Note concerning the citation of Ph D thesis - electronic version:

The author would prefer that this work is not cited, except for the introductory Chapter 1). Each chapter (2 to 7) is redrafted after one or more publications in a scientific journal and we would like that citations are made to these journal articles. Below is a list of the journal articles associated with each chapter.

Chapter 2:

De Schamphelaere KAC, Janssen CR. 2002. A biotic ligand model predicting acute copper toxicity for *Daphnia magna*: the effects of calcium, magnesium, sodium, potassium and pH. Environmental Science and Technology 36:48-54.

Chapter 3:

De Schamphelaere KAC, Heijerick DG, Janssen CR. 2002. Refinement and field validation of a biotic ligand model predicting acute copper toxicity to *Daphnia magna*. Comparative Biochemistry and Physiology C 133:243-258.

Chapter 4 (2 papers):

De Schamphelaere KAC, Janssen CR. 2004. Effects of dissolved organic matter concentration and source, pH and water hardness on chronic toxicity of copper to *Daphnia magna*. Environmental Toxicology and Chemistry 23: 1115-1122.

De Schamphelaere KAC, Janssen CR. 2004. Development and field validation of a biotic ligand model predicting chronic copper toxicity to Daphnia magna. Environmental Toxicology and Chemistry 23: 1365-1375.

Chapter 5:

De Schamphelaere KAC, Janssen CR. 2004. Effects of chronic dietary copper exposure on growth and reproduction of *Daphnia magna*. Environmental Toxicology and Chemistry 23: 2038-2049.

Note: the modelling part of this chapter (pages 165-170) is not adopted in the paper. When referring to this model, this Ph D thesis may be cited..

Chapter 6:

De Schamphelaere KAC, Vasconcelos FM, Heijerick DG, Tack FMG, Delbeke K, Allen HE, CR Janssen. 2003. Development and field validation of a predictive copper toxicity model for the green alga *Pseudokirchneriella subcapitata*. Environmental Toxicology and Chemistry 22:2454-2465.

Chapter 7:

De Schamphelaere KAC, Vasconcelos FM, Allen HE, Janssen CR. 2004. The effect of dissolved organic matter source on acute copper toxicity to *Daphnia magna*. Environmental Toxicology and Chemistry 23:1248-1255.

FACULTEIT
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BIOAVAILABILITY MODELS FOR PREDICTING COPPER TOXICITY TO FRESHWATER ORGANISMS

BIOBESCHIKBAARHEIDSMODELLEN VOOR HET VOORSPELLEN VAN DE TOXICITEIT VAN KOPER VOOR ZOETWATERORGANISMEN

door

KAREL DE SCHAMPHELAERE

Thesis submitted in fulfillment of the requirements for the degree of
Doctor 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**

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De promotor, The promotor,

Prof. Dr. Colin Janssen

De auteur, The author

Karel De Schamphelaere

Physical reality is fundamental, mathematical language is merely a useful approximation.

Author unknown

The enormous usefulness of mathematics in the natural sciences is something bordering on the mysterious.

Eugene P. Wigner

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ONDERZOEKSGROEP
MILIEUTOXICOLOGIE

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Deinze, 12 juni 2003

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List of abbreviations

AAP	Algal assay procedure bottle test
AHA	Aldrich humic acid
ASTM	American Society for Testing and Materials
BLM	biotic ligand model
CA	carbonic anhydrase
CCC	criterion continuous concentration
CL	confidence limit
CMC	criterion maximum concentration
DIC	dissolved inorganic carbon
DOC	dissolved organic carbon
DOM	dissolved organic matter
DW	dry weight
UV	ultra violet
ε_{350}	absorption coefficient at 350 nm
E_bC10	Effect concentration resulting in 10% growth inhibition (algae)
E_bC50	Effect concentration resulting in 50% growth inhibition (algae)
EC50	Effect concentration resulting in 50% effect
EDTA	ethylene diamine tetra acetic acid
EQC	Environmental Quality Criteria
EU	European Union
FIAM	free ion activity model
GSIM	gill surface interaction model
HC5	hazardous concentration for 5% of the organisms
HMTV	hardness-modified trigger value
IC	inorganic carbon
ISO	International Standardization Organization
IWP	Interdepartmental Working Party on Setting Integrated Environmental Quality Standards for Substances
LC50	lethal concentration for 50% of the tested organisms
LOEC	lowest observed effect concentration
MPA	maximum permissible addition

MPC	maximum permissible concentration
MOPS	3-N morpholino-propane-sulfonic acid
NOEC	no observed effect concentration
OCEE	optimal concentration range of essential elements
OECD	Organization for Economic Cooperation and Development
PEC	predicted environmental concentration
PNEC	predicted no effect concentration
QT	quality target
SE	standard error
SEM	standard error of mean
SSD	species sensitivity distribution
SWAD	surface water database
TGD	technical guidance document
TIC	total inorganic carbon
TV	trigger value
US EPA	United States Environmental Protection Agency
WER	water effect ratio
WHAM	Windermere humic aqueous model
WQC	water quality criteria

Introduction

The research conducted in the context of this doctoral thesis is situated in the field of aquatic toxicology, a sub-discipline of ecotoxicology. In aquatic toxicology the effects of natural and anthropogenic substances on the structure and function of freshwater and marine ecosystems are studied (Boudou and Ribeyre, 1989). Based on an interdisciplinary approach between analytical chemistry, biology, toxicology, ecology and physiology, aquatic toxicology aims at providing knowledge that may help in evaluating hazards and risks of chemical substances to aquatic ecosystems and thus in protecting the environment.

