10 mL of the supernatant solution was then pipetted into acid washed polypropylene vials (AAS tubes; Laborimpex, Brussels, Belgium). These samples were analysed for dissolved copper. The remaining supernatant was removed and the algal pellet was re-suspended in 20 mL of 5×10^{-3} M EDTA (Sigma Aldrich Chemie, Steinheim, Germany) and shaken for approximately 30 s to remove the copper adsorbed to the cells (Florence and Stauber, 1986). Franklin *et al.* (2000) have shown that no cell lysis occurred due to this EDTA treatment. The samples were then centrifuged again at 500 g for 20 min and the supernatant removed for copper analysis. These samples are referred to as the adsorbed copper fraction. The remaining algal pellets were left to dry in a laminar flow hood for 2 days and subsequently acid digested with 2 mL of 14 N HNO_3 for 30 minutes. These samples were heated in a microwave oven (Samsung MF245, Korea) at a power setting of 150 W for 5 min. After cooling to room temperature, the copper concentration of these samples - referred to as the absorbed copper fraction – was determined.

2.2.4. Copper measurements

All copper concentrations were analysed using a flame-atomic absorption spectrophotometer (AAS, for Cu > 20 µg Cu L^{-1}, SpectrAA100, Varian, Mulgrave, Australia) or a graphite furnace atomic absorption spectrophotometer (for Cu < 20 µg Cu L^{-1}, SpectrAA800 with Zeeman background correction, Varian, Mulgrave, Australia). Calibration standards (Sigma Aldrich Chemie, Steinheim, Germany) and reagent blank were analysed with every ten samples. The quantification limit of the flame AAS and the graphite furnace AAS is 17 µg Cu L^{-1} and 1.5 µg Cu L^{-1}, respectively (as determined bi-monthly by the method described in Clesceri *et al.*, 1998). Ten mL of the water samples was acidified (pH < 2) with 14 M HNO_3 (p.a., VWR International, Leuven, Belgium) and measured. All reported copper concentrations of the prepared media are dissolved copper concentrations (0.45 µm filtered). Absorbed and adsorbed metal concentrations were reported as g Cu $cell^{-1}$. Analysis of the

nominal zero concentrations could only assure that copper concentrations were < 0.5 µg Cu L^{-1} (detection limit of the graphite furnace AAS). Results of acclimation to nominal zero concentration will therefore be referred to as 0.5 µg Cu L^{-1}.

2.2.5. Pigment determination

Extraction and spectrophotometric determination of algal pigments were based on Clesceri *et al.* (1998). These measurements were performed every week up to week 4 and from then onwards every two weeks. At the moment an inoculum of the acclimated algae was transferred to a sterile medium, 20 mL of algae was taken from each algal culture and filtered over 0.7 µm Whatman® GF/C filter (Whatman International, Maidstone, UK). The filter was placed in a tissue grinder, covered with 3 mL of 90 % aqueous acetone solution (90 mL acetone with 10 mL of 10 g $MgCO_3$ L^{-1}) and macerated for one minute with a grinder. The sample was transferred to a glass centrifuge tube and the volume was adjusted to 10 mL with 90 % aqueous acetone solution. Samples were kept in the dark at 4 °C for 24 h after which the solution was centrifuged (Centra-8, IEC, USA) at 500 g for 20 min. The resulting supernatant was used for spectrophotometric pigment determination (Spectronic 601, Milton Roy, USA). The following formulae were used to calculate chlorophyll and carotenoid concentrations (Rowan, 1989):

$$Chlorophyll\ a\ (mg\ L^{-1}) = \frac{2.67\ (664_b - 665_a) \cdot V_1}{V_2 \cdot L}$$

$$Chlorophyll\ b\ (mg\ L^{-1}) = \frac{\left[21.03 \cdot (OD647) - 5.43 \cdot (OD664) - 2.66 \cdot (OD630)\right] \cdot V_1}{V_2}$$

$$Carotenoid\ (mg\ L^{-1}) = \frac{\left(4.1 \cdot OD480 - 0.043 \cdot C_a - 0.367 \cdot C_b\right) \cdot V_1}{V_2}$$

in which V_1 is the extract volume (L); V_2 the volume of the sample (L); L the length of the cuvette used (m); 664_a and 665_b the optical density of 90 % acetone extract before and after acidification at 664 and 665 nm; *OD480*, *OD647*, *OD664* and *OD630* the optical density at 480, 647, 664 and 630 nm; C_a and C_b concentration of chlorophyll *a* and *b* respectively (mg L^{-1}). Pigment concentrations were reported as g $cell^{-1}$. Autotrophic index (AI) was calculated

as the ratio of the biomass (dry weight) to the chlorophyll *a* concentration. Pigment diversity (PD) was calculated as the ratio of carotenoid concentration to the chlorophyll *a* concentration (Rai *et al.*, 1990).

2.2.6. Statistical analysis

The effect of the various acclimation concentrations on the algal response were compared using one-way analysis of variance (ANOVA) and Duncan's multiple range test (STATISTICA® software, Tulsa, OK, USA). The ANOVA assumptions on homogeneity of variance and normality were tested using Bartlett's and Kolmogorov-Smirnov's test, respectively. In case one or both assumption(s) failed, log10 transformation of the data was sufficient in order to proceed with the ANOVA. Statements of significant differences are based on accepting $p < 0.05$.

2.3. Results

A decrease in algal biomass and growth rate was noted with increasing acclimation concentration after the first week of acclimation (Table 2.1). In contrast, an increase was observed in all pigment concentrations. Algae acclimated to 100 µg Cu L^{-1} had a significantly lower algal biomass (mean ± standard deviation: 4.7 ± 0.7 mg DW L^{-1}; $n = 3$) and growth rate (mean ± standard deviation: 0.489 ± 0.021 day^{-1}; $n = 3$) and high pigment concentrations (84.7, 14.8 and 20.9 × 10^{-14} g $cell^{-1}$ for chlorophyll *a*, *b* and carotenoid concentration) compared to the algae acclimated to 0.5 µg Cu L^{-1}. Algae acclimated to 60 µg Cu L^{-1} showed similar results. The algal biomass and growth rate of algae acclimated to 0.5 µg Cu L^{-1} was significantly higher than those of algae acclimated to higher copper concentrations.

Except for the results obtained during the first (shock) week of acclimation, all parameters did not change significantly during the three months acclimation period. Hence, the mean values ($n = 8$) of the algal biomass, the growth rate, the pigment concentrations, the pigment diversity and the autotrophic index of the different *P. subcapitata* acclimation cultures, are presented in Table 2.2. In weeks 2 to 12 pooled, the highest biomass is observed for algal populations exposed to 1, 5 and 12 µg Cu L^{-1}. Severe reduction of algal biomass is observed in the cultures acclimated to 100 µg Cu L^{-1}. The growth rate (mean ± standard deviation, $n =$

8) of the latter was 0.777 ± 0.038 day^{-1} compared to 0.917 ± 0.015 day^{-1} for algae acclimated to 0.5 µg Cu L^{-1}. A Student's *t* test for independent samples ($p < 0.05$) for algal biomass and growth rate revealed a no observed effect concentration (NOEC) at 35 µg Cu L^{-1} for acclimated algal populations. The autrophic index and pigment diversity gave a NOEC at 60 µg Cu L^{-1}.

Table 2.1: Biomass (mean ± standard deviation (SD), $n = 3$), growth rate (mean ± standard deviation SD, $n = 3$), chlorophyll *a* (Chl *a*) and *b* (Chl *b*) and carotenoid (car) concentration of algae acclimated to seven copper concentration after the first week of acclimation. Values with same letter are not significantly different (analysis of variance, $p < 0.05$).

Concentration µg Cu L^{-1}	**Biomass ± SD mg DW L^{-1}**	**Growth rate ± SD day^{-1}**	**Chl *a* 10^{-14} g $cell^{-1}$**	**Chl *b* 10^{-14} g $cell^{-1}$**	**Car 10^{-14} g $cell^{-1}$**
0.5	$111.6 \pm 2.4_a$	$0.944 \pm 0.001_a$	3.8	0.5	2.2
1	$95.0 \pm 3.1_b$	$0.921 \pm 0.004_b$	4.4	0.6	2.9
5	$93.1 \pm 1.8_b$	$0.919 \pm 0.003_b$	6.7	1.1	3.5
12	$93.4 \pm 3.6_b$	$0.919 \pm 0.005_b$	9.8	1.5	4.1
35	$93.2 \pm 4.1_b$	$0.919 \pm 0.006_b$	10.3	1.1	4.7
60	$30.5 \pm 2.4_c$	$0.759 \pm 0.011_c$	39.3	7.7	9.0
100	$4.7 \pm 0.7_d$	$0.489 \pm 0.021_d$	84.7	20.9	14.8

A significant increase in chlorophyll *a* and carotenoid concentrations is observed in algae acclimated to 60 and 100 µg Cu L^{-1}. Chlorophyll *a* and *b* concentrations increased with a factor of 3.4 and 2.7, respectively, with increasing acclimation concentration. Carotenoid concentrations increased only with a factor of 2. Chlorophyll *a* concentrations were 5 to 6-fold higher than chlorophyll *b* concentrations and 2 to 4-fold higher than carotenoid concentrations. The pigment diversity of algae acclimated to 1 to 35 µg Cu L^{-1} was significantly higher than that of algae acclimated to lower and higher copper concentrations. Similar results were noted for the autotrophic index. A positive linear correlation ($r^2 = 0.76$; $n = 60$) was observed between pigment diversity and autotrophic index (data not shown). A negative logarithmic correlation was observed between chlorophyll *a* ($r^2 = 0.95$; $n = 60$), chlorophyll *b* ($r^2 = 0.87$; $n = 60$) or carotenoid ($R^2 = 0.89$; $n = 60$) concentrations and the algal biomass (Figure 2.1).

Table 2.2: Mean biomass, growth rate, chlorophyll *a* (Chl *a*) and *b* (Chl *b*) concentration, carotenoid concentration (Car), pigment diversity (PD) and autotrophic index (AI) of algae acclimated to seven copper concentrations (mean ± standard deviation (SD), $n = 8$; pooled data of week 2 to 12). Values with the same letter are not significantly different at $p < 0.05$ (analysis of variance).

Concentration	**Biomass ± SD**	**Growth rate ± SD**	**Chl *a* ± SD**	**Chl *b* ± SD**	**Car ± SD**	**PD ± SD**	**AI ± SD**
µg Cu L^{-1}	**mg DW L^{-1}**	**day^{-1}**	**10^{-14} g $cell^{-1}$**	**10^{-14} g $cell^{-1}$**	**10^{-14} g $cell^{-1}$**	**-**	**-**
0.5	$88.1 \pm 8.9_{b}$	$0.917 \pm 0.015_{a}$	$8.4 \pm 3.1_{ac}$	$1.7 \pm 0.5_{b}$	$3.7 \pm 0.8_{c}$	$0.390 \pm 0.065_{b}$	$137 \pm 26_{bc}$
1	$95.6 \pm 6.0_{a}$	$0.920 \pm 0.016_{a}$	$9.4 \pm 2.0_{ac}$	$1.8 \pm 0.6_{b}$	$3.8 \pm 0.2_{c}$	$0.422 \pm 0.081_{ab}$	$178 \pm 34_{a}$
5	$96.4 \pm 7.8_{a}$	$0.919 \pm 0.016_{a}$	$9.9 \pm 3.6_{ac}$	$1.9 \pm 1.0_{b}$	$4.1 \pm 0.6_{bc}$	$0.465 \pm 0.059_{a}$	$183 \pm 39_{a}$
12	$95.6 \pm 4.8_{a}$	$0.920 \pm 0.016_{a}$	$10.6 \pm 2.8_{bc}$	$1.9 \pm 0.7_{b}$	$4.2 \pm 1.1_{bc}$	$0.401 \pm 0.058_{ab}$	$158 \pm 44_{ab}$
35	$88.9 \pm 6.6_{b}$	$0.906 \pm 0.019_{b}$	$10.3 \pm 2.0_{bc}$	$2.0 \pm 0.6_{b}$	$4.4 \pm 0.8_{bc}$	$0.425 \pm 0.045_{ab}$	$150 \pm 29_{abc}$
60	$75.2 \pm 4.4_{c}$	$0.888 \pm 0.008_{c}$	$13.5 \pm 3.2_{b}$	$2.3 \pm 0.8_{b}$	$4.9 \pm 1.1_{b}$	$0.370 \pm 0.051_{b}$	$118 \pm 33_{c}$
100	$35.7 \pm 9.8_{d}$	$0.777 \pm 0.038_{d}$	$28.6 \pm 7.5_{a}$	$4.6 \pm 1.6_{a}$	$7.1 \pm 1.2_{a}$	$0.251 \pm 0.024_{c}$	$56 \pm 14_{d}$

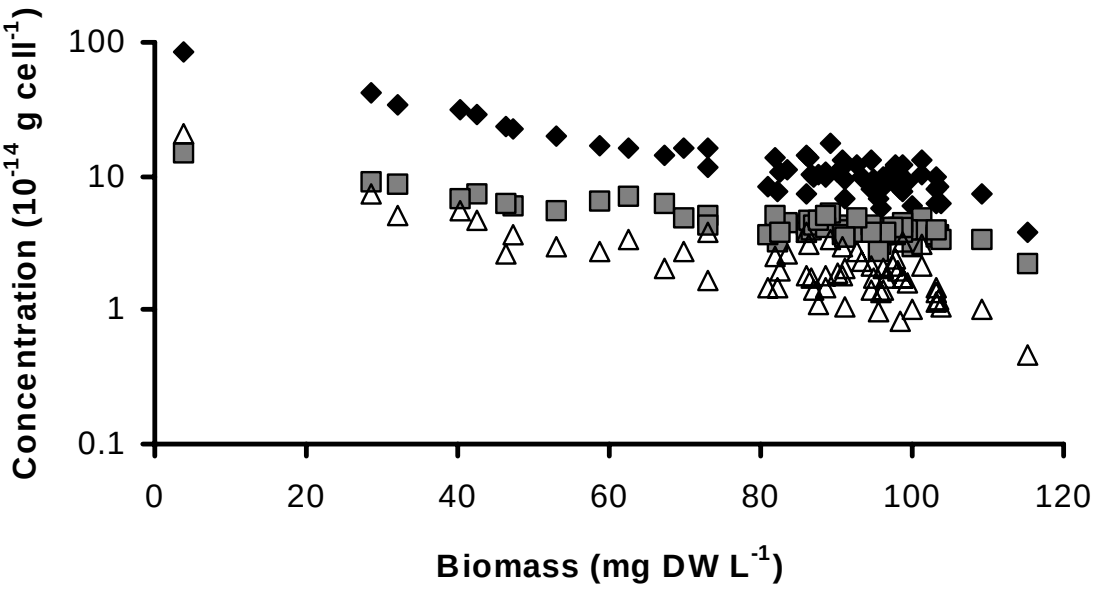

Figure 2.1: Relation between algal biomass and chlorophyll *a* (◆), chlorophyll *b* (Δ) and carotenoid (■) concentration of the acclimated *P. subcapitata*.

Experiments with *P. subcapitata* performed in standard ISO-medium (ISO, 1989) resulted in a 72-h E_rC_{50} of 37 ± 3 µg Cu L^{-1} (mean ± standard deviation; n = 3). The chronic copper sensitivity, performed in modified ISO medium, of *P. subcapitata* acclimated to the seven different copper concentrations is presented in Figure 2.2. An increase in copper toxicity values is observed with increasing copper acclimation concentrations. The 72-h E_rC_{50} (mean ± standard deviation; n = 8) only increased significantly from 88 ± 12 µg Cu L^{-1} to 124 ± 21 µg Cu L^{-1} for algae acclimated to 0.5 and 100 µg Cu L^{-1}, respectively. The data fitted a positive linear regression equation: a = 0.33 × b + 90 (r^2 = 0.94; $p < 0.05$) with *a* the 72-h E_rC_{50} and *b* the acclimation concentration (µg Cu L^{-1}). For each toxicity test, the pH drift was less than 0.5 pH units over 72 h. pH drift in acclimation cultures was sometimes as much as 1 pH unit as a result of the high final algal cell densities (> 10^6 cells mL^{-1}).

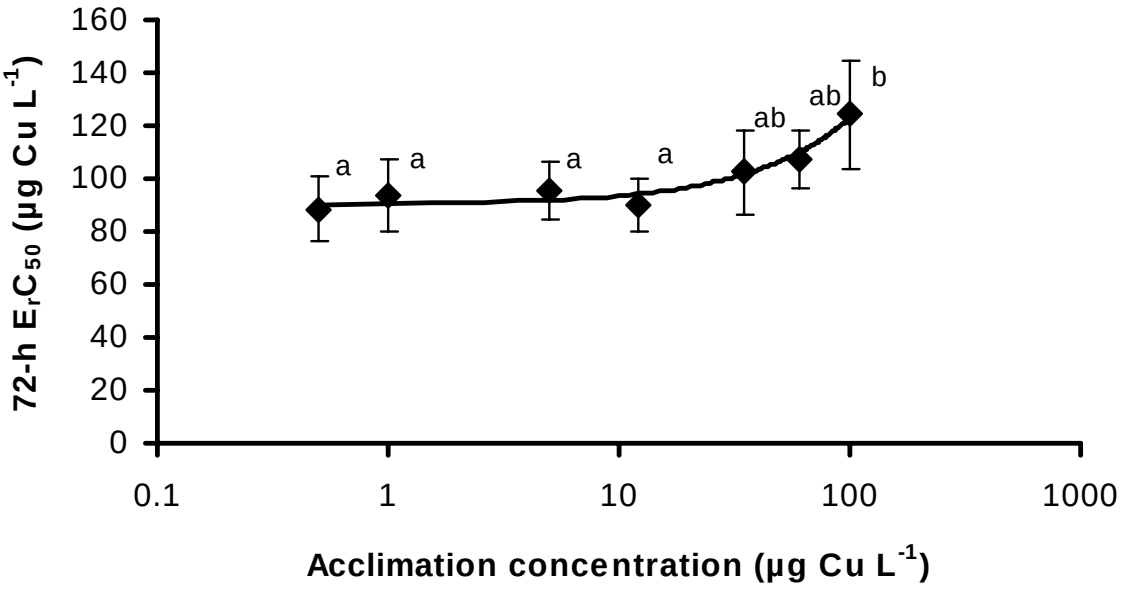

Figure 2.2: Chronic copper tolerance (72-h E_rC_{50}) of *P. subcapitata* acclimated to different copper concentrations. Error bars represent standard deviations. Mean (n = 8) values with the same letter are not significantly different at $p < 0.05$.

Adsorbed and intracellular (absorbed) copper concentrations for the different acclimation cultures are shown in Figure 2.3. An increase in surface-bound and intracellular copper with increasing copper acclimation concentrations is observed. Algae acclimated to the highest copper acclimation concentrations (60 and 100 µg Cu L^{-1}) exhibited a strong increase in both adsorbed and intracellular copper after the first week of acclimation. Intracellular copper concentration for algae acclimated to 0.5 and 100 µg Cu L^{-1} increased from 0.001 to 59.3 × 10^{-15} g Cu $cell^{-1}$, while adsorbed copper concentration ranged from 0.033 to 35.1 × 10^{-15} g Cu $cell^{-1}$. From week 3 onwards, a strong decrease of both parameters is observed. Compared to the first week measurements, intracellular copper concentrations of algae acclimated to 100 µg Cu L^{-1} for 12 weeks decreased from 59.3 × 10^{-15} to 20.6 × 10^{-15} g Cu $cell^{-1}$ and adsorbed copper concentrations from 35.1 × 10^{-15} to 1.8 × 10^{-15} g Cu $cell^{-1}$. Except for the lowest copper acclimation concentration, more than 80 % (50 % for 0.5 µg Cu L^{-1}) of the total cellular copper (adsorbed + absorbed) was located intracellularly.

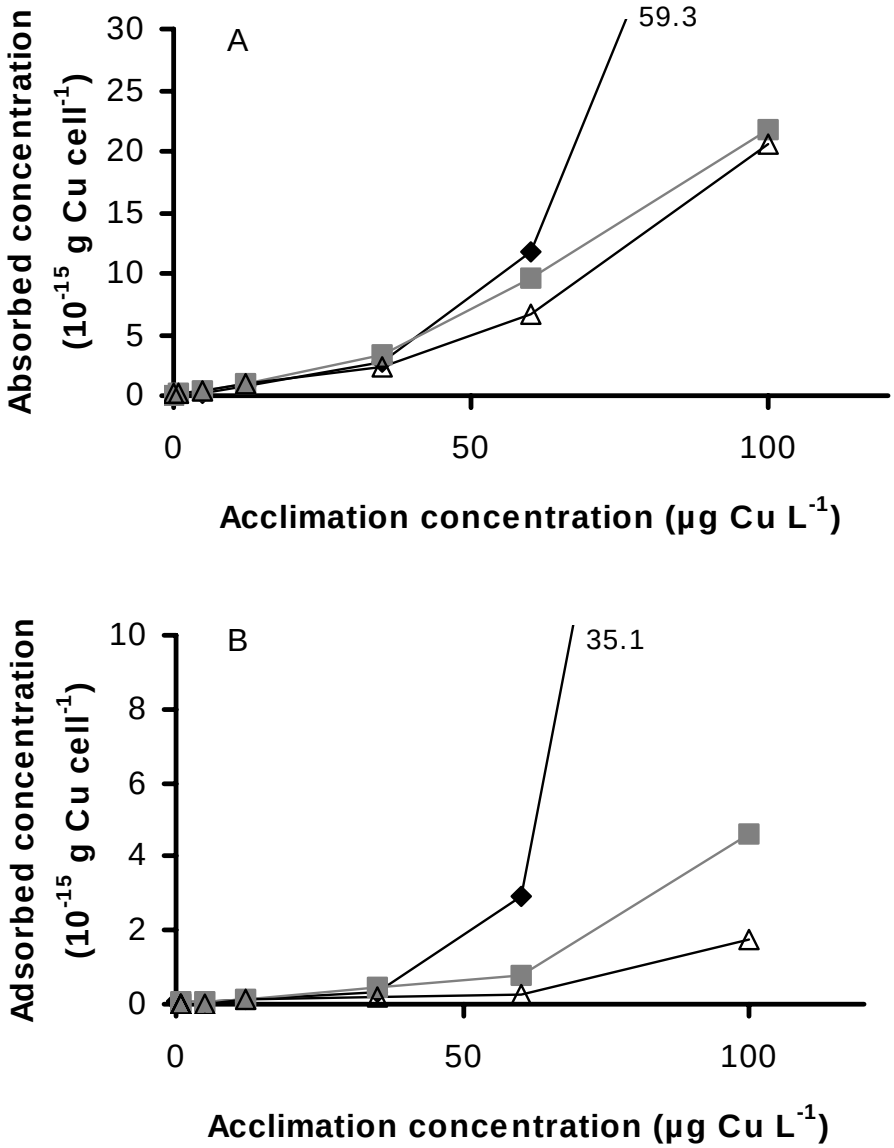

Figure 2.3: Absorbed (A) and adsorbed (B) copper concentration of *P. subcapitata* acclimated to different copper concentrations. (◆: week 1; ■: week 3; Δ: week 12).

Finally, the adsorbed and absorbed copper concentrations were plotted against the algal biomass inhibition of the acclimation cultures (Figure 2.4). This figure gives an indication whether it are mechanisms on the cell wall or in the algal cells which regulate the copper homeostasis at a certain copper exposure concentration. Based on a breakpoint-regression analysis with the log10 transformed values of adsorbed and absorbed copper concentrations, a critical accumulation concentration (with 95 % confidence limits) of copper on the algal cell wall (0.12 (0.09 – 0.17) × 10^{-15} g Cu cell^{-1}; r^2 = 0.9, n = 48, F-test: $p < 0.001$) and in the algal cell (2.9 (2.4 – 3.6) × 10^{-15} g Cu cell^{-1}; r^2 = 0.9, n = 51, F-test: $p < 0.001$) could be determined,

resulting in no algal biomass inhibition. When inhibition of growth was considered, similar results were obtained. Adsorbed and absorbed copper concentrations of 2.6×10^{-15} and 13.7×10^{-15} g Cu cell^{-1} were noted at an algal population biomass inhibition of 50 %. A poor positive correlation was observed between internal copper concentrations of the acclimated algae used in 72 h toxicity assays and their chronic copper sensitivity ($r^2 = 0.10$; $n = 42$, $p > 0.05$).

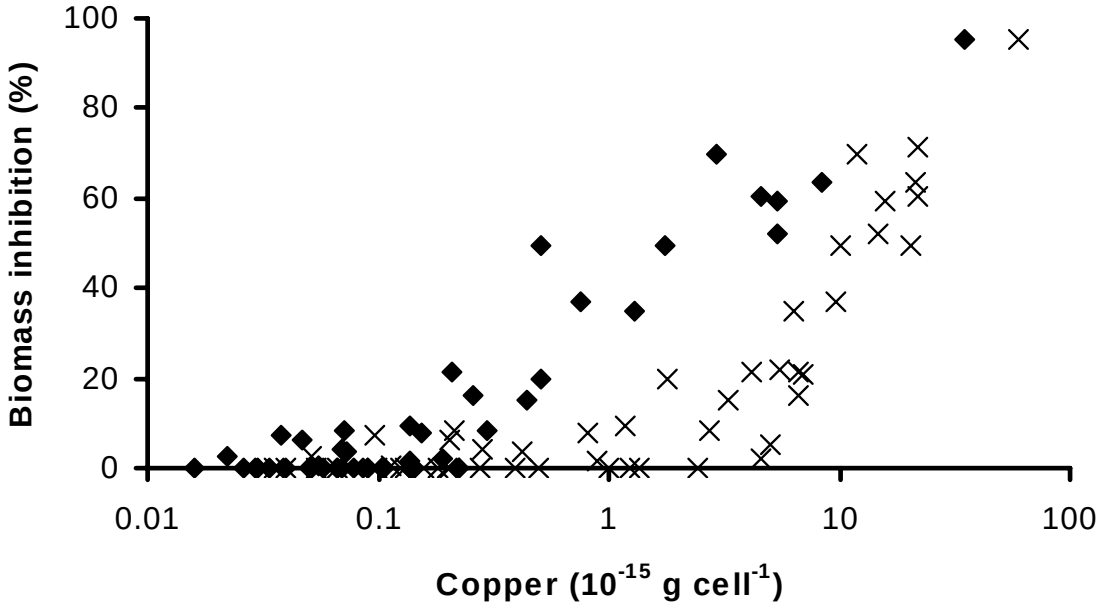

Figure 2.4: Relationship between the algal biomass inhibition and the adsorbed (◆) and absorbed copper (×) concentration in *P. subcapitata* acclimated to different copper concentrations.

2.4. Discussion

The toxicity of copper to *P. subcapitata* has been reported by a number of workers (Bartlett *et al.*, 1974; Christensen *et al.*, 1979; Blaylock *et al.*, 1985; Haley *et al.*, 1986; Franklin *et al.*, 2001, 2002). EC50 values range from 8 to 400 µg Cu L^{-1}. It has been suggested that the main reason for this high variability is attributable to the differences in culture and test media composition affecting both the algal performance and the metal's bioavailability (Janssen and Heijerick, 2003). The EC50s (> 70 µg Cu L^{-1}) obtained in the present study are considerably higher than those derived from tests (37 µg Cu L^{-1}) performed in standard ISO medium (ISO, 1989). This is due to differences in copper complexation capacities of the two media: different DOC sources (AHA versus EDTA) and different concentrations (2 mg C L^{-1} in our

test media compared to 0.32 mg C L^{-1} in standard media). Indeed, several authors have demonstrated that copper toxicity for algae decreases with increasing DOC (Winner and Owen, 1991; Garvey *et al.*, 1991; Janssen and Heijerick, 2003).

It is well recognised that concentrations of a chelator are necessary to prevent iron (Fe) precipitation by forming Fe-complexes in algal culture and test media. Therefore, omitting chelators will lead to nutrient limited algal growth (Lewis, 1995). Consequently, although organic complexes will reduce the copper toxicity, some complexing capacity is needed in culture media if some degree of natural realism is to be attained. Although AHA - used in our study - is not a naturally produced component of aquatic ecosystems, but derived from terrestrial humic acid and brought in the aquatic compartment through run-off, it will better reflect the buffering capacity to metals of natural organic matter in surface waters compared to EDTA. The selection of the DOC concentration used in the test and culture media was based on an analysis of the Surface Water Database (SWAD; Heijerick and Janssen, 2000, update database 2003, personal communication), a database containing the physico-chemistry of approximately 200,000 European surface water monitoring stations. The 10^{th} and 50^{th} percentile of the DOC concentration was around 2 and 5 mg L^{-1}, respectively (Bossuyt and Janssen, 2003).

The influence of essential trace elements on the sensitivity of micro-algae to environmental contaminants has - up to now - not been examined in-depth. This factor may be especially important in toxicity assessments of metals and metal compounds, as it has been reported that pre-exposure to metals during culturing can affect the sensitivity of algae in metal toxicity tests. The copper concentration in standard algal media can differ up to a factor of 10,000 (Table 2.3). Reports of acquired resistance to copper by different algal species growing in copper contaminated freshwater systems are numerous (Stokes *et al.*, 1973; Stokes, 1975; Butler *et al.*, 1980; Foster, 1982; Takamura *et al.*, 1989; Lombardi and Vieira, 1998). Stokes and Dreier (1981) noted that *Scenedesmus* cells cultured in medium with copper addition (500 µg Cu L^{-1}) were - after only 3 generations - more tolerant than those cultured in copper deficient medium. Sandmann and Böger (1980) reported acclimation to copper by *S. acutus* in the first 24 h of exposure. The present study demonstrated that *P. subcapitata* can also acquire increased copper tolerance, but no significant changes were observed at lower – more environmentally realistic – copper concentrations (Figure 2.2). A population NOEC value of 35 µg Cu L^{-1} was observed based on the algal biomass and growth rate of the acclimated

algae. As the test set-up allowed for a gradual acclimation and induction of the homeostasis processes, which is probably naturally occurring in surface waters, this NOEC may be of very high ecological relevance and hence directly comparable to a field situation. A maximum resistance factor (RF = 72-h E_rC_{50} of acclimated organisms divided by the 72-h E_rC_{50} of non-acclimated organisms) of 1.7 was noted. Muyssen and Janssen (2001a) found a maximum RF of 3.2 for *P. subcapitata* and *Chlorella vulgaris* when cultured in medium containing 65 µg Zn L^{-1}. This zinc concentration was, however, higher than background concentrations in European lowland freshwaters (mean: 18.5 µg Zn L^{-1}; range 8.0 – 42.7 µg Zn L^{-1}; Zuurdeeg *et al.*, 1992).

Table 2.3: Essential metal (copper: Cu; zinc: Zn) and EDTA concentrations in culture and test media commonly used in experiments with freshwater Chlorophyceae.

Medium	**Cu**	**Zn**	**EDTA**	**Reference**
	µg L^{-1}	µg L^{-1}	µg L^{-1}	
Chu no 10	0	0	0	Chu, 1942
DSW	0	0	0	Tubbing *et al.*, 1994
JM/5	0	0	3600	Thompson *et al.*, 2001
Volvox	0	7.2	1845	Starr, 1969
OECD/ISO	0.0037	1.4	82	OECD, 1996a; ISO, 1989
ASTM/USEPA	0.004	1.57	246	ASTM, 1998, USEPA, 1994
SAAM	0.0041	15.7	246	Christensen *et al.*, 1979
Fraquil	0.065	0.3	1525	Morel *et al.*, 1975
SANM	0.127	1.56	1100	Kuwabara and Leland, 1986
BBM	400	2000	50000	Nichols and Bold, 1965

From the observed changes in biomass, growth rate, pigment diversity and autotrophic index (Table 2.2) as a function of the copper acclimation concentrations, the Optimal Concentration range (OCEE-curve) or Window of Essentiality can be derived (Van Assche *et al.,* 1997; Hopkin, 1989; respectively). In Table 2.2, significant optimal concentrations were observed in biomass (1 – 12 µg Cu L^{-1}), growth rate (up to 12 µg Cu L^{-1}), pigment diversity (1 – 35 µg Cu L^{-1}) and autotrophic index (1 – 35 µg Cu L^{-1}). Toxicity and deficiency effects (reduced values) were noted at higher and lower concentrations, respectively. Peterson *et al.* (1984) and Reuter

et al. (1987) found an optimal growth for *S. quadricauda* at a Cu^{2+} activity lower than 10^{-11} M. They did not observe limitation of growth at the lowest copper concentrations tested ($10^{-12.5}$ M Cu^{2+}). Knauer *et al.* (1997) determined the optimal copper range for three different species of unicellular green algae: 10^{-15} to 10^{-7} M Cu^{2+} for *S. subspicatus*, 10^{-11} M Cu^{2+} for *Chlamydomonas reinhardtii* and 10^{-13} to 10^{-10} M Cu^{2+} for *Chlorella fusca*. Expressed as Cu^{2+}, the optimal concentration range of *P. subcapitata* obtained in our study was 10^{-14} to 10^{-10} M Cu^{2+} (see further), which is in agreement with those reported in literature. In contrast to the algal growth rate (biomass), pigment concentrations were significantly higher in the highest acclimation concentration. Cid *et al.* (1995) also observed an increase in chlorophyll *a* content in *Phaeodactylum tricornutum* when exposed to copper up to 100 µg Cu L^{-1} (*i.e.* concentration that induced about 50 % growth reduction), followed by a decrease at higher concentrations. At copper concentrations lower than the optimal concentration range, low values of biomass, pigment diversity and autotrophic index were observed and this may be due to possible copper deficiency. However, no significant lower values were demonstrated using the chronic toxicity values and chlorophyll and carotenoid endpoints.

It is suggested that continuous culture over several years in such deficient media may affect the health of the organisms. Standard ISO medium (< 0.1 µg Cu L^{-1}) and other international media vary considerably in essential metal concentrations (Table 2.3) and may be copper deficient for culturing algae, resulting in significantly affected toxicity test results (depending on the parameter investigated).

Measurements of intracellular and adsorbed copper of *P. subcapitata* indicated that 80 % of the total cellular copper was located in the algal cells. Knauer *et al.* (1997) made a similar observation for *S. subspicatus* exposed to copper. Franklin *et al.* (2000) found that 60 % of the copper content was internally bound in *Chlorella* sp. exposed to 10 µg Cu L^{-1}. Remarkable was that - for copper concentrations of 5 up to 100 µg Cu L^{-1} - algae took up 50 % of the copper added to the medium within one week. Franklin *et al.* (2002) noted similar results for *Chlorella* sp. and *P. subcapitata.* Knauer *et al.* (1997) observed that the maximal intracellular copper content for *S. subspicatus* exposed for 5 days to 6.4 mg Cu L^{-1} was 2.5×10^{-14} g Cu $cell^{-1}$. They found that the total cellular copper concentration increased with increasing copper concentration. Hall *et al.* (1989) also observed an increase in cellular copper content in *C. vulgaris* and *Chlamydomonas geitleri* (isolated from a Cu and Zn contaminated lake) when they were transferred from 2.5 to 2860 µg Cu L^{-1}. They found an increase in cellular copper

content from 10^{-15} up to 10^{-12} g Cu cell^{-1} for both *Chlorella* and *Chlamydomonas*, respectively. This is an increase with a factor of 1000, which is similar to that observed in our study (factor 100 – 10,000). According to our results, optimal growth occurs at intracellular copper concentrations of 10^{-17} to 10^{-15} g Cu cell^{-1}. This rather broad range suggests that cells regulate the intracellular copper concentration, presumably through immobilization of excess copper, as demonstrated by Silverberg *et al.* (1976) and Knauer *et al.* (1998).

The internal metal concentrations of culture algae used in the growth inhibition experiments are poorly correlated with the observed copper toxicity in *P. subcapitata*. Franklin *et al.* (2002) observed a relationship between both extracellular and intracellular copper concentrations and 72-h growth inhibition test results for *Chlorella* sp. and *P. subcapitata*. They found that 50 % growth inhibition occurred at 3×10^{-14} g Cu cell^{-1} of intracellular copper and 8×10^{-15} g Cu cell^{-1} of extracellular copper. In our study, the latter copper concentration only occurred in algae acclimated to 100 µg Cu L^{-1}, which did exhibit a strong decrease in biomass production (> 60 %). It has to be noted that, in our study, algae with this high internal copper concentration had an increased copper tolerance. The observed decrease of surface-bound and intracellular copper with longer acclimation duration (Figure 2.3) can be the result of a defence mechanism of the algae such as cell wall exclusion of copper (Knauer *et al.*, 1997) and changes in the permeability of the algal plasma membrane (DeFillipis, 1979). Further research will be needed to determine the exact detoxification mechanism.

From our results, it seems that *P. subcapitata* has different mechanisms to compete with copper stress. From Figure 2.4, it was observed that starting from an adsorbed copper concentration of 0.12×10^{-15} g Cu cell^{-1} (*i.e.* critical accumulation concentration) to the algal cell wall, inhibition of the algal biomass (and growth rate) occurred. This adsorbed copper concentration was noted in algae cultures acclimated to 5 and 12 µg Cu L^{-1}. Ma *et al.* (2003) stated that copper toxicity to unicellular algae could be interpreted by its accumulation at a discrete site or biotic ligand at the algal cell wall. At a copper concentration equal to the 72-h EC50 of *S. subspicatus*, they observed a critical accumulation of adsorbed copper of 6.4×10^{-13} µg Cu cell^{-1}. The lower value in our study (2.6×10^{-15} g Cu cell^{-1}) can be explained by the use of a different algal species and because our critical value is determined on an algal population of 1 week old (high algal density). *Scenedesmus* sp. is already described as more tolerant to copper than *P. subcapitata* (Fargašová *et al.*, 1999). It may be hypothesized that at

copper concentrations lower than the critical accumulation concentration (*i.e.* acclimation concentrations < 12 µg Cu L^{-1}), algae probably have an active copper accumulation in order to fulfil their metabolic requirements (1 and 5 µg Cu L^{-1} are optimal concentrations; Table 2.2). Hence, it may be suggested that - in this acclimation interval of copper - copper uptake in the algae is regulated by the algal cell membrane. Copper uptake is regulated at the cell wall level and will be taken up intracellularly by the cell through specific canals (Harrison *et al.*, 2000). Internal algal copper concentrations (absorbed copper) up to 2.9×10^{-15} g Cu $cell^{-1}$, which occurs in algae acclimated to 12 and 35 µg Cu L^{-1}, resulted in a non-significant inhibition. At higher concentration, significant inhibition of the algal biomass and growth rate occurs (population NOEC = 35 µg Cu L^{-1}). Hence, for copper acclimation concentrations between 12 and 35 µg Cu L^{-1}, copper regulation may be a combination of regulation on the algal cell wall and intracellular copper regulation.

No literature was found on the influence of the cupric ion at relevant background concentrations on the acclimation potential of micro-algae. Free copper activities of the acclimation concentrations used in this study were calculated with the geochemical speciation program Windermere Humic Aqueous Model VI (Tipping, 1994; WHAM 6.0.1, Centre for Ecology and Hydrology, Windermere, UK) and ranged from 10^{-18} to 10^{-8} M Cu^{2+} (1.3×10^{-18}; 2.1×10^{-14}; 1.3×10^{-12}; 1.3×10^{-11}; 3.1×10^{-10}; 4.1×10^{-9}; 1.9×10^{-8} M Cu^{2+}). Based on the results described above, it can be concluded that concentrations ranging from 2.1×10^{-14} to 6.1×10^{-10} M Cu^{2+} are optimal for growth of *P. subcapitata*. Concentrations > 10^{-9} M Cu^{2+} resulted in toxic effects in long-term acclimation experiments, while concentrations ≤ 10^{-14} M Cu^{2+} resulted in deficiency effects. Calculated 10 – 90 percentile Cu^{2+} activity intervals for Europe were 2.1×10^{-14} to 4.6×10^{-8} M Cu^{2+} (Bossuyt and Janssen, unpublished data). This confirms that the concentration range tested in this study is relevant for Europe and that the optimal range for copper is situated within the copper activity range found across Europe. When the total copper concentrations mentioned in Table 2.3, are transformed to copper activities based on WHAM VI and on the physico-chemical water characteristics of the respective standard media, all these media have values lower than 10^{-14} M Cu^{2+}. According to the results of this study, these media are or may become copper deficient for algae and are only relevant for extreme environments, like mountain lakes (Knauer *et al.*, 1997). Results of experiments performed with algae cultured or tested in these media may not be representative for most aquatic ecosystems.

Chapter 3

Multi-generation acclimation to copper of *Daphnia magna* Straus: changes in tolerance, energy reserves and homeostasis

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Chapter
3

Influence of multi-generation acclimation to copper on tolerance, energy reserves and homeostasis of *Daphnia magna* Straus

Abstract - A multi-generation acclimation experiment was performed with *D. magna* exposed to copper to assess possible changes in tolerance and to establish the optimal concentration range of this species (OCEE). The hypothesis was tested that as the bioavailable background concentration of an essential metal increases (within realistic limits), the natural tolerance (to the metal) of the acclimated/adapted organisms and communities will increase. During 18 months the daphnids were exposed to six different, environmentally relevant, copper background concentrations ranging between 0.5 and 100 µg Cu L^{-1} (7×10^{-15} and 3.7×10^{-9} M Cu^{2+}). A modified standard test medium was used as culture and test medium. Medium modifications were: reduced hardness (lowered to 180 mg L^{-1} as $CaCO_3$) and addition of Aldrich humic acid at a concentration of 5 mg DOC L^{-1} (instead of EDTA). An increase in acute (effect concentration resulting in 50 % immobility: 48-h EC50) and chronic copper (effect concentration resulting in 50 % or 10 % reproduction reduction: 21-d EC50, 21-d EC10) tolerance was observed with increasing exposure concentration. The 48-h EC50 (mean ± standard deviation) increased significantly from 204 ± 24 µg Cu L^{-1} to 320 ± 43 µg Cu L^{-1}. A non-significant change in 21-d EC50 (mean and 95 % confidence limits) from 48.0 (47.9 – 48.0) µg Cu L^{-1} to 78.8 (66.3 - 93.6) µg Cu L^{-1} was noted in the chronic toxicity assays. The OCEE was assessed using different biological parameters, *i.e.* net reproduction (R_0), energy reserves (E_a), body length measurements, filtration rates and body burdens. After three generations of acclimation the OCEE ranged between 1 and 35 µg Cu L^{-1} (2×10^{-14} to 80×10^{-12} M Cu^{2+}). It can be concluded that acclimation of *D. magna* to copper does occur in laboratory experiments, even at realistic copper background concentrations (10^{-11} – 10^{-9} M Cu^{2+}). However, it is suggested that this phenomenon is of less importance in the context of regulatory risk assessments. An optimal copper concentration range for *D. magna* was observed between 1 and 35 µg Cu L^{-1} (10^{-14} – 10^{-11} M Cu^{2+}), indicating that copper deficiency can occur in routine laboratory cultures.