The subject of this thesis is the ecotoxicology of metals in freshwater ecosystems and the use of ecotoxicological test data for risk assessment and water quality criteria setting procedures. As opposed to the numerous man-made organic chemicals, metals are naturally occurring substances in the presence of which life has evolved. Some of these metals, the essential metals (e.g. zinc, copper, iron, manganese) have become incorporated into metabolic processes crucial to survival, growth and reproduction of organisms (Linder, 1991; Keen et al., 1993; O'Halloran, 1993).

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 of essential elements (OCEE). When the external concentration of the essential element becomes too low or too high, homeostatic regulation will not be sufficient and deficiency or toxicity can occur, respectively. The focus of this study is on the toxicity of the essential element copper, i.e. on copper concentrations at which the homeostatic regulation capacity of organisms is exceeded. Although copper is essential, its OCEE is relatively small and beyond this range, copper can become toxic, due to its high reactivity with ligands, including functional groups on essential biomolecules of organisms (Mason and Jenkins, 1995).

Nevertheless, copper has many unique properties that justify its use in many wide-spread applications such as electric wires, roof sheeting, water pipes and tubing, biocides and soil fertilizers (Landner and Lindeström, 1999). The wide-spread use, together with its toxicity potential underlines the need for a correct risk assessment and appropriate water quality criteria for this metal.

Authorities all over the world have developed or are developing methods to derive water quality criteria (WQC) and to evaluate the potential environmental risks of metals and man-made substances. To date, WQC and risk assessment of metals are still predominantly based on total or dissolved concentrations (Bergmann and Dorward-King, 1997; Janssen et al., 2000). However, total or even dissolved concentrations of metals are no good predictors of their potential effects on ecosystems. Indeed, several physico-chemical water characteristics such as dissolved organic carbon (DOC), pH and hardness can modify toxicity with several orders of magnitude.

The latter is summarized with the term “bioavailability”. A metal is considered bioavailable when it is free for uptake by an organism and when it can react with its metabolic machinery, which may result in a toxicity response (Newman and Jagoe, 1994; Campbell et al., 1988). The main idea behind “bioavailability”, is that the toxic effect of a metal does not only depend on the total (or dissolved) concentration of that metal in the surrounding environment, but also on the complex interaction between physico-chemical and biological factors. In other words, the same total metal concentration does not result in the same degree of toxic effect under all environmental conditions.

The latter indicates that, if bioavailability is not taken into account, water quality criteria based on total or dissolved concentrations may be under-protective for one type of surface water and over-protective for another. In the context of sustainable development, neither over nor under-protection is desirable as the former will result in increased societal costs involved with emission reduction and environmental sanitation measures, whereas the latter may result in harm to aquatic life and biodiversity.

Despite the body of evidence on the effects of water chemistry on metal toxicity that has been generated during the past decades few regulatory systems have taken this into account. This is mainly due to a lack of quantitative tools. However, in this context, the

recently developed biotic ligand model (BLM) has gained increased interest from both the academic, industrial and regulatory community as this (conceptual) model is able to predict metal toxicity by integrating the most important effects of water chemistry. As such, the BLM can be regarded as a milestone in the ecological risk assessment of metals. Although the foundations were already laid as far back as the early 1970's, the reason of the current success of the BLM is that, for the first time, a model was able to integrate all available state-of-the-science, interdisciplinary knowledge on metal bioavailability into a generalized, visually attractive and easy-to-handle computerized framework.

The BLM was originally developed to predict acute (short-term) toxicity to fish (Di Toro et al., 2001; Paquin et al., 2002a) based on a combination of existing chemical, toxicological, biological and physiological data. At the start of this study, also initial attempts were already available that illustrated the possible use of the BLM concept to predict acute metal toxicity to invertebrate species. However, no real proof for the applicability of the BLM-concepts to this group of organisms was provided (Santore et al., 2001).

Although the acute BLM is currently considered for implementation in the WQC for copper in the U.S.A., it is not suitable for incorporation into the European Union (EU) regulatory frameworks. In the EU, environmental management of metals (and other chemical substances) is addressed through the risk assessment approach (with the exception of Cd, Ni, Hg and Pb, which are currently being managed through WQC derivation) (European Commission, 1993a, 1993b). In this type of risk assessment, according to principles laid down in the technical guidance document (European Commission, 2003) a predicted environmental concentration (PEC) and a predicted no-effect concentration (PNEC) are derived and finally compared to evaluate the risk of the substance.