3.1. Introduction

Life has evolved in the presence of metals of which several, like copper, have essential biological functions (Da Silva and Williams, 1991). For each species, in theory, a bell-shaped concentration-effect curve can be constructed, with deficiency symptoms occurring at low

concentrations and toxic effects occurring at high concentration. Van Assche *et al.* (1997) and Hopkin (1989) called this the Optimal Concentration range for Essential Elements (OCEE) or "window of essentiality", respectively. Between the two extremes there is generally an optimal concentration range within which an organism experiences optimal growth, development and reproduction. When the environmental concentration of an essential element is within the optimal concentration range, organisms can regulate their internal concentrations of the element through binding, detoxification and elimination (Brix and DeForest, 2000). As the natural background concentration of metals in various ecosystems can vary considerably, the range to which the resident species are adapted varies accordingly. This change in tolerance may be due to physiological processes (acclimation) or have a genetic basis (adaptation). Acclimation and/or adaptation to extreme high concentration of metals in the natural environment are well documented for plants and animals (Klerks and Weis, 1987). However, these phenomena can also be induced by metal exposure in the laboratory during experiments and culturing (LeBlanc, 1982; Stuhlbacher *et al.*, 1992, 1993). Organisms cultured in media with relatively high metal concentrations become less sensitive (Kuwabara and Leland, 1986; Maeda *et al.*, 1990; Muyssen and Janssen, 2001d), while the opposite is found in organisms cultured in metal deficient media (Stokes and Dreier, 1981; Muyssen and Janssen, 2001b). Various artificial culture and test media prescribed by international organizations (ISO, 1993; OECD, 1996; ASTM, 1998) consist of only a few salts and/or very low concentrations of essential metals. Consequently, these media may be inadequate to meet the organism's requirement for essential elements (Elendt and Bias, 1990). It can be hypothesized that organisms cultured in these types of media, through acclimation/adaptation processes, may be extremely sensitive to elevated copper concentrations. Consequently, toxicity test results produced under these conditions may not reflect the natural tolerance of these organisms. To our knowledge, no acclimation studies using metal background concentration similar to those encountered in nature, *i.e.*, non polluted water bodies, are described in literature. Indeed, most acclimation studies were performed at unrealistically high metal concentrations.

Monitoring acclimation and subsequent changes in organism sensitivity is generally performed using acute toxicity tests (Stuhlbacher *et al.*, 1992; Bodar *et al.*, 1990; Barata *et al.*, 1998). Reports on the effects of laboratory acclimation to metals on other levels of organisation (biochemical, physiological, population level) and the possible shift in the OCEE are rare. The effect of acclimation duration on subsequent responses is also not well

documented. According to Kuwabara and Leland (1986) and Caffrey and Keating (1997), ultimate acclimation effects were observed after 20 generations for *Pseudokirchneriella subcapitata* and *D. pulex*, respectively. The first authors indicated that the initial effects could be observed within the first five generations. In contrast, LeBlanc (1982) observed acclimation effects after less than 24 h of pre-exposure to a metal. For fish, maximum induced metal tolerance was observed within 5 days up to several weeks (Dixon and Sprague, 1981). Multi-generation metal acclimation experiments with daphnids are scarce. LeBlanc (1982) investigated the survival of *D. magna* during 12 generations of copper exposure. Münzinger (1990) studied the acclimation of *D. magna* to nickel during seven successive generations. Recent research by Muyssen and Janssen (2001b) described the effect of multi-generation zinc acclimation to *D. magna* on energy reserves and juvenile production. They observed an increase in reproduction after acclimation to 500 µg Zn L^{-1} for 5 generations. These authors also determined the OCEE curve, which ranged from 300 to 500 µg Zn L^{-1}, using energy reserves and carapace length.

The possible ecological importance and implications of different metal background concentrations have not been considered in applied ecotoxicology. Especially for essential elements, this variability may have important consequences for the derivation of environmental quality criteria (Janssen *et al.*, 2000). Since organisms are dependent on essential metals for optimal growth and development, all species are acclimated (or adapted) to a range of bioavailable background concentrations occurring in their habitat (Figure 3.1). The importance and consequences of copper acclimation/adaptation of daphnids to bioavailable background concentrations of copper on resulting toxicity data have not been investigated.

The objectives of this research were to assess the influence of multi-generation acclimation of *D. magna* to copper on his acute and chronic sensitivity to this metal and to establish the OCEE of *D. magna* for copper before and after acclimation to copper using physiological and population level test criteria.

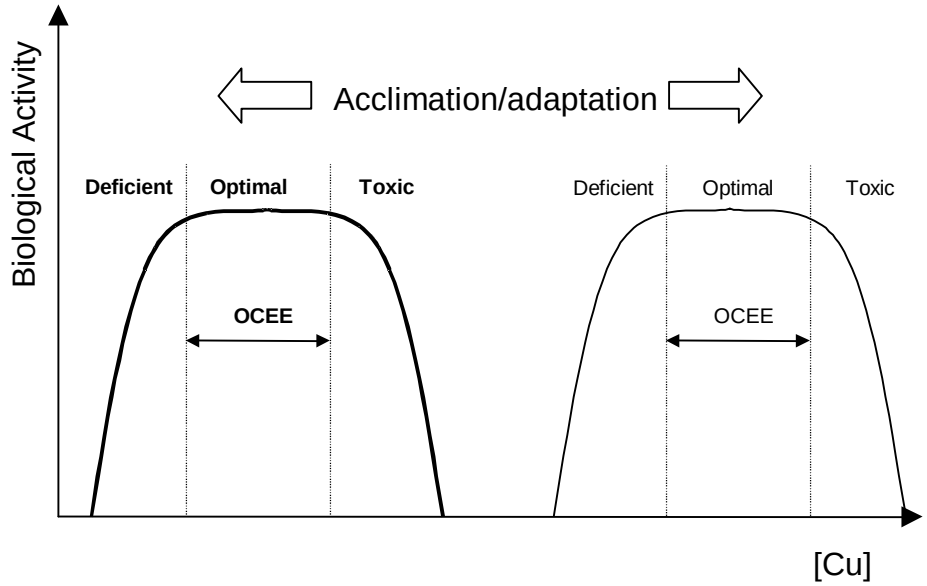

Figure 3.1. Schematic representation of the shift in tolerance and optimal concentration range for essential elements (OCEE) of organisms acclimated or adapted to different concentrations of copper. Adapted from Janssen *et al.* (2000).

3.2. Materials and methods

3.2.1. Experimental test design

Six *D. magna* cultures were simultaneously acclimated to different copper concentrations during an 18 month period. *D. magna* Straus (clone K6) used in all our experiments was originally collected from a pond in Kiel (Antwerp, Belgium) and has been successfully cultured under controlled laboratory conditions for over 15 years. Animals were cultured under semi-static conditions in 2 L polystyrene aquaria containing 2 L of acclimation culture medium. These aquaria contained 200 daphnids, were gently aerated and the medium was renewed twice a week. While cleaning, adults were separated from juveniles with the aid of a 1200 µm and 200 µm sieve, retaining adults and juveniles, respectively. Adults were returned to the culture medium and juveniles were used in experiments or discarded. Cultures and toxicity assays were kept in separate temperature-controlled (20 ± 1 °C) rooms and materials in contact with the culture and test media were soaked for 24 h in 1 % HNO_3 (p.a., VWR International, Leuven, Belgium) and rinsed with deionised water. The acclimation concentrations ranged from 0 to 150 µg L^{-1} dissolved copper. Copper concentrations were prepared by diluting a concentrated stock solution of $CuCl_2$ (0.1 g Cu L^{-1}) in the modified M4-medium (see below).

Acclimation was initiated by transferring juveniles (< 24 h) from the main laboratory culture to six different copper acclimation culture vessels. Offspring of these daphnids are referred to as the first generation (F1) and were used to initiate the second generation (F2) culture. Each generation culture was maintained for 40 days. Reproduction and survival of the daphnids of the different acclimation cultures was monitored during the first three generations. For each acclimation culture, experiments were performed to assess the daphnids sensitivity in acute and chronic toxicity assays and their energy reserves. All toxicity tests were carried out using neonates (< 24 h) originating from third to sixth brood females. Adults were used for determining body concentrations of copper. Juveniles of daphnids acclimated for six generations to 100 µg Cu L^{-1} were subsequently transferred to a 150 µg Cu L^{-1} culture and maintained for an additional five generations.

The chemically defined M4-medium (Elendt and Bias, 1990), was modified and used as both culture and test medium. In the modified medium, ethylenediaminetetraacetic acid (EDTA) was omitted and Aldrich humic acid (AHA, Sigma Aldrich Chemie, Steinheim, Germany) added at a concentration of 5 mg C L^{-1} (see section 3.4.). The hardness was decreased to 180 mg L^{-1} as $CaCO_3$ by adding less $CaCl_2.2H_2O$ and $MgSO_4.7H_2O$ stock solution to the medium and no copper was added to the stock culture medium. The pH (pH meter P407, Consort, Turnhout, Belgium) of the medium was 7.7 ± 0.2. All chemicals were purchased from VWR International (Leuven, Belgium) and were reagent grade. Stock solutions of dissolved organic carbon (DOC) were prepared by dissolving 5 g of AHA in 2 L of deionised water, equilibrating the solution for 24 h at 4 °C and filtering it through a 0.45 µm filter (Gelman Sciences, Ann Arbor, MI, USA). DOC measurements were performed with a TOC-5000 analyser (Shimadzu, Duisburg, Germany).

Stock cultures as well as experimental animals were fed a mixture of the algae *Pseudokirchneriella subcapitata* (Korshikov) Hindak (formerly known as *Selenastrum capricornutum* Printz and *Raphidocelis subcapitata*) and *Chlamydomonas reinhardtii* in a 3:1 ratio. The algae were not cultured in a copper free media (ISO, 1989), resulting in a maximum total copper concentration (adsorbed + absorbed) in the algae of 10^{-15} g $cell^{-1}$ (see section 2.3.). As the organisms grew, increasing amounts of food were supplied: from 0 to 7 days, from 8 to 15 days, and older than 15 days daphnids were fed 8 x 10^6, 12 x 10^6 and 16 x 10^6

cells day^{-1} $daphnid^{-1}$, respectively. The temperature during culturing and testing was 20 ± 1 °C with a light:dark cycle of 12h:12h.

3.2.2. Acute toxicity experiments

Acute toxicity assays were performed following the Test Guideline 202 of the Organisation for Economic Cooperation and Development (OECD, Paris, France) (OECD, 1996b). The test medium used was the modified M4 medium as described above. Five replicates of five juveniles (< 24 h) were exposed to at least six test concentrations ranging from 56 µg Cu L^{-1} to 560 µg Cu L^{-1} and a control. In order to obtain near-equilibrium situations for the copper-DOC reactions, all spiked media were made 24 h prior to the introduction of the daphnids. Each test vessel contained 50 mL of test medium. After 48 h the number of immobilized organisms in each vessel was counted and the 50 % effective concentration (48-h EC50) was calculated following the trimmed Spearman-Karber method (Hamilton *et al.*, 1977). Reported 48-h EC50s are based on measured (dissolved) copper concentrations.

3.2.3. Chronic toxicity experiments

Chronic experiments were performed following OECD Test Guideline 211 (OECD, 1996c). The test medium used was the modified M4 medium as described above. Ten replicates of one juvenile (< 24 h) were exposed to at least six copper concentrations, ranging from no copper addition (control) to 125 µg Cu L^{-1}. Each test vessel contained 50 mL of test medium. Three times a week, the medium was renewed and age-specific survival (l_x) and reproduction (m_x) was recorded. Food was supplied daily at the same age-dependent algal concentration as those used in the acclimation cultures (see section 3.2.1.). The chronic tests were terminated at day 21 and the intrinsic rate of natural increase r_m and net reproduction R_0 were calculated using successive approximations of Lotka's formula (Lotka, 1913) according to Southwood (1976).

$$\sum_{x=0}^{21} l_x \cdot m_x \cdot e^{-r_m \cdot x} = 1$$

$$R_0 = \sum_{x=0}^{21} l_x \cdot m_x$$

The 21-d EC50s and EC10s (*i.e.* the concentration at which 10 % of the organisms is affected) with 95 % confidence intervals were calculated with a logistic fitting in STATISTICA 6 (STATISTICA® software, Tulsa, OK, USA). Calculated values are based on measured copper concentrations.

3.2.4. Feeding behaviour

Relative filtration rates were used as measures of feeding behaviour. Feeding experiments were performed with 7 days old daphnids originating from the 4th generation acclimation cultures in 2 L aquaria. An initial algae concentration of 8×10^6 cells algal mix daphnid^{-1} day^{-1} (= 6×10^6 cells *P. subcapitata* daphnid^{-1} day^{-1}) was added after renewing the media. Daphnids were allowed to feed for 24 h after which the final *P. subcapitata* cell density was measured using a coulter counter (Model DN, Harpenden, Herts, UK). The medium was shaken by hand to re-suspend any settled cells. Each measurement consisted of 2 replicates.

Relative filtration rates were calculated using a simplified version of Gauld's equation (Gauld, 1951; Allen *et al.*, 1995):

$$F = \frac{\ln C_0 - \ln C_t}{t} \cdot \frac{V}{n}$$

C_0 and C_t are the initial and final food concentrations; t = experimental time (hours); V: volume (µL); n: number of daphnids; F = filtration rate (µL ind^{-1} h^{-1}). Results of the feeding experiments are presented as mean values (n = 8) of the filtration rate.

3.2.5. Energy reserves (E_a)

The daphnid's energy reserves were measured following the Cellular Energy Allocation (CEA) method described by De Coen and Janssen (1997). This method is proposed as an alternative for the conventional, more labour-intensive Scope for Growth methodology developed by Warren and Davies (1967). The CEA concept is based on a biochemical assessment, using calorimetric methods, of the organisms' energy consumption (E_c) and energy available for metabolism (E_a).

For each generation, a total of 300 *D. magna* juveniles (< 24 h) originating from each acclimation culture were exposed for 96 h to four copper concentrations (*i.e.* control, acclimation concentration, 75 and 125 µg Cu L^{-1}). The organisms were fed daily at an algal concentration of 6 x 10^6 cells algal mix $daphnid^{-1}$. At the beginning of the exposure (t = 0) and after 96 h, daphnids were collected and shock-frozen in liquid nitrogen (Air Liquide, Luik, Belgium). Blotting prior to freezing was performed with a tissue held on the underside of the sieve to remove excessive water attached to the daphnids. For the determination of each energy fraction 40 organisms were used (except for carbohydrates and proteins which could be measured together using a total of 40 organisms). The different energy fractions of the energy available were transformed into energetic equivalents using the energy of combustion (Gnaiger, 1983): 17,500 mJ mg $glycogen^{-1}$, 2,400 mJ mg $protein^{-1}$ and 39,500 mJ mg $lipid^{-1}$. Increases in energy available during a 96 h exposure period were calculated for each energy fraction as:

$$E_x = E_{x(t96)} - E_{x(t0)}$$

Where $E_{x(t96)}$ is the carbohydrate (or protein or lipid) content after 96 h (in mJ $organism^{-1}$) and $E_{x(t0)}$ is the carbohydrate (or protein or lipid) content at the beginning of the test (in mJ $organism^{-1}$). The total energy available was calculated as:

$$E_a = E_{carbohydrate} + E_{protein} + E_{lipid}$$

3.2.6. Body copper concentration

A total of 60 *D. magna* (3 replicates of 20 daphnids) from each acclimation culture was sampled after 40 days and rinsed with deionised water. The samples were subsequently dried in polypropylene test tubes (Laborimpex, Brussels, Belgium) at 50 °C until no change of the dry weight was observed. The organisms were weighed (Mettler H35, Zürich, Germany) and subsequently digested by adding 1 mL of 14 M HNO_3 (p.a., VWR International, Leuven, Belgium) to the test tubes. The samples were heated in a microwave (Samsung MF245, Korea) using 3 cycles of 5 minutes with increasing power capacity. Cooled samples were

diluted with 9 mL bi-deionised water and stored until analysis with the atomic absorption spectrophotometer (AAS, see section 3.2.8.).

3.2.7. Length measurements

For each acclimation culture, the carapace length of 20 daphnids of the sixth generation was measured. Adult (21 d old) organisms were measured with the aid of a microscope (Kyowa, Tokyo, Japan) equipped with an ocular micrometer ruler. The length of the daphnids was measured in two ways (see section 3.4.): from top of the head to the base of the spine and from top of the head to the tip of the spine.

3.2.8. Copper measurements

Copper concentrations were determined using a flame-atomic absorption spectrophotometer (for Cu > 20 µg Cu L^{-1}, SpectrAA100, Varian, Mulgrave, Australia) or a graphite furnace atomic absorption spectrophotometer (for Cu < 20 µg Cu L^{-1}, SpectrAA800 with Zeeman background correction, Varian, Mulgrave, Australia) as described in section 2.2.4. All reported copper concentrations are dissolved concentrations (0.45 µm filtered). Spectrophotometric analysis of the nominal zero concentration could only assure that copper concentrations were < 0.5 µg Cu L^{-1} (detection limit of the graphite furnace AAS). Results of acclimation to nominal zero concentration will therefore be referred to as 0.5 µg Cu L^{-1}.

Based on the measured copper concentrations in the culture media, copper activities were calculated using the upgraded WHAM V (Windermere Humic Aqueous Model version V) (Tipping, 1994), *i.e.* WHAM 6.0.1 (Natural Environment Research Council, 2001). The used copper acclimation concentrations (0.5, 1, 5, 12, 35, 100 and 150 µg Cu L^{-1}) resulted in the following calculated activities: 7×10^{-15}, 2×10^{-14}, 5×10^{-13}, 4×10^{-12}, 80×10^{-12}, 37×10^{-10} and 12.9×10^{-9} M Cu^{2+}.

3.2.9. Statistical analysis

The effects of the various copper concentrations on the daphnids' response were compared using one-way analysis of variance (ANOVA) with the post-hoc Duncan's multiple range test. Homogeneity of variance and normality was tested using Bartlett's and Kolmogorov-

Smirnov's test, respectively. In case the assumptions were not met, log10 transformation of the data was used to agree with both. If this was not the case, the test endpoints where compared with the non-parametric Mann-Whitney *U* test. Statements of significant differences are based on accepting $p < 0.05$. All statistical comparisons were performed with STATISTICA 6 (STATISTICA® software, Tulsa, OK, USA).

3.3. Results

The organisms maintained in the different copper acclimation concentration cultures did not exhibit reduced fecundity or fitness. Mortality in these cultures did not exceed 10 %. In the acclimation cultures up to 100 µg Cu L^{-1}, juvenile production started at day 8 and a net reproduction of more than 180 juveniles $female^{-1}$ was recorded during 21 days (results not shown). The OECD protocol (OECD, 1996c) accepts a mean number of > 60 of live offspring per parent animal surviving after 21 days as a performance criteria for a healthy daphnid.

All experiments were performed in the modified M4 medium with a DOC concentration of 5 mg C L^{-1}. The results of the acute copper sensitivity of *D. magna* juveniles originating from the 1st, 4th and 14th generation, acclimated to six different copper concentrations are shown in Figure 3.2. Acclimation did not significantly affect first generation sensitivity. Fourth and fourteenth generation organisms acclimated to 35 and 100 µg Cu L^{-1}, did exhibit a significantly reduced copper sensitivity: mean 48-h EC50s (± standard deviation) increased from 245 ± 11 µg Cu L^{-1} and 251 ± 18 µg Cu L^{-1} (1st generation) to 281 ± 14 µg Cu L^{-1} and 354 ± 28 µg Cu L^{-1} (4th generation), respectively. Similarly, 14th generation daphnids had an increase in acute copper toxicity to 290 ± 14 µg Cu L^{-1} and 310 ± 20 µg Cu L^{-1}, respectively. Daphnids acclimated to higher copper concentrations (150 µg Cu L^{-1}) showed no further increase of copper tolerance and after five generations of acclimation their mean 48-h EC50 (± standard deviation) was still 301 ± 7 µg Cu L^{-1} (n = 3). Return of juvenile daphnids acclimated to 100 µg Cu L^{-1} to medium without copper addition, resulted in loss of the increased copper tolerance within one generation. These returned daphnids had a 48-h EC50 of 220 ± 8 µg Cu L^{-1} (mean ± standard deviation; n = 3), which is similar to that of daphnids acclimated to 1 µg Cu L^{-1}. The mean acute copper sensitivity (n = 22) of the daphnids acclimated to copper for 15 generations is presented in Figure 3.3. The values (mean ± standard deviation, n = 22) ranged between 204 ± 24 µg Cu L^{-1} and 320 ± 43 µg Cu L^{-1}. A

significant increase in 48-h EC50 was already observed with daphnids acclimated to 12 µg Cu L^{-1} compared to organisms acclimated to 0.5 µg Cu L^{-1}. Although the data fitted a linear regression equation ($a = 1.1 \times b + 217$, $r^2 = 0.94$) with *a* the 48-h EC50 and *b* the acclimation concentration (µg Cu L^{-1}) there seems to be a plateau between 1 and 12 µg Cu L^{-1} in which there is almost no decrease in copper sensitivity. The 48-h EC50 (mean ± standard deviation, $n = 22$) expressed as copper activities, computed with WHAM VI, ranged between 15.9 ± 2.9 nM Cu^{2+} and 43.8 ± 11.5 nM Cu^{2+}.

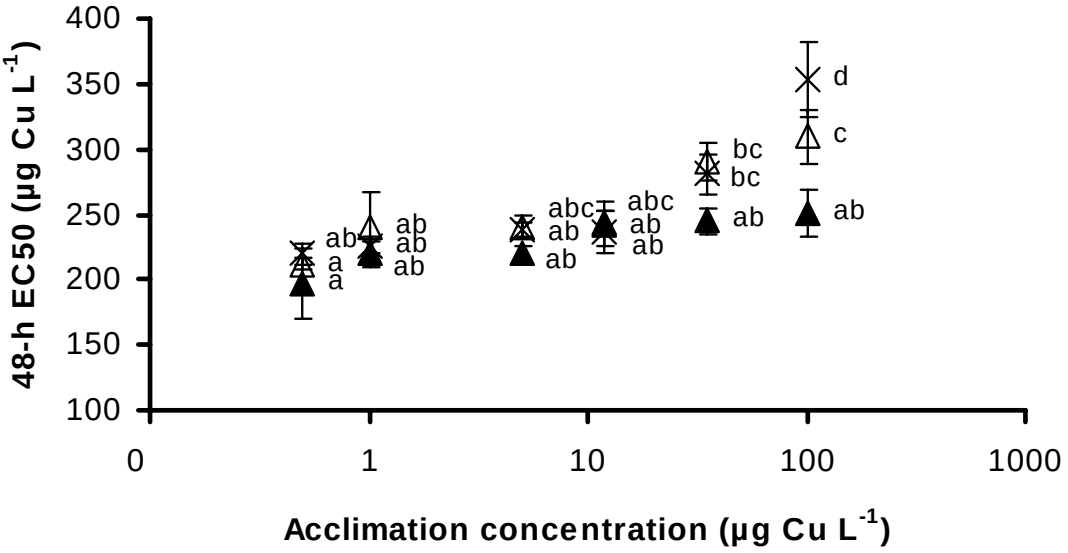

Figure 3.2: Acute copper toxicity (48 h effective concentration; 48-h EC50) of the first (▲), fourth (×) and fourteenth (Δ) generation as a function of the acclimation concentration. Error bars represent standard deviation. Values with the same letter are not significantly different at $p < 0.05$.

The results of the chronic experiments are shown in Table 3.1. For the first generation, the mean 21-d EC50 (± standard deviation, $n = 6$) - in the modified M4 medium with 5 mg C L^{-1} - based on net reproduction was 70.3 ± 3.0 µg Cu L^{-1}. After six acclimation generations, the chronic copper tolerance exhibited an increase with increasing acclimation concentrations. The values ranged between 48.0 and 78.8 µg Cu L^{-1} for daphnids acclimated to 0.5 and 100 µg Cu L^{-1}, respectively. Daphnids acclimated up to 12 µg Cu L^{-1} showed a decrease in their 21-d EC50s compared to those of the first generation organisms. In contrast, daphnids acclimated to 35 and 100 µg Cu L^{-1} exhibited an increased 21-d EC50.

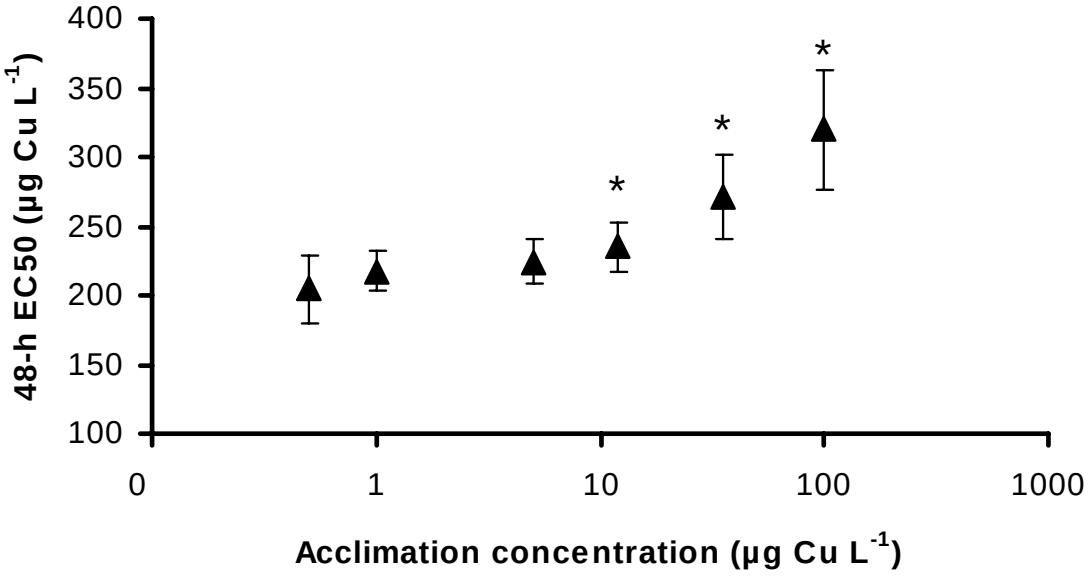

Figure 3.3: Mean acute copper toxicity (48 h effective concentration; 48-h EC50; $n = 22$) with standard deviation of *D. magna* during the acclimation period (18 months) as a function of the bioavailable acclimation concentration. The asterisk (*) denotes results which are significantly different from those of daphnids acclimated to 0.5 µg Cu L^{-1} at $p < 0.05$.

Table 3.1: The chronic 50 % and 10 % effective concentration (21-d EC50, 21-d EC10) based on net reproduction with 95 % confidence intervals for first generation (F1) and sixth (F6) generation *D. magna* acclimated to six different background copper concentrations.

Acclimation concentration (µg Cu L^{-1})	**21-d EC50 (µg Cu L^{-1})**		**21-d EC10 (µg Cu L^{-1})**	
	F1	**F6**	**F1**	**F6**
0.5	68.5 (63.9 – 73.3)	48.0 (47.9 – 48.0)	56.1 (42.7 – 73.8)	46.5 (46.5 – 46.6)
1	70.2 (69.7 – 70.8)	56.4 (52.6 – 60.3)	57.1 (56.1 – 58.1)	50.2 (45.7 – 55.3)
5	72.7 (72.6 – 72.7)	67.6 (66.3 – 68.9)	70.5 (70.3 – 70.7)	56.2 (49.9 – 63.2)
12	65.2 (59.5 – 71.5)	55.6 (52.4 – 59.3)	45.9 (35.4 – 59.5)	38.7 (34.4 – 43.5)
35	70.5 (66.1 – 75.2)	70.7 (53.0 – 94.3)	51.9 (44.2 – 61.0)	45.6 (20.5 - 101.4)
100	74.6 (65.4 – 85.0)	78.8 (66.3 – 93.6)	68.2 (44.9 – 103.4)	51.1 (33.4 – 78.0)

An increase in offspring per female was also observed in the sixth generation compared to the first generation (Figure 3.4). The first generation daphnids had an optimum range situated between 0.5 and 1 µg Cu L^{-1}. For sixth generation daphnids this range was from 1 to 35 µg Cu L^{-1}. Daphnids acclimated to 5 µg Cu L^{-1} exhibited the highest net reproduction (76 ± 8 juveniles $female^{-1}$). In the lowest and the highest acclimation cultures, significantly lower

juvenile production was observed in sixth generation daphnids compared to that in the optimal range. From the third generation onwards, daphnids acclimated to the highest copper concentration started to survive and reproduce in the chronic experiments. The intrinsic growth rate of first, fourth and sixth generation organisms is presented in Table 3.2. The intrinsic growth rate (mean ± standard deviation, n = 10) ranged between 0.11 ± 0.08 day^{-1} and 0.34 ± 0.03 day^{-1}. Daphnids acclimated to 100 µg Cu L^{-1} had always significantly lower intrinsic growth rates compared to daphnids acclimated to 0.5, 1, 5, 12 and 35 µg Cu L^{-1}. Daphnids acclimated to 12 and 35 µg Cu L^{-1} showed a significant decrease in intrinsic growth rate with subsequent generations. No such decreases and no increases were found in intrinsic growth rate for daphnids acclimated to 0.5, 1 and 5 µg Cu L^{-1}. Mortality of daphnids acclimated to 0.5, 1, 5, 12 and 35 µg Cu L^{-1} never exceeded 5 % in the chronic experiments at the acclimation exposure concentration. For daphnids acclimated to 100 µg Cu L^{-1} and exposed in chronic experiment to 100 µg Cu L^{-1}, the mortality decreased from 100 % to less than 20 % in the sixth generation.

Table 3.2: Intrinsic growth rate (mean ± standard deviation, n = 10) of first (F1), fourth (F4) and sixth (F6) generation daphnids acclimated to background copper concentrations. The asterisk (*) denotes results which are significantly different from these of the first generation (F1) at $p < 0.05$.

Generation	**Acclimation concentration (µg Cu L^{-1})**					
	0.5	1	5	12	35	100
	Intrinsic growth rate (day^{-1})					
F1	0.31 ± 0.03	0.31 ± 0.02	0.31 ± 0.03	0.31 ± 0.02	0.32 ± 0.01	-
F4	0.28 ± 0.01	0.34 ± 0.03*	0.33 ± 0.03	0.31 ± 0.04	0.29 ± 0.03*	0.11 ± 0.08*
F6	0.31 ± 0.03	0.31 ± 0.02	0.32 ± 0.03	0.28 ± 0.01*	0.26 ± 0.01*	0.12 ± 0.03*

- : all organisms dead in the chronic experiment

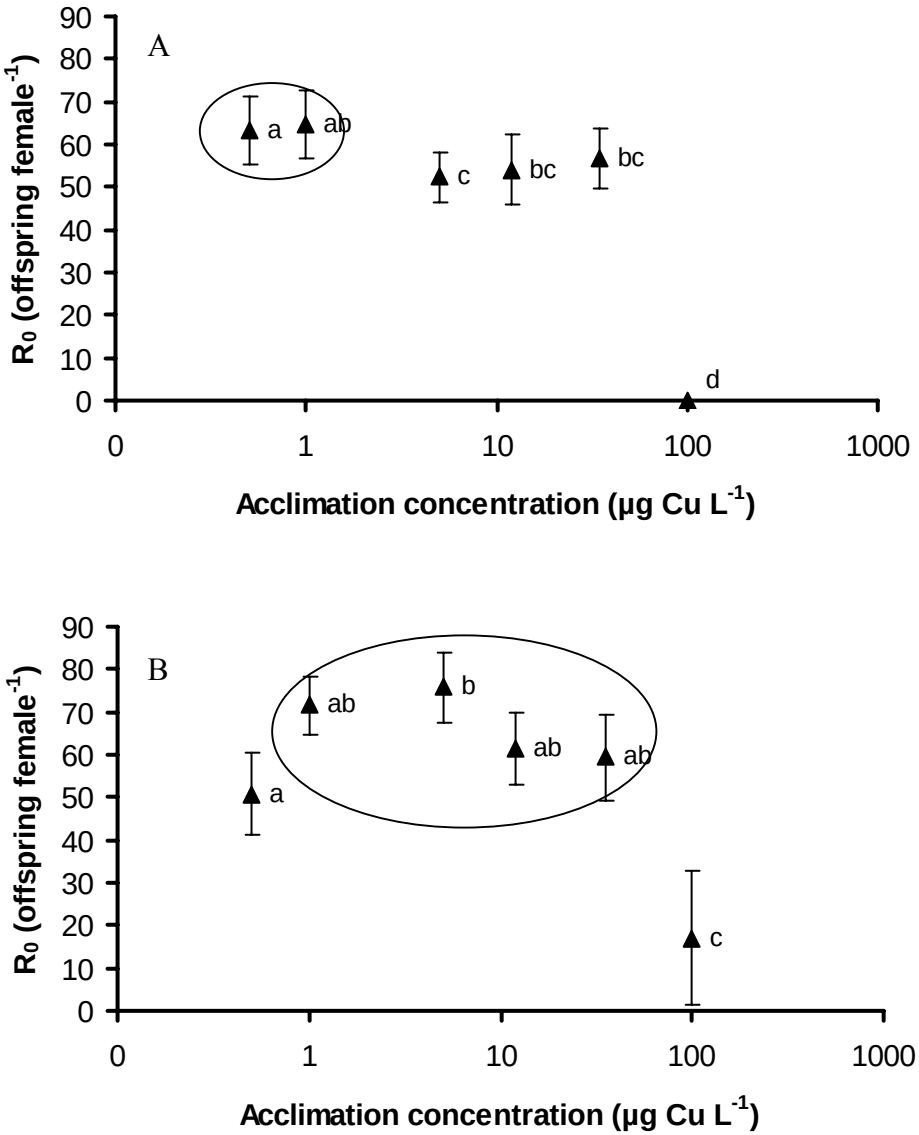

Figure 3.4: Net reproduction (R_0) of acclimated *D. magna* in first (A) and sixth (B) generation. Error bars represent standard deviation. Values with same letter are not significantly different at $p < 0.05$. Ovals indicate the optimal concentration area.

The mean relative filtration rates ($n = 8$) of fourth generation daphnids are presented in Figure 3.5. Daphnids had significantly higher filtration rates at copper concentrations between 1 and 35 µg Cu L^{-1} compared to those at 0.5 and 100 µg Cu L^{-1}. No significant difference was observed between relative filtration rates of daphnids acclimated to 0.5 and 100 µg Cu L^{-1}. The optimal filtration rate (mean ± standard deviation) was 1.3 ± 0.1 mL daphnid^{-1} h^{-1}.

In parallel with the chronic toxicity experiments, the energy reserves of juvenile daphnids, taken from the different acclimation cultures, were determined (Figure 3.6). Compared to first generation measurements, significant increases in energy reserves were observed in all subsequent generations (F3 - F14). In these generations the optimal range was situated between 1 up to 35 µg Cu L^{-1}, compared to 5 up to 12 µg Cu L^{-1} in the first generation. In all generations, significantly lower energy reserves were noted for daphnids acclimated to 0.5 and 100 µg Cu L^{-1}, compared to those of the organisms acclimated to the optimal copper concentrations. In general, energy reserves mainly consisted of lipids (70 %), followed by sugars (20 %) and proteins (10 %) (Table 3.3). These fractions were consistent for all generations. Almost no changes were observed in protein content between acclimation concentrations and generations. Sugar and lipid content were significantly affected by acclimation processes.

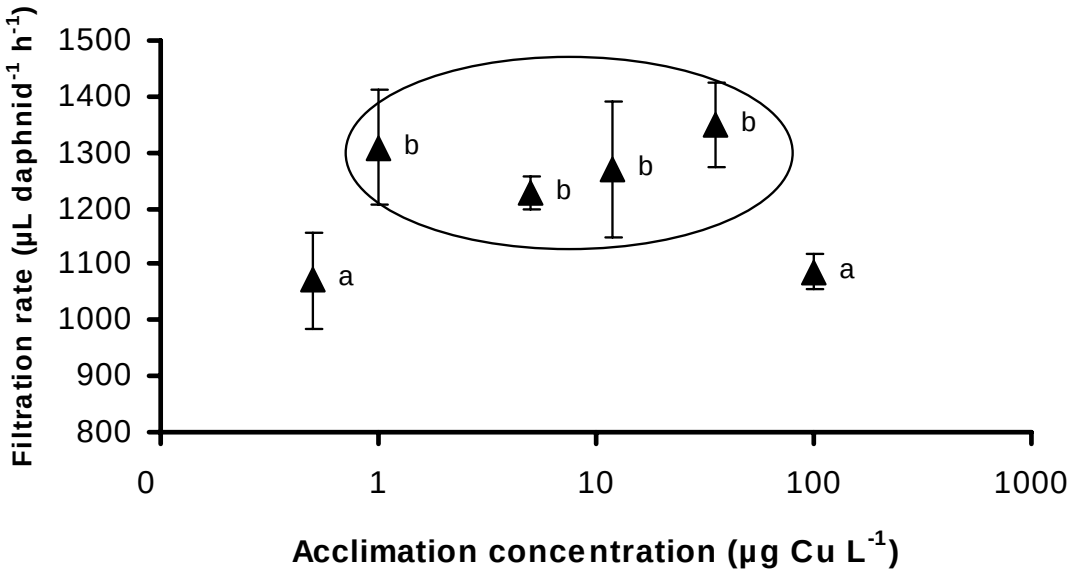

Figure 3.5: Filtration rates (mean ± standard deviation; n = 8) of fourth generation daphnids (7 days old) acclimated to different copper concentrations. Mean values with the same letter are not significantly different at $p < 0.05$. Oval represent optimal concentration range

For each generation, body burdens (absorbed + adsorbed) were measured in the adult daphnids after 40 days of culturing. Significant increases were observed with increasing acclimation concentration (Table 3.4). First generation daphnids showed a high increase in body burdens with increasing acclimation concentration (factor of 25). In all subsequent generations, daphnids acclimated to 35 and 100 µg Cu L^{-1} showed a significant decrease in

their body burdens compared to those of the first generation, while an increase was observed in those acclimated to 0.5 µg Cu L^{-1}. A fairly constant level of body burdens (mean ± standard deviation: 18 ± 5 mg Cu kg DW^{-1}; n = 60) was observed in daphnids acclimated to 1 up to 12 µg Cu L^{-1}, indicating a copper regulation mechanism, during the acclimation period. Daphnids acclimated to 150 µg Cu L^{-1} exhibited a body concentration up to 250 mg Cu kg DW^{-1}.

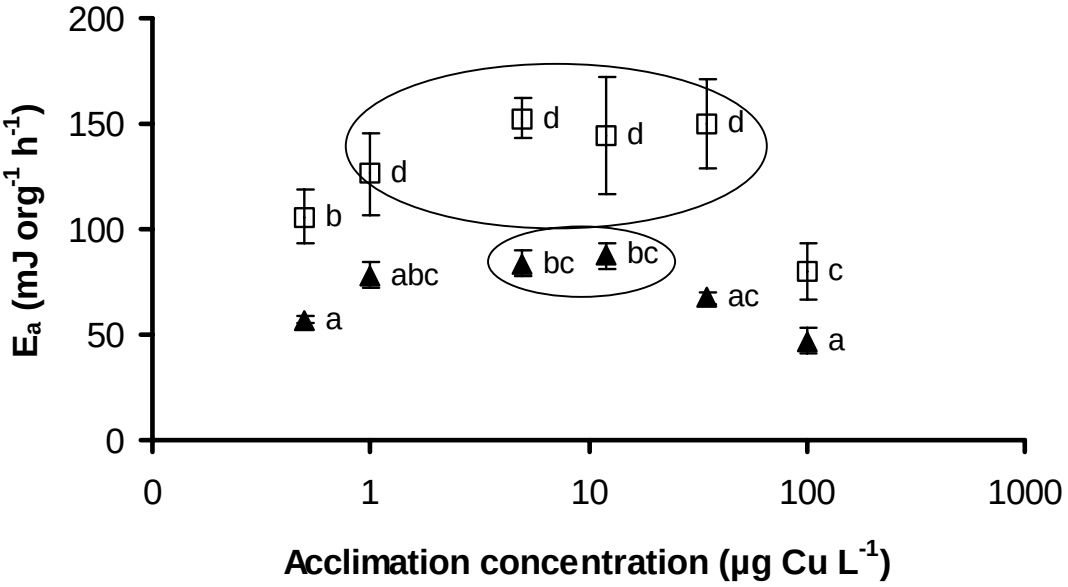

Figure 3.6: Energy reserves (E_a) of first (▲) and mean of F4 to F14 (□) generation *D. magna* acclimated to different copper concentrations. Error bars represent standard deviation. Mean values with the same letter are not significantly different at $p < 0.05$. Ovals represent optimal concentration range.

No significant differences in length of sixth generation adults (n = 20) acclimated to a copper acclimation concentration up to 100 µg Cu L^{-1} were noted (Table 3.5). Daphnids acclimated to 150 µg Cu L^{-1} exhibited a significant decrease in their carapace length. The ratio of the length of the spine to the total length of the daphnids indicated that daphnids acclimated to 0.5 and 150 µg Cu L^{-1} had significantly higher ratios than the daphnids acclimated to 1, 5, 12, 35 and 100 µg Cu L^{-1} (see section 3.4.).

Table 3.3: Sugar, protein and lipid content (mean ± standard deviation, n = 3) of *D. magna* acclimated to six copper concentrations during first (F1) and fourteenth (F14) generation. The asterisk (*) denotes results are significantly different from these of daphnids acclimated to 0.5 µg Cu L^{-1} at $p < 0.05$ within the respective generation; the open circle (º) denotes significant difference between the generations at a certain acclimation concentration.

mJ org^{-1} h^{-1}	**Acclimation concentration (µg Cu L^{-1})**											
	0.5		**1**		**5**		**12**		**35**		**100**	
	Generation											
	F1	**F14**	**F1**	**F14**	**F1**	**F14**	**F1**	**F14**	**F1**	**F14**	**F1**	**F14**
Sugars	21.1 ± 0.4	20.7 ± 0.5	32.4 ± 0.4*	25.9 ± 0.9*º	22.4 ± 0.8	41.8 ± 0.8*º	29.5 ± 0.5*	40.3 ± 1.1*º	21.2 ± 0.9	43.6 ± 1.6*º	16.1 ± 0.6*	10.9 ± 1.1*º
Proteins	15.3 ± 3.3	13.2 ± 0.2	8.1 ± 2.3*	12.8 ± 1.1º	14.9 ± 2.0	16.5 ± 1.4*	13.7 ± 2.3	14.0 ± 0.5	14.2 ± 4.0	11.5 ± 0.3	2.6 ± 0.5*	4.8 ± 0.1*º
Lipids	20.5 ± 1.3	78.3 ± 9.8º	37.7 ± 8.9	67.1 ± 16.4	46.3 ± 8.5*	111.9 ± 16.0*º	44.2 ± 7.4*	126.7 ± 8.0*º	51.0 ± 6.2*	116.0 ± 15.7*º	28.2 ± 7.2	38.0 ± 19.4*

Table 3.4: Body burdens (mean ± standard deviation, n = 3) of first (F1), sixth (F6), ninth (F9) and fourteenth (F14) generation *D. magna* acclimated to different background copper concentrations. Asterisk (*) denotes results are significantly different to those of daphnids acclimated to 0.5 µg Cu L^{-1} at $p < 0.05$ (Mann-Whitney U test).

Generation	**Acclimation concentration (µg Cu L^{-1})**					
	0.5	1	5	12	35	100
	Body concentration (mg Cu kg DW^{-1})					
F1	6.01 ± 0.88	11.49 ± 0.17*	23.29 ± 4.99*	22.97 ± 0.66*	85.62 ± 14.86*	122.50 ± 16.40*
F6	12.53 ± 0.47	17.61 ± 0.11*	21.71 ± 0.69*	27.57 ± 0.03*	42.92 ± 4.88*	84.71 ± 3.24*
F9	14.77 ± 0.72	16.50 ± 0.59	20.66 ± 3.49*	24.40 ± 0.84*	25.03 ± 2.82*	63.29 ± 4.03*
F14	11.43 ± 5.36	18.85 ± 1.04*	22.17 ± 1.05*	23.91 ± 3.90*	37.17 ± 12.42*	90.05 ± 0.30*

Table 3.5: Length (mean ± standard deviation) and ratio of spine to total length (mean ± standard deviation) of adult sixth generation *D. magna* acclimated to six copper concentrations. Mean values (n = 20) with same letter are not significantly different at $p < 0.05$.