PNEC derivation is preferably based on chronic toxicity data obtained with aquatic organisms. Consequently if bioavailability is to be incorporated into the risk assessment of copper in a quantitative manner, models are needed that can predict chronic copper toxicity to different organisms.

The main goal of this study was therefore to develop chronic bioavailability models for organisms belonging to two key-groups, that are generally considered to be very important for aquatic ecosystems: the invertebrate *Daphnia magna* (Crustacea: Cladocera), and the

green alga *Pseudokirchneriella subcapitata* (Chlorophyta: Chlorococcales). Together with some fish species, these two organisms are regarded as the so-called "ecotoxicological base-set" in risk assessment of substances. It was anticipated that bioavailability models developed for these two organisms would help to improve the ecological relevance of currently applied risk assessment procedures for copper.

The thesis consists of 8 chapters covering the following topics:

1. a general introduction and conceptual framework of the study
2. the development of an acute Cu-BLM for *D. magna*
3. the refinement and validation of this BLM
4. the development and validation of a chronic Cu-BLM for *D. magna*
5. the evaluation of the importance of dietary Cu exposure for *D. magna*
6. the development and field-validation of a chronic Cu-toxicity model for *P. subcapitata*
7. the evaluation of the importance of dissolved organic matter source for modelling Cu toxicity
8. a summary of general conclusions and future research perspectives.

Chapter 1

General introduction and conceptual framework of the study

Chapter
1

General introduction and conceptual framework of the study

1.1. Copper in society and the environment

1.1.1. The element and its properties

The atomic number of copper (Cu) is 29 and its atomic weight is 63.546 g mol^{-1}. The density of copper is 8.94 g cm^{-3} at 20°C which ranks them according to Wittmann (1979) into the category of the "heavy" metals, a term that nowadays is less frequently used by most environmental scientists due to its inherently negative connotation.

The electron configuration of copper is $1s^2 2s^2 2p^6 3s^2 3p^6 3d^{10} 4s^1$ and as such it belongs to the first transition series which runs from scandium to zinc and in which the 3d orbital is successively being filled (Stumm and Morgan, 1996). In the periodic system copper is the first element in group IB and belongs to the same group as silver and gold. Therefore it is sometimes considered as one of the "precious metals" (Landner and Lindeström, 1999).

In aerobic freshwater environments Cu mainly occurs in the divalent form, i.e. Cu(II), but inside cells it also exists in the monovalent form, i.e. Cu(I). 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- or 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 and organic complexes 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).

"It is no exaggeration to assert that the single event that has contributed more than any other to the widespread use of copper … was the development of electric power as the major form of energy" (Landner and Lindeström, 1999). Indeed, copper has the highest electrical

conductivity of any metal other than silver. Other properties that explain the wide-spread use of copper in today's society include its high thermal conductivity, ductility and malleability and its good ability to form alloys with other metals (Landner and Lindeström, 1999).

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

Figure 1-1 presents the historical copper production during the last 5,000 years.

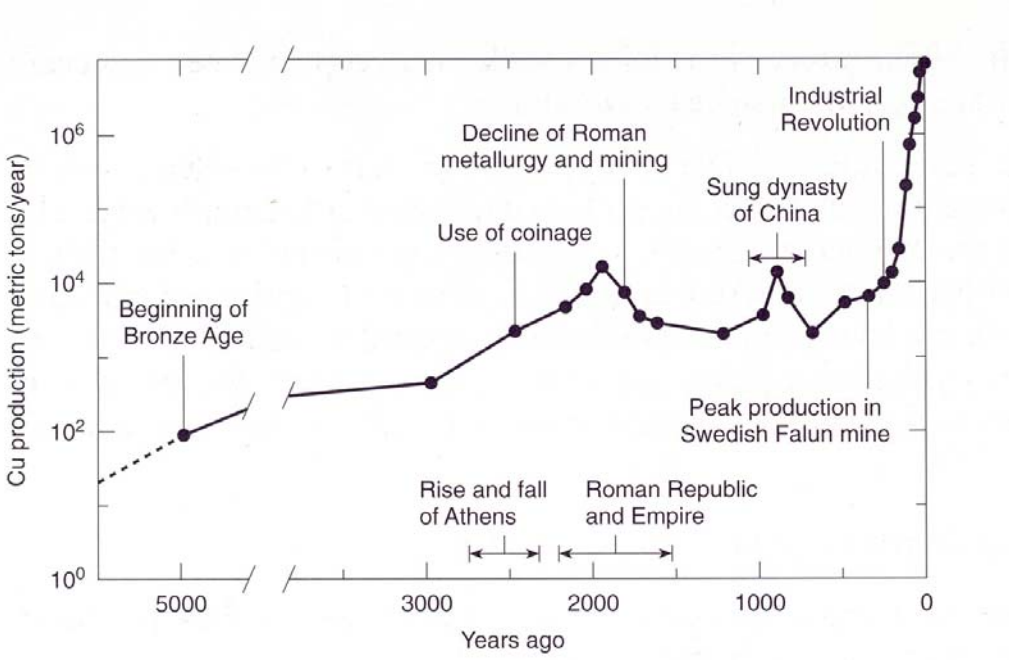

Figure 1-1 Copper production rate during the past 5,000 years, based on a comprehensive review of available literature (Hong et al., 1996).

The use of copper is thought to date back some 9,000 years ago in what is now Turkey, as demonstrated by archaeological findings of decorative artefacts from that period. The discovery of the smelting process to exploit copper carbonate and copper oxide minerals, some two to four thousand years later introduced mankind into the Bronze Age. Mining and production of copper from that time on increased steadily, reaching a peak during the Roman Empire for use in coinage and in alloys, mainly bronzes, for various military and civilian purposes. After the fall of the Roman Empire, copper production greatly declined until the short revival during the Chinese Sung dynasty between the 10^{th} to 12^{th} century. After these early peaks the production and use of copper remained rather limited until its large-scale use

as the preferred material in telegraph wire around 1850. From that time on, copper mining and production has continued to sharply increase for a large number of applications today.

The most important applications of copper and copper alloys today include, but are not restricted to: wire and cable for transmission and electricity, water pipes and tubing, roofing and facing materials on buildings, linings in brakes of vehicles, etc. Copper containing compounds such as Cu(II)-sulphate, Cu(II)-oxide and Cu(II)-naphtenate are used in wood preservatives, algicides, fungicides and boat antifouling paints. These applications exploit the toxicity potential of reactive cupric ions (Cu^{2+}) to "unwanted" organisms. *Vice versa* copper compounds such as Cu(II)-sulphate are frequently used as nutritive additives to livestock feeds and as soil fertilizers in agriculture, in order to prevent copper deficiency, thus exploiting the essentiality of copper (see section 1.2.1.). In this context it should be recognized that inappropriate dosages of copper compounds may result in potentially hazardous copper concentrations in both terrestrial and aquatic environments.

1.1.3. Sources of copper in the environment

1.1.3.1. Occurrence in the earth's crust (Landner and Lindeström, 1999)

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. Among the economically exploitable copper reserves in the world (estimated at about $3 \cdot 10^8$ tonnes in the 1990's), 90% occurs in sulphide ores and 9% in oxide ores. The largest known copper reserves are located in Chile and the U.S.A.

1.1.3.2. Natural and anthropogenic sources

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.). Copper fluxes to the oceans are mainly natural (about 80%, mostly transport from rivers) with 20% anthropogenic (i.e. mostly diffuse sources).

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, 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 that originate from natural sources and that potential increased risk stems from anthropogenic sources that cause increases in metal concentrations above critical levels that may result in adverse effects on ecosystems (Van Tilborg, 2002; see also section 1.2.1).

Natural background concentrations of copper mainly depend on biogeochemical cycling and are, according to Zuurdeeg (1992), between 0.5 and 2.5 μg Cu L^{-1} for Northern Europe. According to an analysis of monitoring data in Europe (data from Belgium, Netherlands, Sweden, United Kingdom, Germany and Spain) environmental concentrations in Europe range between 0.5 to 35 μg Cu L^{-1} (10^{th} and 90^{th} percentiles, respectively), indicating a clear anthropogenic input in an important portion of the monitored freshwater bodies (Heijerick et al., unpublished data).

Anthropogenic sources can be classified as point sources or diffuse (non-point) sources (Landner and Lindeström, 1999). Point sources include, among others, mining 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, releases of copper from paints, wood preservatives and other pesticides, erosion and leaching from agricultural soils (fertilizers and cattle manure). As mentioned earlier the relative importance of these sources is geographically very variable.

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

1.2.1. Essentiality

Life has evolved in the presence of metals, some of which – the essential metals (e.g. copper and zinc) – have become incorporated into metabolic processes crucial for the survival, growth and reproduction of organisms (Linder, 1991; Keen et al., 1993; O'Halloran, 1993). An element is considered essential when: (1) it is determined to be present in all

healthy 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 are linked to a distinct biochemical defect (at the molecular level) (Wittmann, 1979). Some metals such as Fe, Mn, Zn, Cu, Co and Mo are essential for all living organisms. Copper is required for the functioning of a variety of enzymes such as superoxide dismutase (a scavenger of toxic oxy-radicals), cytochrome c-oxidase (part of the electron transport system in eukaryotic organisms), several oxidases (e.g. amine oxidase, ascorbate oxidase), mono-oxygenases and di-oxygenases (Cass and Hill, 1980). 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).

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 of essential elements (OCEE). 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 1-2).