Acclimation concentration µg Cu L^{-1}	**Length mm**	**Ratio spine/total length %**
0.5	$2.9 \pm 0.1_a$	$10.0 \pm 2.4_a$
1	$2.9 \pm 0.1_a$	$8.3 \pm 0.8_{ab}$
5	$3.0 \pm 0.1_a$	$7.4 \pm 1.6_{ab}$
12	$3.0 \pm 0.1_a$	$8.1 \pm 1.1_{abc}$
35	$3.1 \pm 0.1_a$	$6.4 \pm 1.0_b$
100	$2.9 \pm 0.1_a$	$11.1 \pm 1.8_{ac}$
150	$2.5 \pm 0.1_b$	$20.2 \pm 2.5_d$

3.4. Discussion

The influence of copper acclimation of laboratory daphnid cultures on the results of acute and chronic toxicity tests has important consequences for the evaluation of existing test data. Internationally recommended culture and test media for daphnids contain only a few salts (ISO, 1993; USEPA, 1993; OECD, 1996; ASTM, 1998) and may consequently be deficient in

essential trace elements such as copper (Table 3.6). Several authors (Keating, 1985; Elendt and Bias, 1990) proposed other media for culturing and testing with daphnids as they recognized the organism's need for these micronutrients. On the other hand, daphnids are frequently cultured in (natural) waters (carbon-filtered tap water, filtered surface or well water) of which the composition is unknown and which may fluctuate with time (Biesinger and Christensen, 1972; Münzinger and Monicelli, 1991). Fluctuations in copper concentrations of culture media resulted in different copper sensitivities for *D. pulex* (Ingersolland and Winner, 1982). Comparison of inter-laboratory copper sensitivity may be difficult due to the acclimation/adaptation of the organisms to the different copper concentrations in these different media. Beside the natural copper concentrations, DOC is also an ubiquitous component in surface waters. This is generally neglected in these standard media or is often replaced by EDTA (up to 2500 μg L^{-1}; Elendt, 1986; Elendt and Bias, 1990, Samel *et al.*, 1999), which is a very strong metal chelator. These metal chelators determine metal speciation by binding the copper ion and thus influence the metal bioavailability. If acclimation/adaptation of organisms is influenced by ion activity, comparison of copper toxicity data derived in laboratories and in the field will be difficult. In this study, daphnids are cultured in a more environmentally realistic medium (for Europe) than the standard M4 medium and with realistic copper concentration, in order to assess the influence of these different acclimation concentrations on the metal sensitivity. These copper concentrations are based upon an analysis of the Surface Water Database (SWAD, Heijerick and Janssen, unpublished data, update 2003; see section 1.1.3.3.). Out of this analysis, the 5, 20, 75, 90, 98 and 99 percentile were chosen as the acclimation concentrations (0.5 – 100 μg Cu L^{-1}), thus covering a wide range of naturally occurring copper concentrations. A similar analysis was performed for DOC, resulting in an European 50 percentile value of 4 or 5 mg C L^{-1} based on a log-logistic or a log-normal distribution, respectively.

According to Klerks and Levinton (1989) two phenomena can account for an increased tolerance in organisms. Exposure to a pollutant can lead to physiological acclimation or behavioural changes within the life span of the exposed organism. However, exposure to a pollutant can also lead to natural selection for an increased tolerance, resulting in genetic adaptation to the pollutant. If physiological acclimation is the cause of the increased tolerance, it will not be passed on to offspring of the exposed animals. But in the case of genetic adaptation, the resistance will be maintained in the offspring. Loss of the increased copper tolerance after returning acclimated organisms to medium without copper addition, confirm

that the former mechanism was responsible for the results obtained in this multi-generation study. No further increase in copper tolerance was observed in daphnids acclimated to 150 µg Cu L^{-1}, compared to that of daphnids acclimated to 100 µg Cu L^{-1}. Similar results of the existence of a certain acclimation plateau are also observed in acclimation experiments with zinc for *D. magna* (Muyssen and Janssen, 2001b) and rainbow trout (Stubblefield *et al.*, 1999). It may be hypothesized that the increased copper tolerance is levelling off when daphnids are acclimated to a concentration higher than half of the EC50 concentration. From our results it is suggested that the increase in copper tolerance (Figure 3.2) may be divided in 4 parts. In the first part, there is a slight decrease in daphnids copper sensitivity due to the loss of copper deficiency effects (< 1 µg Cu L^{-1}). This is followed by a certain plateau in which there is no increase in 48-h EC50 up to 12 µg Cu L^{-1}. In the third part there is a significant increase in copper tolerance followed by the last part in which the copper tolerance is levelling off (> 100 µg Cu L^{-1}). LeBlanc (1982) also found that daphnids acclimated for 12 generations to high (bioavailable) copper (30 µg Cu L^{-1} in reconstituted hard water) concentrations exhibited an increased copper tolerance, which was lost within one generation after return to the control medium. He used the resistance factor method (*i.e.* 48-h EC50 of copper acclimated organisms divided by the 48-h EC50 of non-acclimated organisms) to illustrate the increase of copper tolerance. In this multi-generation study, the resistance factor of 1.3 for first generation organisms increased up to 2.8 for fourth generation daphnids acclimated to 100 µg Cu L^{-1} (3.7 nM Cu^{2+}), when based on calculated copper ion activities. However, when expressing the EC50 as total copper concentration, the mean resistance factor was only 1.6, which is similar to the one found by LeBlanc (1982). In our study, the chronic resistance factor for sixth generation daphnids was similar to the acute one and ranged between 0.9 and 1.8 for 21-d EC50 with a mean of 1.5. Still it has to be noted that no clear trend was observed in chronic tolerance. According to our results, it is suggested that gaining an increased (acute) tolerance is a very rapid process, but gaining maximal increased tolerance may be, for *D. magna*, a slow process of several generations. Organisms/communities exposed to low (environmentally relevant) copper concentrations can have an increased acute copper tolerance, which will render the organisms less sensitive than those living in an ecosystem with a lower copper concentration.

Table 3.6: Copper and zinc concentrations (µg L^{-1}) in culture and test media commonly used in experiments with freshwater Cladocera.

Medium	Cu	Zn
ISO (EEC)[1]	-	-
ASTM [2]	-	-
EPA [3]	-	-
DSW [4]	-	-
COMBO [5]	0.25	5.0
DT [6]	0.6	0.1
M7 [5]	1.6	6.3
M4 [7]	6.3	6.3
NW [8]	10.0	10
MS [9]	25.0	25

[1] ISO (1982), [2] ASTM (1998), [3] Horning and Weber (1985), [4] NNI (1980), [5] Samel *et al.* (1999), [6] Span *et al.* (1988), [7] Elendt and Bias (1990), [8] Elendt (1986), [9] Keating (1985).

As a validation of the increased acute tolerance observed in our laboratory experiments, daphnids collected from a copper microcosm experiment were tested. The microcosm study was performed in the Fraunhöfer Institute (IUCT) in Schmallenberg, Germany by Dr. Christoph Schäfers (Schäfers and Delbeke, 2002) and consisted of seven different copper concentrations: 5, 10, 20, 40, 80, 160 µg Cu L^{-1} and a control. To test our hypothesis, juvenile daphnids were collected at the end of the study, after 4 months of exposure, and were used in acute experiments. Although the daphnids of the microcosm system were cultured in a nutrient poor ecosystem, similar resistance factors as those observed in our laboratory experiments were noted. For daphnids collected from 20 µg Cu L^{-1} exposure, a resistance factor of 3.2 ± 0.1 (mean ± standard deviation, n = 3) was noted (results not shown). A significant ($p < 0.05$) increase (compared to the control) in acute copper tolerance was also observed for the daphnids exposed to 5 µg Cu L^{-1} (mean resistance factor ± standard deviation: 2.5 ± 0.5). Daphnids acclimated to 40, 80 and 160 µg Cu L^{-1} could not be tested, due to their low density. The ecosystem no-observed-effect concentration (NOEC) for the microcosm was around 20 µg Cu L^{-1} (6100 pM Cu^{2+}) (Schäfers and Delbeke, 2002). Increased acute copper tolerance can thus probably occur in field organisms occurring in aquatic systems with a copper concentration above 12 µg Cu L^{-1} (4×10^{-12} M Cu^{2+}). Nevertheless, this

difference in sensitivity due to acclimation to copper background concentrations is very small compared to that resulting from inter-clonal sensitivity differences. A factor of 2.4, 6.7 and up to 200 was found in toxicity studies with different clones of *D. magna* for zinc, copper and cadmium, respectively (Baird *et al.*, 1990; Baird *et al.*, 1991; Barata *et al.*, 1998). Bossuyt and Janssen (unpublished data) observed up to a factor 10 difference in copper sensitivity due to inter-clonal variation.

The fact that acclimation to copper concentrations up to 35 µg Cu L^{-1} did not affect survival indicates that different copper background concentrations may not be important for the long-term health of daphnid populations. This observation, *i.e.* that acute toxicity is more sensitive to metal acclimation/adaptation than chronic toxicity, has already been reported by Baird *et al.*, (1990). They found relative small differences in chronic cadmium tolerance between daphnid clones, while acute toxicity differed with more than a factor 10. They postulated that this might be related to the relative roles played by specific mechanisms (*e.g.* metallothionein detoxification of metals) and general response (of both the 'supply' and 'demand' type). Winner and Farrell (1976) observed no significant differences in survivorship curves, no delayed reproduction, no change in frequency of reproduction and hence no reduction in intrinsic growth rate when *D. magna* was exposed to copper concentrations up to 40 µg Cu L^{-1} (half of the 48-h EC50). Adverse effects were noted at a concentration of 60 µg Cu L^{-1}. The test medium they used was a local pond water (hardness 130-160 mg L^{-1} as $CaCO_3$, pH 8.2-9.5).

Increased survival observed in daphnids acclimated to 100 µg Cu L^{-1} during three or more generations is in accordance with the findings of LeBlanc (1982). He also found that survival progressively improved with each generation. After three generations of acclimation to 10 µg Cu L^{-1} 100 % survival (21-days test) at the highest test concentration (30 µg L^{-1}) was noted, while only 50 % of the first generation daphnids survived this exposure. Several authors have concluded that chronic endpoints such as survival and reproduction are not as sensitive as other criteria such as *e.g.* filtration rates, body length of juveniles and phototaxis (Flickinger *et al.*, 1982; Janssen *et al.*, 1993; Fernandez-Casalderrey *et al.*, 1994). Flickinger *et al.* (1982) reported reductions in feeding rate of daphnids exposed to 10 and 20 µg Cu L^{-1}, while no effects were observed on reproduction and survival. Although the performance of the parental daphnids acclimated to 100 µg Cu L^{-1} was excellent, the fact that first and second generation offspring did not survive at this acclimation concentration can be explained by the population

density. High density in the cultures induced an increase in DOC (factor of 3) within 4 days. Low density (chronic assays) only gave a twofold increase in DOC within 4 days (unpublished data). Moreover, in the chronic assays water exchange was more frequently (thrice a week) and hence the concentration of organic carbon remains low, resulting in less complexation and a more toxic medium.

Except for the reports on zinc acclimation by Muyssen and Janssen (2001b, c) and Muyssen *et al.* (2002), no literature is available on optimal metal concentration ranges for *D. magna*. In our study, the observed optimal concentration range for copper using the energy reserves as endpoint, is in agreement with the results observed for chronic reproduction and feeding behaviour, *i.e.* between 1 and 35 µg Cu L^{-1} ($2 \times 10^{-14} - 80 \times 10^{-12}$ M Cu^{2+}). The shift of the window of essentiality towards lower and higher copper concentrations observed in the third and subsequent generations can be a consequence of acclimation to the background copper concentration. Our initial hypothesis was that organisms acclimated/adapted to different copper background concentrations will shift their optimal concentration range. If daphnids are acclimated to a specific copper concentration, their optimal concentration (= culture concentration) will vary. The existence of an optimum copper concentration range demonstrates that most of the standard media, presently used in several laboratories, are probably deficient in copper (Table 3.6) and may affect the health of the test organisms.

Copper deficiency and toxicity effects observed outside this range can be linked to the observed lower energy reserves, which may, in turn, be related to the activation of energy consuming processes. Indeed, outside this established OCEE, the daphnids probably used more energy reserves to maintain their homeostasis and to compete with deficiency and toxicity stress, resulting in less energy available for reproduction (Figure 3.6). This phenomenon has been previously described and termed as the cost of tolerance (Wilson, 1988) or physiological cost (Calow, 1991). In this context it may be suggested that at high copper concentration, daphnids probably produce metallothioneins to detoxify the copper inside their body (Roesijadi, 1992) or produce copper granules (Taylor, 1995) to store copper in an inactive form. Rainbow and White (1989) found copper rich granules in hepatopancreatic cells of decapods exposed to high copper concentrations. Conversely, at low copper concentrations (below essentiality concentration), daphnids may increase their energy consumption, as they have to actively increase copper uptake rates to fulfil their copper requirement. Flickinger *et al.* (1982) assumed that lower feeding rates in *D. magna* were

linked with increased chronic copper stress. Ferrando and Andreu (1993) also observed copper induced effects on the filtration rate of *D. magna*. Up to a copper concentration of 20 µg L^{-1} they observed an increase in filtration rate; a significant decrease was noted at concentrations ≥ 50 µg L^{-1}.

According to De Coen and Janssen (1997) the CEA biomarker can clearly be linked to population level. They observed a highly significant relationship between CEA and r_m and R_0 of daphnids exposed to sub-lethal cadmium concentrations. We performed a Spearman rank order correlation test on our data and found a good correlation ($r^2 = 0.9$; $p < 0.05$) between the mean energy reserves (F3 – F14) and the mean 21 days chronic reproduction (F3 – F14). The correlation between energy reserves and intrinsic growth rate had a lower significance and the determination coefficient dropped from 0.8 to 0.3 when the data point of 100 µg Cu L^{-1} was omitted, indicating that this correlation was less important.

The energy fraction data indicate that approximately the same percentage of all three energy sources is used to combat copper stress (deficiency and toxicity). Expressed as energy consumption, more lipids are used to compete with the stress, followed by glycogen and proteins. The highest increases for lipids and sugars are observed mainly in the optimal range (2.4×10^{-14} – 8.0×10^{-11} M Cu^{2+}). At higher (toxicity) and lower (deficiency) copper acclimation concentrations decreases were observed in all three parameters. Bodar *et al.* (1988) concluded, that of the three energy reserve fractions analysed, glycogen, probably followed by lipids was first depleted in cadmium stressed daphnids. This discrepancy with our study can possibly be explained by the fact that copper (essential) and cadmium (non-essential) affect daphnids differently.

From our results, it may be hypothesized that at lower copper acclimation concentrations (deficiency), organisms try to regulate their internal copper by depleting the copper from the medium, but never reach the same level as in the optimal copper concentration range. A possible indication for this active accumulation is the increase of copper body concentrations daphnids acclimated to 0.5 µg Cu L^{-1} compared to these of the first generation. At higher copper concentrations (> 100 µg Cu L^{-1}), this regulation mechanism fails with body concentrations up to 250 mg Cu kg DW^{-1} resulting ultimately in toxicity. The high body concentrations may suggest that at least some of the accumulated copper is in detoxified form as there was no hard evidence of metallothionein induction (unpublished data). Similar

results, for zinc, were observed in *D. magna* (Muyssen and Janssen, 2002) and in mussels (Rainbow, 1993; Kraak *et al.*, 1993). Both reports concluded that with increased active regulation and increased withstanding of toxicity organisms need to put more energy in their metabolism, resulting again in lower reproduction rates, as mentioned above.

Only daphnids acclimated to 150 µg Cu L^{-1} (12.9 nM Cu^{2+}) had a decrease in size, indicating that length of daphnids was not a very sensitive parameter in our study. Winner (1981) observed also a decrease in body size of *D. magna* with increasing copper concentration. The length of the spine is according to ecological studies dependent on stress (*e.g.* predators, food concentration) (Peters and Bernardi, 1987). According to our results, the spine length is related to the experienced copper stress (deficiency – toxicity). The decrease of the ratio of the length of spine to total body length of the daphnids is in agreement with the optimal concentration range found in reproduction, energy reserves and body burdens.

3.5. Conclusions

Within the selected, environmentally relevant, copper acclimation range the acute copper tolerance of *D. magna* increased with increasing copper acclimation concentrations. Based on the net reproduction, an optimal concentration range was observed between 1 and 35 µg Cu L^{-1} (0.02 – 80 pM Cu^{2+}). Lower and higher copper acclimation concentrations resulted in reduced reproduction due to copper deficiency and toxicity, respectively. Similar observations were made using the daphnid's energy reserves and filtration rates as endpoints. A fairly constant body concentration was observed between 1 and 12 µg Cu L^{-1} (0.007 – 4 pM Cu^{2+}), indicating a possible copper regulation mechanism. It can be concluded that acclimation can occur in the field at environmentally relevant copper concentrations.

Chapter 4

Multi-generation acclimation of *Daphnia magna* Straus to different bioavailable copper concentrations

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Chapter
4

Multi-generation acclimation of *Daphnia magna* Straus to different bioavailable copper concentrations

Abstract - A large acclimation experiment was performed with *Daphnia magna* in which two different copper bioavailability (as Cu^{2+}) groups (N and M) were used. In the N group the cupric ion activity increased with increasing dissolved copper acclimation concentration, while in the M group the ion activity decreased with increasing dissolved copper concentration. The dissolved copper acclimation concentrations of the culture medium used for both groups were 1, 12 and 100 µg Cu L^{-1}; *i.e.* 20, 300 and 2000 pM Cu^{2+} for the N group and 2000, 300 and 20 pM Cu^{2+} for the M group. The activity of copper carbonates and hydroxides was up to an order of magnitude lower than the cupric ion activity. After five generations of acclimation, the acute copper sensitivity (48-h EC50, mean ± standard deviation) of the N group ranged from 193 ± 24 µg Cu L^{-1} to 296 ± 50 µg Cu L^{-1} and for the M group from 198 ± 27 µg Cu L^{-1} to 315 ± 38 µg Cu L^{-1} for daphnids acclimated to 1 and 100 µg Cu L^{-1}, respectively. The internal copper concentration also exhibited a significant increase with increasing dissolved copper concentration. An optimal copper concentration for first generation daphnids was determined around 12 µg Cu L^{-1} using energy reserves as an endpoint. Acclimation of the daphnids for five consecutive generations to the three dissolved copper concentrations, resulted in a shift in the optimal concentration range towards 1 µg Cu L^{-1}. On the contrary, daphnids exposed to 100 µg Cu L^{-1} can survive and reproduce, but never reached an optimal energy reserve status. Our results suggest that copper acclimation and accumulation are more related to the dissolved copper concentration of the culture medium, than to the copper activity.

4.1. Introduction

Life has evolved in the presence of metals of which several, like copper, have essential biological functions (Da Silva and Willliams, 1991). For each species, in theory, a bell-shaped concentration-effect curve can be constructed with deficiency symptoms occurring at low concentrations and toxic effects occurring at high concentrations. Between these two extremes there is generally an optimal concentration range within which an organism experiences optimal growth, development and reproduction (Muyssen and Janssen, 2001b, c; Bossuyt and Janssen, 2004; see chapter 3). Van Assche *et al.* (1997) and Hopkin (1989) called this range the optimal concentration range for essential elements (OCEE) and window of essentiality, respectively. When the environmental concentration of an essential element is within the

optimal concentration range, organisms can regulate the internal concentrations of the element through binding, detoxification and elimination (Brix and Deforest, 2000). As the natural background concentration of metals in various aquatic systems can vary considerably, the range to which the resident species are adapted should vary accordingly.

Acclimation and/or adaptation to (extremely) high concentration of metals in the natural environment are well documented for both plants and animals (Klerks and Weis, 1987). Metal-induced acclimation in the laboratory during long-term experiments and/or culturing has also been reported. Indeed, changes in metal sensitivity due to laboratory acclimation have already been studied by LeBlanc (1982), Maeda *et al.* (1990), Stuhlbacher *et al.* (1992), Stuhlbacher *et al.* (1993) and Muyssen and Janssen (2001b). Most of these studies express their results in total metal concentrations; indeed only a few calculated the free metal ion activity in attempt to account for metal bioavailability (Muyssen and Janssen, 2001b; Bossuyt and Janssen, 2003: see chapter 3). Recent studies have already shown that the bioavailable metal concentration determines the acute and chronic toxicity and not the total or dissolved metal concentration. De Schamphelaere and Janssen (2002, 2004) and De Schamphelaere *et al.* (2002) e.g. demonstrated that not only the cupric ion but also the copper carbonates and hydroxides influence the toxicity of copper to *D. magna*.

The possible ecological importance and implications of different bioavailable metal background concentrations have not been considered in applied ecotoxicology. Especially for essential elements this variability may have important consequences for the derivation of environmental quality criteria (Janssen *et al*., 2000). Therefore the objectives of this research were (i) to study the ability of the freshwater crustacean *D. magna* to acclimate to different bioavailable concentrations of copper in laboratory conditions and (ii) to determine to what extent the bioavailability of copper influences accumulation and sensitivity to copper of *D. magna* and (iii) to evaluate the possible consequences of these findings for metal risk assessments.

4.2. Materials and methods

4.2.1 Experimental design

Six *D. magna* cultures were simultaneously acclimated to different copper concentrations for five consecutive generations. *D. magna* Straus (clone K6) used in all our experiments was originally collected from a pond in Kiel (Antwerp, Belgium) and has been successfully cultured under controlled laboratory conditions for over 15 years. Animals were cultured as described in section 3.2.1.

D. magna acclimation populations were cultured simultaneously in two sets of three copper concentrations. The culture medium used in the first set (further denoted as the N group) was the chemically defined M4 (Elendt and Bias, 1990) with lowered hardness (180 mg L^{-1} as $CaCO_3$) and EDTA (ethylenediaminetetraacetic acid) replaced by Aldrich humic acid (AHA, Sigma Aldrich Chemie, Steinheim, Germany) at a concentration of 5 mg C L^{-1}. For a discussion on the importance and relevance of this modification we refer to Bossuyt and Janssen (2003) or section 3.4. The dissolved copper concentrations used for the acclimation experiment were 1, 12 and 100 µg Cu L^{-1}. The pH (pH meter P407, Consort, Turnhout, Belgium) of the medium was 7.7 ± 0.2. The second set (further denoted as the M group) used the same dissolved copper concentrations, but the medium composition was manipulated so that the lowest dissolved copper concentration had the highest copper bioavailability and the highest dissolved concentration had the lowest bioavailability. This was done by modifying the pH, DOC and hardness of the M4 medium within the optimal range of *D. magna* (Heijerick *et al.*, 2003): the composition of the N and M media is presented in Table 4.1. All media were buffered with MOPS (3-N morpholino propane sulfonic acid, Sigma-Aldrich, Steinheim, Germany, 750 mg L^{-1}). MOPS was chosen because it is completely non-complexing for metals (Kandegedara *et al.*, 1999) and it has been demonstrated that addition of 750 mg MOPS L^{-1} does not alter the copper or zinc toxicity to *D. magna* (De Schamphelaere *et al.*, 2004). This original test design resulted in ion activities, based on WHAM V (Windermere Humic Aqueous Model version V, Tipping, 1994) calculations, of 20, 300 and 2000 pM Cu^{2+} for both groups. The activity of the copper carbonates and hydroxides was up to an order of magnitude lower than the cupric ion activity and were not included in the further analysis in this study. The addition of copper to the culture media was

done 6 hours prior to the introduction of the daphnids. Ion activity was measured with an ion selective electrode (Cu ISE, model 94-29, Orion Research, Boston, MA; see section 4.2.7).

All chemicals were purchased from VWR International (Leuven, Belgium) and were reagent grade. Copper concentrations were prepared by diluting a concentrated stock solution of $CuCl_2$ (0.1 g Cu L^{-1}) in the assigned medium. Stock solutions of dissolved organic carbon (DOC) were prepared by dissolving 5 g of AHA in 2 L of deionised water, equilibrating the solution for 24 h at 4 °C and filtering it through a 0.45 µm filter (Gelman Sciences, Ann Arbor, MI, USA). The DOC concentration of the stock solutions was measured with a TOC-5000 analyser (Shimadzu, Duisburg, Germany).

Table 4.1: Physico-chemical characteristics of the different media (N and M group). DOC: dissolved organic carbon; Cu_t: nominal dissolved copper concentration; Cu^{2+}: calculated ion activity with WHAM V.

	Unit	N1	N12	N100	M1	M12	M100
pH		7.7	7.7	7.7	6.5	7.1	8.4
Hardness	mg $CaCO_3$ L^{-1}	180	180	180	180	180	125
DOC	mg C L^{-1}	5	5	5	0.5	7	25
Cu_t	µg L^{-1}	1	12	100	1	12	100
Cu^{2+}	pM	20	300	2000	2000	300	20

Stock cultures as well as experimental animals in chronic assays were fed a mixture of the algae *Pseudokirchneriella subcapitata* (Korshikov) Hindak (formerly known as *Selenastrum capricornutum* Printz or *Raphidocelis subcapitata*) and *Chlamydomonas reinhardtii* in a 3:1 ratio. The algae were cultured in the standard ISO (International Organisation for Standardisation, 1989) medium, which contains trace amounts (3.7 ng Cu L^{-1}) of copper. This resulted in a maximum total copper concentration (adsorbed + absorbed) in the algae of 10^{-15} g $cell^{-1}$ (Bossuyt and Janssen, unpublished data). As the organisms grew, increasing amounts of food were supplied: from 0 to 7 days, from 8 to 15 days, and older than 15 days daphnids were fed 8 x 10^6, 12 x 10^6 and 16 x 10^6 cells day^{-1} $daphnid^{-1}$, respectively. The temperature during culturing and testing was 20 ± 1 °C with a light: dark cycle of 12h:12h.

4.2.2 Acute toxicity experiments

Acute toxicity experiments with daphnids of each generation were performed as described in section 3.2.2. The test medium used for all acute experiments was the modified M4 medium similar to that used for the N group (without added copper).

4.2.3 Chronic toxicity experiments

Chronic experiments with fifth generation daphnids were performed as described in section 3.2.3. The test medium of all chronic experiments was the modified M4 medium similar to that used for the N group (without added copper). Based on R_0, the 50 % (21-d EC50) and 10 % (21-d EC10) effective concentrations with their 95 % confidence intervals were calculated in STATISTICA 6 (STATISTICA® software, Tulsa, OK, USA) using the equation proposed by Van Ewijk and Hoekstra (1993). A parameter for hormesis is included in this model:

$$y = \frac{k \cdot (1 + g \cdot x)}{1 + (2 \cdot g \cdot x_{50} + 1) \cdot \left(\frac{x}{x_0} \right)^s}$$

The parameter y represents the response of the measured endpoint; x is the test concentration; k represents the response of measured endpoint at $x = 0$; g is the hormesis parameter: if $g > 0$, the curve shows an increase for low doses; s is the slope parameter. The parameter x_0 is the EC50. Calculated values are based on measured copper concentrations.

4.2.4 Energy reserves (E_a)

The energy reserves of first and fifth generation daphnids were measured as described in section 3.2.5. A total of 300 *D. magna* juveniles (< 24 h) originating from each acclimation culture were exposed for 96 h in 2 L aquaria containing 1 L of the modified M4 medium as that used for the N group (without added copper) spiked with different copper concentrations (*i.e.* control, 1, 12, 100 and 125 µg Cu L^{-1}).

4.2.5 Internal copper concentration

For each generation 60 *D. magna* (3 replicates of 20 organisms) from the different acclimation cultures were sampled after 40 days, rinsed with deionised water and transferred to 5 $\times 10^{-3}$ M EDTA for 20 min to remove copper adsorbed to the daphnid's carapace. The samples were subsequently destructed as described in section 3.2.6.

In a second series of experiments, juvenile (< 24 h) daphnids of the parental culture and fifth generation daphnids from the acclimation cultures (N1, N100, M1 and M100) were transferred to the N1, N100, M1 and M100 media for 21 days. Each day, the daphnids were fed a mixture of the algae at the same age-dependent algal concentration as those used in the acclimation cultures (cf. above). At the start (t = 0), after 2h and after 1, 2, 4 days and every two days until day 21, daphnids were collected and their internal copper concentrations and body weight was measured. Internal body concentrations are expressed as mg Cu $(kg\ DW)^{-1}$.

4.2.6. Length measurements

For each generation and treatment, the carapace length of 10 juvenile (< 24 h) and 10 adult (21 d) daphnids was measured. Organisms were measured with the aid of a microscope (Kyowa, Tokyo, Japan) equipped with an ocular micrometer ruler. The length of the daphnids was measured from top of the head to the tip of the spine.

4.2.7. Copper measurements

Copper concentrations in test and culture media were determined as described in section 2.2.4. All measured copper concentrations were within 10 % of the nominal concentrations. All reported copper concentrations are dissolved concentrations (0.45 µm filtered).

Cu^{2+} activities were determined using a cupric ion selective electrode (Cu-ISE, model 94-29, Orion Research, Boston, Michigan, USA) and a double junction Ag/AgCl reference electrode (Model 90-02, Orion research). Before each use, the Cu-ISE was polished with polishing paper to restore the electrode to good operating conditions. The electrode was then rinsed with double deionised water (DI water) and soaked in 0.01 M H_2SO_4 (p.a.) for 5 minutes and subsequently with DI water for 30 minutes. The Cu-ISE was calibrated before each use with a

Cu-ethylenediamine (Cu-EN) buffer over the pCu range of 5 to 11. Cu^{2+} activity was calculated for each calibration point with visual MINTEQ (free download from http://amov.ce.kth.se/people/gustafjp/vminteq.htm) using stability constants from Martell *et al.* (1997). The observed slope for all calibrations was 29.1 ± 0.4 mV/pCu, which is close to the theoretical slope at 20 °C (Cu-ISE manual, Orion Research).

4.2.8. Statistical analysis

The effects of the various treatments on the daphnids' response were compared using one-way analysis of variance (ANOVA) and Duncan's multiple range test (STATISTICA® software, Tulsa, OK, USA). Bartlett's and Kolmogorov-Smirnov's test were used to test homogeneity of variance and normality, respectively. In case these assumptions were not met, the data were compared with the non-parametric Mann-Whitney *U* test. Statements of significant differences are based on accepting $p < 0.05$.

4.3. Results

At the start of the acclimation experiment, the copper ion activity in the different acclimation media was measured daily to ensure that our test set-up followed our envisaged design (Figure 4.1). Similar ion activities were noted for N1 and M100, for N12 and M12, and for N100 and M1. For all acclimation concentrations, a 2 to 40-fold decrease was observed with increasing time. The highest decrease (more than one order of magnitude) was observed for acclimation cultures with the highest copper activities (N100 and M1). Despite this decrease, the copper activities observed on day 4 still followed our envisaged test design (*i.e.* N1 and M100 had the lowest ion activity, while N100 and M1 had the highest). As the cupric ion activity was two orders of magnitude higher than that of the copper carbonates and hydroxides, and more than 95 % of the copper was situated on the DOC, the cupric ion activity was assumed to be the main bioavailable copper fraction in this study.

The mean (5 generations; $n = 15$) acute copper sensitivity of *D. magna*, acclimated to the different copper concentrations in the N and M group is shown in Figure 4.2. Based on the cupric ion activity, the 48-h EC50s of the daphnids of the N group increased with an increasing ion activity. In contrast, the M group exhibited a decrease in 48-h EC50s. When the EC50s of both groups were plotted against the dissolved copper acclimation concentrations,

they both showed an increase in 48-h EC50 values with increasing acclimation concentration. Daphnids acclimated to 100 µg Cu L^{-1} (for both groups) had a significantly lower sensitivity to copper than daphnids acclimated to 1 µg Cu L^{-1}. The values (mean ± standard deviation, n = 15) ranged from 193 ± 24 µg Cu L^{-1} to 296 ± 50 µg Cu L^{-1} for the N group, and from 198 ± 27 µg Cu L^{-1} to 315 ± 38 µg Cu L^{-1} for the M group. An ANOVA between the two groups (N and M) and between the acclimation concentrations revealed no significant differences in copper sensitivity between the N1 and M100, between N12 and M12 and between N100 and M1 daphnids. Return of the fifth generation daphnids acclimated to N100 and M100 to medium without copper resulted in loss of the increased tolerance within one generation: 48-h EC50 (mean ± standard deviation, n = 3) dropped from 356 ± 57 µg Cu L^{-1} to 210 ± 15 µg Cu L^{-1} and from 354 ± 4 µg Cu L^{-1} to 211 ± 9 µg Cu L^{-1}. The toxicity values of the return generation were not significantly different (Student's t test for independent samples, $p > 0.05$) from those of the N1 and M1 acclimation culture.

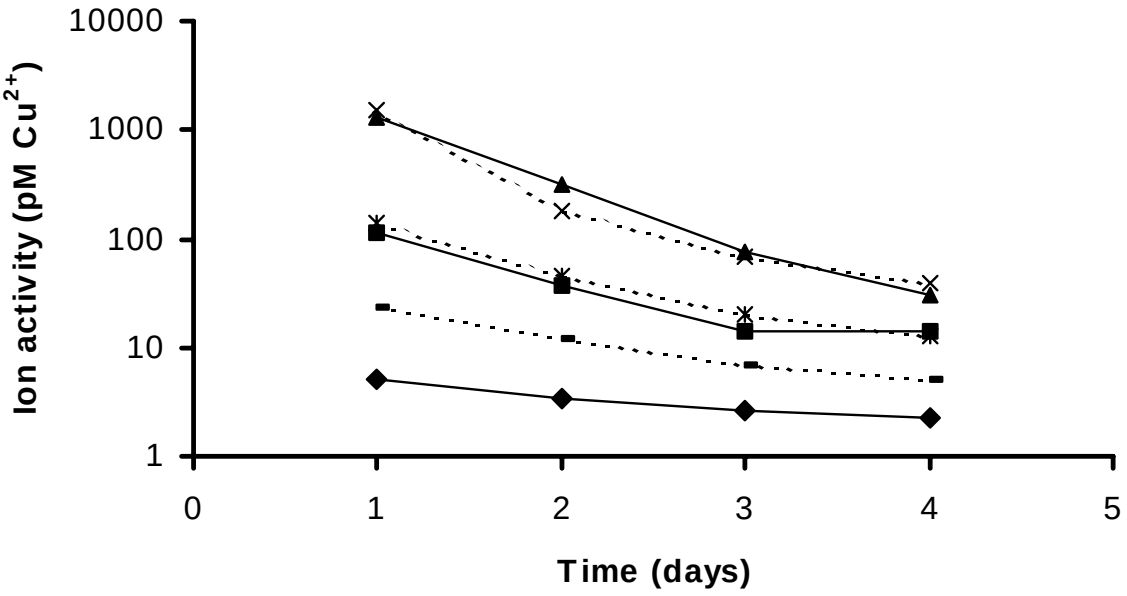

Figure 4.1: Ion activity of the different acclimation culture media during a 4 day period. Symbols represent N1 (◆), N12 (■), N100 (▲), M1 (×), M12 (∗) and M100 (-).

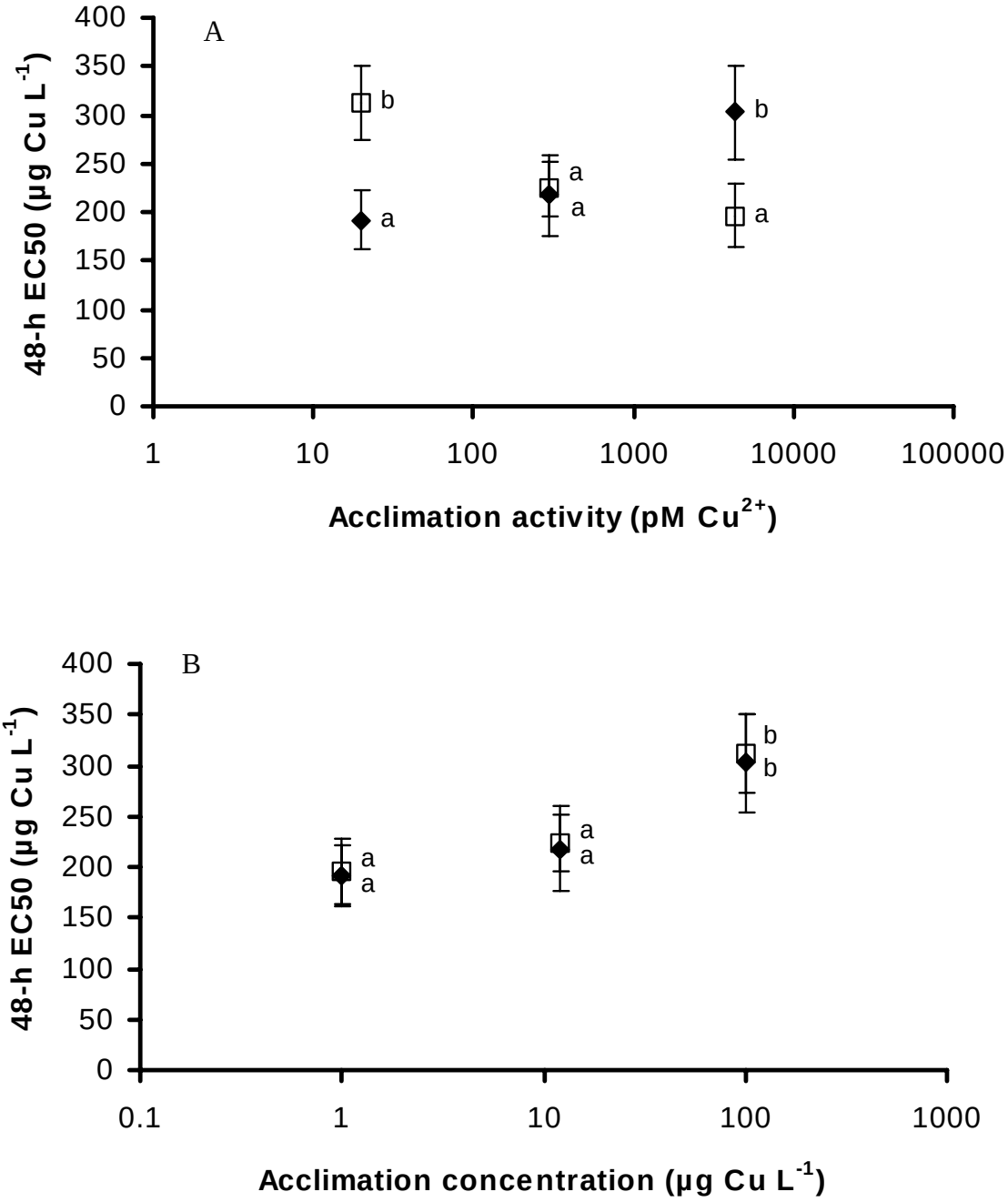

Figure 4.2: Mean (5 generations, n = 15) acute copper toxicity of *D. magna* of N (◆) and M (□) group as a function of the cupric ion activity (A) and as a function of the dissolved copper concentration (B). Error bars represent standard deviation. Mean values with the same letter are not significantly different at $p < 0.05$ (ANOVA).

The results of the chronic toxicity experiments with fifth generation daphnids are shown in Table 4.2. No clear change in chronic sensitivity was observed with increasing acclimation concentration. Also 21-d EC10s followed no clear trend. Net reproduction (R_0, mean ±

standard deviation, n = 10) at the test concentration of 1 and 12 µg Cu L^{-1} (= acclimation concentration) was 95 ± 9, 87 ± 6, 106 ± 12 and 86 ± 11 for daphnids of N1, N12, M1 and M12, respectively. No significant (Student's t test for independent samples, $p > 0.05$) differences were noted between these values. Daphnids exposed to a test concentration of 100 µg Cu L^{-1} did not reproduce.

Table 4.2: The chronic 21-d EC50 and EC10 with 95 % confidence intervals for fifth generation *D. magna* acclimated to the different media.

Culture	**21-d EC50 µg Cu L^{-1}**		**21-d EC10 µg Cu L^{-1}**	
N1	79.6	75.0 - 84.5	68.7	64.4 - 73.3
N12	81.2	75.8 - 86.9	72.7	68.0 - 77.6
N100	75.7	57.4 - 99.8	71.3	65.4 - 77.6
M1	67.8	62.3- 73.7	58.2	43.7 - 77.5
M12	76.3	64.6 - 90.1	53.8	52.5 - 54.9
M100	74.8	67.5 - 82.9	69.0	66.2 - 71.8

The internal copper concentrations of first and fifth generation adults (n = 3) taken from the different acclimation cultures increased with increasing dissolved copper concentrations (Table 4.3). No significant differences were noted between the internal concentration of first and fifth generation daphnids. Mean (± standard deviation, 5 generations, n = 15) internal concentrations ranged from 10.6 ± 2.0 mg Cu kg DW^{-1} (at 1 µg Cu L^{-1}) to 64.7 ± 20.0 mg Cu kg DW^{-1} (at 100 µg Cu L^{-1}) for N group daphnids, and from 13.9 ± 4.0 mg Cu kg DW^{-1} (at 1 µg Cu L^{-1}) to 38.9 ± 4.2 mg Cu kg DW^{-1} (at 100 µg Cu L^{-1}) for M group daphnids. No significant differences (ANOVA, $p > 0.05$) were observed between the mean body concentrations of the N and M group for a dissolved copper exposure concentration of 1 and 12 µg Cu L^{-1}. The internal copper concentration of daphnids acclimated to N100 and M100 were significantly higher than those of N1 and M1, respectively. Return of the copper-acclimated daphnids to a medium without copper addition for one generation, resulted in a significant decrease of the internal copper concentration: body concentrations (mean ± standard deviation, n = 3) dropped from 72.2 ± 10.8 mg Cu kg DW^{-1} to 9.7 ± 0.9 mg Cu kg DW^{-1} and from 35.9 ± 2.8 mg Cu kg DW^{-1} to 10.5 ± 0.4 mg Cu kg DW^{-1} for daphnids

acclimated to N100 and M100, respectively. No significant difference (Student's *t* test for independent samples, $p > 0.05$) was noted for body concentrations of the returned daphnids compared to those of daphnids of the N1 and M1 group.

The length of adults ($n = 10$) of the first and fifth generation of the acclimation cultures showed no significant differences between the N and M group (Table 4.3). A similar observation was made for the length of the juveniles ($n = 10$). Also no significant differences were observed between the length of daphnids cultured in the three different acclimation copper concentrations (1, 12 and 100 µg Cu L^{-1}). Adults of the fifth generation were significantly (Student's *t* test for independent samples, $p < 0.05$) larger than those of the first generation.

Next to the acclimation studies, a 21 day accumulation experiment was performed. For the parental generation, daphnids exposed to the medium of the N and M group exhibited a similar copper accumulation pattern (Figure 4.3A). A decrease in internal copper concentrations was observed up to day 5. From day 16 onwards, an increase in copper body concentration was observed in the N100 and M100 treatments. In contrast, body concentrations of fifth generation daphnids of N100 and M100 exhibited no increase after day 16. N1 and M1 daphnids seemed to have a similar body concentration, but the body concentration of M100 organisms were lower than those of N100. Body concentrations of daphnids exposed to N12 and M12 followed a similar pattern and were situated between the N1 (M1) and N100 (M100). For fifth generation daphnids, a fairly constant internal copper concentration was observed in all groups from day 8 onwards (Figure 4.3B).

Energy reserves of first and fifth generation daphnids are shown in Figure 4.4. The N and M group seemed to have a similar response. In first generation daphnids, the highest energy reserves were noted in daphnids acclimated to 12 µg Cu L^{-1} and clearly lower energy reserves in those acclimated to 1 and 100 µg Cu L^{-1}. Fifth generation daphnids exhibited a significant increase (ANOVA, $p < 0.05$) in their energy reserves. For both groups, significantly higher energy reserves were observed in daphnids acclimated to 1 and 12 µg Cu L^{-1} compared to daphnids acclimated to 100 µg Cu L^{-1}.

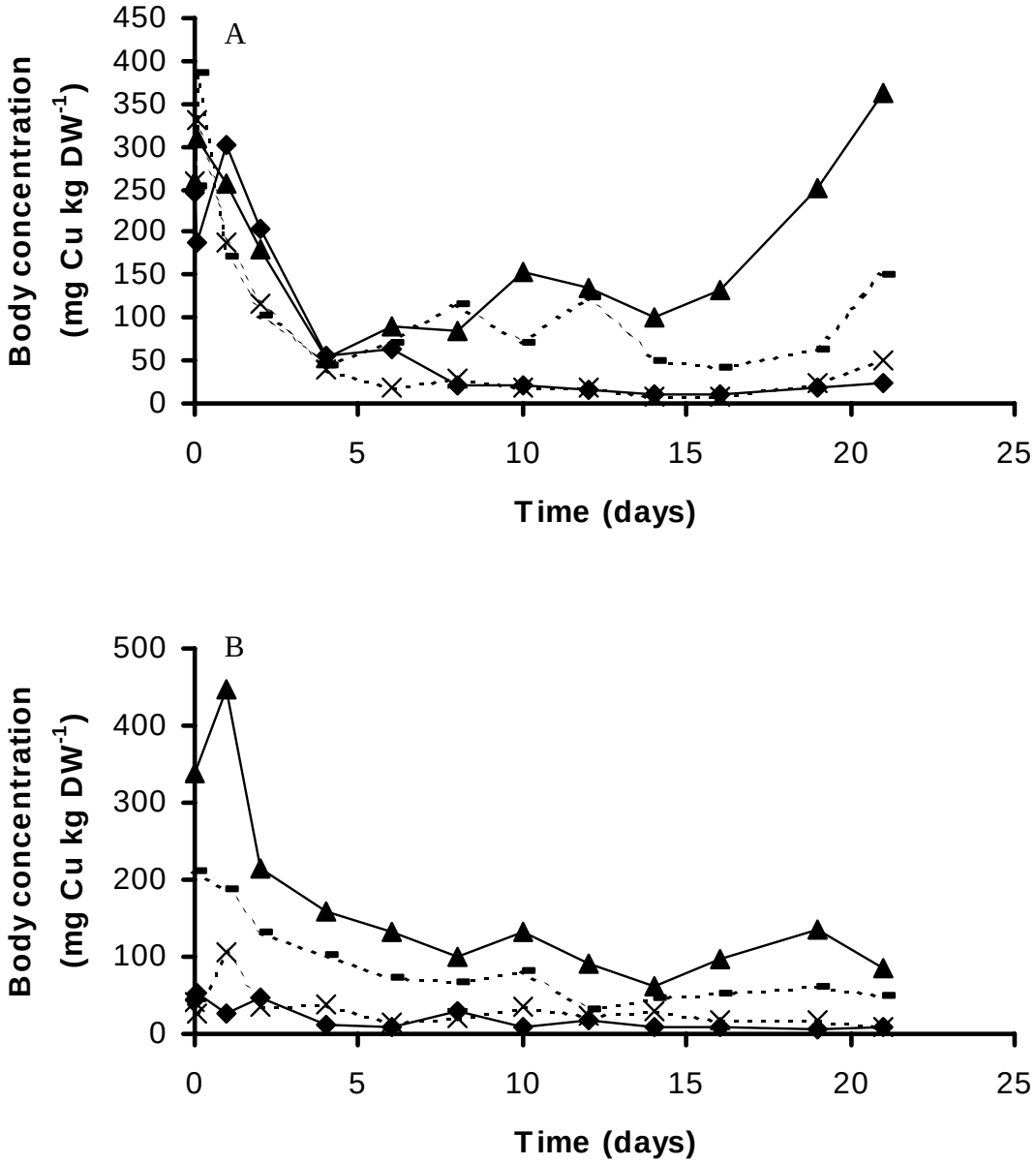

Figure 4.3: The copper accumulation in parental (A) and fifth (B) generation *D. magna* acclimated to the different copper concentrations during 21 days. Symbols represent N1 (◆), N100 (▲), M1 (×) and M100 (-).

Table 4.3: Internal copper concentrations (mean ± standard deviation, *n* = 3), juvenile length (mean ± standard deviation, *n* = 10) and adult length (mean ± standard deviation, *n* = 10) of first (F1) and fifth (F5) generation daphnids acclimated to different copper exposures (N and M group with 1, 12 and 100 µg Cu L^{-1}). The asterisk (*) denotes a significant difference (Student's *t* test for independent samples, $p < 0.05$) between F5 and F1.

	Unit	**N1**	**N12**	**N100**	**M1**	**M12**	**M100**
Internal concentration	mg Cu kg DW^{-1}						
F1		10.8 ± 3.8	17.6 ± 4.5	70.4 ± 7.3	12.8 ± 6.4	25.5 ± 6.3	42.1 ± 1.4
F5		12.5 ± 1.3	18.2 ± 0.5	72.2 ± 10.8	22.1 ± 3.4	23.6 ± 0.4	35.9 ± 2.8*
Juvenile length	mm						
F1		1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1
F5		1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.4 ± 0.1
Adult length	mm						
F1		3.8 ± 0.1	3.8 ± 0.1	3.7 ± 0.1	3.8 ± 0.1	3.7 ± 0.2	3.6 ± 0.1
F5		4.4 ± 0.1*	4.1 ± 0.2*	4.3 ± 0.1*	3.9 ± 0.1	4.0 ± 0.1*	4.2 ± 0.1*

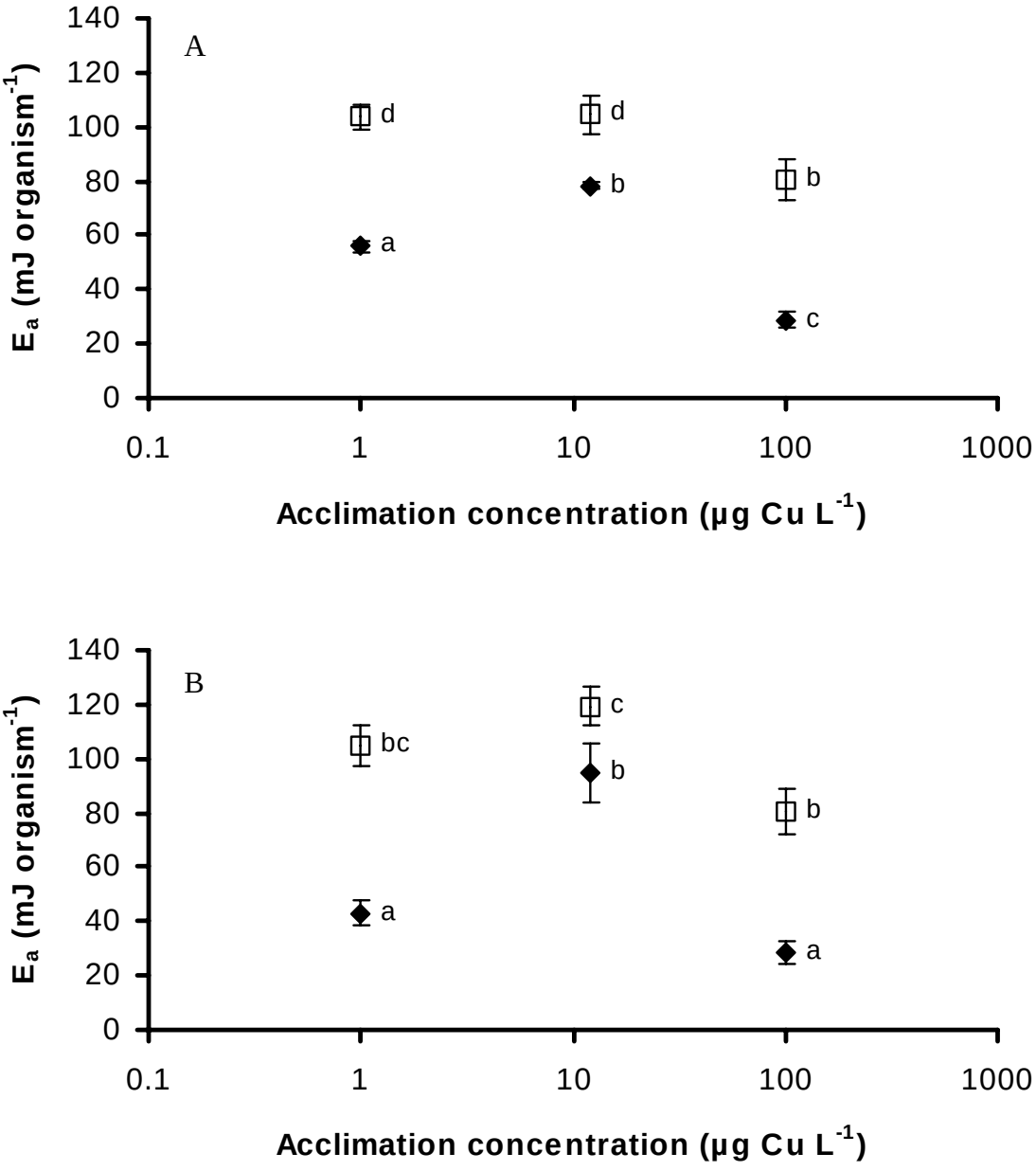

Figure 4.4: Energy reserves (*Ea*) of first (◆) and fifth (□) generation *D. magna* of N (A) and M (B) group. Error bars represent standard deviation. Mean values with same letter are not significantly different at $p < 0.05$ (ANOVA).

4.4. Discussion

The acute results of our study revealed a resistance factor of 1.6 (*i.e.* ratio of 48-h EC50 of copper acclimated organisms / the 48-h EC50 of non-acclimated organisms) for both groups. The lack of an increased tolerance for daphnids of M1 (2000 pM Cu^{2+}) may suggest that

acclimation is not influenced by the cupric ion. In contrast, organisms cultured in 100 µg Cu L^{-1} in the M medium, *i.e.* with a cupric ion activity of 20 pM Cu^{2+}, showed a significant increase in acute copper tolerance. Increase of copper tolerance in *D. magna* seems thus more influenced by the dissolved copper concentration than by the ion activity. No clear acclimation effects were observed in the chronic copper tolerance of fifth generation daphnids (Table 4.2). Overall, this seems to be in agreement with previous results (Bossuyt and Janssen, 2004; see section 3.3.), although in that study a slight increasing trend (factor of 1.6) was also noted in chronic tolerance after five generation of acclimation to 100 µg Cu L^{-1}. This slight difference could be due to the use of a different calculation method for the EC50 in this study, *i.e.* here, the hormesis effect, based on the equation reported by Van Ewijk and Hoekstra (1993), was taken into account if it occurred in the chronic assays, while in the former study the logistic fit was used.

Baird *et al.* (1990) already demonstrated that the variation in acute metal toxicity is greater than that in chronic toxicity. Also, Taylor *et al.* (2000) observed acclimation effects on acute copper tolerance and an absence of tolerance shifts in chronic toxicity tests with rainbow trout. Muyssen and Janssen (2001) also observed no acclimation effects on chronic zinc tolerance of *D. magna*. It can thus be concluded that according to the results of these studies and those of the present study, acclimation does affect acute tolerance, but does not influence the sensitivity of organisms in chronic assays.

An increase in the internal copper concentrations with increasing dissolved copper concentrations rather than with increased copper activities was observed in the acclimated daphnids. Hence, accumulation seemed to be more influenced by the dissolved copper concentration in the medium than by its copper activity. This was also confirmed during the 21-day accumulation experiment with *D. magna*. Fifth generation daphnids cultured in N1 and M1, which differed in copper activities, followed a similar trend in copper accumulation during the exposure period. Additionally, the copper accumulation trend of fifth generation daphnids acclimated to N100 was comparable to that of daphnids acclimated to M100. However, it should be noted that a significant difference was observed between the internal concentrations of daphnids acclimated to N100 and M100. Although this requires further investigation, it may be suggested that the high DOC concentration in the M100 treatment (*i.e.* 25 mg C L^{-1}), resulting in increased copper-DOC complexes, may have limited the uptake and incorporation of copper in the daphnids.

A decrease in internal copper concentration is observed for both parental and fifth generation daphnids up to day 5 or 8, respectively. It is suggested that this is mainly determined by the decrease in the specific surface/volume ratio of the juvenile daphnids as they grow and possibly by the binding of copper to the active sites (e.g. gills). It has to be noted that the parental daphnids exhibited high internal copper concentration at day 0. This could be related to the relatively high copper concentration in the main laboratory culture medium (*i.e.* 7 ± 2 µg Cu L^{-1}), continuously used to maintain the laboratory daphnid culture during the last 15 years. The fairly constant body concentration from day 8 onwards may indicate a possible regulation mechanism in the daphnids. The increase in body concentrations observed in N100 and M100 parental daphnids aged ≥ 16 days, may indicate the failure of this regulation mechanism and hence the onset of toxicity. As this increase was not observed in fifth generation organisms, this may indicate that the acclimation process also influences the uptake and regulation of copper in *D. magna*. Bossuyt and Janssen (2003) already demonstrated that maximum acclimation effects occurred after three generations. They observed an increase in acute tolerance, juvenile production and energy reserves and a decrease in total body burdens in acclimated daphnids.

The energy reserves of the juvenile N and M group daphnids were not different based on dissolved copper concentrations. These results demonstrate the existence of an optimal physiological concentration range (OCEE) of copper. For the first generation daphnids, the optimal copper concentration based on the energy reserve status was around 12 µg Cu L^{-1}. The lower energy reserves in daphnids cultured in 1 µg Cu L^{-1} and 100 µg Cu L^{-1} probably occurred due to copper deficiency and toxicity, respectively.

The shift of the optimal copper range towards lower concentrations (*i.e.* towards 1 µg Cu L^{-1}) for fifth generation daphnids of the N and M group is in agreement with previous results (Bossuyt and Janssen, 2003; see section 3.3.). Copper-acclimated daphnids (fifth generation) of both the N and M group exhibited an optimal energy reserve status between 1 and 12 µg Cu L^{-1}, while a lower energy reserve status was noted at the highest acclimation concentration (100 µg Cu L^{-1}). Hence, optimal copper concentrations for *D. magna* are also more influenced by the dissolved copper concentrations of the culture medium, than by its copper activity. Except for our studies, no literature has been found on optimal copper concentrations for daphnids. The existence of an OCEE for zinc in laboratory-acclimated and field-collected *D.*

magna has been demonstrated by Muyssen and Janssen (2001) and by Muyssen *et al*. (2002). In the latter study, the observed optimal concentration was in agreement with the (dissolved) metal concentration measured in the field.

The complete experimental design was based on WHAM V (Tipping, 1994) calculations. Although the measured copper activities were in the same order as the calculated ones, differences of up to a factor of 4 were noted (on day 1, Figure 4.1). However, it has to be kept in mind that the calculated Cu^{2+} activities are only estimations representing the (pseudo)-equilibrium situation at the moment daphnids are transferred to fresh medium. Daphnids and algae are able to excrete organic copper-complexing agents, which may reduce the copper activity (Xue and Sigg, 1990; Fish and Morel, 1983). Bossuyt and Janssen (2003) noted an increase (factor of 3) in DOC concentration during three days of culturing *D. magna* at 100 µg Cu L^{-1}. The authors are aware that a newer version of the geochemical speciation program (WHAM VI, Tipping, 1998) is currently available. Although WHAM VI calculations give a different output in copper activities, the general trend is similar to that of WHAM V. Copper activities with WHAM VI were 0.024, 4.13 and 3700 pM Cu^{2+} (N group) and 90.1, 8.15 and 0.5 pM Cu^{2+} (M group) for copper concentrations of 1, 12 and 100 µg Cu L^{-1}. The highest dissolved copper concentration in the M group (M100) still had the lowest copper activity (0.5 pM Cu^{2+}) of the M group. Hence the conclusions presented above remain valid.

From the above, it seems that copper acclimation and bioaccumulation is related to the dissolved copper concentration in the medium. Heijerick and Janssen (2002) already demonstrated that the internal zinc concentrations in *D. magna* were related to the dissolved zinc concentration in the medium. In our study an increase in internal copper concentrations was noted with increasing dissolved copper (acclimation) concentration. It is suggested that at these low copper concentrations different mechanisms in copper uptake may occur compared to those taking place at high copper concentrations, *i.e.* concentrations where toxicity occurs. Hence, the cupric ion is probably not the only species which is bioavailable during the copper acclimation exposure. As described by some authors, the hydroxides and carbonates (De Schamphelaere and Janssen, 2002, 2004) and copper-DOC complexes (Erickson *et al.*, 1996; Tao *et al.*, 2001; De Schamphelaere and Janssen, 2004) may also be bioavailable. Since acclimation and accumulation both seemed to be a function of the dissolved concentration, it could be suggested that acclimation is related to the internal copper concentrations.

Daphnids exposed to 100 µg Cu L^{-1} exhibited a lower energy reserve status than daphnids exposed to optimal copper concentrations. The low energy reserves at the high copper concentration is probably due to increased energy expenditure to keep copper outside the organism or to detoxify it internally. A combination of both processes can result in an increased tolerance. After acclimation (5 generations), this process has become more efficient resulting in higher energy reserves (but still lower than in the optimal range) and lower internal copper concentration (Figure 4.3, after day 16). According to Taylor *et al.* (2003) increased tolerance in fish is related to a reduced sodium loss upon copper exposure although this mechanism was not reflected by the amount of copper at the gill surface. As no single mechanism of copper tolerance exists among organisms (Mulvey and Diamond, 1991), further research on the exact mechanism of acclimation of *D. magna* to copper is needed.

For the establishment of water quality criteria (WQC) and ecological risk assessment, it has to be kept in mind that organisms do have an optimal range for essential metals. According to this study, optimal copper concentrations for *D. magna* are between 1 and 12 µg Cu L^{-1}. This optimal range has also been noted in previous multi-generation acclimation studies by the authors (optimal range between 1 and 35 µg Cu L^{-1}: Bossuyt and Janssen, 2003; Bossuyt and Janssen, 2004; see section 3.3.). Also for *P. kirchneriella* (tested in a medium with 30 mg L^{-1} as $CaCO_3$, see section 2.3.) an optimal concentration range between 1 and 35 µg Cu L^{-1} (10^{-14} – 10^{-10} M Cu^{2+}) was observed. According to Landner and Lindeström (1999), different freshwater WQCs for copper are e.g. 12, 3, 2 and 1.3 µg Cu L^{-1} for the US, Norway, Canada and The Netherlands, respectively. Although these current WQC are situated within the optimal concentration range of both organisms (although some of them are close to the deficiency side), attention has to be paid to this in the derivation of future WQC and ecological risk assessments of essential metals.

4.5. Conclusions

The results confirm that *D. magna* is able to acclimate to copper and that the acclimation process seems to be dependent on the dissolved copper concentration, rather than on the cupric ion activity. Increases in acute copper tolerance were observed, while no change was observed in the chronic copper tolerance of fifth generation daphnids. Measurement of internal copper concentrations and energy reserves in the daphnids also revealed that the acclimation process is dependent on the dissolved copper concentration. From this it is clear

that more research is needed to fully understand the physiological mechanisms of copper acclimation and its relation to metal bioavailability. Our results suggest that when laboratory toxicity data for essential elements such as copper are used for WQC derivation, they must be carefully evaluated prior to use. Only studies with test organisms cultured in media containing sufficient levels of essential elements and preferable acclimated for a sufficient period of time (at least 3 generations) should be used. Where applicable, deficiency data, in addition to toxicity data, should be incorporated into the derivation of WQCs.

Chapter 5

Copper regulation and homeostasis of *Daphnia magna* and *Pseudokirchneriella subcapitata*: influence of acclimation

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Chapter
5

Copper regulation and homeostasis of *Daphnia magna* and *Pseudokirchneriella subcapitata*: influence of acclimation

Abstract - This study aimed to evaluate (1) the capacity of the green alga *Pseudokirchneriella subcapitata* and the waterflea *Daphnia magna* to regulate copper when exposed to environmentally realistic copper concentrations and (2) the influence of multi-generation acclimation to these copper concentrations on copper bioaccumulation and homeostasis. Active copper regulation was observed in algae up to 5 µg Cu L^{-1} and in daphnids up to 35 µg Cu L^{-1}. Constant internal copper concentrations (13 ± 4 µg Cu g DW^{-1}) were observed in algae exposed to 1 through 5 µg Cu L^{-1} and in daphnids exposed to 1 through 12 µg Cu L^{-1}. At higher exposure concentrations, there was an increase in internal copper, while no increase was observed in bioconcentration factors, suggesting the presence of a storage mechanism. At copper concentrations of 100 µg Cu L^{-1} (*P. subcapitata*) and 150 µg Cu L^{-1} (*D. magna*), the significant increases observed in internal copper concentrations and in bioconcentration factors may be related to a failure of this regulation mechanism. For both organisms, internal copper concentrations lower than 13 µg Cu g DW^{-1} may result in copper deficiency. For *P. subcapitata* acclimated to 0.5 and 100 µg Cu L^{-1}, internal copper concentrations ranged (mean ± standard deviation) between 5 ± 2 µg Cu g DW^{-1} and 1300 ± 197 µg Cu g DW^{-1}, respectively. For *D. magna*, this value ranged between 9 ± 2 µg Cu g DW^{-1} and 175 ± 17 µg Cu g DW^{-1} for daphnids acclimated to 0.5 and 150 µg Cu L^{-1}. Multi-generation acclimation to copper concentrations ≥ 12 µg Cu L^{-1} resulted in a decrease in internal copper concentrations for both organisms compared to the internal copper concentration of the first generation. It can be concluded that *P. subcapitata* and *D. magna* can regulate their internal copper concentration to maintain copper homeostasis within their optimal copper range.

5.1. Introduction

Copper is a co-factor in a wide range of biochemical redox reactions involving intracellular enzymes/proteins such as cytochrome oxidase, superoxide dismutase, dopamine-hydroxylase, lysyl oxidase, and extracellular ceruloplasmin (Harris and Gitlin, 1996). The redox nature of copper which makes it essential to processes such as cellular respiration, free-radical defence, and cellular iron metabolism, also makes copper a very potent toxicant.

Consequently, for each species a bell-shaped concentration-effect curve can be observed, with deficiency symptoms occurring at low concentrations and toxic effects occurring at high concentrations (Van Assche *et al.*, 1997; Hopkin, 1989; Bossuyt and Janssen, 2003). Given that copper is essential but potentially toxic, it is not surprising that this metal is highly regulated in some organisms. Copper metabolism has been especially extensively studied in mammals due to the genetically linked disorders such as Wilson's disease and Menke's syndrome (Linder and Hazegh-Azam, 1996). Grosell *et al.* (1998, 2001) reported extensively on the copper homeostasis in copper-acclimated freshwater fish, while Solioz and Odermatt (1999) and Wunderli-Ye and Solioz (1999) investigated this in the bacteria *Enterococcus hirae*. However, literature concerning the metabolism of essential metals in invertebrates is scarce. Winner (1985), Winner and Gauss (1986) and Bossuyt and Janssen (2003, 2004a, see section 3.3. and 4.3.) described the bioaccumulation of copper in *Daphnia magna*. Muyssen and Janssen (2002a) investigated zinc accumulation and homeostasis in *D. magna* and linked this with an established optimal concentration range for zinc (Muyssen and Janssen, 2001b).

Aquatic biota can regulate their internal concentrations of essential metals in three ways: active regulation, storage, or a combination of both mechanisms. Active regulators are organisms that maintain stable tissue concentrations by excreting the metal at rates comparable to the intake rate (Brix and DeForest, 2000). Other biota store metals in detoxified forms, such as in inorganic granules (Brown, 1982) or bound to metallothioneins (Roesijadi, 1992). However, literature on copper regulation in aquatic organisms is rather ambiguous. In general, essential metals such as copper and zinc tend to be actively regulated by organisms such as decapod crustaceans, algae and fish (Amiard *et al.*, 1987; Rainbow and White, 1989; Kraak *et al.*, 1993). Conversely, organisms such as bivalve molluscs, barnacles and aquatic insects tend to store these metals in detoxified forms (Amiard *et al.*, 1987; Krishnakumar *et al.*, 1990). No literature was found on copper regulation mechanisms in the branchiopod crustacean *D. magna* and the green alga *Pseudokirchneriella subcapitata*.

In natural aquatic systems, organisms are exposed to varying concentrations of natural background copper and possible anthropogenic addition of copper. Adaptation or acclimation to these concentrations can result in an increased tolerance (Klerks and Weis, 1987; Bossuyt and Janssen, 2003, 2004a; see section 3.3.). The mechanisms to achieve this can vary among organisms. For example, reduction of copper uptake and exclusion of copper have been identified as possible mechanisms of copper tolerance in algae after a long-term

acclimation/adaptation to sub-lethal copper concentrations (Foster, 1977; Mulvey and Diamond, 1991). Grosell *et al.* (2001) noted that copper-acclimated rainbow trout exhibited a reduced accumulation of copper in plasma compared to non-acclimated rainbow trout. Copper elimination was stimulated in acclimated European eels (Grosell *et al.*, 1998). No literature data were found on the influence of copper acclimation on its accumulation in *D. magna* and *P. subcapitata*.

Given the above, the present study aims at investigating the regulation capabilities of the freshwater unicellular green algae *P. subcapitata* and the cladoceran *D. magna* exposed to environmentally realistic copper concentrations; and to investigate if multi-generation acclimation to these copper concentrations influences copper accumulation/regulation and homeostasis of both organisms.

5.2. Materials and methods

5.2.1. Acclimation cultures

Experiments were performed with the unicellular green algae *P. subcapitata* (Korshikov) Hindak (CCAP 278/4, formerly known as *Selenastrum capricornutum* Printz and *Raphidocelis subcapitata* Korshikov) obtained from the Culture Collection of Algae and Protozoa (CCAP; CEH, Ambleside, UK). Preparation of the synthetic ISO culture medium and the maintenance of algal cultures followed procedures described in ISO protocol 8692 (ISO, 1987) and in section 2.2.1. Seven different copper acclimation treatments were simultaneously started: 0.5 (without copper addition), 1, 5, 12, 35, 60 and 100 µg Cu L^{-1} (Table 5.1).

The waterflea *D. magna* Straus, which was originally collected from a pond in Kiel (Antwerp, Belgium) has been successfully cultured under standard laboratory conditions for over 15 years. This species was exposed to three different acclimation sets (Table 5.1). The first set consisted of multi-generation acclimation experiments as described by Bossuyt and Janssen (2004a) (see section 3.2.1.). Daphnids were acclimated to copper concentration of 0.5 (without copper addition), 1, 5, 12, 35 and 100. Juveniles of daphnids acclimated for six generations to 100 µg Cu L^{-1} were subsequently transferred to a 150 µg Cu L^{-1} medium and maintained for an additional five generations. The second set consisted of similar acclimation

experiments (1, 12 and 100 µg Cu L^{-1}) as the first set and was performed simultaneously with the third set in which the media had different pH, hardness and DOC levels as described in section 4.2.1. The second set will be further on referred to as the N group (N1, N12 and N100) and the third set as the M group (M1, M12 and M100) experiments.

Table 5.1: Overview of the different acclimation experiments. Copper activities are calculated with WHAM VI (Tipping, 1998).

		Culture medium						
P. subcapitata								
Acclimation concentration	µg Cu L^{-1}	0.5	1	5	12	35	60	100
Cu^{2+}	pM	0.001	0.02	1.3	13	310	4100	19000
Hardness	mg $CaCO_3$ L^{-1}				30			
DOC	mg C L^{-1}				2			
pH					7.8			
***D. magna* multi-generation**								
Acclimation concentration	µg Cu L^{-1}	0.5	1	5	12	35	100	150
Cu^{2+}	pM	0.007	0.02	0.5	4	80	3700	12900
Hardness	mg $CaCO_3$ L^{-1}				180			
DOC	mg C L^{-1}				5			
pH					7.7			
***D. magna* N group**								
Acclimation concentration	µg Cu L^{-1}	1	12	100				
Cu^{2+}	pM	0.02	4	3700				
Hardness	mg $CaCO_3$ L^{-1}		180					
DOC	mg C L^{-1}		5					
pH			7.7					
***D. magna* M group**								
Acclimation concentration	µg Cu L^{-1}	1	12	100				
Cu^{2+}	pM	90	8	0.5				
Hardness	mg $CaCO_3$ L^{-1}	180	180	125				
DOC	mg C L^{-1}	0.5	7	25				
pH		6.5	7.1	8.3				

5.2.2. Copper concentration in acclimated *P. subcapitata* and *D. magna*

Absorbed (or internal) and adsorbed copper of *P. subcapitata* were measured as described by Franklin *et al.* (2000) and reported in section 2.2.3.. These measurements were performed every week up to week 4 and subsequently every two weeks.

Measurements of absorbed and adsorbed copper in *D. magna* is described in Bossuyt and Janssen (2004a) (see section 3.2.6. and 4.2.5.). For each generation, 60 *D. magna* (3 replicates of 20 daphnids) from the different copper acclimation cultures were sampled after 40 days and rinsed with deionised water to determine the total copper concentration (adsorbed and absorbed) of the daphnids. Concurrently, another 60 (3 × 20) daphnids were rinsed with deionised water and transferred to 5×10^{-3} M EDTA during 20 minutes to remove adsorbed copper. The samples were subsequently dried in polypropylene test tubes (Laborimpex, Brussels, Belgium), weighed (Mettler H35, Zürich, Germany), digested with 14 N HNO_3, heated in a microwave and finally the copper content was determined atomic absorption spectrophotometry (AAS, see further).

The bioconcentration factors (BCF = copper concentration in tissue / copper concentration in water) were based on absorbed copper concentrations of *P. subcapitata* and *D. magna*. As all measured copper acclimation concentrations were within 10 % of the nominal ones, the latter were used to derive the BCFs. For each acclimation concentration, the BCFs of the algae - based on the weekly absorbed copper measurements - were pooled for statistical analysis. Similarly, this was done for the BCFs of the daphnids. Regressions were based on the log10 transformed BCF values and the log10 transformed acclimation concentrations.

As described in Bossuyt and Janssen (2004a) (see section 3.2.3.), chronic toxicity assays were performed with first through sixth generation acclimated daphnids. The surviving daphnids of these assays were used for absorbed copper analysis at the end of the test.

5.2.3. Time-dependent accumulation experiment with *D. magna*

Juvenile (< 24 h) daphnids of third and fourth generation of second and third set of acclimation experiments (Table 5.1, N and M group) were transferred into 2 L aquaria with 2 L of their respective acclimation medium during 21 days. The medium was renewed every

two days. The daphnids were fed a mixture of the algae *P. subcapitata* and *Chlamydomonas reinhardtii* in a 3:1 ratio daily. As the organisms grew, increasing amounts of food were supplied: from 0 to 7 days, from 8 to 15 days, and older than 15 days daphnids were fed 8 x 10^6, 12 x 10^6 and 16 x 10^6 cells day^{-1} $daphnid^{-1}$, respectively. At the start (t = 0), after 2h, 1, 2, 4 days and subsequently every two days until day 21, daphnids (n = 10) were collected. The total and absorbed copper concentrations of the daphnid samples were determined as described previously. The length (from top of the head to base of the spine) and weight of the daphnids was measured prior to the copper analysis.

At day 21, adult daphnids (fourth generation) exposed to 100 µg Cu L^{-1} (N100 and M100) were transferred to a medium with 1 µg Cu L^{-1} (N1) for 4 days. Absorbed copper concentrations were measured after 1, 2, 24, 48, 72 and 96 h. A similar experiment was performed with adults taken from 1 µg Cu L^{-1} exposure (N1 and M1) and transferred to medium containing 100 µg Cu L^{-1} (N100).

5.2.4. Copper measurements and speciation calculations

Copper concentrations in test and culture media were determined as described in section 2.2.4. All measured copper concentrations were within 10 % of the nominal concentrations. All reported copper concentrations are dissolved concentrations (0.45 µm filtered). Total, absorbed and adsorbed concentrations of the organisms were reported as µg Cu g DW^{-1}. Based on the measured copper concentrations in the culture media, copper activities were calculated using WHAM VI (Windermere Humic Aqueous Model version VI; Tipping, 1998). The resulting copper activities for the different acclimation media are presented in Table 5.1.

5.2.5. Statistical analysis

The effects of the various copper concentrations on the daphnids' accumulation response were compared by one-way analysis of variance (ANOVA) with the post-hoc Duncan's multiple range test. Homogeneity of variance and normality was tested using the Bartlett's and Kolmogorov-Smirnov's test, respectively. If ANOVA assumptions were not met, the test endpoints were compared with the non-parametric Mann-Whitney U test. Acceptance of regressions was based on the F-test. The comparison of data between generations was based

on the Student's *t* test for independent samples. Statements of significant differences were based on accepting $p < 0.05$. All statistical comparisons were performed with STATISTICA 6 (STATISTICA® software, Tulsa, OK, USA).

5.3. Results

In the experiments with *P. subcapitata*, increasing absorbed copper concentrations as a function of increasing acclimation concentrations were observed. The internal concentrations reported in Table 5.2 are representative for the values measured during the other weeks. Algae acclimated to 35, 60 and 100 µg Cu L^{-1} exhibited higher absorbed copper concentrations in the first week compared to the subsequent weeks (up to > 40 % decrease). The absorbed copper concentration of algae acclimated to 0.5 and 1 µg Cu L^{-1} did not exhibit this trend, while in those acclimated to 5 and 12 µg Cu L^{-1} even an increase in internal concentrations was noted. Week 1 can be considered as a shock week, *i.e.* a period in which the algae adjust rapidly to the elevated copper concentrations. Hence, the data of week 2 through 12 can be pooled ($n = 8$). A significant increase of absorbed copper concentrations in *P. subcapitata* is observed with increasing acclimation concentration starting from 5 µg Cu L^{-1} (Figure 5.1A). Between 1 and 5 µg Cu L^{-1}, no significant changes were noted, while algae acclimated to 0.5 µg Cu L^{-1} exhibited significantly lower absorbed copper concentrations. The absorbed copper concentration (mean ± standard deviation, $n = 8$) in the algae ranged from 5 ± 2 µg Cu g DW^{-1} to 1300 ± 197 µg Cu g DW^{-1} for algae acclimated to 0.5 and 100 µg Cu L^{-1}, respectively. The fairly constant absorbed copper concentration (mean ± standard deviation, $n = 16$) between 1 and 5 µg Cu L^{-1} was 13 ± 4 µg Cu g DW^{-1}. A linear relationship was observed between absorbed (a) and total (b) copper concentrations of the algae: $a = 0.8 \times b$ ($n = 51$; $r^2 = 0.99$; $p < 0.05$; Figure 5.2). In the BCF-acclimation concentration plot, a significant linear decrease (slope: -1; $n = 3$; $r^2 = 0.86$; $p < 0.05$) was observed up to 5 µg Cu L^{-1} (Figure 5.1B). No significant changes were observed between 5 and 60 µg Cu L^{-1} (slope = 0.3; $n = 4$, $r^2 = 0.96$, $p > 0.05$). At higher copper acclimation concentrations (100 µg Cu L^{-1}) a significant increase in the BCF value was noted.

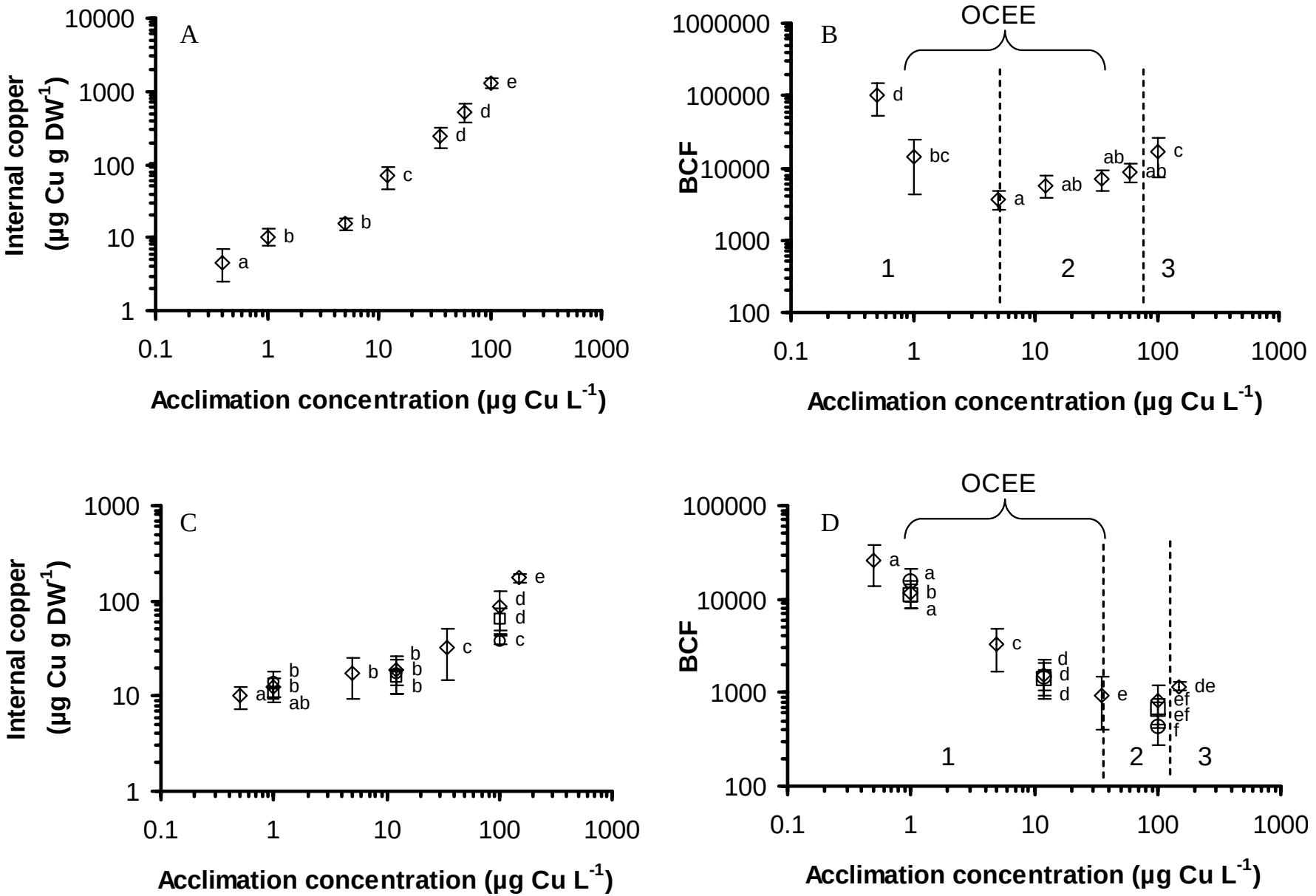
A
B
C
D
OCEE
Internal copper (µg Cu g DW-1)
BCF
Acclimation concentration (µg Cu L-1)
1
2
3

Figure 5.1 (left side): Mean (± standard deviation, n = 8) internal body concentrations (A) and bioconcentration factors (B) of *P. subcapitata* acclimated to seven copper concentrations. Mean (± standard deviation, n = 18) internal body copper concentrations (C) and bioconcentration factors (D) of *D. magna* (40 days old) acclimated to seven copper concentrations. ◇: derived from multi-generation experiment; □: derived from N group experiment; ○: derived from M group experiment. Mean values with same letter are not significantly different at $p < 0.05$. Dashed lines represents different areas; 1: active regulation; 2: storage; 3: regulation failure. The optimal copper concentration range determined by Bossuyt and Janssen (2004a, b; see chapter 2 and 3) is indicated on the graph.

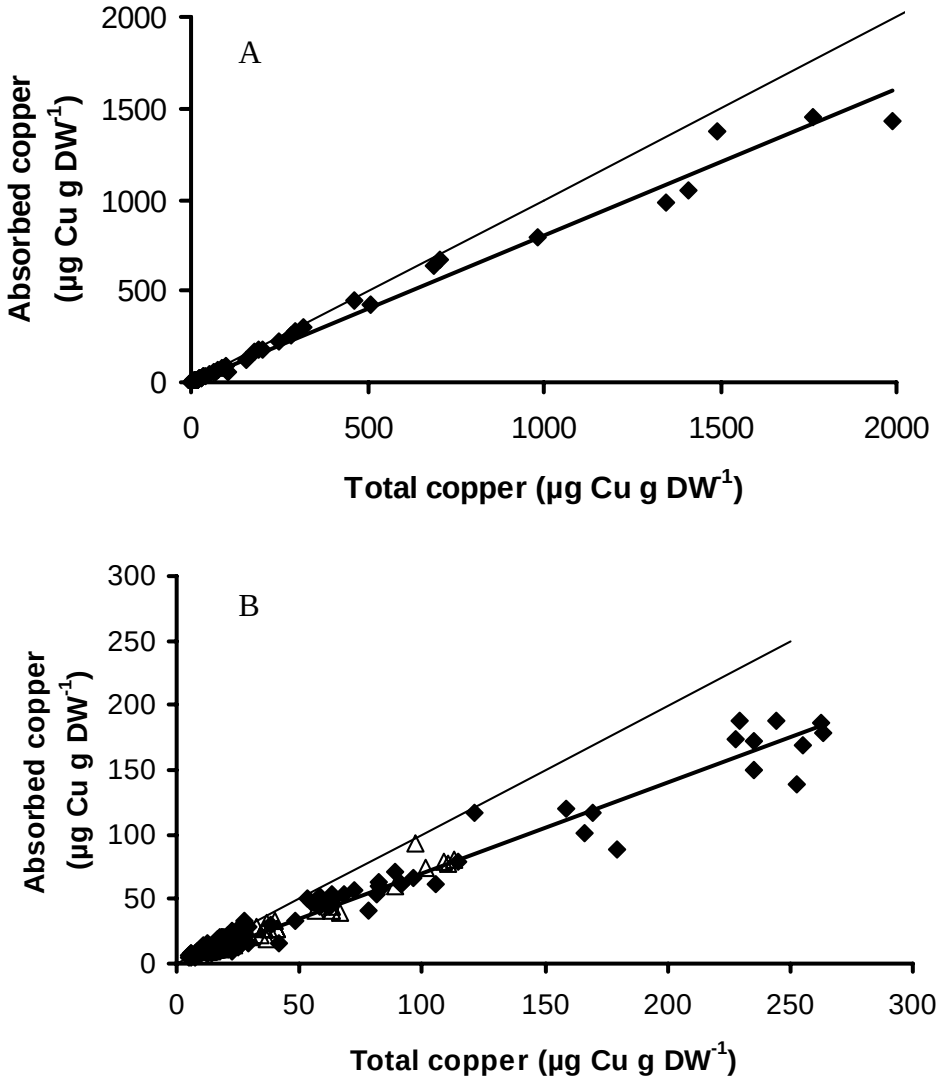

Figure 5.2: Relationship between total and absorbed copper in *P. subcapitata* (A) and *D. magna* (B). The thin black line is the 1:1 reference line. Open triangles are results of the N and M group experiments.