Recently, studies have demonstrated the existence of an OCEE for invertebrates and algae for both zinc and copper (Bossuyt and Janssen, 2003a, 2003b, 2003c; Muyssen and Janssen, 2001; Muyssen and Janssen, 2002). These studies have also indicated that the OCEE may shift to lower or higher concentrations upon acclimation to lower or higher concentrations (Figure 1-2). However, based on acute and chronic toxicity test data these authors only report a maximum of a factor of two to three decrease in sensitivity of *D. magna* and *P. subcapitata* in laboratory acclimation studies with these metals. Although the importance of this acclimation still needs to be addressed under field circumstances, acclimation effects only account for sensitivity differences up to factor two to three (within one species), whereas bioavailability has been demonstrated to account for differences well over 2 orders of magnitude within the same species (Erickson et al., 1996; Di Toro et al.,

2001). The latter clearly illustrates the high importance of bioavailability as compared to acclimation for correctly assessing the environmental risks of (essential) metals (see section 1.4).

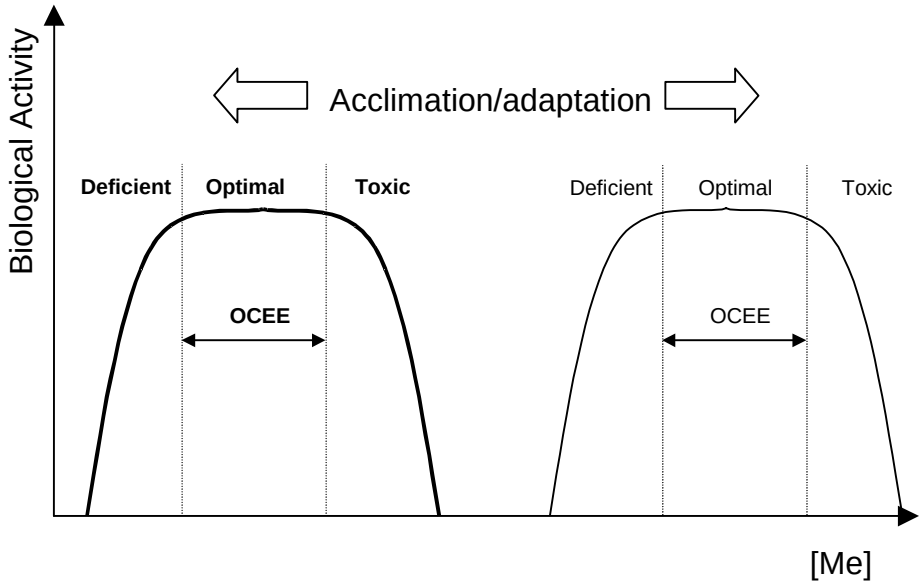

Figure 1-2 The OCEE-concept (Optimal Concentration range of Essential Elements; Van Assche et al., 1997). Biological activity is maximal within the OCEE and decreases at lower (deficiency) or higher concentrations (toxicity). Acclimation and adaptation to lower or higher concentrations can shift the OCEE to lower or higher concentrations, respectively.

1.2.2. Mechanisms of copper toxicity in fish and invertebrates

1.2.2.1. General

Despite the obvious complexity of the toxicological processes of metal toxicity, some generalizations can be inferred. Ochiai (1977) proposed that toxicity is due to non-specific binding of metal ions causing one of the following: 1) blocking of the essential biological functional groups of biomolecules, 2) displacing essential metal ions in biomolecules, and 3) modifying the active conformation of biomolecules. Additionally, copper can also undergo redox cycling within the cell, resulting in the production of reactive oxygen radicals and

leading to tissue damage (e.g. oxidation of lipid membranes) and molecule dysfunction (e.g. DNA damage) (Mason and Jenkins, 1995).

Another point of interest is the location of the sites of toxic action, which may be linked to the route of metal uptake. When considering waterborne and dietary exposure to metals, the gill and gut tissue, respectively, are commonly considered to be the primary target for metal uptake and/or toxicity (Paquin et al., 2002). Basically, there are three possibilities: 1) the metal reacts with biomolecules on the apical membrane of epithelial tissue, causing tissue damage and/or inhibition of transport channels, 2) the metal enters the epithelial tissue and reacts with transport channels on the basolateral membrane, and 3) the metal enters the extracellular fluids (blood or haemolymfe) from where it is distributed into other tissues.

The mechanisms of dietary toxicity are generally less investigated than those of waterborne toxicity. Reported examples include Cd accumulation in gut diverticula of *D. magna* causing a reduced Ca-uptake (Munger et al., 1999), accumulation of metals in copepod (Hg, Cd, Co, Ag and Zn) and cladoceran (Ag) ovary tissues resulting in decreased reproduction (Hook and Fisher, 2001a, 2001b; Hook and Fisher, 2002) and inhibition of feeding rate upon dietary Cd exposure (hypothesized to be the result of Cd binding to gut tissue, causing damage and hence inhibiting food assimilation, Allen et al., 1995; Taylor et al., 1998). The issue of dietary metal toxicity (especially for essential metals) is currently a matter of intensive debate and research and is dealt with in more detail in chapter 5.

The sections below will focus on the mechanisms of acute copper toxicity (in terms of mortality) and on some aspects that may determine chronic copper toxicity to fish and invertebrates. Whereas the mechanisms of acute toxicity are well understood, this is certainly not the case for chronic toxicity as the latter may be the result of a complex combination of different mechanisms.

1.2.2.2 Acute toxicity in fish and invertebrates

Fish and invertebrates have similar ion regulation mechanisms, which are the main target of acute (short-term) metal toxicity (Grosell et al., 2002). The fish gill is not only the tissue that is responsible for oxygen uptake and regulation of major ion balances (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, Cl^{-}, NH_3, H^{+}) but is also the main route of waterborne metal uptake and toxicity.

Gill-like structures also occur in freshwater invertebrates and there is growing evidence that these structures have similar functions (Kikuchi, 1983; Kikuchi and Shiraishi, 1997; Grosell et al., 2002a). Therefore, it is often assumed that metal toxicity to these organisms occurs via similar mechanisms, and in fact this is the premise that is taken in extrapolating the BLM concept from fish to invertebrates (see section 1.3.3.). For this reason, fish and invertebrates will be treated together in the discussion hereunder. Comparability or differences between these two groups of organisms will be clarified were necessary.

The gill is a multi-functional organ which serves many purposes such as respiration, nitrogenous waste excretion, acid-base balance and osmoregulation. It has also been demonstrated that the gill serves a role in trace element absorption (Spry et al., 1988; Kamunde et al., 2002). The key target for both copper and silver toxicity in freshwater fish appears to be sodium homeostasis, although chloride absorption and nitrogenous waste excretion can also be influenced (Grosell et al., 2002). Hereunder we will only focus on sodium balance, as this is the most important process with regard to acute copper toxicity, for a discussion on the other processes we refer to Grosell et al. (2002a). Sodium uptake from water across the gills is essential for all water breathing freshwater organism as it compensates for the diffusive loss of sodium from its concentrated extracellular fluids (~ 100 to 200 mM Na) to the surrounding dilute environment (typically Na concentrations ~ 1 mM) (Grosell et al., 2002).

Figure 1-3 summarizes the current understanding of sodium transport across the freshwater gill epithelium. Sodium entry across the apical membrane appears to be in exchange for protons or ammonia (Krogh, 1938) via either a sodium-proton exchanger or a sodium channel coupled to a proton pump (Potts, 1994). These mechanisms have been documented for both fish (Bury and wood, 1999; Grosell and Wood, 2002) and invertebrate species (Zetino et al., 2000). Sodium uptake is indirectly linked with the hydration of CO_2 by cellular carbonic anhydrase which facilitates the conversion of CO_2 to bicarbonate and protons and thus fuels the proton extrusion and the sodium uptake (Perry, 1986). Upon entry into the gill tissue, Na is actively pumped into the extracellular fluid (i.e. plasma in vertebrates, haemolymfe in invertebrates) via the Na^+/K^+ ATPase located in the basolaterale membrane (Skou and Essman, 1992 for review).

Copper induced disturbance of sodium balance was first demonstrated in *Daphnia magna*, which exhibited reduced whole-body concentrations of sodium after only a few hours of exposure (Holm-Jensen, 1948). Later findings of reduced plasma osmolarity, Cl and Na concentrations in various freshwater fish exposed to copper confirmed that this metal is an osmoregulatory toxicant (McKim et al., 1970; Stagg and Shuttleworth, 1982).