Table 5.2: The absorbed (internal) copper concentrations *of P. subcapitata* after 1, 3, 6, 12 weeks of acclimation to different copper concentrations.

week	**Acclimation concentration (µg Cu L^{-1})**						
	0.5	1	5	12	35	60	100
	Absorbed concentration (µg Cu g DW^{-1})						
1	9	14	14	54	278	789	3953
3	4	6	19	79	220	671	1455
6	4	11	14	82	256	440	1372
12	6	11	14	43	163	443	988

In the first set of experiments with *D. magna*, *i.e.* the multi-generation experiment, absorbed copper concentrations in *D. magna* increased significantly with increasing acclimation concentrations for each generation. The internal concentrations reported in Table 5.3 are representative for the values measured during the other generations. For daphnids acclimated to 35 and 100 µg Cu L^{-1}, a decrease in absorbed copper concentrations was observed after six generation compared to those of the first generation. After 9 generations, daphnids acclimated to 12 µg Cu L^{-1} also exhibited a decrease in internal copper concentrations compared to those of the first generation. Only daphnids acclimated to 0.5 µg Cu L^{-1} exhibited a significant increase in absorbed copper in all consecutive acclimation generations. Omitting the first generation (*i.e.* shock generation), all data of subsequent generations were pooled. A significant increase in the mean absorbed copper concentration (n = 21) of acclimated 40 day old *D. magna* was observed with increasing acclimation concentrations starting at 12 µg Cu L^{-1} (Figure 5.1C). Between 1 and 12 µg Cu L^{-1}, no changes were noted (mean ± standard deviation: 13 ± 4 µg Cu g DW^{-1}, n = 63), while absorbed copper concentrations in daphnids acclimated to 0.5 µg Cu L^{-1} tend to be lower. The body concentrations (mean ± standard deviation, n = 21) ranged from 9 ± 2 µg Cu g DW^{-1} to 175 ± 17 µg Cu g DW^{-1} for daphnids exposed to 0.5 and 150 µg Cu L^{-1}, respectively. A linear relationship was observed between absorbed (a) and total (b) copper concentrations of the daphnids: $a = 0.7 \times b$ (n = 166; r^2 = 0.97; $p < 0.05$; Figure 5.2B). A linear decrease of the BCFs as a function of the acclimation concentration was noted between 0.5 and 35 µg Cu L^{-1} (Figure 5.1D; slope –0.86; n = 5; r^2 = 0.99; $p < 0.05$). A significant increase of the BCF was observed for daphnids exposed to 150 µg Cu L^{-1} compared to that of organisms exposed to 100 µg Cu L^{-1}. No significant differences were observed between the BCFs and body concentrations of the multi-generation experiment

(diamonds) and those of the N and M experiments (circles and squares, Figure 5.1D). However, when the acclimation concentrations are expressed as copper activities, the multi-generation and N group experiments exhibit an increase in BCF and body concentration with increasing copper activity, while the M group experiments show a decrease with increasing copper activity (data not presented).

Table 5.3: The absorbed copper concentrations (mean ± standard deviation, $N = 3$) of first (F1), sixth (F6), ninth (F9) and fourteenth (F14) generation *D. magna* acclimated to different copper concentrations. Asterisk (*) denotes results that are significantly different from those of daphnids acclimated to 0.5 µg Cu L^{-1} at $p < 0.05$ (Student's *t* test for independent samples). °: denotes significant (Student's *t* test for independent samples, $p < 0.05$) differences compared to first generation daphnids (F1).

Generation	**Acclimation concentration (µg Cu L^{-1})**					
	0.5	1	5	12	35	100
	Internal concentration (µg Cu g DW^{-1})					
F1	5.5 ± 0.49	10.9 ± 0.3*	15.0 ± 3.7*	19.3 ± 0.9*	77.1 ± 6.5*	90.7 ± 15.4*
F6	12.8 ± 0.1°	17.5 ± 0.3*°	21.9 ± 0.8*°	24.0 ± 0.7*°	32.0 ± 1.3*°	61.7 ± 6.2*°
F9	8.9 ± 0.4°	10.2 ± 0.8	12.0 ± 1.2*	13.7 ± 1.2*°	14.8 ± 0.2*°	50.3 ± 4.4*°
F14	9.1 ± 2.0°	11.4 ± 1.9*	12.6 ± 2.1*	11.1 ± 0.3*°	21.6 ± 1.5*°	63.6 ± 1.6*°

In the multi-generation acclimation experiment, chronic toxicity assays were conducted with first, third, fifth and sixth generation daphnids acclimated to 100 µg Cu L^{-1} (Bossuyt and Janssen, 2004a; see section 3.3.). The absorbed copper concentrations of the surviving daphnids were measured after 21 days of exposure to the various test concentrations (Table 5.4). Body concentrations increased as a function of the test concentration. Except for the control daphnids, a decrease in the absorbed copper concentration is observed in fifth and sixth generation daphnids compared to those of the first generation.

Table 5.4: The absorbed copper concentration of first (F1), third (F3), fifth (F5) and sixth (F6) generation *D. magna* acclimated to 100 µg Cu L^{-1} and exposed for 21 days to different copper concentrations.

Generation	**Test concentration (µg Cu L^{-1})**					
	control	25	50	75	100	125
	Absorbed concentration (µg Cu g DW^{-1})					
F1	23	47	176	-	NS	NS
F3	39	66	136	-	NS	NS
F5	13	21	44	55	-	NS
F6	30	35	47	60	165	NS

NS: no survival; -: survival < 20 %.

In the time-dependent accumulation experiment, absorbed body concentrations (for third and fourth generation daphnids acclimated to three different copper concentrations; N group experiment) exhibited a decreasing trend and large fluctuations up to day 8 (Figure 5.3). From day 8 on, fairly constant body concentrations, depended on the exposure concentration, were maintained. These body concentrations were significantly lower than those measured on day 0. Similar results were observed with daphnids acclimated to the copper concentrations in the M group experiments.

Transferring adult daphnids (21 days old) of the accumulation experiment with 1 and 100 µg Cu L^{-1} to a medium containing 100 µg Cu L^{-1} and 1 µg Cu L^{-1}, respectively, resulted in a rapid change in internal copper concentrations (Table 5.5). Daphnids (N1 and M1) increased their internal copper concentration with a factor of 7.0 (N1) or 3.6 (M1) within 24 h when exposed to a copper concentration of 100 µg Cu L^{-1}. Conversely, the daphnids transferred from 100 to 1 µg Cu L^{-1} decreased their internal concentration only with a factor of 1.4 (N100) or 1.7 (M100).

Considering all data of the 21 days accumulation experiments, an inverse relationship was observed between the absorbed copper concentration (a) and the weight (b) of the daphnids (n = 306; $a = 677 \times b^{-0.6}$; $r^2 = 0.64$; $p < 0.05$). Although the daphnids (N and M group) were acclimated for four generations to different copper concentrations in different media, a similar positive logarithmic relationship was noted between length (a) and weight (b) of the differently acclimated daphnids during the 21 days exposure: $a = 0.6 \times \ln(b) - 0.1$ (n = 155; r^2

= 0.91; $p < 0.05$). Juveniles (< 24 h) had a mean length of 1.19 ± 0.05 mm and a dry weight 9.5 ± 3.3 µg DW. Adults (21 days) had a mean length of 3.59 ± 0.13 mm and a dry weight of 355 ± 40 µg DW.

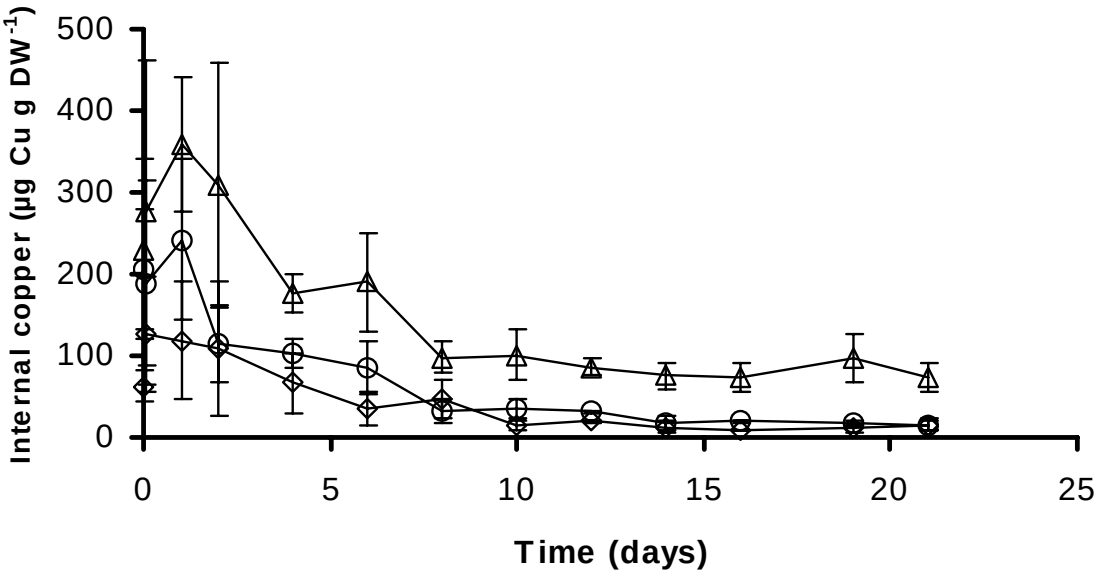

Figure 5.3: Mean (3rd and 4th generation) internal copper concentration of *D. magna* (N group experiments) acclimated to different copper concentrations and exposed during 21 days to the acclimation concentrations. ◇: 1 µg Cu L^{-1}; ○: 12 µg Cu L^{-1}; △: 100 µg Cu L^{-1}.

Table 5.5: The absorbed copper concentration of 21 day old *D. magna* (acclimated for four generations) after transfer to another medium: N1 and M1 daphnids transferred to medium with 100 µg Cu L^{-1}; N100 and M100 daphnids transferred to medium with 1 µg Cu L^{-1}.

Transfer	**Time (h)**						
	0	2	4	24	48	72	96
	Absorbed concentration (µg Cu g DW^{-1})						
N group							
1 → 100	13	34	43	91	79	68	75
100 → 1	65	46	48	47	36	36	28
M group							
1 → 100	17	27	63	61	78	83	66
100 → 1	43	51	39	26	29	28	21

5.4. Discussion

Many aquatic organisms can regulate essential metals like copper to varying degrees. Consequently, an inverse relationship between water concentrations of essential metals and the corresponding BCF is often observed (Brix and DeForest, 2000; McGeer *et al.*, 2003). This is because at low metal concentrations organisms are actively accumulating essential metals to meet their metabolic requirements. At higher water concentrations, organisms with active regulatory mechanisms are able to excrete excess metals or limit uptake (Rainbow and Dallinger, 1993). If an organism actively regulates a metal, the slope of the relationship between water concentration and corresponding BCF is expected to be near –1, while the slope is expected to be near zero in an organism which stores it. In their literature review, McGeer *et al.* (2003) recently demonstrated this phenomenon for both essential and non-essential metals. In our study, a negative slope of –1 for *P. subcapitata* up to 5 µg Cu L^{-1} (1.3 pM Cu^{2+}) and of –0.9 for *D. magna* up to 35 µg Cu L^{-1} (80 pM Cu^{2+}) was noted, indicating the presence of an active copper regulation mechanism. The results at higher copper concentrations suggest a possible copper storage mechanism. The significant higher BCF at the highest acclimation concentration (100 µg Cu L^{-1} for algae, 150 µg Cu L^{-1} for daphnids) may be related to a failure of this regulation mechanism, resulting in an excess internal copper concentrations. It can be concluded from the BCFs that *P. subcapitata* and *D. magna* can both regulate absorbed copper concentrations at environmentally relevant copper concentrations. Borgmann (2000) suggested that measurement of the body concentration of metals is a powerful tool for predicting metal effects. However, he noted that this was especially for non-essential and non-regulated metals. Hence, the accumulation-toxicity relationships, as proposed by Borgmann (2000) are not appropriate for predicting copper effects.

Bossuyt and Janssen (2004b; see section 2.3.) already observed a decreased algal biomass and growth rates in *P. subcapitata* acclimated to a copper concentration of 60 µg Cu L^{-1}. Although a strong decrease was observed during the first week of acclimation, this decrease became smaller during subsequent acclimation weeks. A final no observed effect concentration of 35 µg Cu L^{-1} was recorded. Based on these parameters, an optimal concentration range between 1 and 35 µg Cu L^{-1} (*i.e.* between 10^{-14} and 10^{-10} M Cu^{2+} based on WHAM VI calculation in the respective media), while at a concentration of 0.5 µg Cu L^{-1} lower biomass and growth rates were observed (deficiency). For *D. magna*, Bossuyt and Janssen (2003, 2004a; see section 3.3.) observed an optimal copper concentration between 1 and 35 µg Cu L^{-1} (10^{-14} to

10^{-11} M Cu^{2+}) based on reproduction, carapace length, energy reserves and filtration rates as toxicity endpoints while a negative response was observed in daphnids acclimated to 0.5 (deficiency) and to 100 µg Cu L^{-1} (toxicity). Based on these results, *D. magna* seems to be able to actively regulate the internal copper concentration within this optimum range, while in *P. subcapitata* a combination of active regulation and storage seems to occur.

The significantly lower copper body concentrations of *P. subcapitata* acclimated to 0.5 µg Cu L^{-1}, compared to those acclimated to the concentrations where no changes in body concentrations occurred (1 to 5 µg Cu L^{-1}), may indicate copper deficiency (< 13 µg Cu g DW^{-1}). Similarly, the decreased absorbed copper concentration in daphnids acclimated to 0.5 µg Cu L^{-1} compared to those acclimated to 1 up to 12 µg Cu L^{-1} (*i.e.* optimal copper concentrations; Bossuyt and Janssen, 2003) may indicate that *D. magna* needs at least 13 µg Cu g DW^{-1} to avoid copper deficiency. Both organisms thus seem to exhibit an active copper accumulation when exposed to concentrations < 1 µg Cu L^{-1}. As Bossuyt and Janssen (2003, 2004a; see section 3.3.) observed lower energy reserves in daphnids acclimated to 0.5 µg Cu L^{-1}, this seems to be evidence for the fact that active accumulation is an energy consuming process.

Bossuyt and Janssen (2004a, b; see section 2.3 and 3.3.) demonstrated that both organisms exhibited an increased copper tolerance with consecutive generations of acclimation to the highest copper concentrations, while in this study we observed that the internal copper concentrations decreased with consecutive generations. Whether acclimation results in a reduced copper uptake or an enhanced copper excretion, could not be concluded from our results. It can only be noted that daphnids acclimated for 4 generations to 100 µg Cu L^{-1} and returned to 1 µg Cu L^{-1} reduced their internal copper concentration with only a factor of two within 96 h, while daphnids acclimated to 1 µg Cu L^{-1} transferred to 100 µg Cu L^{-1} more than tripled their internal copper concentration within 4 h, indicating a possible changed copper uptake mechanism. Additionally, fifth generation daphnids exposed to copper concentrations in the chronic assay exhibited lower absorbed copper concentrations and survived at higher copper exposure concentrations compared to first generation daphnids. Hence, the BCFs are thus not good predictors of chronic toxicity (higher BCFs at lower metal concentrations) and their use in hazard and/or risk assessment of metals is not appropriate. This corroborates the findings of McGeer *et al.* (2003). However, the BCFs provided a view on different copper

regulation strategies. Additionally, the observed excess in absorbed copper confirms the observed decrease in some endpoints as described in Bossuyt and Janssen (2004a).

It could be argued that the measured absorbed copper concentrations in the daphnids consisted mainly of copper related with the undigested algae present in the gut of the organisms. Total (adsorbed + absorbed) copper concentrations of *P. subcapitata* ranged between 10^{-15} - 10^{-14} g Cu cell^{-1}. Bossuyt and Janssen (2004a; see section 3.3.) measured the filtration rates of acclimated *D. magna* (mean ± standard deviation: $2 \times 10^5 \pm 0.3$ cells ind^{-1} h^{-1}), while De Coen (1999) observed a maximal gut filling of daphnids within 30 minutes. Taken this into account, the maximal amount of copper due to algae ranged between 0.01 and 2.4 ng Cu daphnid^{-1} in the acclimation experiments of 0.5 and 100 µg Cu L^{-1}, respectively. This represents < 1 % to 20 % for daphnids exposed to 0.5 and 100 µg Cu L^{-1}, respectively. Additionally, daphnids in our study were transferred to an EDTA solution for 20 minutes so that the algae concentration in the gut tract may be reduced due to excretion. Consequently, it may be suggested that the measured absorbed copper concentrations are largely determined by copper incorporated in the soft tissues of the daphnids.

Organisms can detoxify metals by isolating them within their tissues as granules or as insoluble metal precipitates, or by excretion (Mulvey and Diamond, 1991). Some crabs and shrimps accumulate metals in their chitinous exterior, which is periodically shed as they grow and moult (Al-Mohanna and Nott, 1986). Muyssen and Janssen (2002a) found that the zinc body burdens of zinc acclimated daphnids were influenced by the moulting cycle, *i.e.* they observed a two to three day fluctuation in zinc body burden. They concluded that 38 % of the total zinc content was in and on the exoskeleton. Hall (1982) and Carney *et al.* (1986) reported that exoskeletal metal concentration of *D. magna* was 20 to 40 % of the total metal body content. In these studies, no discrimination was made between the metal incorporated in the exoskeleton and that adsorbed to it. In our experiments, daphnids were washed with EDTA, resulting in no adsorbed copper on the carapace. As observed from the linear relationship between total and absorbed copper concentrations, 30 % of the total copper concentration was situated on the carapace. As no 2 – 3 days fluctuations in absorbed copper concentrations were observed in our study, it is suggested that copper is not be excreted during the moulting.

When organisms are exposed to elevated levels of metals, low molecular-weight proteins and peptides (*i.e.* metallothioneins or phytochelatins: Roesijadi, 1992; Grill *et al.*, 1985; Ahner and Morel, 1995) are induced to bind and detoxify excess metals. These molecules are also involved in the homeostasis, storage, transport and in the metabolism of essential trace metals such as zinc and copper (Brady, 1982; Dallinger, 1993). Hence, organisms may show some degree of tolerance to the metal toxicity (Chapman and Wang, 2000). Bodar *et al.* (1990) observed increased metallothionein-like protein concentrations in *D. magna* acclimated to sub-lethal cadmium concentrations. As we observed an increased tolerance combined with increased absorbed copper concentrations, we have performed preliminary experiments aimed at determining metallothioneins using the modified mercury saturation assay (Klaverkamp *et al.*, 2000). In daphnids acclimated to 100 µg Cu L^{-1} no indication of the presence of metallothioneins was observed. Hence, further research is needed to optimize and verify this method for daphnids.

From the above, it can be concluded that the unicellular green algae *P. subcapitata*, tested in standard soft water, and the freshwater cladoceran *D. magna*, tested in standard hard water, can both actively regulate their absorbed copper concentrations up to 5 and 35 µg Cu L^{-1} (1.3 and 80 pM Cu^{2+}), respectively. Absorbed concentrations were constant between 1 and 5 µg Cu L^{-1} and between 1 and 12 µg Cu L^{-1} for *P. subcapitata* and *D. magna*, respectively. At higher copper concentrations, there was an increase in absorbed copper, indicating the presence of a storage mechanism. At copper concentrations of 100 µg Cu L^{-1} (*P. subcapitata*) and 150 µg Cu L^{-1} (*D. magna*), this storage mechanism seems to be overwhelmed as adverse effects were observed. Optimal concentration ranges for copper were within the concentration intervals in which the organism actively regulates/stores copper. Multi-generation copper acclimation resulted in a decrease of the absorbed copper concentrations in both organisms.

Chapter 6

Copper toxicity to field-collected cladoceran species: intra- and inter-species sensitivity, species sensitivity distributions and community sensitivity

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Redrafted from:

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Bossuyt BTA, Muyssen BTA, Janssen CR. (2004). Relevance of generic and site-specific species sensitivity distributions in the current risk assessment procedures for copper and zinc. Environmental Toxicology and Chemistry (submitted February 2004).

Chapter
6

Copper toxicity to field-collected cladoceran species: intra- and inter-species sensitivity, species sensitivity distributions and community sensitivity

Abstract - The acute copper sensitivity of several freshwater cladoceran species collected in six European aquatic systems was assessed. The aim of this research was to study the relative acute cladoceran community sensitivity in different aquatic systems. The collected species belonged to 4 different families (Daphniidae, Bosminidae, Macrothricidae, Chydoridae) and 13 different genera: *Daphnia*, *Ctenodaphnia*, *Ceriodaphnia*, *Simocephalus*, *Scapholeberis*, *Bosmina*, *Acantholeberis*, *Alona*, *Acroperus*, *Chydorus*, *Eurycercus*, *Disparalona* and *Pleuroxus*. To establish the comparative copper sensitivity a standard test medium (ISO) was used. The 48-h EC50s of the field-collected organisms tested in this standard laboratory water ranged from 5.3 to 70.6 µg Cu L^{-1}. One genus (*Ctenodaphnia*) exhibited significant intra-species differences (factor of 2). Among seven common genera a significant variation in copper sensitivity was demonstrated (factor of 3). Including the other genera, this raised up to a factor of 12. It was observed that most of the field-collected cladoceran species were more sensitive than the standard laboratory clone of *D. magna*. A positive relationship was noted between the cladoceran 48-h EC50 of the different aquatic systems and the ambient copper concentration. A positive relationship was also observed between the 48-h EC50 of the field-collected cladoceran species (without the Chydoridae family) and the length of the organisms. A generic species sensitivity distribution (SSD; with log-normal distribution) based on toxicity data obtained in the standard ISO medium of all species (collected at all sites) was constructed and resulted in a hazardous concentrations which protects 95 % of the species occurring in a (hypothetical) ecosystem (*i.e.* HC5) of 6.7 µg Cu L^{-1} (90 % confidence limits: 4.2 – 10.8). This generic SSD was not significantly different from the obtained site-specific SSDs (*i.e.* constructed with species only occurring at a specific site). The community sensitivity (geometric mean of 48-h EC50 values of species within a community) among sites varied within a factor of 2 (between 17.3 and 23.6 µg Cu L^{-1}), while HC5s varied within a factor of 4 for copper (between 4.5 and 17.3 µg Cu L^{-1}). The community sensitivity of our generic SSD was significantly lower than the one based on literature toxicity data of cladoceran species (which were re-calculated to the hardness of our standard medium). This research indicates that attention has to be paid when extrapolating data results from standard organisms to field situations and that copper sensitivity difference do exist between various cladoceran species. It is suggested that the community sensitivity of different cladoceran populations is similar among aquatic systems and is not dependent on the species composition.

6.1. Introduction

Cladocera, especially daphnids, are widely used in aquatic toxicology. In addition to their ecological significance, the advantages of using cladoceran species as test organisms include their short life cycle, the ease of laboratory culturing, their low space and water volume requirements, and their sensitivity to chemicals (Parkhurst *et al.*, 1981; Presing, 1981; Adema *et al.*, 1982; Baudo, 1987; Münzinger and Monicelli, 1991). Among freshwater animals, *Daphnia magna* Straus is probably the most commonly used test organism in ecotoxicological studies, although its use has been criticised by several authors (Chapman, 1983, Mount and Norberg, 1984; Forbes and Depledge, 1993; Koivisto, 1995). Indeed, *D. magna* has a limited geographical range and even within this range it is confined to small water bodies (Brooks, 1957; Koivisto, 1995). Since *D. magna* is not a common organisms in zooplankton communities, continued use of this species in research designed to formulate water quality criteria can be justified only if it can be demonstrated that *D. magna* has a sensitivity comparable to that of more widespread, important members of zooplankton communities or if its biological variance (intra- and inter-species variations) is not significantly different.

Species-to-species variation was discussed by Chapman (1983). He suggested that among other factors, the selection of the test species may influence the accuracy of toxicity predictions based on laboratory data. Several authors demonstrated that the sensitivity to metals and other compounds (organic and inorganic) within a genus is very similar (Winner and Farrell, 1976; Canton and Adema, 1978; Elnabarawy *et al.*, 1986; Versteeg *et al.*, 1997; Vaal *et al.*, 1997a). However, studies with several species (belonging to different genera) of the Daphniidae were rather ambiguous. Hickey (1989) assessed the toxicity of a variety of pure compounds and discharges to five cladoceran species (*D. magna*, *D. carinata*, *Simocephalus vetulus*, *Ceriodaphnia dubia* and *C. pulchella*). He found that at the genus level differences in acute toxicity values were small, generally less than a factor of 3 (maximum 8). The genus of *Simocephalus* and *Ceriodaphnia* were more sensitive than *Daphnia* with *C. dubia* being more sensitive than *C. pulchella*. Also Mount and Norberg (1984) and Koivisto *et al.* (1992) demonstrated that different cladoceran species can exhibit considerable differences (up to a factor of 13) in sensitivity. They observed that the sensitivity to copper of small species was higher than that of *D. magna* and *D. pulex*. Using literature toxicity data of 48 compounds (30 organic, 12 inorganic and 6 effluents) Versteeg *et al.* (1997) demonstrated that *Ceriodaphnia* sp. was on average 2.4 times more sensitive than *D. magna*, *D. pulex* or *S.*

vetulus. A major shortcoming in all these studies is the use of a limited number of species (3 to 5 species) and the fact that these mainly belong to the family of the Daphniidae. Toxicity data obtained with cladoceran species belonging to other families are scarce or non-existing (Ghosh *et al.*, 1990; Hatakeyama and Sugaya, 1989).

Several studies demonstrated that Daphniidae are more sensitive to metal toxicity than vertebrates and insects (Vaal *et al.*, 1997a, b). Additionally, they are also very sensitive to some organic substances (*e.g.* aniline, malathion, parathion; Vaal et al, 1997b) and inorganic compounds (*e.g.* chlorite; Fisher *et al.*, 2003). Variation in species sensitivity to metals is difficult to explain. Considerable differences exist between species with respect to uptake, distribution, storage, elimination, receptor sites and interaction with biomolecules. A metal may also affect the organism through more than one mode of action. Additionally, other factors such as taxonomic relationship, physical scale, acclimation/adaptation to a certain environment (background or ambient copper concentrations), etc. contribute to the observed sensitivity differences between species. Vaal *et al.* (1997b) already indicated that to gain a more thorough insight into the role of taxonomic similarity at lower hierarchical levels than phylum and class, considerable more species and taxa should be included in this type of analysis an that the limiting factor for this is the lack of useful toxicity data.

In most studies describing the ecotoxicity of chemicals to the cladoceran species, the organisms were cultured in “standard” laboratory water (mostly reconstituted water) which was probably different from the surface waters from which the organisms originated from (Winner and Farrell, 1976; Koivisto *et al.*, 1992; Mount and Norberg, 1984). Differences in water characteristics and ambient metal concentrations can lead to changes in tolerance. Bossuyt and Janssen (2003) demonstrated significant changes in acute copper tolerance of *D. magna* after long-term acclimation to various copper concentrations in the laboratory. To our knowledge, no copper toxicity studies have been performed with resident cladoceran species collected in different types of aquatic systems. As such, there is no data available on the acclimation/adaptation of resident species to the background or ambient copper concentration of these systems. Additionally, information on intra- and inter-species sensitivity differences between resident cladoceran species is also lacking. Consequently, possible differences in community sensitivities of cladoceran species have not yet been evaluated.

Because of some clear deficiencies in the derivation of environmental quality criteria, species sensitivity distributions (SSDs) are increasingly being used to complement or replace the use of arbitrary assessment factors in the risk assessment of chemicals (Posthuma *et al.*, 2002; TGD, 2003). The development of SSDs, proposed in both North America (Stephan *et al.*, 1985) and Europe (Kooijman, 1987), involve fitting a statistical distribution to data obtained from toxicity assays with various species for a particular substance. At lower risk assessment tiers, this may involve selecting a threshold level that represents a safe concentration of the substance, *i.e.* protecting most organisms (usually 95 %) in an assemblage of species (Aldenberg and Slob, 1993; Van Straalen and Denneman, 1989; Wagner and Løkke, 1991). Because the species used to construct sensitivity distributions are usually chosen from the available literature or databases, (1) they typically originate from a range of aquatic systems (sometimes freshwater and marine ecosystems are pooled for this analysis), and (2) their number and identity varies among chemicals. Therefore there is little reason to expect a consistent relationship between SSDs that have been constructed to date and toxicant impacts on natural communities or aquatic systems (Forbes and Calow, 2002).

Another problem associated with the use of literature-based SSDs is the fact that the data used to construct this SSD are obtained from toxicity assays performed using different test media. Hence, different (metal) bioavailabilities may contribute to the variation in observed sensitivities. In an attempt to take into account these differences in bioavailability, the USEPA (1996) re-calculated the observed effect concentrations to a certain hardness (*e.g.* 50 mg $CaCO_3$ L^{-1}). However, recent studies have shown that not hardness, but dissolved organic carbon (DOC) plays the largest role in copper bioavailability (De Schamphelaere *et al.*, 2002). Consequently, literature-based SSDs may not represent the true sensitivity of the (hypothetical) ecosystem. No SSDs for copper were identified in literature based on organisms tested in a single (standard) medium, *i.e.* making re-calculations unnecessary.

To increase the diversity of collected cladoceran species we sampled six European aquatic systems with different water characteristics. This research is divided in two parts. In the first part, we investigated the single species sensitivity, while in the second part community sensitivities were investigated. Consequently, the objectives of this research were to (i) determine the acute copper sensitivity of field-collected cladoceran species in a standard medium (ISO, same metal bioavailability); (ii) determine intra- and inter-species variation in copper sensitivity within and among the different genera; (iii) relate the observed 48-h EC50

values to the water characteristics of the surface waters; (iv) compare the site-specific community sensitivities, using the toxicity test results of field-collected cladoceran species originating from different aquatic systems and tested in one standard media; and finally (v) compare the different community sensitivities to the generic cladoceran SSD (based on all field-collected species) and to that derived with literature toxicity data.

6.2. Materials and methods

6.2.1. Sampling of natural water and organisms

Six unpolluted surface waters with different physico-chemical water characteristics (Table 6.1) were sampled at different locations in Europe. These surface waters encompass a representative range of the main factors affecting metal bioavailability (pH, hardness, DOC, Na, alkalinity) in Europe. This selection was based on the Surface Water Database (SWAD), a database containing the physico-chemistry of approximately 200,000 surface water monitoring stations, spread over 6 European countries (Heijerick and Janssen, 2000, updated 2003, personal communication). Samples were taken in three countries between May 2001 and October 2002 and the sampling sites were given the following labels: Ankeveen, Bokrijk, Leuven, Markermeer, Oberkirchen and Teut (Table 6.2, Figure 6.1). At each site, water samples (75 L) were collected in pre-washed (0.14 N HNO_3, 24 hours, followed by three rinses with deionised water) 25 L polyethylene vessels and transferred to our laboratory within 12 hours. The samples were stored at 4°C in total darkness.

Table 6.1: Water characteristics of the sampling sites. DOC: dissolved organic carbon; C: conductivity; T: temperature; ε_{350} = UV absorbance at 350 nm; Chl *a*: chlorophyll *a*; Cu^{2+}: calculated with WHAM VI (Tipping, 1998).

Site	Sampling	DOC	Na	Ca	Mg	K	Cl	Fe	Al	Cu	Cu^{2+}	pH	C	T	ε_{350}	Chl a
	dd-mm-yyyy	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	µg L^{-1}	pM		µS cm^{-1}	°C	L mg^{-1} cm^{-1}	mg m^{-3}
Ankeveen	14-06-2002	37.7	8.0	26.6	4.5	2.0	13.3	0.6	0.06	3.2	0.5	6.8	367	18.5	ND	1.2
	12-09-2002	27.5	7.6	19.4	4.0	1.1	16.9	0.3	0.03	9.2	3.7	7.4	412	19.0	ND	1.1
Bokrijk	22-08-2002	5.7	14.7	7.5	1.3	1.6	18.0	0.2	0.04	3.3	475	5.5	ND	18.0	ND	2.5
	01-10-2002	3.0	13.1	9.7	1.4	BDL	26.2	0.1	0.04	2.5	2966	5.0	141	11.8	0.00300	2.7
Leuven	06-07-2002	7.2	25.8	58.1	6.2	1.3	29.8	BDL	0.20	4.4	32.4	8.2	ND	17.0	ND	1.6
	23-10-2002	9.8	23.6	71.1	5.1	2.2	40.4	BDL	0.04	3.3	2.6	8.3	476	12.1	0.00520	64.7
Markermeer	26-05-2001	8.2	76.8	66.5	17.1	9.5	127	BDL	0.04	5.1	8.7	8.3	ND	ND	0.00574	ND
	24-09-2002	10.4	67.6	55.1	14.5	6.4	124	0.3	0.34	11.0	34.0	8.0	780	ND	0.00413	13.4
Oberkirchen	19-06-2002	2.3	2.9	14.9	2.9	BDL	3.4	0.1	0.09	3.2	100	7.7	120	13.6	ND	9.3
	10-09-2002	1.6	2.6	15.2	3.1	BDL	3.3	BDL	0.02	3.6	354	7.4	81	11.8	ND	BDL
Teut	22-08-2002	4.0	5.4	3.0	1.8	1.2	7.5	0.4	0.23	3.3	6084	4.3	ND	21.0	ND	3.8
	01-10-2002	3.7	5.7	2.9	1.5	BDL	13.1	BDL	0.04	2.0	3718	4.1	100	14.9	ND	80.1

ND: not determined; BDL: below detection limit (K: < 1 mg L^{-1}; Fe: < 0.05 mg L^{-1}; Chl *a*: < 1 mg m^{-3})

Table 6.2: Overview of the different sampled aquatic systems, their location and a short description.

Label	Name	Location	Description
Ankeveen	Ankeveense Plassen	Nederhorst den Berg, The Netherlands	National park consisting of grassland with ditches and ponds ± 2 m deep with organic underground Eutrophic
Bokrijk	Educational Ponds	Bokrijk, Belgium	Part of national park of Midden Limburg, surrounded with pines and reed ± 1 m deep with humic underground Oligotrophic
Leuven	Abdij van t'Park	Heverlee, Belgium	Consist of 4 connected lakes surrounded with reed, grass and foliage trees ± 2 m deep with sandy underground Mesotrophic
Markermeer	Markermeer	Marken, The Netherlands	Inland lake surrounded with grasslands 5 m deep with sandy sediment
Oberkirchen	Lüttmecke	Oberkirchen, Germany	Artificial pond (1960) with in- and outflow surrounded with hills of grassland and forest ± 2 m deep with clay sediment Oligotrophic
Teut	De Teut	Termolen, Belgium	Lake, part of national park surrounded with heath and trees ± 2 m deep with sandy sediment Eutrophic

A B

C D

E F

Figure 6.1: The different sampling sites. A: Ankeveen; B: Bokrijk; C: Leuven; D: Markermeer; E: Oberkirchen; F: Teut.

The physico-chemistry of the surface waters was characterised (Table 6.1). Concentrations of Ca, Mg, Na and K were analysed using flame emission spectrometry (ELEX 6361, Eppendorf, Cologne, Germany), Cl and SO_4 using ion chromatography (2000i/SP, Dionex, Sunnyvale, CA, USA) and DOC with a TOC analyser (TOC-5000, Shimadzu, Duisburg, Germany). The concentration of Cu, Fe and Al were measured with atomic absorption spectrophotometer (AAS, Varian, Mulgrave, Australia). The conductivity was measured with a conductivity meter (WTW Cond 315i, Weilheim, Germany). The copper activity in the different surface waters was calculated with WHAM VI (Windermere Humic Aqueous Model version VI, Tipping, 1998) based on the measured physico-chemical characteristics of these waters. Chlorophyll *a* concentration was measured according to Clesceri *et al.* (1998) with a spectrophotometer (Spectronic 601, Milton Roy, USA). Prior to analysis and use of the natural surface waters as culture and test medium for the resident cladoceran species, all surface waters were filtered over a 0.45 µm filter (Sapor® 450 membrane filter, Gelman laboratory, Ann Arbor, MI, USA) in order to remove particulate matter.

Together with the surface water, the sampled organisms were brought to the laboratory within 12 hours. These field species were collected by filtering 50 m^3 of the surface waters through a zooplankton net with a mesh size of 80 µm. During transport, the surface waters were gently aerated to prevent oxygen deficiency. The organisms were identified in the laboratory using cladoceran keys (Leentvaar, 1978; Scourfield and Harding, 1994; Flössner, 2000). The different cladoceran species were then isolated and transferred to different aquaria containing the respective surface water. These cladoceran species were kept in a temperature-controlled room (20 ± 1 °C) with a light : dark cycle of 12 : 12h, until they were used for testing (within one month). They were daily fed with 5×10^5 algal cells mL^{-1} of a mixture of *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* at a ratio of 3:1. Twice a week, the aquaria were cleaned and the respective surface water was renewed.

6.2.2. Acute toxicity experiments

Acute toxicity assays were performed following the OECD guideline 202 (OECD, 1996b) with minor modifications for the field-collected cladoceran species concerning age of the test organisms and the test volume. Next to acute assays with the field-collected cladoceran species (< 48 h old), assays with juveniles (< 24 h) of laboratory-reared *D. magna* and *C. dubia* were also performed. For each species, five replicates of five organisms were exposed

to at least five different metal concentrations and a control. Experiments were performed in standard ISO medium (nr. 6341; ISO, 1996) with a pH of 7.8, a hardness of 250 mg $CaCO_3$ L^{-1} and containing four major salts (2.0 mM as $CaCl_2.2H_2O$; 0.5 mM as $MgSO_4.7H_2O$; 0.75 mM as $NaHCO_3$ and 0.075 mM as KCl). The medium was prepared 24 h prior to the start of the assays. All chemicals were purchased from VWR International (Leuven, Belgium) and were reagent grade. Each test vessel contained 50 mL of test medium. For the smallest organisms (*Chydorus* sp., *Acroperus* sp., *Alona* sp.) multi-well plates with 2 mL wells were used. The experiments were carried out in a temperature-controlled room (20 ± 1 °C) with a light : dark cycle of 12 : 12 h. After 48 h of exposure the number of immobilized organisms in each vessel was counted and the 50 % effective concentration (48-h EC50) with its 95 % confidence limits was calculated using the trimmed Spearman-Karber method (Hamilton *et al.*, 1977). Reported 48-h EC50s are based on measured (dissolved) copper concentrations.

6.2.3. Copper measurements

Copper concentrations were determined as described in section 2.2.4. All measured copper concentrations were within 10 % of the nominal concentrations. All reported copper concentrations are dissolved concentrations (0.45 µm filtered). Copper in controls of all tests using the ISO medium were below the detection limit.

6.2.4. Length measurements

For each cladoceran species, the carapace length of 10 organisms - used in the acute assays - was measured. They were measured with the aid of a microscope equipped with an ocular micrometer ruler (Kyowa, Tokyo, Japan). The length of the daphnids was measured from the top of the head to the tip of the spine.

6.2.5. Statistics and species sensitivity distributions

SSDs were constructed based on Aldenberg *et al.* (2002). The generic SSD was established using the toxicity data of all communities collected at the different locations. As the type of distribution is of major importance determining the outcome of the SSD extrapolation, the Anderson-Darling goodness-of-fit test for normality (Aldenberg *et al.*, 2002) was applied to test the compliance of the test results to the distribution. The data were fitted to different

distributions (n = 21) commonly used in literature to construct SSDs (BestFit 2.0, Palisade, London, UK).

After evaluating the different distribution models, the community sensitivity of each site was also evaluated by assuming a log-normal distribution of species sensitivity within a community. The community sensitivity was then defined as the arithmetic mean of the log-transformed EC50 values of the species in that community (equals the geometric mean of non-transformed EC50s) or the HC50 (hazardous concentration which affects 50 % of the species in a hypothetical ecosystem). Based on the log-normal distribution the hazardous concentration that protect 95 % of the species (HC5) of a (hypothetical) ecosystem with its confidence limits can be calculated. The 90 % confidence limits of the SSD were calculated according to Verdonck *et al.* (2001) using a parametric bootstrapping method. Multiple toxicity data for the same species were summarized as arithmetic means. These values were superimposed on the SSD graphs with the Hazen plotting method (Cunnane, 1978).

Intra- and inter-species variation in sensitivity and comparison of the sensitivity of the different communities were performed by one-way analysis of variance (ANOVA) followed by the post-hoc Duncan's multiple range test. Homogeneity of variance and normality is tested using Cochran's and Kolmogorov-Smirnov's test, respectively. In case the assumptions were not met, the test endpoints where compared with the non-parametric Mann-Whitney *U* test. Regressions were based on the F-test. Statements of significant differences are based on accepting $p < 0.05$. All statistical comparisons were performed with STATISTICA 6 (STATISTICA® software, Tulsa, OK, USA).

6.3. Results

6.3.1. Single species sensitivity

Table 6.1 summarizes the water characteristics of the 6 sampling sites. A large variability in water characteristics between the sampled sites can be observed. DOC concentrations ranged from 1.6 (Oberkirchen) to 37.7 (Ankeveen) mg C L^{-1}; pH ranged from 4.1 (Teut) to 8.3 (Markermeer, Leuven); hardness ranged from 13 (Teut) to 236 (Markermeer) mg $CaCO_3$ L^{-1}. Dissolved copper concentrations ranged from 2.0 (Teut) to 11.0 (Markermeer) µg Cu L^{-1}, *i.e.* copper activities from 0.5 (Ankeveen) to 6084 (Teut) pM Cu^{2+}. No large differences in water

characteristics were observed between the two sampling periods, except for the DOC concentration and pH in Ankeveen. Chlorophyll *a* measurements revealed that three sampling stations (Leuven, Markermeer and Teut) had relative high concentrations during autumn indicating an algal bloom.

A total of 22 different field-collected cladoceran species were collected at the different sampling sites and their acute copper sensitivity was assessed (Table 6.3). Some of these species occurred at multiple sites. Hence, the total number of cladoceran organisms to be tested was 44. These species belonged to 4 different families (Daphniidae, Bosminidae, Macrothricidae, Chydoridae) and 13 different genera: *Daphnia, Ctenodaphnia, Ceriodaphnia, Simocephalus, Scapholeberis, Bosmina, Acantholeberis, Alona, Acroperus, Chydorus, Eurycercus, Disparalona* and *Pleuroxus* (Figure 6.2). Most of the cladoceran species occurred at several sampling sites. However, 50 to 70 % of the species collected at the Ankeveen and Teut sites only occurred in these waters. The control mortality in all acute toxicity assays performed in standard medium (ISO) was < 10 % for all tested species. The 48-h EC50s ranged from 5.3 (*Scapholeberis mucronata*) to 70.6 (*Disparalona rostrata*) µg Cu L^{-1}. The 48-h EC50 (mean ± standard deviation, $n = 4$) of the laboratory clone of *D. magna* was 32.0 ± 2.9 µg Cu L^{-1}, while that of laboratory-reared *C. dubia* was 11.8 ± 0.9 µg Cu L^{-1}. Almost all species had a relative sensitivity (*i.e.* the ratio of 48-h EC50 of the local species to the 48-h EC50 of the laboratory clone of *D. magna*) lower than 1, except for *D. rostrata* (Ankeveen, Teut), *Pleuroxus truncatus* (Ankeveen), *D. magna* (Markermeer) and *Chydorus ovalis* (Teut). For 9 out of 11 cladoceran species which were collected during both sampling periods, the difference in copper sensitivity was smaller than a factor of 1.3.