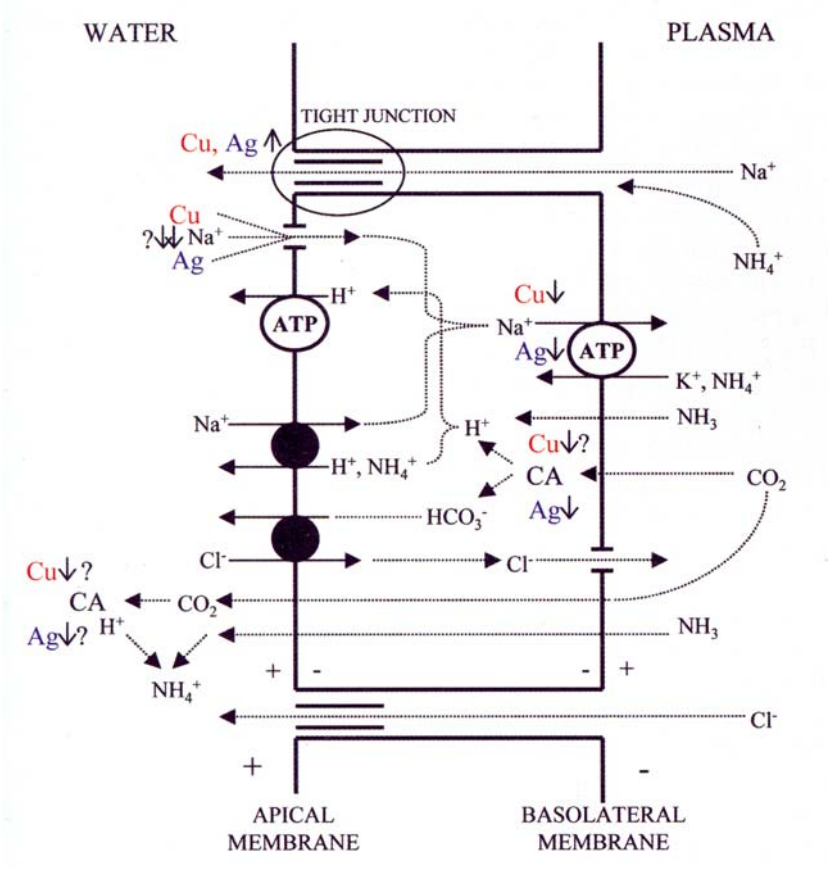

Figure 1-3 Schematic representation of a general model of acid-base, sodium, chloride and ammonia transport across the gill epithelium of freshwater organisms and the transport channels involved (after Grosell et al., 2002a). The depicted transport processes may well occur in different cell types, but they are most resolved in gill tissue. Dotted lines represent diffusive transport events; solid lines depict all other events. Carriers marked with "ATP" perform active transport depending on ATP. Carbonic anhydrase is abbreviated CA. The symbols "↑" and "↓" denote that the metal (Cu and/or Ag) increases or decreases the relevant processes, respectively. See text for further details.

Laurén and McDonald (1985) demonstrated that the reason for copper-induced disturbance of sodium homeostasis in rainbow trout was a reduction of branchial sodium uptake at lower copper concentrations and a combination of this and increased sodium efflux at higher copper concentrations. The latter was attributed to the displacement of calcium by copper in the tight junctions, which partly control the permeability of the branchial epithelium (Laurén and McDonald, 1985). Since then, numerous reports have been published on the mechanisms of this decreased Na uptake in fish.

First, copper appears to inhibit the basolateral Na^+/K^+ ATPase (e.g. Laurén and McDonald, 1987; Pelgrom et al., 1995), related to increased copper concentration in the gill tissue (Li et al., 1998; De Boeck et al., 2000) and invoked by interference of Mg binding to this enzyme (Li et al., 1996).

Second, at the apical side, inhibition of sodium channels and sodium-proton exchangers has been reported to be targets for copper toxicity (Grosell and Wood, 2002). In addition, it has been suggested that copper may inhibit carbonic anhydrase and as such deplete the proton substrate for the sodium-proton exchanger (Vitale et al., 1999; Grosell et al., 2002a).

Finally, although the exact mechanisms of chloride uptake inhibition are not as well understood, decreases of sodium levels upon copper exposure are often accompanied with a decrease in chloride levels (Laurén and McDonald, 1985; Wilson and Taylor, 1993). According to Grosell et al. (2002a), given the fact that sodium and chloride uptake are linked by carbonic anhydrase, this may point to this enzyme also being a likely target for copper toxicity.

To our knowledge, no such mechanistic/molecular studies with copper and invertebrates have been published. Silver, a metal with similar toxicity action mechanisms as copper in fish (see Grosell et al., 2002a for review) is demonstrated to inhibit Na^+ / K^+ ATPase and increase sodium efflux in the crayfish *Cambarus Diogenes* (Grosell et al., 2002b).

The overall etiology of copper and silver-induced mortality seems to be very similar (Hogstrand and Wood, 1998; Grosell et al., 2002a). The net loss of sodium (and chloride)

creates an osmotic imbalance between plasma and tissues. Via a complex cascade of events, this eventually leads to cardiovascular collapse resulting in death. Mortality occurs when plasma sodium concentrations are reduced by approximately 30% (Wilson and Taylor, 1993; Wood et al., 1996). A similar etiology is expected for invertebrates, although invertebrates may have different tolerance for reduced haemolymfe concentrations (Grosell et al., 2002a). Bianchini and Wood (2002) have even observed *D. magna* surviving a 60% sodium loss upon a 21-day exposure to silver.

1.2.2.3 Chronic toxicity to fish and invertebrates

The mechanisms described above explain the acute mortality in both fish and invertebrates exposed to silver and copper. However, it is unclear how ionoregulatory disturbance affects organisms in long-term exposures. Paquin et al. (2002b) indicate that in chronic exposures, one should also take into account that organisms may exhibit acclimation effects. For example, it has been shown that, upon silver exposure, rainbow trout were able to restore plasma sodium after 28 days after an initial, sublethal sodium loss (Galvez et al., 1998). On the other hand, even longer-term studies (18 months) demonstrate an increased mortality accompanied with a very slow sodium loss rate (Paquin et al., 2002b). Moreover, as suggested by Kooijman (2000) an organisms' survival time will decrease with increasing body concentrations of toxic oxy-radicals, which may occur due to long-term copper accumulation resulting in redox cycling.