Table 6.3: The 50 % effective concentration (48-h EC50: mean ± standard deviation (SD)) of various field-collected cladoceran species tested in their respective natural water (field; see chapter 7) and in standard medium (ISO). WER: water effect ratio (see chapter 7); *n*: number of toxicity assays; RS. Relative sensitivity compared to that of the laboratory clone *Daphnia magna* in standard medium (mean ± standard deviation: 32.0 ± 2.9 µg Cu L^{-1}).

Site	**Species**	**Sampling**	**Field**			**ISO**					
			48-h EC50	**SD**	***n***	**48-h EC50**	**SD**	***n***	**WER**	**SD**	**RS**
		dd-mm-yyyy	**µg Cu L^{-1}**			**µg Cu L^{-1}**			**-**		**-**
Ankeveen	*Ceriodaphnia reticulata*	14-06-2002	473.2	8.1	3	13.3	1.1	3	26.8	1.8	0.4
		12-09-2002	276.7	37.1	4	17.7	1.0	3	20.3	1.8	0.6
	Daphnia longispina	14-06-2002	319.7	40.6	2	9.89	-	1	23.2	8.9	0.3
		12-09-2002	203.1	21.6	3	11.9	4.5	4	20.5	2.2	0.4
	Disparalona rostrata	12-09-2002	137.6	-	1	70.6	-	1	1.9	-	2.2
	Pleuroxus truncatus	12-09-2002	491.9	58.5	3	51.6	11.9	2	10.2	0.9	1.6
	Scapholeberis mucronata	14-06-2002	133.3	25.1	3	5.3	0.9	2	34.1	10.9	0.2
	Simocephalus exspinosus	14-06-2002	464.0	-	1	16.6	-	1	32.6	-	0.5
		12-09-2002	343.7	25.8	3	20.4	17.7	3	20.8	1.6	0.6
Bokrijk	*Acroperus harpae*	01-10-2002	18.4	5.6	3	11.5	2.6	3	1.6	0.5	0.4
	Chydorus sphaericus	01-10-2002	33.3	-	1	23.1	-	1	1.4	-	0.7
	Eurycercus lamellatus	01-10-2002	12.9	-	1	24.3	2.1	2	0.5	-	0.8
	Simocephalus vetulus	22-08-2002	9.6	-	1	29.8	-	1	0.3	-	0.9
		01-10-2002	25.0	10.4	2	10.3	0.3	2	2.4	0.9	0.3
Leuven	*Alona quadrangularis*	23-10-2002	103.6	55.6	3	28.2	2.8	4	3.2	0.7	0.9
	Ceriodaphnia dubia	23-10-2002	115.4	-	1	-	-	-	-	-	-
	Chydorus sphaericus	23-10-2002	150.0	28.8	4	20.2	2.9	3	7.3	2.6	0.6

Table 6.3 (continued)

	Daphnia galeata	23-10-2002	123.6	10.4	3	22.6	3.4	3	5.6	1.1	0.7
	Daphnia magna	06-07-2002	-	-	-	26.8	3.5	4	-	-	0.8
		23-10-2002	178.4	29.9	4	30.0	13.6	3	7.6	4.3	0.9
	Simocephalus vetulus	23-10-2002	151.8	47.2	2	18.4	11.5	2	9.2	3.2	0.6
Markermeer	*Alona* sp.	26-05-2001	173.1	53.3	3	22.7	12.2	4	8.0	4.3	0.7
	Ceriodaphnia pulchella	24-09-2002	75.8	16.0	3	12.0	5.5	3	7.6	3.7	0.4
	Ceriodaphnia quadrangula	24-09-2002	34.6	6.5	2	-	-	-	-	-	-
	Daphnia longispina	26-05-2001	76.1	22.6	3	10.0	1.7	3	7.9	3.2	0.3
	Daphnia magna	26-05-2001	170.3	48.7	3	53.2	21.5	4	5.1	0.7	1.7
		24-09-2002	208.1	59.4	3	40.6	5.4	3	4.0	1.5	1.3
	Simocephalus exspinosus	26-05-2001	128.4	14.0	3	20.7	10.2	4	8.8	3.5	0.6
		24-09-2002	121.1	28.2	3	19.1	6.0	3	6.5	1.4	0.6
Oberkirchen	*Acroperus harpae*	19-06-2002	36.9	1.5	2	14.4	3.0	2	2.6	0.7	0.5
	Chydorus sphaericus	19-06-2002	37.2	7.8	4	38.0	15.7	2	2.6	0.3	1.2
		10-09-2002	30.0	14.9	2	14.1	1.3	2	2.4	1.0	0.4
	Daphnia longispina	10-09-2002	11.0	2.5	3	11.3	1.3	3	1.0	0.2	0.4
	Simocephalus vetulus	19-06-2002	31.4	4.4	3	16.1	3.8	3	2.1	0.8	0.5
		10-09-2002	17.6	3.1	3	18.8	8.6	3	1.0	0.3	0.6
Teut	*Acantholeberis curvirostris*	01-10-2002	852.9	337.9	4	11.9	4.6	4	74.4	20.3	0.4
	Acroperus elongatus	22-08-2002	69.4	13. 8	3	15.2	2.5	3	4.6	0.5	0.5
		01-10-2002	22.6	-	1	17.1	-	1	1.3	-	0.5
	Bosmina longirostris	01-10-2002	135.1	90.4	3	9.2	3.8	3	13.7	6.4	0.3
	Ceriodaphnia pulchella	01-10-2002	139.2	45.6	3	16.4	6.9	3	8.7	1.2	0.5

Chydorus ovalis	01-10-2002	162.3	27.8	3	33.4	11.7	3	5.2	1.6	1.0
Disparalona rostrata	01-10-2002	136.8	129.9	2	43.3	0.1	2	3.2	3.0	1.4
Scapholeberis microcephala	22-08-2002	108.3	13.8	2	11.2	2.7	2	10.1	3.6	0.4
	01-10-2002	110.3	-	1	20.3	-	1	5.4	-	0.6

-: could not be calculated.

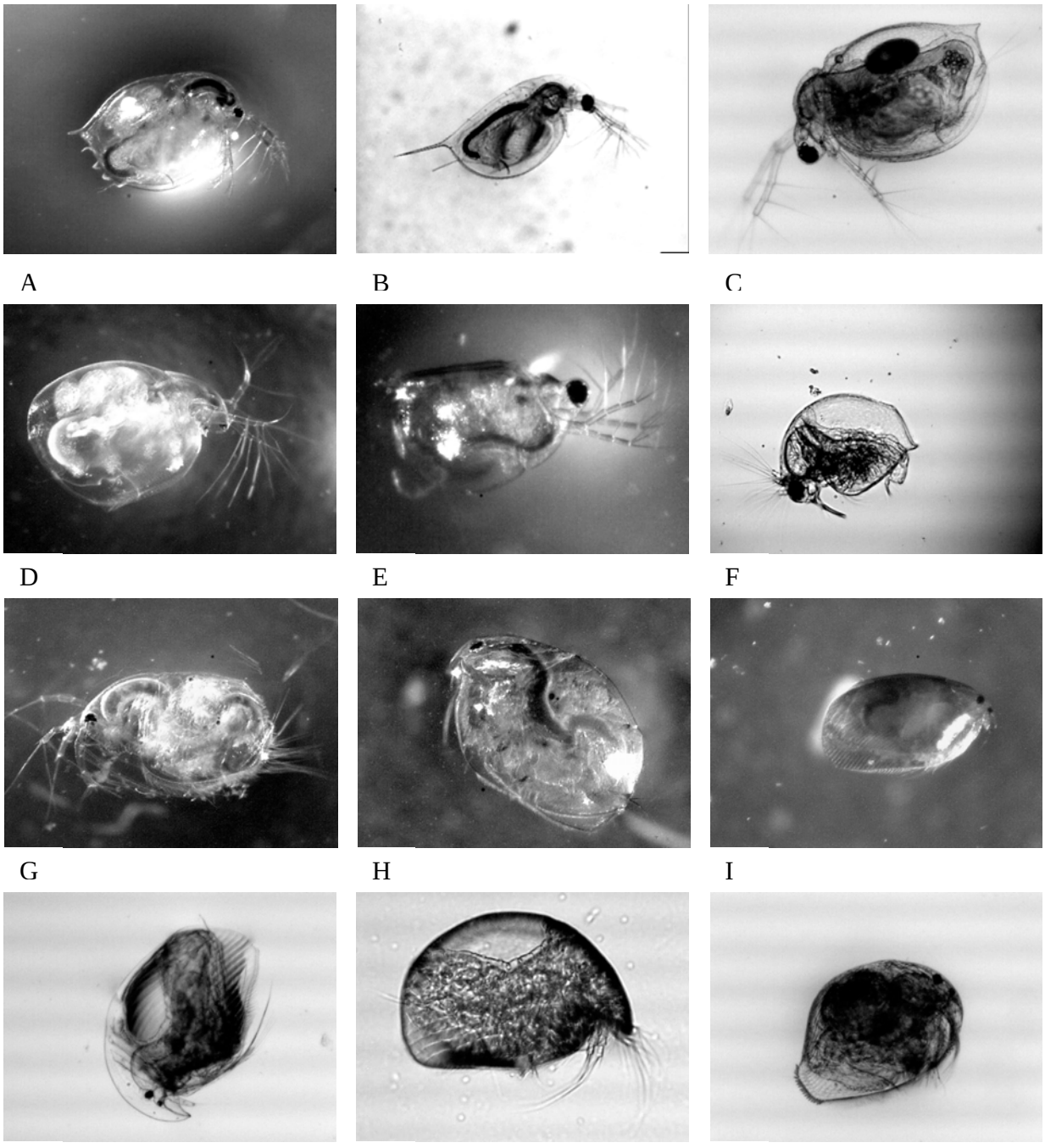

Figure 6.2: Picture of several field-collected cladoceran species. A: *Daphnia magna*; B: *Daphnia longispina*; C: *Ceriodaphnia pulchella*; D: *Simocephalus vetulus*; E: *Scapholeberis microcephala*; F: *Bosmina longirostris*; G: *Acantholeberis curvirostris*; H: *Eurycercus lamellatus*; I: *Acroperus elongatus*; J: *Acroperus harpae*; K: *Alona quadrangularis*; L: *Pleuroxus truncatus*; M: *Chydorus sphaericus*. Magnification: A, B, D, G, H: ×10, C, F, I, J, K, L, M: × 20.

Figure 6.3 shows the variation in copper sensitivity within a genus (or sub-family for Aloninae). No statistically significant differences within the genus of *Ceriodaphnia* and *Simocephalus,* collected at different sites, were noted. Also no significant difference in copper sensitivity was observed between the laboratory clone of *C. dubia* and the *Ceriodaphnia* sp. collected in the field. Within the *Daphnia* genus, the sensitivity of *D. longispina* collected at different sites (Ankeveen, Markermeer, Oberkirchen) was not different. Another *D. longispina* clone was collected from the Rhine (Germany) and tested. The 48-h EC50 (mean ± standard deviation, n = 3) was 10.1 ± 1.0 µg Cu L^{-1}, which was not significantly different from the mean value for *D. longispina* (11.0 ± 2.6 µg Cu L^{-1}). *D. magna* collected from Markermeer seemed to have a significantly higher 48-h EC50 compared to that of the laboratory clone and the Leuven site. In the sub-family *Aloninae* sp. there seemed to be small differences in inter-genus sensitivity. Both *Alona* sp. (collected in Leuven and Markermeer) had higher 48-h EC50 than the *Acroperus* sp. (collected in Bokrijk, Oberkirchen and Teut). Within these genera, no significant differences were noted. No statistics could be performed with *Chydorus* sp. due to low number of toxicity assays which could be performed with organisms from Bokrijk and Oberkirchen (n < 3). *C. ovalis* from Teut had a higher 48-h EC50 than *C. sphaericus*.

Figure 6.4 shows the inter-genus sensitivity of the most frequently occurring (n > 3) resident cladoceran species in this study. Because no significant differences were observed within the genera of *Acroperus* sp, *Alona* sp., *Ceriodaphnia* sp., *Simocephalus* sp. and *Chydorus* sp., the different species within a genus were pooled. Similarly, this was also performed for *Daphnia* and *Ctenodaphnia*, keeping in mind that there were small significant differences within the genus/species. The 48-h EC50 (mean ± standard deviation) of the different genera ranged between 13.5 ± 5.5 (n = 14) µg Cu L^{-1} and 36.6 ± 14.2 (n = 18) µg Cu L^{-1} for *Daphnia* and *Ctenodaphnia*, respectively. Other species like *Acantholeberis curvirostris* (Teut), *Bosmina longirostris* (Teut) and *Scapholeberis* sp. (Ankeveen, Teut) had a mean 48-h EC50s lower than 15 µg Cu L^{-1} (down to 5.3 ± 0.6 µg Cu L^{-1} for *S. mucronata*). *D. rostrata* (Ankeveen, Teut) and *P. truncatus* (Ankeveen) had significantly higher 48-h EC50 values compared to the other field organisms.

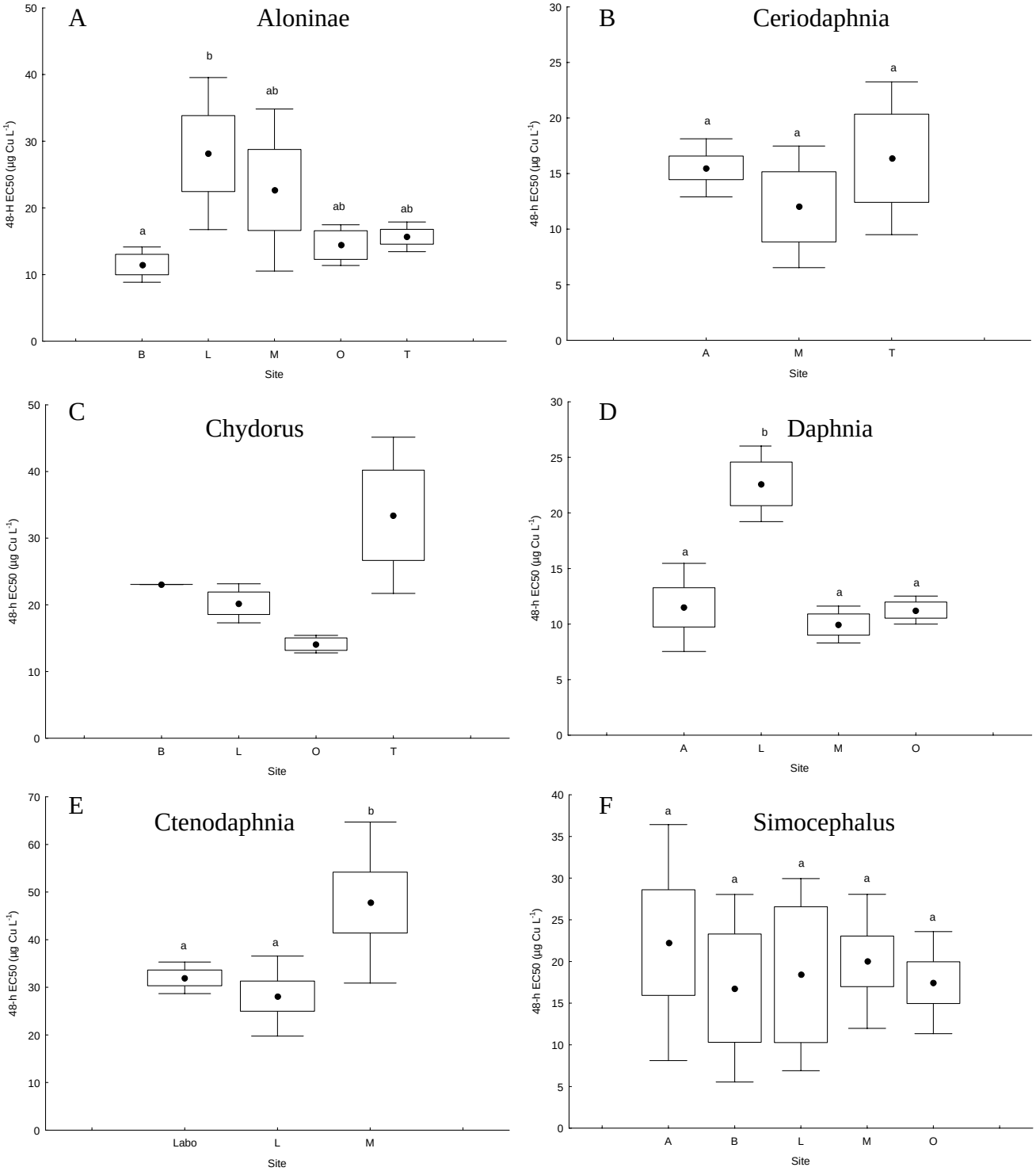

Figure 6.3: Intra-species acute copper sensitivity of 6 different cladoceran genera. For the Aloninae, two genera were included in the figure. Black dots represent the arithmetic mean, the boxes the standard error with standard deviation. Values with same letter are not significantly different (ANOVA, $p < 0.05$). Sites are indicated on the X-axis: A: Ankeveen; B: Bokrijk; L: Leuven; M: Markermeer; O: Oberkirchen; T: Teut; Labo: laboratory clone.

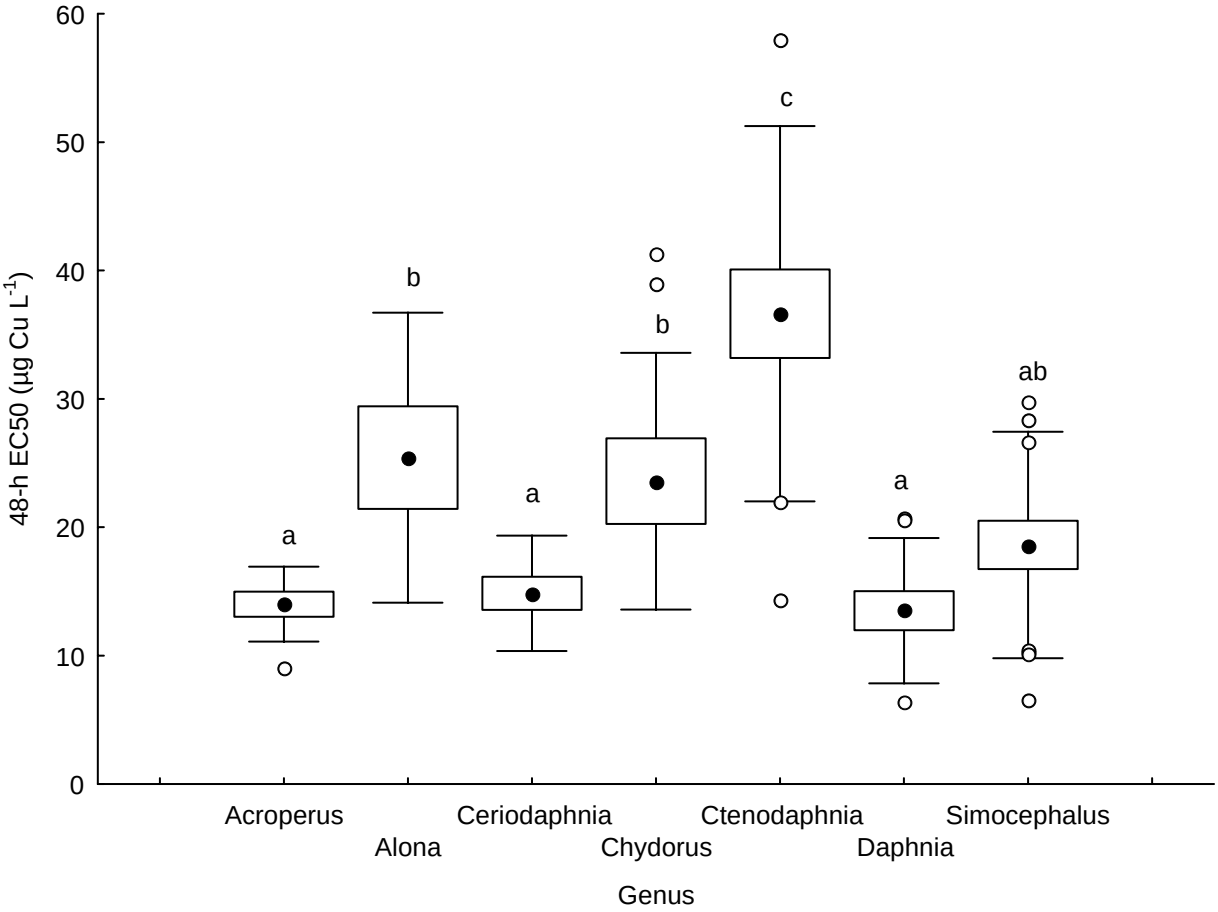

Figure 6.4: Inter-genus acute copper sensitivity of the field-collected cladoceran species. Black dots represent the arithmetic mean, the boxes the standard error with standard deviation and open dots represent the outliers. Values with same letters are not significantly different (ANOVA, $p < 0.05$).

To compare the observed 48-h EC50s with the water characteristics of the different surface waters, the EC50 values of the different species of an aquatic system were pooled (based on log transformed values, *i.e.* geometric) to generate a mean cladoceran EC50 of that aquatic system. A weak increasing trend between the mean cladoceran EC50 of the aquatic systems and the ambient copper concentration was observed ($r^2 = 0.35$, $p = 0.07$, $n = 10$; Figure 6.5). No increasing trend was noted with increasing copper activities. The other water characteristics showed no relationship with the EC50s ($r^2 < 0.2$).

No relationship was observed between the cladoceran EC50 and the chlorophyll *a* concentration in the surface waters, indicating that the food availability did not alter the copper sensitivity of the various cladoceran species.

Concurrently with the acute toxicity assays, the length of the used field-collected cladoceran species was measured. The length ranged between 0.3 (*C. ovalis*) and 1.5 mm (*D. magna*). No relationship was noted between all 48-h EC50s and the length of the cladoceran species (Figure 6.6). However, when the toxicity values of the Chydoridae (open squares) were omitted, a significant positive linear relationship was observed (r^2 = 0.43, p = 0.02, n = 12).

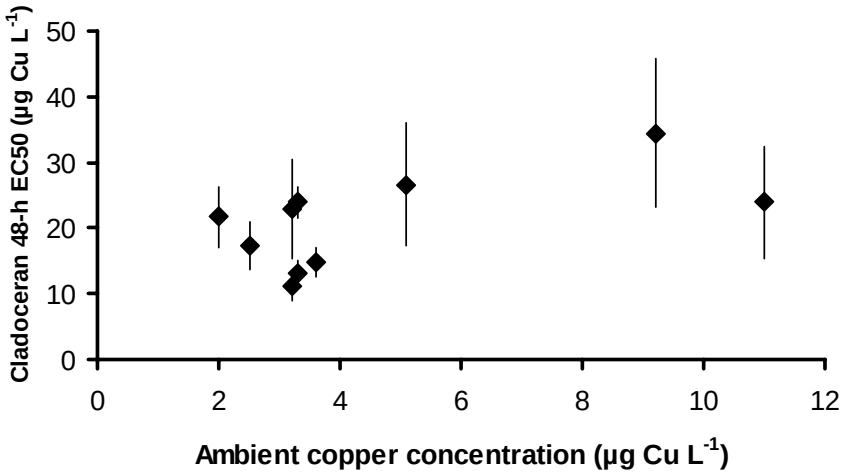

Figure 6.5: Relation between the ambient copper concentration and the mean cladoceran sensitivity of the different aquatic systems. Error bars represent the standard error.

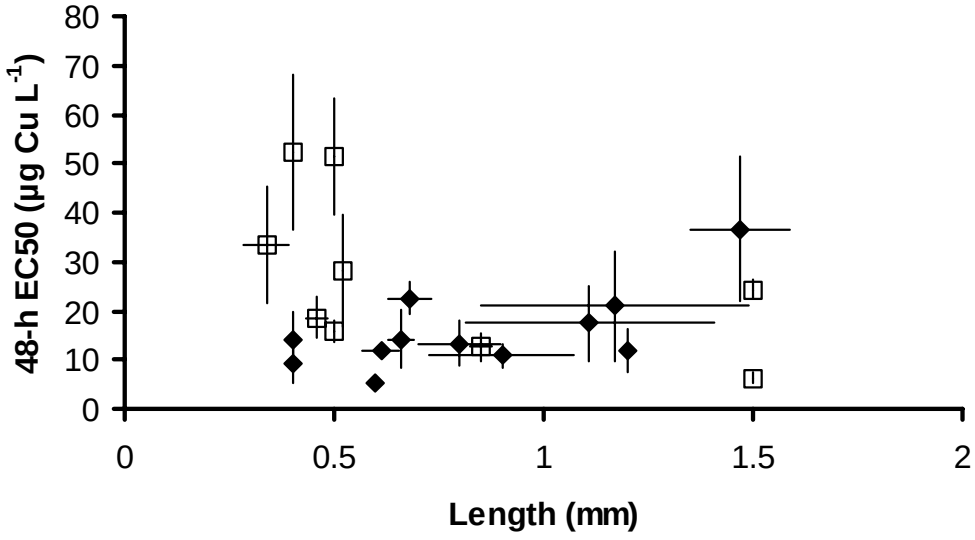

Figure 6.6: Relationship between the size and the observed 48-h EC50 of the field-collected cladoceran species. Error bars represent the standard deviation. ☐: the family of Chydoridae; ◆: other cladoceran species.

6.3.2. Community sensitivity

The generic acute SSD (*i.e.* based on toxicity data obtained in a single standard test medium) for copper in ISO medium was constructed using toxicity data of 22 cladoceran species (Figure 6.7). The generic copper SSD also included the species *Eurycercus glacialis*, obtained from an additional site (Bihain, Belgium) and tested in ISO medium (mean 48-h EC50 with standard deviation: 6.3 ± 0.8). *D. magna* was one of the least sensitive species, while *C. dubia* was one of the most sensitive. Of 21 distributions tested, the Anderson-Darling test ($p > 0.15$) indicated that the log-normal distribution provided the best fit. The distributions most frequently used in literature and which resulted in the best fit for our data are summarized in Table 6.4. The acute HC5 with its 90 % confidence limits based on the log-normal distribution is 6.7 µg Cu L^{-1} (4.2 - 10.8). The construction of the generic SSD based on other distribution models resulted in up to a factor of 3.0 difference in HC5 values (Table 6.4). Based on the HC50, this difference was only a factor of 1.6.

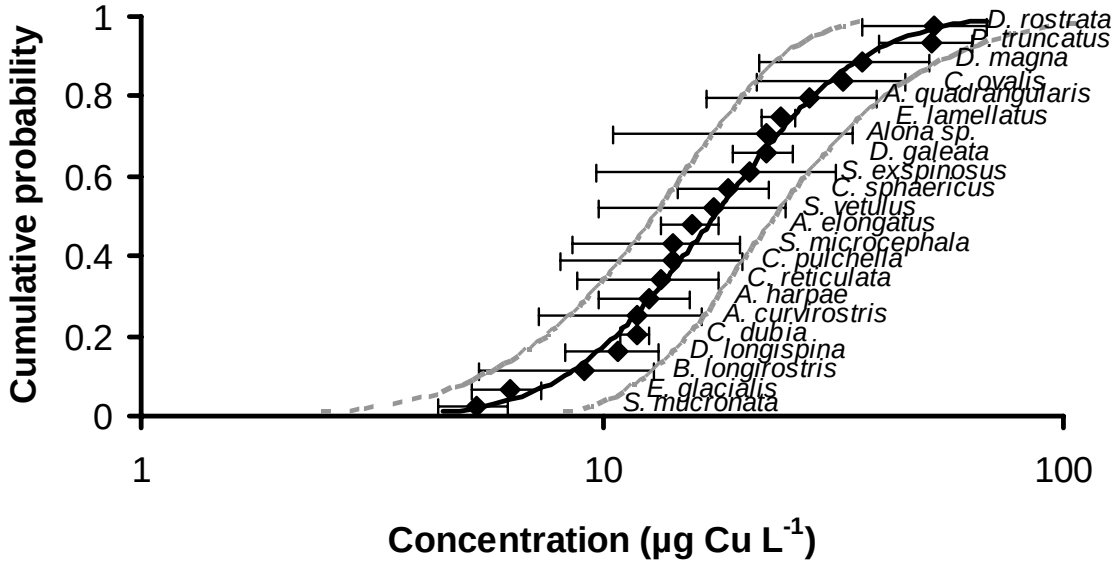

Figure 6.7: Generic species sensitivity distribution (log-normal distribution) based on field-collected cladoceran species tested in standard medium with 90 % uncertainty limits. ◆: acute toxicity data points with standard deviation.

Table 6.4: The different distributions that resulted in the best fit for the toxicity data of field-collected cladoceran species in a standard medium (with their rank number of best fit according to the Anderson-Darling test) and the effect on the calculated HC5 and HC50 (i.e. the hazardous concentrations which protect 5 and 50 % of the species in the hypothetical ecosystem).

Distribution	**Copper (µg Cu L^{-1})**		
	Rank	HC5	HC50
Log-normal	1	6.7	18
Log-logistic	6	11	18
Triangular	5	9.4	22
Uniform	7	7.7	29
Weibull	3	4.2	19
Beta	4	5.6	18
Extreme value	2	3.7	19

Using the log-normal distribution, SSDs based on the toxicity data of the resident species tested in standard medium for each sampling site were constructed (Ankeveen, Bokrijk, Leuven, Markermeer, Oberkirchen and Teut; Figure 6.8). The community sensitivity of the different sites is reported in Table 6.5. No significant difference (Student's *t* test for independent samples, $p > 0.05$) in community sensitivity between the data of the two sampling periods was observed. Hence, summer and autumn data were pooled for Ankeveen, Markermeer and Oberkirchen.

No significant differences were observed among the site-specific community sensitivities for copper. The HC50s ranged from 17.3 to 23.6 µg Cu L^{-1}. In other words, a maximal difference in community sensitivity of ≤ 2 was noted. The HC5s (with 90 % confidence limits) of the different aquatic systems were 4.5 (1.6 – 11.9), 10.8 (6.8 – 17.0), 17.3 (13.7 – 21.4), 7.5 (3.6 – 14.8), 10.8 (8.4 – 13.7), 7.5 (4.3 – 12.7) µg Cu L^{-1} for Ankeveen, Bokrijk, Leuven, Markermeer, Oberkirchen and Teut, respectively. Hence, the difference in HC5s between the different aquatic systems was 3.8. As an indication of the slope of the site-specific SSD, the ratio of the HC50 and the HC5 is calculated: this ranged from 1.4 (Leuven) to 4.0 (Ankeveen). The lower this value, the steeper the slope. However, according to the ANOVA assumptions, no significant differences (Cochran's test, $p > 0.05$) were observed between the

variances of the mean species EC50s (which is a measure of the slope of the log-normal distribution) of the different sites. From Table 6.5, it can also be noted that the site-specific SSDs are not significantly different from the generic one.

Based on all toxicity data obtained with the field-collected cladoceran species summarized in the generic SSD, we simulated the influence of species composition on the value of HC5. Random samples of 4, 5 or 6 species (1000 iterations) were taken and assumed that these species represented a real aquatic system. The factor difference of the highest to the lowest HC5 in this simulation ranged from 4 (systems with 6 species) to 10 (systems with 4 species). Compared to the observed HC5s of the different aquatic systems, a factor difference of 2 to 9 was observed. However, no significant differences were noted between the "simulated" SSD (based on a random selection of species) and the observed ones obtained for the 6 sites (Mann-Whitney *U* test, $p < 0.05$).

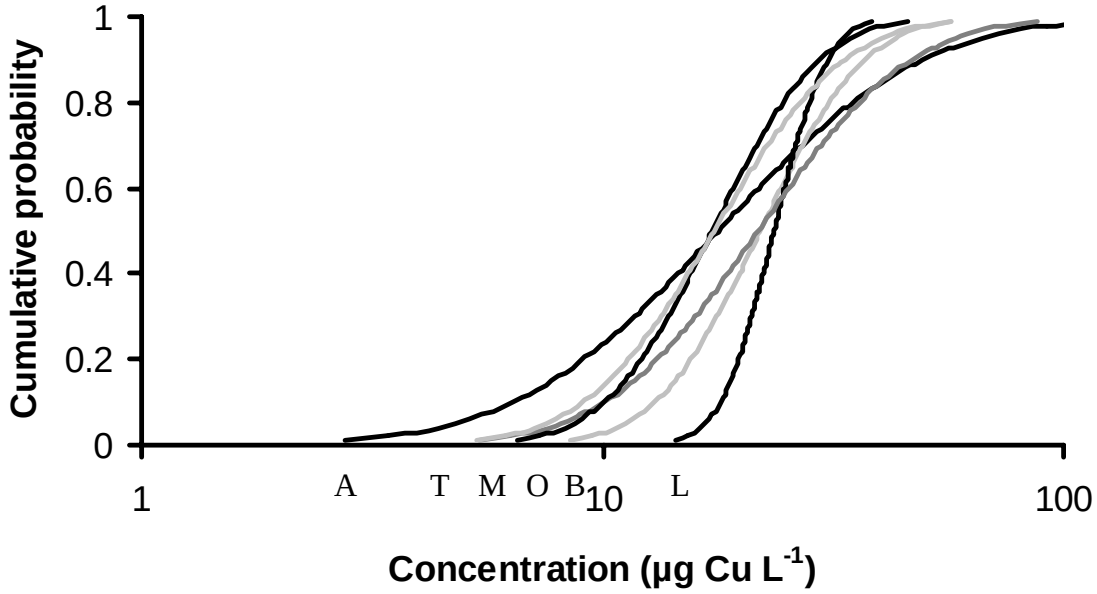

Figure 6.8: The calculated (log-normal) site-specific species sensitivity distributions of the 6 different sites based on their resident cladoceran species. O: Oberkirchen; L: Leuven; B: Bokrijk; T: Teut; M: Markermeer; A: Ankeveen.

As there was no difference in community sensitivity between the two sampling periods, the observed HC50s of the two sampling moments were pooled together and, based on the complete cladoceran system, seemed to exhibit an increasing trend with increasing calcium

(r^2 = 0.94, p = 0.006, n = 5) and sodium (r^2 = 0.45, p = 0.22, n = 5) concentration of the different aquatic systems. No such trend was observed with measured ambient copper concentration (r^2 = 0.11) and all other water characteristics (Na, pH, DOC and hardness). However, if the sampling moments were taken separately, no linear relationship was observed between the HC50s and the different water characteristics ($r^2 < 0.2$), except with the ambient copper concentration (r^2 = 0.35, p = 0.07, n = 10, cf. above). No relationship was observed between the HC5s and the measured water characteristics.

Table 6.5. The parameters of the log-normal distribution (mean and standard deviation (SD) of the natural logarithmic values of the 48-h EC50s) fitted to the toxicity data for each site separately and to the toxicity data of all field-collected species (generic). Value between brackets represents the community sensitivity in µg Cu L^{-1}.

Site	**Copper**	
	Mean	**SD**
Ankeveen	2.88 (17.8)	0.80
Bokrijk	3.09 (20.9)	0.41
Leuven	3.16 (23.6)	0.21
Markermeer	3.08 (21.8)	0.60
Oberkirchen	2.85 (17.3)	0.42
Teut	2.86 (17.5)	0.51
Generic	2.93 (18.7)	0.60

6.4. Discussion

6.4.1. Single species sensitivity

In aquatic toxicity testing, the most frequently used standard invertebrate species are *D. magna*, *D. pulex* and *C. dubia*. Several reports have demonstrated that differences in sensitivity exist among these cladocerans (Winner and Farrell, 1976; Mount and Norberg, 1984; Elnabarawy *et al.*, 1986; Hatakeyama and Sugaya, 1989; Koivisto *et al.*, 1992; Zou and Bu, 1994; Magliette *et al.*, 1995; Mark and Solbé, 1998). No reports were identified in which

the metal sensitivity of several field-collected cladoceran species was compared based on tests performed in a single standard medium. As can be seen in Figure 6.7 and Table 6.3, *D. magna* is one of the least sensitive cladoceran species tested in this study. *C. dubia* is, in contrast, one of the most sensitive species. This is in agreement with previous studies with laboratory-reared organisms, in which the sensitivity of *Ceriodaphnia* sp. was compared with that of *D. magna* (Mount and Norberg, 1984; Elnabarawy *et al.*, 1986; Magliette *et al.*, 1995). Winner and Farrell (1976) observed a significantly higher acute copper sensitivity (factor of 1.3) of *D. parvula* and *D. ambigua* compared to *D. magna* or *D. pulex*. Elnabarawy *et al.* (1986) noted 48-h EC50 values (mean with 95 % confidence intervals) of 41 (37 – 49), 31 (30 – 34) and 23 (22 – 26) µg Cu L^{-1} for *D. magna*, *D. pulex* and *C. reticulata*, respectively. Hickey (1989) found that *S. vetulus*, *C. dubia* and *C. pulchella* were more sensitive than *Daphnia* sp. (factor of 3 to 8). Koivisto *et al.* (1992) noted 48-h EC50 of 1.4, 3.3, 4.1, 3.4 and 11.3 µg Cu L^{-1} for *B. longirostris*, *C. sphaericus*, *D. galeata*, *D. pulex* and *D. magna*, respectively. The cladoceran species tested in our study, revealed a factor of 12 difference in copper sensitivity, although this difference was within the family of the Daphniidae limited to a factor of 7 and within the genus of *Daphnia* only factor of 3. This is in line with the results of the above mentioned studies.

From Figure 6.3E it is clear that the *D. magna* species collected at Markermeer had a significantly higher 48-h EC50 (factor of 1.7) than the laboratory clone or the one collected at the Leuven site. Bossuyt and Janssen (2003, 2004; see chapter 3) demonstrated that *D. magna* can acclimate/adapt to environmentally relevant copper concentrations and observed an increased acute copper tolerance of *D. magna* when acclimated to copper concentrations ≥ 12 µg Cu L^{-1} (4 pM Cu^{2+}). At acclimation concentrations lower than 12 µg Cu L^{-1}, no increase in copper tolerance was observed. In our study, the laboratory clone was cultured in a standard medium with 7 µg Cu L^{-1} (< 0.1 pM Cu^{2+}), suggesting that the daphnids exhibited no increased copper tolerance.

From Table 6.1, it can be seen that the copper concentrations in Markermeer can reach up to 11 µg Cu L^{-1}, while in Leuven this reached up to 4.4 µg Cu L^{-1}. However, for both sites, the copper activity in the surface water was similar and ranged between 2 and 34 pM Cu^{2+}. No significant differences were observed between copper sensitivity of *D. magna* at the sampling moments: 26.8 ± 3.5 and 30.0 ± 13.6 µg Cu L^{-1} for *D. magna* collected in Leuven with a

measured ambient copper concentration of 4.4 (32.4 pM Cu^{2+}) and 3.3 (2.6 pM Cu^{2+}); 53.2 ± 21.5 and 40.6 ± 5.4 µg Cu L^{-1} for *D. magna* collected in Markermeer with a measured ambient copper concentration of 5.1 (8.7 pM Cu^{2+}) and 11.0 (34 pM Cu^{2+}). Based on this, it could be suggested that acclimation/adaptation is better related to the dissolved copper concentration than to the copper activity of the surface waters. This corroborates with the results of Bossuyt *et al.* (submitted, see chapter 4).

Although not significant, an increase could be observed in the copper tolerance of *C. pulchella* collected at Teut (6000 pM Cu^{2+}, *i.e.* 3.3 µg Cu L^{-1}) compared to those collected in Markermeer. However, Potts and Freyer (1979) demonstrated that organisms living in acidic waters can better regulate their sodium uptake/loss resulting in a higher copper tolerance. No significant increase in copper tolerance was observed in other species collected at the Markermeer site (*D. longispina*, *Alona* sp., *S. exspinosus*), although a positive linear relationship was observed between the mean cladoceran EC50s of the aquatic systems and the ambient copper concentration present in the surface waters ($r^2 = 0.35$, $p = 0.07$). Muyssen and Janssen (2002b) noted that acclimation of *C. dubia* to zinc was very limited and occurred only at low zinc concentrations, compared to a similar study with *D. magna* (Muyssen and Janssen, 2001b). Hence, it is possible that various cladocerans/organisms may react differently to chronic copper exposure resulting in different copper tolerances. Brix *et al.* (2001) related this to three primary reasons, *i.e.* homeostatic regulatory strategies for copper, membrane permeability and allometric consideration.

However, the authors are aware that as a result of adaptation (*i.e.* genetic changes or species selection), different clones can occur within the same and in different aquatic systems. These clonal differences may result in sensitivity differences of to a factor of 10 as demonstrated for cadmium by Baird *et al.* (1991). In experiments with a Belgian, a German (IUCT) and an Italian clone of *D. magna* exposed to copper, we noted a sensitivity difference of a factor of 6 (Bossuyt *et al.*, unpublished data). Muyssen *et al.* (2002) also reported clonal differences in zinc toxicity to *D. magna*. Further research will be necessary to differentiate adaptation and acclimation in the field.

Although in the present study the cladocerans were collected at various sampling sites having water characteristics which were clearly different from those of the test medium, the control mortality in the acute toxicity assays was always < 10 %. It could be hypothesized that all

species of a certain aquatic system exhibited a similar stress when tested in the standard medium. However, the different species of an aquatic system did not exhibit similar relative sensitivities: they ranged between 0.2 and 2.2 for Ankeveen, 0.3 and 0.9 for Bokrijk, 0.6 and 0.9 for Leuven, 0.3 and 1.7 for Markermeer, 0.4 and 1.2 for Oberkirchen and 0.3 and 1.4 for Teut. Hence, it is suggested that the standard medium used in our study resulted in no or a similar additional stress to the field-collected cladoceran species. However, the standard medium used in this study (ISO, 1996) maximises bioavailability and thus, in risk assessment terms, represents a worst case condition. Indeed, the medium does not contain any DOC (except background of 276 ± 104 µg C L^{-1} as assumed by De Schamphelaere and Janssen, 2002), and consist only of four salts. Of the six sites, the Teut site with its low pH, low hardness and low DOC (viz. Teut, Table 6.1), resulting in a high copper bioavailability could be considered as the most sensitive. However, here, specific species, adapted to these conditions may occur. *A. curvirostris*, *S. microcephala* and *C. ovalis*, for example, are three species that are typical for waters with pH between 4.0 and 5.6 and low hardness (< 15 mg $CaCO_3$ L^{-1}) (Leentvaar, 1978; Flössner, 2000). Potts and Freyer (1979) demonstrated that *A. curvirostris* has a higher affinity for sodium ions to compete with this acidity and hence retains this ion better than *D. magna* (mostly present in hard waters with a high pH). Grosell *et al.* (2002) already indicated that sodium loss is one of the most important effects of acute copper toxicity.

Grosell *et al.* (2002) demonstrated that smaller animals (*e.g.* daphnids) are more sensitive than large animals (*e.g.* fish) because they exhibit higher sodium turnover rates. They postulated that the same relative inhibition of sodium uptake results in a faster depletion of the internal sodium in animals with higher sodium turnover. Hence, they found an increase in copper toxicity values (*i.e.* less sensitive) with increasing body mass. We tested this hypothesis using the results of the present study and observed no relationship between the size of the field-collected cladoceran species and their copper sensitivity. However, when the toxicity data of the family of the Chydoridae was omitted, a positive relationship between the cladocerans size and their sensitivity was observed. Koivisto *et al.* (1992) reported also a higher copper sensitivity of smaller organisms. However, in their study *C. sphaericus* exhibited a higher 48-h EC50 (factor of 2) value than *B. longirostris*, which is a larger organism. Similar findings were done in our toxicity data. The family of the Chydoridae consist mainly of sediment dwellers, and *D. rostrata* even lives in the sediment and detritus layer (Flössner, 2000). All other cladoceran species are mainly euplanktonic species. It is suggested that different

bioavailability modifying mechanisms may be present in these epibenthic organisms. Further research is required to elucidate these observations.

6.4.2. Community sensitivity

As reported in literature, different distributions can be fitted to toxicity data for deriving an SSD. Log-normal, log-logistic, triangular, uniform, exponential, Weibull, etc. are frequently used (Versteeg *et al.*, 1999; Newman *et al.*, 2000; Brix *et al.*, 2001; van Straalen, 2002). However, several users of the SSD approach assume a distribution without testing whether it provides a good fit to the data (Steen *et al.*, 1999). Brix *et al.* (2001) noted that cladocerans exposed to copper are clearly the most sensitive taxonomic group and they assumed that the cladoceran-based SSDs fits a log-logistic distribution. In our study, the acute toxicity data for copper (n = 22) obtained with the various cladoceran species tested in the standard ISO medium fitted a log-normal distribution best. Compared to this distribution, the log-logistic, uniform and triangular distribution gave over-estimation in the lower tail. In contrast, the Weibull, beta and extreme value distribution gave under-estimation in the lower tail. As reported by Forbes and Calow (2002), the choice of distribution is important since the differences among the most commonly employed distributions are largely in their tails, and it is there where the critical effect concentration is estimated. Although the data for copper were best fitted with a log-normal distribution, it should be considered that this metal is essential for all living organisms; this is not taken into account by this function (and other frequently used distributions). Van Straalen (2002) proposed a threshold model, which incorporates a true no-effect level, for SSDs based on essential metals. He suggested that this is a better principle for environmental protection in comparison to the approach based on “95 % protection” and concluded that the 5 % level can hardly be distinguished from the 0 % level.

The second problem with literature-based SSDs is that the toxicity data summarized in this SSD are obtained from tests in which different standard or natural waters are used as test medium. This results in a confounded SSD as the observed variation in sensitivity are both due to the species differences and metal bioavailability differences. Indeed, physico-chemical water characteristics (pH, DOC, hardness) are important in determining acute and chronic toxicity of metals. In some risk assessment practices and for some metals, an attempt is made to account for the influence of water hardness on toxicity data by re-calculating all data to a certain hardness using a correction factor (USEPA, 1996). However, at least for copper, DOC

is the most important parameter affecting the toxicity to *D. magna* (De Schamphelaere *et al.*, 2002). As a result, the use of copper toxicity data obtained using different test media and without considering all relevant water characteristics may result in an incorrect assumption on the distribution of the species sensitivity and hence may lead to over- or under-estimation of HC5. Although an environmentally unrealistic medium was used, it is suggested that the toxicity data presented in this study may contribute to the assessment of the true relative sensitivity of the different cladoceran species and hence allow a correct extrapolation of this type of data to different aquatic systems using the biotic ligand model (Bossuyt *et al.*, 2004; see chapter 7).

Due to the above shortcomings, different acute HC5s based on toxicity databases (all organisms) have been reported. These range between 3.4 (hardness not mentioned; Wheeler *et al.*, 2002) and 8.6 µg L^{-1} (50 mg L^{-1} as $CaCO_3$; Fisher and Burton, 2003). Re-calculating the data from Fisher and Burton (2003) to a hardness of 250 mg L^{-1} as $CaCO_3$ (equal to the hardness of the ISO medium) results in HC5 values of 36 µg Cu L^{-1}. The HC5 value found in our study - based on cladoceran data only and obtained in an environmentally unrealistic medium - was clearly lower: 6.7 µg Cu L^{-1}.

We constructed a literature-based SSD using the acute copper toxicity data of cladoceran species reported in Brix *et al.* (2001). The "community" sensitivity of the literature-based SSD (transformed to 250 mg $CaCO_3$ L^{-1}; Figure 6.9) was significantly higher than that of our generic SSD (Mann-Whitney *U* test, $p < 0.001$). However, in our study we did not observe any significant differences in community sensitivity between the various aquatic systems. This again indicates that, especially for copper, bioavailability is a more important factor than inter-community differences (species composition) and that the SSDs based on toxicity data derived in one standard media (ISO) for all species are more useful for further use in risk assessment procedures. We also found that the generic SSD is not significantly different from the site-specific SSDs. Hence, the generic SSD can be used as a model for a range of aquatic systems. For risk assessment purposes, this SSD can then easily be extrapolated to site-specific situations using biotic ligand models (*e.g.* De Schamphelaere *et al.*, 2002). Additionally, the reports from which toxicity data are obtained to construct the literature-based SSD may sometimes not provide all the essential information (physico-chemical characteristics) needed for these bioavailability models.

The authors are aware that most current environmental risk assessment procedures are based on chronic toxicity data. It could be hypothesised that transformation of our acute toxicity data can deliver chronic data. Using a constant ACR for *D. magna* of 1.8 (Bossuyt *et al.*, unpublished data), our estimated chronic HC5 in standard laboratory medium resulted in 3.7 µg Cu L^{-1}, keeping in mind that this was obtained in an environmentally unrealistic medium. Further research is needed on chronic toxicity data of field-collected cladoceran species to construct a generic SSD and derive a chronic HC5.

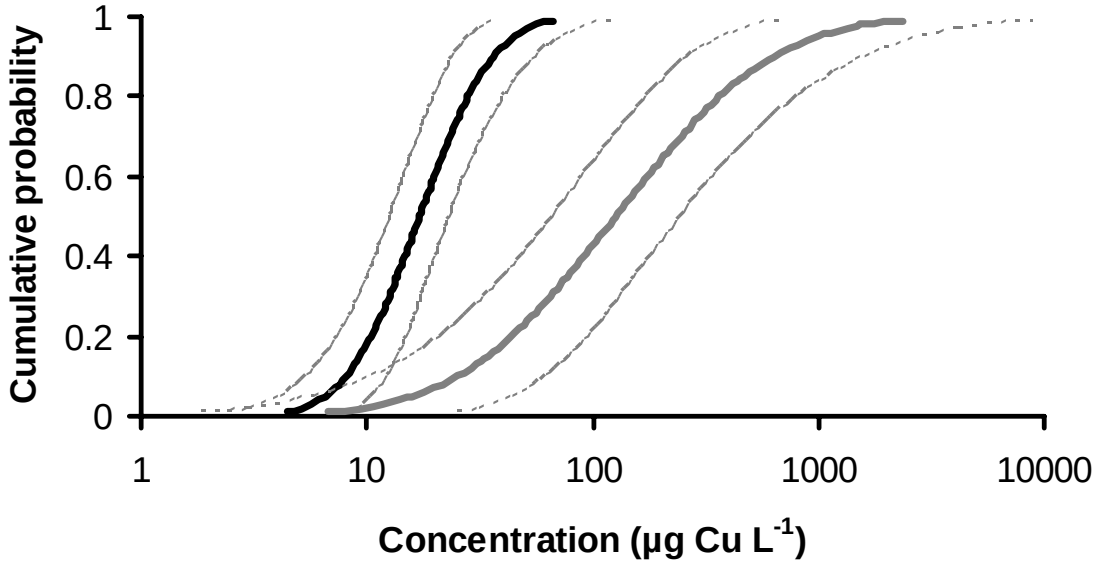

Figure 6.9: The observed acute species sensitivity distribution (thick black line) compared to the literature-derived SSD (thick grey line) based on cladoceran data (n = 10) taken from Brix *et al.* (2001). Thin lines represent the 90 % uncertainty limits.

Chapter 7

Using the biotic ligand model for predicting the acute sensitivity of cladoceran dominated communities to copper in natural surface waters

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Redrafted from:

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Chapter
7

Using the biotic ligand model for predicting the acute sensitivity of cladoceran dominated communities to copper in natural surface waters

Abstract - In this study, the acute copper sensitivity of field-collected cladoceran species was determined using their natural surface waters and a standard reconstituted test water as test medium. A total of 44 species were collected at two occasions from six different sites, representing different water types and chemistries in Europe. The collected species belonged to 4 different families (Daphniidae, Bosminidae, Macrothricidae, Chydoridae) and 13 different genera: *Daphnia*, *Ctenodaphnia*, *Ceriodaphnia*, *Simocephalus*, *Scapholeberis*, *Bosmina*, *Acantholeberis*, *Alona*, *Acroperus*, *Chydorus*, *Eurycercus*, *Disparalona*, *Pleuroxus*. The 48-hour median effective concentrations (48-h EC50) for the cladoceran species ranged from 5.3 to 70.6 µg Cu L^{-1} in standard test water and from 9.6 to 853 µg Cu L^{-1} in natural waters. The community sensitivity (the geometric mean of 48-h EC50 values of species within a community) ranged from 10.4 to 27.4 µg Cu L^{-1} in standard water and from 16.4 to 281 µg Cu L^{-1} in natural water. This indicates that bioavailability is more important than inter-community (species composition) differences in determining the variability of copper toxicity across different aquatic systems. For the four surface waters which had a pH within the range for which the acute *Daphnia magna* biotic ligand model (BLM) has previously been successfully validated, the BLM predicted 48-h EC50s for 27 of the 28 tested cladoceran species within factor of 2 of the observed values. For the same sites all community sensitivities were predicted within a factor of 2.3. The BLM was clearly overprotective for the two acidic surface waters tested. Hence, the BLM can be considered a valuable tool for estimating the potentially harmful effects of copper to natural cladoceran communities, but more research will be needed for acidic surface waters.

7.1. Introduction

Cladocerans, are widely used in aquatic toxicology due to their ecological significance, short life cycle, ease of laboratory culturing, and low space and water volume requirements (Baudo, 1987; Münzinger and Monicelli, 1991). Among the cladocerans, *Daphnia magna* Straus is probably the most commonly used test organism in ecotoxicological studies, although its use is criticised by several authors because its limited geographical range and its confinement to small water bodies (Brooks, 1957; Chapman, 1983; Mount and Norberg, 1984; Forbes and Depledge, 1993; Koivisto, 1995). Hence, the continued use of *D. magna* and other infrequently found zooplankters in testing aimed at setting water quality standards should

ideally be accompanied by testing of more ecologically relevant/representative species. Ideally, the toxicity of a chemical to a given species in a specific body of water would be estimated better by toxicity assays using the indigenous species (Chapman, 1985; Rand and Petrocelli, 1985) and the specific surface water under consideration. To our knowledge, no detailed copper toxicity studies were performed with resident cladoceran species originating from several aquatic systems and tested in the surface water of origin.

For the further enhancement of the ecological relevance in setting environmental standards, species sensitivity distributions (SSDs) are increasingly recommended to complement or replace the use of arbitrary assessment factors in the risk assessment of chemicals (OECD, 1992; Posthuma *et al.*, 2002). The SSD approach, proposed in both North America (Stephan *et al.*, 1985) and Europe (Kooijman, 1987), involves fitting a statistical distribution to point effect estimates obtained from toxicity assays with different species. At lower risk assessment tiers, this may involve selecting a threshold level that represents a safe concentration of the substance, *i.e.* protective for most organisms (usually 95 %) in an assemblage of species (Van Straalen and Denneman, 1989; Wagner and Løkke, 1991; Aldenberg and Slob, 1993;). For example, Brix *et al.* (2001) constructed acute SSDs for copper based on literature toxicity data, mostly obtained with species that were maintained in the laboratory under ideal/controlled circumstances for several years. The latter is a common problem of the SSD approach, as this type of toxicity data may not be fully representative for a certain ecosystem, albeit this is one of the main assumptions underlying the SSD approach (Forbes and Calow, 2002). Indeed, we have previously shown that field-collected cladoceran species may be slightly more sensitive to copper than laboratory maintained organisms (Bossuyt and Janssen, unpublished data; see chapter 6).

Another problem with literature-based toxicity data on copper is that they are usually obtained in test media in which the bioavailability is near maximal (*i.e.* test results obtained in media containing no or very low concentrations of dissolved organic carbon (DOC), a major factor controlling copper bioavailability, *e.g.* Meador, 1991 and De Schamphelaere *et al.*, 2002). Hence, the often applied hardness correction for metal toxicity data (*e.g.* Brix *et al.*, 2001) is probably not sufficient to make an SSD based on literature data representative for that occurring in natural environments. However, the biotic ligand model (BLM) could, by taking into account all water chemistry parameters affecting copper toxicity (*e.g.* DOC, Ca, Mg, Na, pH and alkalinity), allow the normalization of laboratory-derived toxicity data to field

situations (Santore *et al.*, 2001; De Schamphelaere *et al.*, 2002). This implies that the BLM should not only be applicable to the organisms for which the model was originally developed/calibrated (*i.e. D. magna* and *D. pulex*), but also for a larger number of species.

Given the above-mentioned shortcomings and the ecological importance of the cladocerans in aquatic food webs, the present study was aimed at investigating the sensitivity of cladoceran dominated freshwater communities to copper in both standard laboratory test water and in their natural water of origin. Three hypotheses were tested: (1) inter-community differences in copper sensitivity are less important than differences in copper bioavailability in determining the overall sensitivity of cladoceran dominated communities, (2) the BLM can be used to normalize toxicity data obtained in artificial laboratory test waters to data obtained in natural surface waters for field-collected cladoceran species, (3) the BLM-predicted community sensitivity does not differ from the observed community sensitivity in natural surface waters. These hypotheses are investigated using the SSD approach, allowing an easy interpretation of the results in a regulatory context.

7.2. Materials and Methods

7.2.1. Sampling of natural water and organisms

Six pristine surface waters with different physico-chemical water characteristics (Table 6.1) were sampled at different locations in Europe (see section 6.2.1). Samples were taken in three countries between May 2001 and October 2002 and the sampling sites were given the following labels: Ankeveen, Bokrijk, Leuven, Markermeer, Oberkirchen and Teut (Table 6.2). Prior to analysis and use of the natural surface waters as culture and test medium for the resident cladoceran species, all surface waters were filtered over a 0.45 µm filter (Sapor® 450 membrane filter, Gelman laboratory, Ann Arbor, MI, USA) in order to remove particulate matter.

Together with the surface water, the sampled organisms were brought to the laboratory within 12 hours as described in section 6.2.1. The different cladoceran species were then isolated and transferred to different aquaria containing the respective surface water. These cladoceran species were kept in a temperature-controlled room (20 ± 1 °C) with a light : dark cycle of 12 : 12h, until they were used for testing (within 1 month). They were daily fed with 5×10^5 algal

cells mL^{-1} of a mixture of *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* in a ratio of 3:1. Twice a week, the aquaria were cleaned and the respective surface water was renewed.

7.2.2. Acute toxicity experiments

Acute toxicity assays were performed as described in section 6.2.2. Experiments with all collected organisms and with our laboratory maintained *D. magna* clone (as a reference) were performed in (1) standard ISO medium (International Organisation for Standardisation, 1996) with a pH of 7.8 and containing four major salts (2.0 mM as $CaCl_2.2H_2O$; 0.5 mM as $MgSO_4.7H_2O$; 0.75 mM as $NaHCO_3$ and 0.075 mM as KCl) and (2) in their natural water of origin. Tests with juveniles (< 24 h) of our laboratory *D. magna* clone were performed in ISO medium and in all natural waters. Reported 48-h EC50s are based on measured (dissolved) copper concentrations.

7.2.3. Copper measurements

Copper concentrations were determined as described in section 2.2.4. All measured copper concentrations were within 10 % of the nominal concentrations. All reported copper concentrations are dissolved concentrations (0.45 µm filtered). Copper in controls of all tests using the ISO medium were below the detection limit.

7.2.4 Biotic ligand model calculations

For a detailed description of the BLM framework we refer to published BLM work (Di Toro *et al.*, 2001; De Schamphelaere *et al.*, 2002) and the excellent review by Paquin *et al.* (2002). The BLM used in this study was the acute Cu-BLM developed with *D. magna* as described in De Schamphelaere *et al.* (2002). Although other versions of the acute Cu-BLM exist (De Schamphelaere *et al.*, 2002; Santore *et al.*, 2001), our choice was inspired by the fact that it is the only model that has been developed based on toxicity data in reconstituted artificial test waters, refined based on tests in which copper speciation was explicitly measured and successfully validated with an independent toxicity dataset obtained with spiked natural surface waters using published assumptions with respect to copper binding characteristics of

natural DOM. Calculations were carried out using the BLM software (Windows version 1.0.0., Hydroqual, 2002).

To test if the BLM developed for predicting acute copper toxicity to *D. magna* could also be used for other cladoceran species, the following analyses were carried out. It was assumed that the stability constants for toxic copper species (Cu^{2+}, $CuOH^{+}$ and $CuCO_3$) and the competing cations (Ca^{2+}, Mg^{2+}, Na^{+} and H^{+}) are equal for all cladocerans. The only difference between species is assumed to be the $f_{CuBL}^{50\%}$, the fraction of biotic ligand sites occupied at 50 % effect. This value can be considered, in the absence of any competition effects, as the inherent sensitivity of a species to copper.

The BLM can be used in two directions. If the dissolved EC50 of a species in a water with known chemistry is known, the $f_{CuBL}^{50\%}$ can be calculated ("speciation mode"). If the water chemistry and the $f_{CuBL}^{50\%}$ of a species are known, the dissolved EC50 for that species in that water can be calculated ("toxicity mode"). One way the BLM could be used in future Water Quality Criteria (WQC) setting and risk assessment procedures is that the $f_{CuBL}^{50\%}$ for several species are estimated based on literature-based toxicity data for which also the chemistry of the test water is known. Once the $f_{CuBL}^{50\%}$ for several species is known, the dissolved EC50s can be estimated for any given water chemistry.

In this study we wanted to simulate the latter scenario by using the toxicity data obtained in artificial reconstituted water (see chapter 6) to calculate the $f_{CuBL}^{50\%}$ values for all field-collected cladoceran species and use these values to predict dissolved EC50s in their natural waters of origin.

Following steps were taken. First, since DOC is a crucial factor for determining copper toxicity, even in artificial waters where no DOC was added deliberately (*i.e.* only background DOC present) (De Schamphelaere and Janssen, 2002), a sound estimate needed to be made of the average background copper-binding DOC in the standard ISO medium. The latter was achieved by the method described in De Schamphelaere *et al.* (2004) by applying the acute Cu-BLM (De Schamphelaere *et al.*, 2002) to the reference toxicity data with *D. magna* obtained in ISO medium. The average (± standard deviation, n = 4) estimated DOC concentration was 380 ± 110 µg L^{-1}, with 50 % assumed active fulvic acid (AFA) and 50 % assumed inert for copper complexation. This is very close to and not significantly different

from our earlier reported value of 276 ± 104 µg L^{-1} (De Schamphelaere and Janssen, 2002). The former was used as the DOC-input for all further calculations with the standard ISO medium.

For all cladoceran species collected at each sampling site, the $f_{CuBL}^{50\%}$ was calculated for *n* replicate tests. These *n* $f_{CuBL}^{50\%}$ values were then used to predict *n* dissolved EC50s in the natural water for a given species. For the natural waters it was assumed that the DOC consisted of 50 % AFA and of 50 % inert organic matter (De Schamphelaere *et al.*, 2002, Dwane and Tipping, 1998). For three samples an additional prediction was made using *a* % AFA estimated from a linear relation with the UV-light absorption coefficient at 350 nm (Perkin Elmer, Lambda/Bio, Überlingen, Germany; De Schamphelaere *et al.*, 2004). The average predicted EC50 was then compared to the average observed EC50. The predicted EC50s were further used to calculate predicted community sensitivities.

7.2.5. Statistics and species sensitivity distributions

Species sensitivity distributions (SSD) were constructed, based on Aldenberg *et al.* (2002), for all communities taken at the different locations and at different times as described in section 6.2.5. SSDs were constructed with the test results obtained with both standard test medium (ISO) and surface waters and also with BLM-predicted toxicity data. Multiple data for the same species were summarized as arithmetic means prior to inclusion in the SSD. The reported SSDs are fitted to a log-normal distribution since the Anderson-Darling goodness-of-fit test for normality (Aldenberg *et al.*, 2002) indicated that this distribution gave the best fit ($p > 0.15$) compared to other statistical distributions ($n = 21$) commonly used for constructing SSDs (see section 6.3) (calculated by BestFit 2.0 software, Palisade, London, UK).

Comparison across different sites/sampling periods was performed by analysis of variance (ANOVA) with the post-hoc Duncan's multiple range test. Comparison between standard test medium, observed and predicted toxicity in natural water was tested by one-sided Student *t* tests for independent samples. Homogeneity of variance and normality is tested using Bartlett's and Kolmogorov-Smirnov's test, respectively ($p < 0.05$). All statistical comparisons were performed with STATISTICA 6 (STATISTICA® software, Tulsa, OK, USA).

As the ANOVA assumptions failed for the water effect ratio (WER) calculations of the different aquatic systems, comparisons were performed with the non-parametric Mann-Whitney *U* test ($p < 0.05$).

7.3. Results and discussion

7.3.1. Species and community sensitivity in standard and natural water

A total of 22 different cladoceran species were collected from the six locations. Six species (*Alona* sp., *Acroperus harpae*, *Ceriodaphnia pulchella*, *D. magna*, *Simocephalus exspinosus*, *Disparalona rostrata*) were present at 2 sites, 3 (*Chydorus sphaericus*, *Daphnia longispina*, *Simocephalus vetulus*) at 3 sites. In total, more than 200 acute toxicity experiments were carried out. The acute copper toxicity (48-h EC50) of all cladoceran species tested in standard water and in their natural surface water is presented in Table 6.3. Community sensitivities of the different aquatic systems are reported in Table 7.1. Acute copper toxicity in standard ISO water ranged from 5.3 µg Cu L^{-1} (*Scapholeberis mucronata*) to 70.6 µg Cu L^{-1} (*Disparalona rostrata*). Control mortality in all assays with field-collected cladoceran species was lower than 20 %. Although the ANOVA revealed a significant difference between the community sensitivity of Ankeveen at the different sampling moments (10.4 µg Cu L^{-1} on 14-06-2002 compared to 27.4 µg Cu L^{-1} on 12-09-2002), this was not observed when compared with the Student's *t* test for independent samples ($p > 0.05$; see section 6.3.2.). Without the second sampling of Ankeveen, community EC50s were within a factor of 2.3, *i.e.* between 10.4 µg Cu L^{-1} (Ankeveen, 14-06-2002) and 23.6 µg Cu L^{-1} (Leuven). Tested in natural waters, species 48-h EC50 values varied about 90-fold, *i.e.* between 9.6 µg Cu L^{-1} (*S. vetulus*) and 852.9 µg Cu L^{-1} (*Acantholeberis curvirostris*). Community sensitivities (as 48-h EC50) in natural water varied between 16.4 µg Cu L^{-1} (Oberkirchen) and 281 µg Cu L^{-1} (Ankeveen), a 20-fold range. Compared to the 2-fold range observed in standard water, this indicates that the factor bioavailability (water chemistry) in natural waters is far more important for determining the community threshold than the inherent species sensitivity differences. In Figure 7.1 the SSDs based on toxicity assays in standard ISO water and in natural surface waters are visually compared. Except for the results of one sampling period at the Oberkirchen site (Figure 7.1B) and the Bokrijk results (Figure 7.1H), clear differences in community sensitivity due to the different water characteristics of the sampling sites are noted.

Table 7.1: Parameters of the log-normal species sensitivity distributions (mean and standard deviation (SD) of log transformed toxicity values; original values between brackets in µg Cu L^{-1}) of the six aquatic systems based on experiments performed in standard medium (ISO) and in their respective natural water (field) and based on BLM predictions with 50 % active fulvic acid (predicted) and with an optimal percentage of active fulvic acid (opt % AFA). SSD parameter values with same letters are not significantly different (one-way analysis of variance, Duncan's test, $p < 0.05$); WER values (water effect ratio) were compared with the non-parametric Mann-Whitney *U* test ($p < 0.05$); significant difference between species sensitivity distributions of field and standard medium at $p < 0.001$ (°°°) and $p < 0.05$ (°) with Student's *t* test for independent samples; significant difference between predicted and observed at $p < 0.001$ (***), $p < 0.01$ (**) and $p < 0.05$ (*).

Site	**Sampling date**	**ISO**		**Field**		**WER**		**Predicted**		**Predicted (opt % AFA)**	
	dd-mm-yyyy	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Ankeveen	14-06-2002	2.34_{a} (10.4)	0.50	5.64_{a}°°° (281)	0.60	29.1_{a}	7.9	5.72	0.49	-	-
	12-09-2002	3.31_{b} (27.4)	0.75	5.62_{a}°°° (276)	0.44	20.0_{b}	9.6	6.44^{*}	0.32	-	-
Bokrijk	01-10-2002	3.04_{a} (20.9)	0.41	3.01_{bc} (20.3)	0.39	1.7_{ce}	0.8	1.68^{*}	0.72	0.96^{**}	0.64
Leuven	23-10-2002	3.16_{ab} (23.6)	0.21	4.91_{d}°°° (136)	0.38	6.5_{d}	3.2	5.56^{*}	0.11	5.02	0.12
Markermeer	26-05-2001	3.11_{a} (22.4)	0.68	4.84_{d}°°° (126)	0.43	7.5_{d}	3.2	5.47	0.44	5.16	0.45
	24-09-2002	3.05_{a} (21.1)	0.62	4.80_{d}°°° (122)	0.48	6.1_{d}	2.6	5.64^{*}	0.32	4.91	0.40
Oberkirchen	19-06-2002	3.03_{a} (20.7)	0.53	3.54_{c} (34.5)	0.15	2.4_{c}	0.6	3.91	0.27	-	-
	10-09-2002	2.64_{a} (14.4)	0.26	2.80_{b}° (16.4)	0.47	1.6_{e}	1.0	3.25	0.16	-	-
Teut	22-08-2002	2.57_{a} (13.1)	0.22	-	-	6.8_{d}	3.6	-	-	-	-
	01-10-2002	2.95_{a} (19.1)	0.54	5.17_{ad}°°° (176)	1.09	7.4_{d}^{w}	5.1	2.55^{***}	0.73	2.09^{***}	0.75

-: could not be calculated; w: data without *Acantholeberis curvirostris*: WER = 74 ± 20

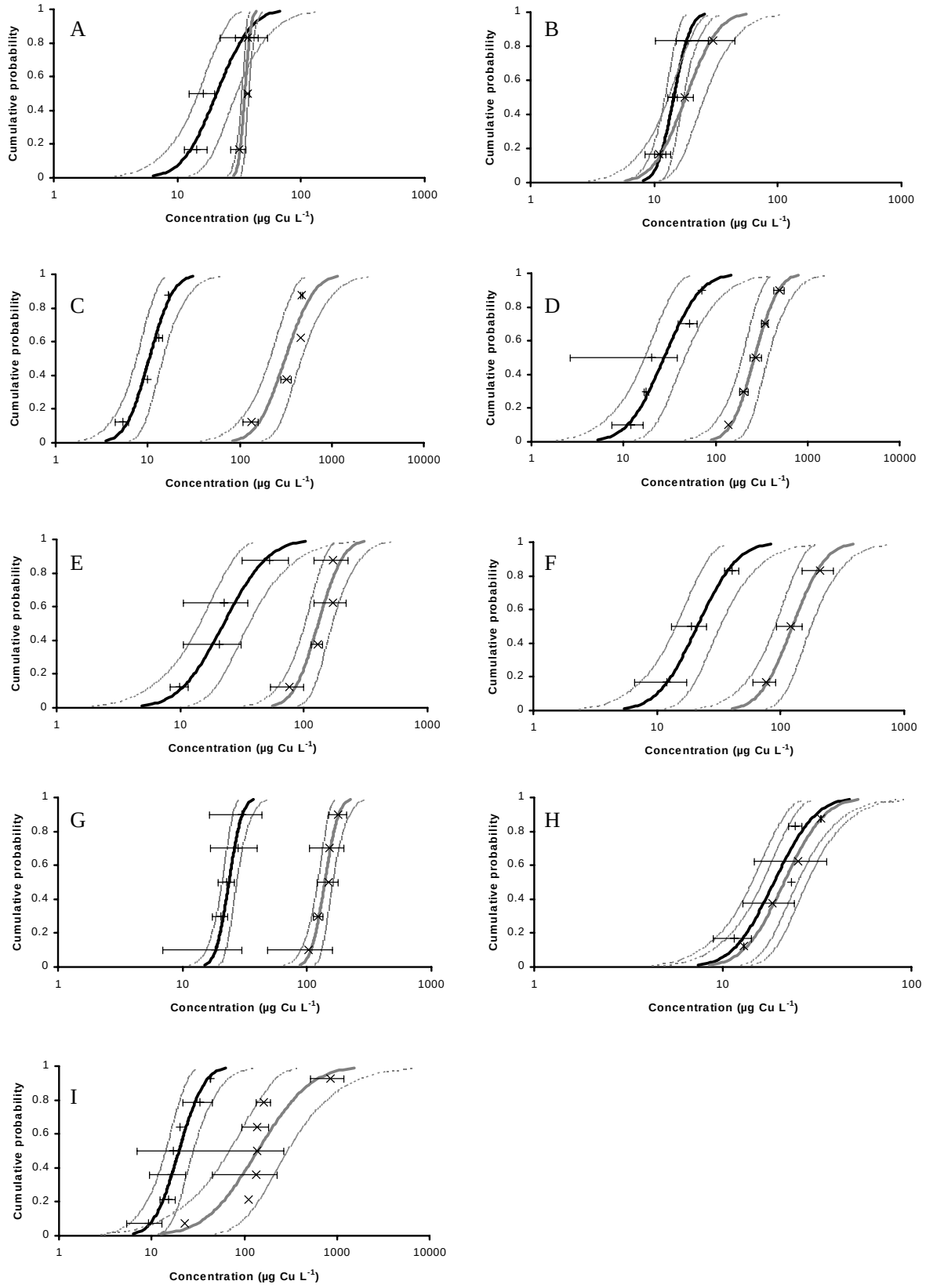

Figure 7.1: Species sensitivity distributions for the different aquatic systems and different sampling periods. Results are based on experiments in standard medium (ISO, thick black line) and in the natural water of origin (thick grey line). The fine grey broken lines represent the 90 % confidence

limits around the species sensitivity distribution. +: data points with standard deviation derived from tests with standard medium; ×: data points with standard deviation derived from tests with the natural water of origin. A: Oberkirchen in June; B: Oberkirchen in September; C: Ankeveen in June; D: Ankeveen in September; E: Markermeer in May; F: Markermeer in September; G: Leuven in October; H: Bokrijk in October; I: Teut in October.

With a few exceptions, species EC50s and community EC50s in natural water were higher than the EC50s in standard water. The differences between both test media can, for the ease of interpretation, be summarized as water effect ratios (WER), a commonly applied approach for regulating potentially toxic discharges in USA (USEPA, 1994). In this approach the WQC is multiplied with the WER to obtain the site-specific WQC. In this study, WERs ranged between 0.3 (*S. vetulus*) and 74 (*A. curvirostris*) when individual species are considered (Table 6.3). When individual WERs were averaged for a community, they (mean ± standard deviation) ranged between 1.6 ± 1.0 (Oberkirchen) and 29.1 ± 7.9 (Ankeveen) (Table 7.1). A significant positive linear relationship (F-test, $p < 0.001$, $n = 10$; $r^2 = 0.95$; Figure 7.2) was observed between WERs and DOC concentration of the natural surface waters, as already demonstrated by Heijerick *et al.* (unpublished data) and again pointing to DOC being the most important factor determining copper toxicity (De Schamphelaere *et al.*, 2002). Although the WER approach is useful in setting site-specific WQC, it still requires costly full site-to-site toxicity testing. However, if the BLM proves to be applicable to a wide range of species, the BLM prediction of 48-h EC50s could replace the WER approach. This is tested below.

7.3.2. Predictive capacity of the BLM for field-collected cladoceran species

At this point it is useful to split our sampling locations into two groups. Four of the six sites (Ankeveen, Leuven, Markermeer and Oberkirchen) had pH levels > 5.5 (Table 6.1) and will further be designated as ‘normal’ sites. The other two sites (Teut and Bokrijk) will be referred to as acidic sites (pH < 5.5). This division is based on the fact that the BLM for *D. magna* has so far not been calibrated for predicting copper toxicity at pH levels below 5.5. The reason for this is that *D. magna* cannot survive at these low pH levels. Indeed, in the *D. magna* experiments with surface waters of Teut and Bokrijk more than 80 % mortality occurred in the control water (no copper added) (Table 7.2).

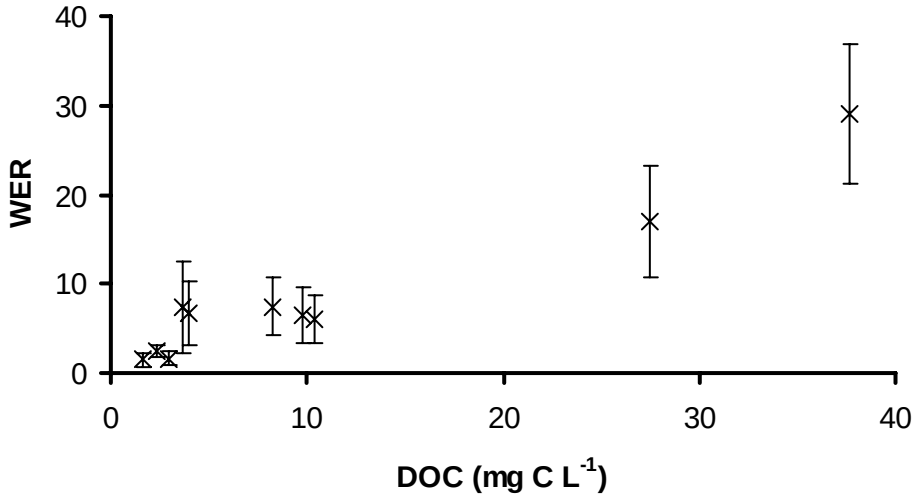

Figure 7.2: Relationship between dissolved organic carbon (DOC) concentration in the different aquatic systems and the calculated water effect ratio (WER) of the system.

Table 7.2: The observed acute effective concentration (48-h EC50) with standard deviation of laboratory-reared *Daphnia magna* tested (*n*: number of tests) in natural surface waters of the six sampling sites. Biotic ligand model predictions with the assumption of 50 % active fulvic acid and with an optimal percentage of active fulvic acid (opt % AFA).

Site	**date**	**48-h EC50**	***n***	**predicted**	**Predicted opt % AFA**
	dd-mm-yyyy	µg Cu L^{-1}		µg Cu L^{-1}	µg Cu L^{-1}
Ankeveen	14-06-2002	1086 ± 22	4	708	-
	12-09-2002	755 ± 24	2	721	-
Leuven	23-10-2002	205 ± 8	2	313	188
Markermeer	01-05-2001	257[a]	1	357	181
	24-09-2002	188[a]	1	322	217
Oberkirchen	19-06-2002	81 ± 8	2	65	-
	10-09-2002	103 ± 2	2	43	-
Teut	01-10-2002	†	3	-	-
Bokrijk	01-10-2002	†	3	-	-

-: could not be calculated; †: more than 80 % control mortality; a: only 1 test performed; no standard deviation calculated.

Before evaluating if the BLM can be used for predicting 48-h EC50s of field-collected cladoceran species tested in natural surface waters, it was necessary to ensure that the toxicity of copper in these water to the *D. magna* clone of our laboratory can be correctly predicted. Table 7.2 presents the acute toxicity data of the laboratory-reared *D. magna* tested in the collected natural surface waters. The observed 48-h EC50s in the four normal waters ranged between 81 and 1086 µg Cu L^{-1}. Table 7.2 also presents the BLM-predicted 48-h EC50s. When using the 50 % AFA assumption, EC50s were predicted within factor 1.7 of the observed values, except for the Oberkirchen sample collected in September 2002. For the latter sample, EC50s were under-estimated by a factor of 2.4 (*i.e.* toxicity was over-estimated). For three samples (Leuven and Markermeer on both sampling occasions), UV-absorbance was measured to derive an optimal % AFA (Table 6.1). When using this optimal % AFA, the predictive performance of the BLM increased for all samples and increased from an average prediction error of factor 1.5 to 1.2. This confirms the earlier demonstrated usefulness of this approach (De Schamphelaere *et al.*, 2004).

Next, using the approach mentioned in the materials and methods section, 48-h EC50s of field-collected cladoceran species in their natural water of origin were predicted (Figure 7.3). When applying the 50 % AFA assumption to the normal waters, 67 % of the EC50s were predicted within factor of 2 error, and 96 % within factor of 3 error (n = 27). The mean (± standard deviation) prediction error for the normal waters was a factor of 1.9 ± 1.1. The EC50 for one species (*Disparalona rostrata* from Ankeveen) was over-estimated by a factor of 6.6. This organism is a littoral organism (Flössner, 2000) and lives on and in the sediment or detritus layer, while all other sampled cladocerans occur mainly in the pelagic environment, like *D. magna*. Hence different bioavailability modifying mechanisms may be at work in "(epi)benthic" as opposed to "pelagic" cladoceran species. Further research is necessary to elucidate these observations.

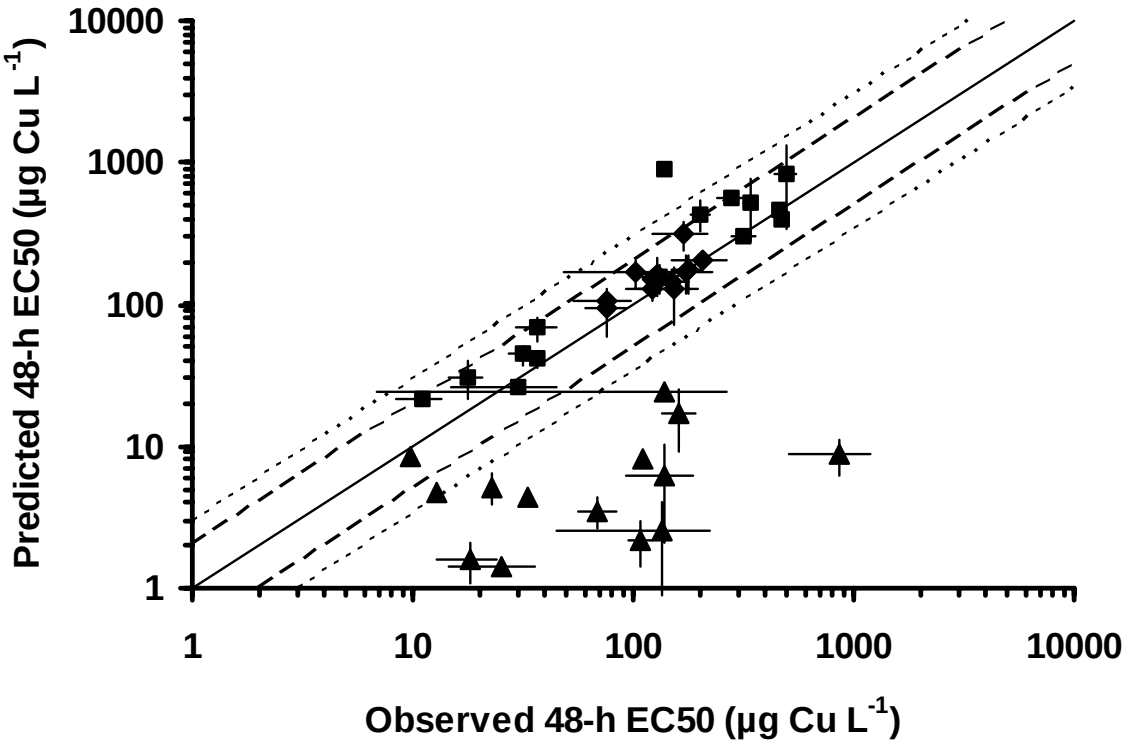

Figure 7.3.: The relationship between the observed and predicted 48-h mean effective concentration (48-h EC50) of copper to field-collected cladoceran species of normal sites (pH > 5.5) with 50 % active fulvic acid (■) or with optimal percentage fulvic acid (◆) and of acidic sites (pH < 5.5) with optimal percentage fulvic acid (▲). Error bars represent the standard deviation. The solid lines indicate a perfect match between the observed and predicted EC50. The dashed lines indicate a factor-of-two and a factor-of-three difference between the observed and predicted EC50.

When examining the acute data of the Markermeer and Leuven surface waters, only 50 % of the species EC50s was predicted within a factor of 2 error (mean ± standard deviation = 2.0 ± 0.5, n = 12). However, when for these samples the optimal % AFA was used, all EC50s were predicted within a factor of 2 (mean ± standard deviation = 1.2 ± 0.3, n = 12). This again indicates the usefulness of the UV-absorption measurements for improving BLM predictions in natural waters.

In contrast, when acidic sites (Bokrijk and Teut) were considered, all 48-h EC50s were under-predicted. For 13 out of the 14 tested species from these sites, the predictions were more than a factor of 2 lower than the observed EC50s, with a mean under-estimation of the EC50s of factor 13 and a maximum of factor 61 (for *A. curvirostris* from Teut). It is known that acute

copper toxicity to freshwater organisms occurs through an inhibition of Na uptake and/or an increase of Na efflux (Grosell *et al.*, 2002). Usually the exposure of freshwater organisms to high acidity triggers the same effects, as shown for example for trout by McWilliams and Potts (1978). However, it may be hypothesized that the species occurring in the Teut and Bokrijk, may be adapted to the low pH/low hardness levels occurring in these water bodies. Indeed, at least three species, *i.e. A. curvirostris*, *Scapholeberis microcephala* and *Chydorus ovalis*, are typically for surface waters with a pH between 4.0 and 5.6 and hardness lower than 10 mg L^{-1} as $CaCO_3$. Potts and Freyer (1979) observed that *A. curvirostris* retains sodium better in acidic waters than *D. magna* and that the uptake system of the former has a higher affinity for sodium ions than that of *D. magna*. It may thus be suggested that the adaptation to acidic conditions may result in an associated lower than expected toxicity of copper in acidic waters for these organisms. Further research is needed to test this hypothesis.

7.3.3. Predictive capacity of the BLM for community sensitivity

For each of the six sampling sites, SSDs and community EC50s were calculated based on the log-normal distribution of the predicted and observed (in natural water) EC50 values. The parameters (mean and standard deviation of the log transformed toxicity values) on which these distributions are based, are shown in Table 7.1. With the summer sampling of the Teut, no community sensitivity could be calculated as only two species could be tested in the natural surface water. Predictions, based on the assumption of 50 % AFA, resulted in 4 (of 9) values that were not significantly (Student's *t* test for independent samples) different from those measured. Based on the large under-estimation of species EC50s, this was expected for the three sampling occasions of the acidic sites for which community sensitivity was over-estimated, not only with the 50 % AFA assumption, but also with the optimal % AFA assumption.

The significant over-estimation of community sensitivity for Leuven and Markermeer (September 2002 sampling) may be explained by the low binding capacity of the natural DOM, as with the optimal % AFA assumption, those differences were not significant anymore. This leaves us with one sample in which we cannot explain the over-estimated community sensitivity, *i.e.* the Ankeveen sampling of September 2002. The observed community sensitivity was 276 µg Cu L^{-1}, while we predicted 626 µg Cu L^{-1} (factor of 2.3). However, when *D. rostrata* was eliminated from the dataset, the observed and predicted

community EC50s were 311 and 567 µg Cu L^{-1}, respectively. Although still statistically significantly different (p = 0.04), this difference was now only a factor of 1.8.

For the above, it is concluded that for normal waters (pH > 5.5) community sensitivity to copper can be accurately predicted within a factor of 2.3 when assuming 50 % AFA (mean ± standard deviation = 1.7 ± 0.4, n = 7). When only those normal sites where UV-absorption was measured are considered, the prediction errors were a factor of 2.0 ± 0.2 (n = 3) with 50 % AFA assumed and a factor of 1.2 ± 0.2 (n = 3) when the optimal % AFA was taken.

7.4. Conclusions and research recommendations

In this study we demonstrated that bioavailability is far more important than inherent community sensitivity differences in determining the effect of copper to cladoceran dominated communities in natural surface waters. We have also shown that the acute Cu-BLM, as developed with *D. magna* can be used to predict copper toxicity to most field-collected cladoceran species occurring in normal waters (pH > 5.5). The BLM was not predictive for organisms living in acidic waters (pH < 5.5). The BLM can be used for estimating community sensitivity to copper of resident communities tested in the spiked natural surface waters. Although this study clearly demonstrates, for the first time, the applicability of the BLM concept for predicting acute copper toxicity in natural waters, future research should investigate if this holds for the recently developed chronic Cu-BLM too (De Schamphelaere and Janssen, 2004). The present and the suggested future research will improve the currently applied risk assessment procedures of copper and its substances in freshwater environments.

Chapter 8

General conclusions and
future research perspectives

Chapter
8

General conclusions and future research perspectives

Like all chemicals, metals may present risks to the environment. Presently, these risks are being managed through the establishment of environmental quality criteria or risk assessment procedures. Although copper is considered to be a very potent toxicant, it is also an essential element required for a variety of biochemical and physiological processes. As stated in chapter 1, current risk assessment procedures do not take this essentiality into account. Additionally, copper is a natural component of the Earth and is thus ubiquitously present at low (background or sub-lethal) concentrations. The influence of these varying low copper concentrations occurring in aquatic ecosystems on the sensitivity and the physiological processes of the aquatic organisms/communities present has, prior to this study, not been investigated. Additionally, the standard organisms used in laboratory studies are not always present in each ecosystem. Hence, differences in species sensitivity may result in differences in community sensitivity. Due to all these shortcomings, it may be difficult to predict the copper sensitivity of natural species in an aquatic ecosystem based on laboratory-derived toxicity data.

In the first part of this dissertation, long-term experiments were performed in the laboratory. Two different freshwater species, the green algae *Pseudokirchneriella subcapitata* and the cladoceran *Daphnia magna*, were acclimated to a range of copper concentrations using environmentally relevant artificial media. This range was based on a study of the naturally occurring copper concentrations in Europe (section 1.1.3.3.). During these acclimation experiments, an optimal copper concentration range (OCEE) was observed for both organisms using different ecotoxicological endpoints. *P. subcapitata* and *D. magna* both exhibited optimal concentration ranges between 1 and 35 µg Cu L^{-1}. Transforming this range to copper ion activities with WHAM VI speciation program and based on the physico-chemical water characteristics of the respective media used in this study, the range was situated between 2.1×10^{-14} and 3.1×10^{-10} M Cu^{2+} and between 2×10^{-14} and 8×10^{-11} M Cu^{2+}, respectively. At lower and higher copper concentrations, both organisms exhibited a decrease in the measured ecotoxicological endpoints indicating deficiency and toxicity responses, respectively. During acclimation, the OCEE shifted towards lower and higher copper concentrations. For both

organisms, it was concluded that **optimal copper concentrations ranged from approximately 1 to 35 µg Cu L^{-1} or 10^{-14} to 10^{-11} M Cu^{2+}**.

Both species exhibited an increased copper tolerance after acclimation to a total copper concentration ≥ 12 µg Cu L^{-1} (90^{th} percentile of copper in Europe) corresponding to a bioavailable copper concentration (expressed as copper activity based on WHAM VI calculations) of 0.13 and 4 pM Cu^{2+} for *P. subcapitata* and *D. magna*, respectively. At lower, more common copper concentrations, no increases in tolerance were observed. Acclimation to ambient copper concentration thus resulted in **increases in tolerance by a maximum factor of 2**. Hence, these increases are **negligible in the copper risk assessment procedures** compared to the observed differences due to the variation in bioavailability in natural surface waters.

However, several generations of exposure to (very) low copper concentrations (< 0.5 µg Cu L^{-1}, *i.e.* 1×10^{-18} M Cu^{2+} and 7×10^{-15} M Cu^{2+} for *P. subcapitata* and *D. magna*, respectively) resulted in a decreased copper tolerance and general fitness compared to that of organisms acclimated to more environmentally common copper concentrations. As already demonstrated above, this copper concentration was situated outside the OCEE of both organisms. Hence, toxicity data obtained with organisms cultured in standard artificial media in the laboratory should be carefully evaluated as these organisms may have become over-sensitive. It is thus recommended to **avoid the use of artificial standard culture media which contain no or very little essential elements**.

Experiments with *D. magna* exposed to different bioavailable copper concentrations - *i.e.* performed by changing DOC, pH and hardness - during 5 generations revealed that the **acclimation process was a function of the dissolved copper concentration** present in the culture media. Copper tolerance, accumulation in the daphnids and the OCEE seemed also to be dependent on the dissolved copper concentration. Although this is in contrast with the fact that toxicity is dominated by the bioavailable copper concentration and not by the total/dissolved concentration, it is suggested that at these low copper concentrations (background or ambient) different mechanisms are responsible for copper uptake. Further research on the exact mechanisms of copper uptake at these low copper concentrations would improve the knowledge of the physiological interactions between the organisms and the ambient copper concentrations.

Copper accumulation measurements with both organisms revealed that only a small part of the body concentration is situated on the cell wall (20 %) or on the carapace (30 %). Hence, the total body concentrations consisted mainly of copper absorbed in the organisms. Both organisms exhibited a similar constant absorbed concentration (13 ± 4 µg Cu g DW^{-1}) within a certain copper concentration range, while a decrease was observed at lower and an increase at higher water concentrations. In *P. subcapitata* this range was situated between 1 and 5 µg Cu L^{-1}; in *D. magna* between 1 and 12 µg Cu L^{-1}. Long-term acclimation to the higher copper concentrations (≥ 12 µg Cu L^{-1}) resulted in a decrease in the absorbed copper concentration of both organisms, indicating a change in the copper homeostasis. This decrease was correlated with the increase in copper tolerance. Further research is needed to determine the exact relationship between copper accumulation and acclimation (increase in tolerance).

Based on the calculated bioconcentration factors (BCF), different copper regulation mechanisms are proposed and linked to the previously determined OCEE. *P. subcapitata* exhibited active copper regulation mechanisms up to 5 µg Cu L^{-1}, followed by copper storage mechanisms between 5 and 60 µg Cu L^{-1}. At higher copper concentration, this regulation mechanism fails and higher BCFs (and body burdens) were noted. For *D. magna*, active copper regulation was observed up to 35 µg Cu L^{-1}, followed by a copper storage mechanism between 35 and 100 µg Cu L^{-1}. Failure of the copper regulation was noted at concentrations higher than 100 µg Cu L^{-1}. Based on these results, it was concluded that the **OCEE coincides with active copper regulation and storage** for *P. subcapitata* and only with active copper regulation for *D. magna*.

In the second part of this doctoral thesis, the copper sensitivity of various field-collected cladoceran species of different aquatic ecosystems tested in a standard laboratory medium was assessed. A total of 22 different cladoceran species, belonging to 4 different families, were collected. A factor of 12 inter-genus difference in copper sensitivity was noted. Within the genera of the *Daphnia* sp., the inter-species difference in copper sensitivity was only a factor of 3. ***D. magna* was less sensitive than most of the field-collected cladoceran species**. Significant intra-species differences were only noted for the genus of *Ctenodaphnia* and subfamily of *Aloninae*. Not considering the (benthic) family of Chydoridae, an increased copper tolerance was found with the organism's size.

As the organisms were sampled in six different aquatic systems dominated by cladocerans, species sensitivity distributions (SSD) could be constructed and the community sensitivities assessed. A generic SSD, based on all cladoceran species used in our research (n = 22) and tested in one standard laboratory medium, indicated that the **toxicity data fitted a log-normal distribution**. Hence, the site-specific SSDs were based on this distribution, although only a limited data set could be used for each aquatic system. Although small differences were observed in the species sensitivity (cf. above), no differences were found between the community sensitivity of the different aquatic systems. Additionally, no differences were noted between the site-specific SSDs and our generic one. The site-specific hazardous concentration protecting 95 % of the species (HC5) varied within a factor of 4 (between 4.5 and 17.3 µg Cu L^{-1}), while the HC50 varied within a factor of 2 (between 17.3 and 23.6 µg Cu L^{-1}). Although the copper concentration ranged between 2.0 and 11 µg Cu L^{-1} in the sampled surface waters, **no increased community tolerance was observed** as a function of the ambient copper concentration.

Field-collected cladoceran species were tested in a standard medium, as well as in their natural surface water of origin. While the species sensitivity in standard laboratory water varied within a 12-fold range, the sensitivity of the various cladoceran species tested in the natural surface water varied with a factor of 90. Similarly, the community sensitivity differed with a 20-fold range (compared to the 2-fold range in standard laboratory medium). This indicates that **the factor bioavailability (water chemistry) in natural waters is far more important for determining the community threshold than inherent species sensitivity differences**.

As demonstrated in chapter 2 through 4, the influence of acclimation to relevant background or ambient copper concentrations is of minor importance for the organism's sensitivity. Additionally, species sensitivity differences were rather small compared to sensitivity differences caused by bioavailability. Differences in community sensitivity were smaller than a factor of 2 (chapter 6). Hence, to account for differences in bioavailability, normalization of the toxicity data was performed with the current biotic ligand model (BLM). For the four surface waters with a pH within the range for which the acute *D. magna* BLM has previously been developed and validated (pH 5.5-8.5), the BLM predicted the 48-h EC50s for 27 of the 28 tested cladoceran species within factor of 2 of the observed values. The community sensitivities were predicted within a factor of 2.3. The BLM was clearly over-protective for

the two acidic surface waters (pH < 5.5) tested. This demonstrates that the **BLM is able to predict the acute copper sensitivity of various cladoceran species** and hence the community sensitivity of different aquatic systems. However, further research is needed to determine the reason(s) why the resident organisms of acidic sites are not as sensitive as expected. This could be related to different toxicity mechanisms at these low pH and possible adaptation (or acclimation) to the physico-chemical water characteristics resulting in different physiology. As the BLM is based on the stability constants established with our *D. magna* laboratory clone, further work on the determination of these constants for the different cladoceran species is required. This research has been initiated (B. Bossuyt) in the Laboratory of Environmental Toxicology and Aquatic Ecology.

Although the acute *D. magna* BLM is currently considered for implementation in the water quality criteria for copper in the USA, it is not suitable for incorporation into the European Union regulatory frameworks. In the EU, the environmental management of metals (and other chemical substances) is addressed through a risk assessment approach (with the exception of Cd, Ni, Hg and Pb, which are currently being managed through WQC derivation in the context of the Water Framework Directive). In this type of risk assessment, according to the principles laid down in the technical guidance document (TGD, 2003) a predicted environmental concentration (PEC) and a predicted no effect concentration (PNEC) are derived and finally compared to evaluate the risk of substances. Additionally for data-rich substances (like metals), this approach is based upon chronic toxicity data. Therefore, it would be valuable if the chronic toxicity of copper to field-collected cladoceran species is studied. This research has been initiated (B. Bossuyt) in the Laboratory of Environmental Toxicology and Aquatic Ecology and the initial results seem to confirm our hypothesis.

In chapter 1 (section 1.4.), an overview was given of the current short-comings in the current risk assessment procedures of (essential) metals. Most important were the problems associated with (1) accounting for the effects of background or ambient copper concentrations on the species/community sensitivity and the OCEE; and (2) the species sensitivity differences among field-collected organisms and between laboratory and field species. In these two research lines, copper bioavailability was taking into account through (1) expressing the copper concentrations (acclimation) as bioavailable copper concentrations (or the assumed bioavailable fraction Cu^{2+}, based on WHAM VI calculations with the water characteristics of the used media); (2) the selection of environmentally relevant total (and

bioavailable) acclimation concentrations that were chosen; and (3) testing field-collected cladoceran species in a standard medium and in natural surface waters.

In general, the main conclusions of this doctoral research can be summarized as follows: (1) acclimation to copper does occur at environmentally relevant copper concentrations (≥ 12 µg Cu L^{-1}), but is of less importance in the context of regulatory risk assessments as the sensitivity of the model organisms was affected by only a factor of 1.6; (2) culture and test media containing no or only very limited amounts of essential elements should be avoided as they render the test organisms over-sensitive; (3) optimal concentrations of copper can shift during the acclimation and are thus dependent on the ambient copper concentration of the medium; (4) different sensitivity to copper is observed among different cladoceran species; (5) different aquatic systems have a similar community sensitivity and (6) the copper sensitivity of field-collected cladoceran species can be predicted in natural surface waters using the BLM approach.

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Summary

This doctoral thesis is situated in the field of aquatic toxicology, *i.e.* the study of the effects of natural and anthropogenic substances on the structure and functioning of freshwater and marine ecosystems. More specifically, the subject of this dissertation is the ecotoxicology of copper in freshwater ecosystems.

Contrary to the numerous man-made organic chemicals, metals are naturally occurring substances and life has evolved in the presence of these elements. Some of these, the essential metals (like copper), have become incorporated into metabolic processes crucial to survival, growth and reproduction of organisms. Each species has for each essential element an optimal concentration range in which it can satisfy its metabolic requirements and develop and perform in an optimal way. However, when the external concentration of the essential element becomes too low or too high, homeostatic regulation will fail and deficiency or toxicity can occur, respectively. This study focuses on the optimal range of the essential element copper, *i.e.* copper concentrations at which homeostatic regulation occurs and interacts with the organism on a physiological level.

The present research aimed at studying some ecological factors that, to date, have not been taken into account in the environmental risk assessment procedures for metals: (1) in natural aquatic systems, organisms/communities can be adapted or acclimated to the background or ambient copper concentration present in the surface waters; and (2) the copper sensitivity of field-collected species can be different from those of standard organisms (intra- and inter-species differences). If the influence/magnitude of these data gaps can be assessed, they can be incorporated in the BLM which could improve the ecological relevance of BLM-predicted metal toxicity. This research is divided in an experimental part using standard organisms (chapter 2 to 5) and in a part using field-collected cladoceran species (chapter 6 and 7). Each chapter describes a well-defined series of experiments on a specific topic.

In **chapter 2**, the freshwater green algae *Pseudokirchneriella subcapitata* were cultured at seven - five of which are ecologically relevant for European surface waters - copper concentrations ranging from < 0.5 (no addition of copper) to 100 µg Cu L^{-1} (*i.e.* 1.3×10^{-18} to 1.9×10^{-8} M Cu^{2+}). After 12 weeks of acclimation copper tolerance increased significantly

from 88 ± 15 to 124 ± 25 µg Cu L^{-1} for *P. subcapitata* acclimated to 0.5 and 100 µg Cu L^{-1}, respectively. Based on the algal biomass, the growth rate, the pigment diversity and the autotrophic index, an optimal concentration range was observed between 1 and 35 µg Cu L^{-1} (2.1×10^{-14} to 6.1×10^{-10} M Cu^{2+}). Lower and higher copper concentrations were considered to be sub-optimal, *i.e.* deficient and toxic, respectively. Based on the algal biomass and growth rate a population NOEC of 35 µg Cu L^{-1} was observed.

In **chapter 3**, the freshwater cladoceran *Daphnia magna* was acclimated to six different copper concentration during 18 months ranging from < 0.5 (no copper addition) to 100 µg Cu L^{-1} (*i.e.* 7×10^{-15} to 37×10^{-10} M Cu^{2+}). Their copper tolerance changed as a function of the copper concentration present in the culture medium. The 48-h EC50 increased significantly from 204 ± 24 µg Cu L^{-1} to 320 ± 43 µg Cu L^{-1}. A non-significant change in 21-d EC50 (mean and 95 % confidence limits) from 48.0 (47.9 – 48.0) µg Cu L^{-1} to 78.8 (66.3 - 93.6) µg Cu L^{-1} was noted in the chronic toxicity assays. In the first generation, the OCEE ranged between 5 and 12 µg Cu L^{-1} based on physiological and population level parameters studied (net reproduction, energy reserves, body length measurements and filtration rates). After three generations of acclimation a shift towards lower and higher copper concentrations occurred and the OCEE was then situated between 1 and 35 µg Cu L^{-1} (2×10^{-14} to 80×10^{-12} M Cu^{2+}). Long-term copper deficiency resulted in a decreased fitness of the daphnids, expressing itself in a reduced copper tolerance, lower net reproduction and energy reserves.

In **chapter 4**, a second multi-generation acclimation experiment with *D. magna* was performed during 5 consecutive generations. In this study different bioavailable copper concentrations were used divided in two groups: N and M group experiments. The dissolved copper acclimation concentrations of the culture medium used for both groups were 1, 12 and 100 µg Cu L^{-1} corresponding to 20, 300 and 2000 pM Cu^{2+} for the N group and 2000, 300 and 20 pM Cu^{2+} for the M group. This was acquired by changing the dissolved organic carbon concentration, pH and hardness levels. After five generations of acclimation, an increase in the acute copper tolerance was observed for both groups in which daphnids were acclimated to 100 µg Cu L^{-1}. The internal copper concentration also increased with increasing dissolved copper concentration. An optimal copper concentration range for first generation daphnids was determined around 12 µg Cu L^{-1} using energy reserves as an endpoint. Acclimation of the daphnids for five consecutive generations to the three dissolved copper concentrations,

resulted in a shift in the optimal concentration range towards 1 µg Cu L^{-1}. Our results suggest that copper acclimation and accumulation are more related to the dissolved copper concentration of the culture medium, than to the copper activity of the medium.

In **chapter 5**, copper accumulation and regulation in the acclimated *P. subcapitata* (as described in chapter 2) and in *D. magna* (as described in chapter 3 and 4) was examined and the results were linked to the previously established OCEE for copper and both organisms. Constant body concentrations were found between 1 and 5 µg Cu L^{-1} for *P. subcapitata* and between 1 and 12 µg Cu L^{-1} for *D. magna*. The bioconcentration factors (BCFs) indicated that copper was actively regulated (including active accumulation) in the deficient and optimal range for *D. magna*, while for *P. subcapitata* a part of the optimal range included a copper storage mechanism. At the highest (toxic) acclimation concentration, internal copper concentrations increased significantly indicating the failure of the copper regulation mechanisms. Absorbed copper concentrations lower than 13 µg Cu g DW^{-1} resulted in deficiency in both *P. subcapitata* and *D. magna*.

In **chapter 6**, the copper sensitivity of various cladoceran species collected in six European surface waters was assessed. The collected species belonged to 4 different families (Daphniidae, Bosminidae, Macrothricidae, Chydoridae) and 13 different genera. The 48-h EC50 of the various cladoceran species, tested in a standard laboratory medium (ISO), ranged from 5.3 to 71 µg Cu L^{-1}. Among seven common genera a significant variation in copper sensitivity was demonstrated (factor of 3). Including the other genera, this raised up to a factor of 12. Most of the field-collected cladoceran species were more sensitive than the standard laboratory clone of *D. magna*. A positive relationship was also observed between the 48-h EC50 of the field-collected cladoceran species (without the Chydoridae family) and the length of the organisms. The generic SSD (log-normal distribution) based on toxicity data obtained in this standard medium for all species (collected at all sites) resulted in a hazardous concentrations which protects 95 % of the species occurring in a (hypothetical) ecosystem (*i.e.* HC5) of 6.7 µg Cu L^{-1} (90 % confidence limits: 4.2 – 10.8 µg Cu L^{-1}). This generic SSD was not significantly different from the site-specific SSDs. Community sensitivity (the geometric mean of 48-h EC50 values of species within a community) among sites varied within a factor of 2 (between 17.3 and 23.6 µg Cu L^{-1}), while HC5s varied within a factor of 4 (between 4.5 and 17.3 µg Cu L^{-1}). It is concluded that the community sensitivity of different

cladoceran populations is similar among aquatic systems and is not dependent on the species composition.

In **chapter 7**, the acute copper sensitivity of the field-collected cladoceran species (chapter 6) was determined using their natural surface waters. The 48-h EC50 of the various cladoceran species ranged from 9.6 to 853 µg Cu L^{-1} in natural waters. The community sensitivity ranged from 16.4 to 281 µg Cu L^{-1}. Comparing this to the results obtained in chapter 6, indicates that bioavailability is more important than inter-community (species composition) differences in determining the variability of copper toxicity across different aquatic systems. For the four surface waters which had a pH within the range for which the acute *D. magna* biotic ligand model (BLM) has previously been successfully validated, the BLM predicted 48-h EC50s for 27 of the 28 tested cladoceran species within factor of 2 of the observed values. For the same sites all community sensitivities were predicted within a factor of 2.3. The BLM was generally protective for the "normal" systems and clearly over-protective for the two acidic surface waters tested.

In **chapter 8**, general conclusions and future perspective were formulated. The main conclusions of all chapters described above include (1) acclimation to copper does occur at environmentally relevant copper concentrations (12 µg Cu L^{-1}), but is of less importance in the context of regulatory risk assessments as the sensitivity of the model organisms was affected by only a factor of 1.6; (2) culture and test media containing no or only very limited amounts of essential elements should be avoided as they render the test organisms over-sensitive; (3) optimal concentrations of copper can shift during the acclimation and are thus dependent on the ambient copper concentration of the medium; (4) different sensitivity to copper is observed among different cladoceran species; (5) different aquatic systems have a similar community sensitivity and (6) the copper sensitivity of field-collected cladoceran species and the community sensitivity of different aquatic systems can be predicted in natural surface waters using the BLM approach.

Samenvatting

Deze doctoraatsthesis is gesitueerd in het domein van de aquatische toxicologie, *i.e.* de studie van de effecten van chemische stoffen op de structuur en de werking van zoetwater en mariene ecosystemen. Het doel van de aquatische ecotoxicologie is het evalueren van de risico's van natuurlijke en antropogene stoffen voor aquatische ecosystemen. Meer specifiek is het onderwerp van deze thesis de ecotoxicologie van metalen in zoetwater ecosystemen.

In tegenstelling tot de ontelbare synthetische organische chemicaliën zijn metalen natuurlijk voorkomende stoffen en het leven is geëvolueerd in de aanwezigheid van deze elementen. Enkele van deze metalen, de essentiële metalen (zoals koper), zijn geïncorporeerd in metabolische processen die cruciaal zijn voor overleving, groei en reproductie van organismen. Elke soort kent voor elk essentieel element een optimaal concentratiebereik (OCEE) waarbinnen ze kan voldoen aan de metabolische behoeften en optimaal kan functioneren. Wanneer de externe concentratie echter te hoog of te laag wordt, zal deze homeostatische regulatie falen en zal er respectievelijk toxiciteit en deficiëntie optreden. Deze studie zal zich focussen op het optimaal bereik van het essentieel element koper, *i.e.* koper concentraties waarbij homeostatische regulatie en interacties met het organisme op fysiologisch niveau optreden.

Het voorliggend onderzoek bestudeert enkele ecologische factoren die, tot op vandaag, nog niet in rekening gebracht zijn in de milieu-risicoanalyse procedures voor metalen: (1) in natuurlijke systemen kunnen organismen/gemeenschappen geadapteerd of geacclimatiseerd zijn aan achtergrond- of milieuconcentraties van koper, aanwezig in de oppervlaktewateren; en (2) de kopergevoeligheid van soorten, in het veld verzameld, kan verschillen van deze van standaard gebruikte organismen (intra- en inter-soorten verschillen). Als de invloed/grootte van deze tekortkomingen kan vastgesteld worden, kunnen ze geïncorporeerd worden in het biotisch ligand model (BLM), zodanig dat de ecologische relevantie van de voorspelde metaaltoxiciteit kan verbeterd worden. Dit onderzoek is onderverdeeld in een experimenteel deel, waarbij gebruik gemaakt wordt van standaard organismen (hoofdstuk 2 tot 5) en in een deel, waarbij gebruik gemaakt wordt van cladoceren, gecollecteerd in het veld (hoofdstuk 6 en 7). Elk hoofdstuk beschrijft een goed gedefinieerde reeks van experimenten over een specifiek onderwerp.

In **hoofdstuk 2**, werd de zoetwater groenwier *Pseudokirchneriella subcapitata* gekweekt bij zeven koperconcentraties – waarvan vijf ecologisch relevant voor Europese oppervlaktewateren – variërend tussen < 0.5 (geen kopertoevoeging) en 100 µg Cu L^{-1} (*i.e.* 1.3 ± 10^{-18} tot 1.9 ± 10^{-8} M Cu^{2+}). Na 12 weken van acclimatisatie verhoogde de kopertolerantie significant van 88 ± 15 tot 124 ± 25 µg Cu L^{-1} voor *P. subcapitata* geacclimatiseerd respectievelijk aan 0.5 en 100 µg Cu L^{-1}. Gebaseerd op de algenbiomassa, de groeisnelheid, de pigmentdiversiteit en de autotrofe index werd een optimaal concentratiebereik geobserveerd tussen 1 en 35 µg Cu L^{-1} (2.1 ± 10^{-14} tot 6.1 ± 10^{-10} M Cu^{2+}). Lagere en hogere koperconcentraties werden beschouwd als suboptimaal, respectievelijk deficiënt en toxisch. Gebaseerd op de algenbiomassa en groeisnelheid werd een populatie NOEC van 35 µg Cu L^{-1} genoteerd.

In **hoofdstuk 3** wordt de zoetwater cladoceer *Daphnia magna* geacclimatiseerd aan zes verschillende koperconcentraties variërend van < 0.5 (geen kopertoevoeging) tot 100 µg Cu L^{-1} (*i.e.* 7×10^{-15} tot 37×10^{-10} M Cu^{2+}) gedurende 18 maanden. Hun kopertolerantie veranderde in functie van de koperconcentratie, aanwezig in het kweekmedium. De 48-h EC50 verhoogde significant van 204 ± 24 µg Cu L^{-1} tot 320 ± 43 µg Cu L^{-1}. Een niet-significante stijging werd gevonden in de 21-d EC50 (gemiddelde met 95 % confidentie limieten) van 48.0 (47.9 – 48.0) tot 78.8 (66.3 – 93.6) µg Cu L^{-1} in de chronische experimenten. In de eerste generatie situeerde de OCEE zich tussen 5 en 12 µg Cu L^{-1}, gebaseerd op fysiologische en populatieniveau parameters (netto reproductie, energie reserves, lichaamslengte en filtratiesnelheden). Na drie acclimatisatiegeneraties trad er een verschuiving naar lagere en hogere koperconcentraties op en de OCEE was dan gesitueerd tussen 1 en 35 µg Cu L^{-1} (2×10^{-14} tot 80×10^{-12} M Cu^{2+}). Langdurige koperdeficiëntie resulteerde in een verlaagde fitness van de daphnia's, tot uiting komende in verlaagde kopertolerantie, lagere netto reproductie en energiereserves.

In **hoofdstuk 4**, werd een tweede multi-generatie acclimatisatie experiment met *D. magna* uitgevoerd gedurende vijf opeenvolgende generaties. In deze studie werden verschillende biobeschikbare koperconcentraties gebruikt verdeeld over 2 groepen: de experimenten van de N en M groep. De opgeloste koperconcentraties van het acclimatisatiekweekmedium gebruikt voor de twee groepen waren 1, 12 en 100 µg Cu L^{-1}, overeenkomend met 20, 300 en 2000 pM Cu^{2+} voor de N groep en met 2000, 300 en 20 pM Cu^{2+} voor de M groep. Dit werd bereikt door de opgeloste organische koolstofconcentratie, pH en hardheidsniveau te veranderen. Na

vijf acclimatisatie generaties werd een verhoging in de acute kopertolerantie gevonden voor beide groepen waarin de daphnia's geacclimatiseerd waren aan 100 µg Cu L^{-1}. De interne koperconcentratie verhoogde ook met stijgende opgeloste koperconcentraties. Een optimaal koperconcentratiebereik voor de eerste generatie daphnia's situeerde zich rond 12 µg Cu L^{-1} met energiereserves als eindpunt. Acclimatisatie van de daphnia's, gedurende vijf opeenvolgende generaties blootgesteld aan de drie verschillende koperconcentraties, resulteerde in een verschuiving van het optimaal concentratiebereik naar 1 µg Cu L^{-1}. Onze resultaten suggereerden dat koperacclimatisatie en -accumulatie meer gerelateerd waren aan de opgeloste koperconcentratie dan aan de koperactiviteit van het medium.

In **hoofdstuk 5** werd de koperaccumulatie en -regulatie in de geacclimatiseerde *P. subcapitata* (als beschreven in hoofdstuk 2) en in *D. magna* (als beschreven in hoofdstuk 3) bestudeerd en de resultaten werden gerelateerd aan de vroeger gevonden OCEE voor koper en voor beide organismen. Constante lichaamsconcentraties werden gevonden tussen 1 and 5 µg Cu L^{-1} voor *P. subcapitata* en tussen 1 en 12 µg Cu L^{-1} voor *D. magna*. De bioconcentratiefactoren (BCFs) toonden aan dat koper actief gereguleerd (inclusie actieve accumulatie) is in het deficiënt en optimaal bereik voor *D. magna*, terwijl bij *P. subcapitata* binnen een deel van het optimaal bereik ook een koperopslag mechanisme voorkomt. Bij de hoogste (toxisch) acclimatisatieconcentratie steeg de interne concentratie significant, aantonend dat de koperregulatie mechanismen faalden. Geabsorbeerde koperconcentraties, lager dan 13 µg Cu g DW^{-1}, resulteerden in deficiëntie voor zowel *P. subcapitata* als *D. magna*.

In **hoofdstuk 6** werd de kopergevoeligheid van verschillende cladocerensoorten, gecollecteerd in zes Europese oppervlaktewateren, bepaald. De gecollecteerde soorten behoorden tot 4 verschillende families (Daphniidae, Bosminidae, Macrothricidae, Chydoridae) en 13 verschillende genera. De 48-h EC50 van de verschillende soorten, getest in een standaard laboratoriummedium (ISO), situeerden zich tussen 5.3 en 71 µg Cu L^{-1}. Tussen zeven algemene genera werd een significante variatie in kopergevoeligheid aangetoond (factor 3). Inclusief de andere genera verhoogde dit tot een factor 12. De meeste in het veld gecollecteerde cladocerensoorten waren gevoeliger dan de standaard *D. magna* laboratoriumkloon. Een positieve relatie tussen de 48-h EC50 van de in het veld gecollecteerde cladocerensoorten (zonder de Chydoridae) en de lengte van de organismen werd gevonden. De generieke soortgevoeligheidsdistributie (SSD; log-normaal verdeling),

gebaseerd op de toxiciteitsdata van alle species (gecollecteerd in alle sites), verkregen in het standaardmedium, resulteerde in een HC5 waarde van 6.7 µg Cu L^{-1} (90 % confidentie limieten: 4.2 – 10.8 µg Cu L^{-1}), *i.e.* de concentratie die 95 % van de soorten voorkomend in een (hypothetisch) ecosysteem beschermt. Deze generieke SSD was niet significant verschillend van de site-specifieke SSDs. De gemeenschapsgevoeligheid (*i.e.* geometrisch gemiddelde van de 48-h EC50 waarden van de species binnen een gemeenschap) varieerde tussen de sites binnen een factor 2 en schommelde tussen 17.3 en 23.6 µg Cu L^{-1}, terwijl de HC5 waarden varieerden binnen een factor 4 en schommelden tussen 4.5 en 17.3 µg Cu L^{-1}. Er werd geconcludeerd dat de gemeenschapsgevoeligheid van de verschillende cladocerenpopulaties gelijkaardig is tussen aquatische systemen en onafhankelijk van de soortensamenstelling.

In **hoofdstuk 7** werd de acute kopergevoeligheid van de in het veld gecollecteerde cladocerensoorten (hoofdstuk 6) bepaald in hun natuurlijk water. De 48-h EC50 van de verschillende soorten situeerde zich tussen 9.6 en 853 µg Cu L^{-1}. De gemeenschapsgevoeligheid lag tussen 16.4 en 281 µg Cu L^{-1}. Vergeleken met de resultaten uit hoofdstuk 6, wordt duidelijk dat biobeschikbaarheid een belangrijkere factor is dan inter-gemeenschapsverschillen (soortensamenstelling) bij het bepalen van de variabiliteit van kopertoxiciteit tussen verschillende aquatische systemen. Voor vier oppervlaktewateren die een pH hadden, liggend binnen het bereik waarin het acute *D. magna* BLM succesvol gevalideerd was, lagen de BLM-voorspelde 48-h EC50s, voor 27 van de 28 geteste cladocerensoorten, binnen een factor 2 van de geobserveerde waarden. Voor dezelfde sites werden de gemeenschapgevoeligheden voorspeld binnen een factor 2.3. Het BLM was over het algemeen beschermend voor de 4 “normale” systemen en duidelijk overbeschermend voor de twee zure oppervlaktewateren.

In **hoofdstuk 8** werden de algemene conclusies en vooruitzichten geformuleerd. De hoofdconclusies van alle hierboven beschreven hoofdstukken waren (1) acclimatisatie aan koper komt voor bij milieurelevante koperconcentraties (12 µg Cu L^{-1}), maar is van minder belang in de context van regelgevende risicobepalingen omdat de gevoeligheid van de modelorganismen beïnvloed waren met slechts een factor van 1.6; (2) kweek- en testmedia, met geen of zeer lage hoeveelheden aan essentiële elementen moeten vermeden worden omdat zij zorgen voor overgevoelige testorganismen; (3) optimale koperconcentraties verschuiven gedurende de acclimatisatie en zijn dus afhankelijk van de aanwezige

koperconcentratie in het medium; (4) verschillende kopergevoeligheid is geobserveerd tussen de verschillende cladocerensoorten; (5) verschillende aquatische systemen hebben een gelijkaardige gemeenschapsgevoeligheid; en (6) de kopergevoeligheid van in het veld gecollecteerde cladocerensoorten en de gemeenschapsgevoeligheid van verschillende aquatische systemen kunnen voorspeld worden in natuurlijke oppervlaktewateren met de BLM-benadering.

Curriculum vitae

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Oral Presentations

Bossuyt BTA, De Schamphelaere, KAC, Janssen, CR. Copper sensitivity of field-collected cladoceran communities: species sensitivity distributions, acclimation and bioavailability. 14th Annual Meeting of SETAC Europe, 18-22 April 2004, Prague, Czech republic.

Deleebeeck NME, De Schamphelaere KAC, Heijerick DG, Bossuyt BTA, Janssen CR. Influence of water quality characteristics on the chronic toxicity of nickel to freshwater organisms: development and validation of chronic biotic ligand models. 14th Annual Meeting of SETAC Europe, 18-22 April 2004, Prague, Czech republic.

De Schamphelaere KAC, Bossuyt, BTA, Deleebeeck N, Heijeick DG, Van Sprang PA, Janssen CR. The biotic ligand model for metals: from development and field validation to uncertainty reduction in ecological risk assessment. Special Symposium: RA of metals: Bioavailability to reduce uncertainty. 14th Annual Meeting of SETAC Europe, 18-22 April 2004, Prague, Czech republic.

Burton GA, Custer K, Geyer S, Nguyen L, Janssen C, McWilliam R, Bossuyt B, Beltrami M, Baudo R. Identification of stressor effects using in situ exposures of species, populations and communities. Special Symposium: RA of metals: Bioavailability to reduce uncertainty. 14th Annual Meeting of SETAC Europe, 18-22 April 2004, Prague, Czech republic.

Bossuyt BTA, De Schamphelaere KAC, Janssen CR. Using the biotic ligand model for predicting the acute sensitivity of cladoceran dominated population to copper in natural surface waters. BELTOX meeting, April 1st, 2004, Liège, Belgium.

Janssen CR, De Schamphelaere KAC, Bossuyt BTA. Copper in aquatic environments: recent advances in acclimation and bioavailability research. Fifth International Conference devoted to Economics, the Mineral Processing, the extractive Metallurgy, the Environment and Applications of Copper, Copper 2003 – Cobre 2003, November 30 – December 3 2003, Santiago, Chile.

De Schamphelaere KAC, Heijerick DG, Bossuyt BTA, Van Sprang P, Deleebeeck N, Muyssen BTA, Janssen CR. The biotic ligand model: mechanisms and mathematics. SETAC 24th Annual Meeting in North America, 9-13 November 2003, Austin, Texas, USA.

Burton GA, Nguyen LT, Janssen C, McWilliam R, Bossuyt B, Beltrami M, Baudo R, Green A. Field validation of zinc effects and the SEM-AVS model: Benthic colonization and *in situ* toxicity. SETAC 24th Annual Meeting in North America, 9-13 November 2003, Austin, Texas, USA.

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Janssen C, De Schamphelaere K, Heijerick D, Bossuyt B. Biotic ligand model development and validation: predicting acute and chronic toxicity. Workshop Bioavailability of metals in surface waters " Integrating Science and Regulation". 7-8 February 2002, Het Pand, Ghent, Belgium.

Janssen C, Heijerick D, De Schamphelaere K, Bossuyt B. Copper toxicity in European surface waters: observed vs. predicted toxicity as a function of water characteristics. Sixth International Conference on the Biogeochemistry of trace elements, ICOBTE 2001 conference proceedings, July 29-August 2, University of Guelph, Guelph, Ontario, Canada.

Heijerick DG, Bossuyt BT, Janssen CR. Copper toxicity for *Daphnia magna*, *Raphidocelis subcapitata* and local crustaceans in six European surface waters. 11th Annual Meeting of SETAC Europe. 6-10 May 2001, Madrid, Spain.

De Schamphelaere KA, Heijerick DG, Bossuyt BT, Janssen CR. Refinement and validation of a biotic ligand model predicting acute copper toxicity for *Daphnia magna*. 11th Annual Meeting of SETAC Europe. 6-10 May 2001, Madrid, Spain.

Janssen C, Heijerick D, Lock K, Muyssen B, De Schamphelaere K, Bossuyt B, Vangheluwe M, Van Sprang P. Ecological considerations in the environmental risk assessment of metals: considering uncertainties in current practices. 6th International Symposium on Metal Ions in Biology and Medicine. San Juan – Puerto Rico. May 2000.

Poster presentations

Bossuyt BTA, De Schamphelaere KAC, Janssen CR. Effects of sodium, calcium and magnesium on the sensitivity of field-collected cladocerans to copper. 14th Annual Meeting of SETAC Europe, 18-22 April 2004, Prague, Czech republic.

Bossuyt BTA, Janssen CR. Species sensitivity distributions: cladoceran dominated communites exposed to copper. 14th Annual Meeting of SETAC Europe, 18-22 April 2004, Prague, Czech republic.

Bossuyt BTA, Muyssen BTA, De Schamphelaere KAC, Janssen CR. Species sensitivity distributions of field-collected crustacean populations: a reality check of current risk assessment practice. BELTOX meeting, April 1st, 2004, Liège, Belgium.

Bossuyt BTA, Muyssen BTA, De Schamphelaere KAC, Janssen CR. Species sensitivity distributions of field-collected crustacean populations: a reality check of current risk assessment practice. SETAC 24th Annual Meeting in North America, 9-13 November 2003, Austin, Texas, USA.

Bossuyt, B.T.A. and Janssen, C.R. Acclimation of *Daphnia magna* Straus to copper: Effects on sensitivity and accumulation. Seventh International Conference on the Biogeochemistry of trace elements, ICOBTE 2001 conference proceedings, June 15-19, University of Uppsala, Uppsala, Sweden.

De Schamphelaere KA, Heijerick DG, Muyssen BT, Bossuyt BT, Janssen CR. How important is dietary exposure for predicting toxicity of essential metals to *Daphnia magna*? SETAC 23rd Annual Meeting in North America, 16-20 November 2002, Salt Lake City, Utah.

Bossuyt BT, Janssen CR. Influence of bioavailability on acclimation of *Daphnia magna* to copper. 8th symposium on Applied Biological Sciences, 9 October 2002, Het Pand, Ghent, Belgium.

Bossuyt BT, Janssen CR. Reducing uncertainty: (1) Uncertainty factors may lead to copper deficiency. Copper Risk Assessment Workshop, 13 & 14 June 2002, Renaissance Hotel Brussels, Belgium.

Bossuyt BT, Janssen CR. Reducing uncertainty: (2) Ecosystem-dependent species sensitivity differences. Copper Risk Assessment Workshop, 13 & 14 June 2002, Renaissance Hotel Brussels, Belgium.

Bossuyt BT, Janssen CR. Influence of multi-generation acclimation to copper on tolerance, energy reserves and homeostasis of *Daphnia magna*. 12th Annual Meeting of SETAC Europe, 12-16 May 2002, Vienna, Austria.

Bossuyt BT, Janssen CR. Copper toxicity to field-collected aquatic organisms. 12th Annual Meeting of SETAC Europe, 12-16 May 2002, Vienna, Austria.

Bossuyt BT, Janssen CR. Acclimation of the freshwater crustacean *Daphnia magna* to copper: Changes in tolerance and energy allocation. 7th FLTBW PhD Symposium, 10 October 2001, Ghent, Belgium.

Bossuyt BT, Cornelis BJ, Janssen CR. Bioavailability and ecotoxicity of nickel: effect of water quality characteristics. 7th FLTBW PhD Symposium, 10 October 2001, Ghent, Belgium.

Bossuyt B, Janssen CR. Acclimation of *Daphnia magna* to different background concentrations of copper. SETAC Europe 11th Annual Meeting, May 6th-10th 2001, Madrid, Spain.

Bossuyt B, Heijerick D, De Schamphelaere KA, Janssen CR. Comparative sensitivity of laboratory-reared and field-collected organisms. SETAC Europe 11th Annual Meeting, May 6th-10th 2001, Madrid, Spain.

Conferences, workshops and symposia

14th Annual Meeting of SETAC Europe, 18-22 April, Prague, Czech republic.

BELTOX, Role of Bioavailability in Environmental Risk Assessment, 1st of April 2004, ISSeP, Liège, Belgium.

7th International Conference on the Biogeochemistry of Trace Elements, ICOBTE 2003 conference proceedings, June 15-19, University of Uppsala, Uppsala, Sweden.

8th Symposium on Applied Biological Sciences, 9 October 2002, Het Pand, Ghent, Belgium.

Copper Risk Assessment Workshop, 13-14 June 2002, Brussels, Belgium.

12th Annual Meeting of SETAC Europe, 10-16 May 2002, Vienna, Austria.

Workshop on “Bioavailability of metals in surface waters: integrating science and regulations”, 7-8 February 2002, Ghent, Belgium.

7th FLTBW PhD Symposium, 10 October 2001, Ghent, Belgium.

11th Annual Meeting of SETAC Europe, 6-10 May 2001, Madrid, Spain.

7th FECS Conference on Science and the Environment. "Metal Speciation in the Aquatic Environment", 27-30 August 2000, Porto, Portugal.

21st Congress of the European Society for Comparative Physiology and Biochemistry. 24-28 July 2000, Liège, Belgium.

Foreign research visits

August 5, 2003. Institut National de l'Environment Industriel et des Risques (INERIS), Verneuil-en-Halatte, France, under the supervision of Francois Le Goff.

July 2, 2002. Institut National de l'Environment Industriel et des Risques (INERIS), Verneuil-en-Halatte, France, under the supervision of Dr. Jean-Marc Bonzom.

June 10-16, 2001. Fraunhöfer Institut, Schmallenberg, German, under the supervision of Dr. Christoph Schäfers.

November 1-2, 2000. Lancaster University, Lancaster, United Kingdom, under the supervision of Dr. Hao Zhang.

October 30, 2000. Water Research Centre (WRc), Medmenham, United Kingdom, under the supervision of Dr. Sean Comber.

Membership in professional communities

Society of Environmental Toxicology and Chemistry (SETAC) (since 2000).