Mortality is a typical endpoint considered in short-term toxicity tests, but in long-term (chronic) exposures reproductive success is also an important measure of a substances' toxicity, as this also determines the ecological effects on populations and communities. To our knowledge, no studies have been performed investigating the possible effect of ionoregulatory malfunctioning on reproductive success. Bianchini and Wood (2002) suggest that a decrease of whole body Na^+ concentrations in *D. magna* chronically exposed to silver may have been responsible for the observed decreased reproduction. Although high sodium losses may indeed result in an overall decreased fitness of the organism and in an enhanced energy requirement for maintenance purposes, there is no evidence that this is the only mechanism causing reduced reproductive success in chronic exposures.

According to Kooijman (2000) effects of toxicants on reproduction can either be direct or indirect. With a direct effect on reproduction this author means that the toxicant affects the survival probability of the developing ovum, which has, to our knowledge, not yet been demonstrated for copper. Indirect effects are the result of either a decreased food assimilation rate or increased energetic costs of growth and/or maintenance. Decreased algal ingestion rates of daphnids have been observed upon copper exposure of *D. magna* (Flickinger et al., 1982; Ferrando and Andreu, 1993) but it was unclear if these effects were caused by dietary or waterborne exposure.

Next to this complexity of possible toxic action mechanisms, it should also be taken into account that exposure to increased metal concentration may induce several detoxification mechanisms, such as metallothionein synthesis or the sequestration of toxic metal ions in electron-dense granules (Mason and Jenkins, 1995). These mechanisms may also result in very different toxicity patterns compared to acute exposures.

Finally, chronic exposures are always accompanied with providing food to the test organisms and this creates a possibility for dietary metal exposure. This issue has insufficiently been studied to fully evaluate its ecological importance (see chapter 5).

All of the above illustrates the complexity of processes involved in the chronic toxicity caused by metals. This is probably even more complex for essential metals like copper. It is not the first purpose of this study to unravel the chronic copper toxicity mechanisms for *D. magna* in detail. Rather, it was attempted to develop a tool to predict copper bioavailability based on concepts developed for acute copper toxicity. As such it was envisaged to obtain insights in the differences between acute and chronic metal toxicity which would help us to formulate future research needs.

1.2.2.4 Copper toxicity to unicellular algae

It is commonly accepted that mechanisms of metal toxicity in algae are very different from those observed in fish and invertebrates. This seems logical, since in the latter the gill epithelial cells, the border between the external water and the plasma, are the first target of waterborne metal uptake into the organism and toxicity. The border between the intra- and

extra-cellular environment in algae, however, is not a gill but is generally composed of a polymeric cell wall and a plasma-membrane.

On approaching the surface of an algal cell, a metal ion will normally first encounter a protective polysaccharide or glycoprotein layer (the cell wall) (Campbell, 1995). The macromolecules making up this external layer contain a variety of functional groups, resulting in a matrix of negatively charged sites at which the metal can accumulate and through which the metal must migrate before eventually meeting the plasma membrane and being transported into the cytoplasm. It is commonly accepted that the binding to the cell wall and the cell membrane is kinetically fast and that the uptake into the cytoplasm via facilitated diffusion is the rate-limiting step of the uptake process (Campbell, 1995). Similar mechanisms may occur in wall-less algae, where rapid equilibrium binding between metal in solution and the cell membrane is assumed (Macfie et al., 1994).

A number of copper toxicity mechanisms to algae have been reviewed by Stauber and Davies (2000), some of which are summarized below. At the cell-membrane, copper may cause changes in membrane potential and permeability or may compete with essential metals for binding and uptake (Sunda and Huntsman, 1983; Cid et al., 1996; Franklin et al., 2001). Interactions between copper and manganese and copper and zinc have been reported (Sunda and Huntsman, 1983; Reuter and Morel, 1981). Following transport into the cytoplasm, copper may inhibit enzymes such as esterase and β-galactosidase (Peterson and Stauber, 1996; Franklin et al., 2001) and cause changes in intracellular pH (Cid et al., 1996). Copper is also reported to affect organelles such as chloroplasts and mitochondria. Wong et al. (1994) reported structural alterations to thylakoid membranes of *Chlorella* species and inhibition of photosynthesis. Cid et al. (1995) reported a disrupted mitochondrial electron transport upon copper exposure.

Perhaps the most ecologically relevant effect of copper is its inhibition of growth caused by the disruption of the glutathione metabolism. Copper was shown to oxidize thiol groups on enzymes or free thiols such as glutathione, leading to a lowering of the reduced to oxidized glutathione ratio and subsequent inhibition of cell division (Stauber and Florence, 1987). Changes in an algal species' growth rate may influence species competition, succession, community structure and ecosystem functions (Stauber and Davies, 2000). For

this reason growth is also the most frequently used endpoint in standard toxicity testing with algae.

1.3. Bioavailability

1.3.1. What is bioavailability?

During the last decades numerous definitions have been put forward for the term "bioavailability", but it seems that no single sentence is capable of fully covering its entire meaning. The following set of definitions gives a relatively good summary of what the term "bioavailability" includes.

> *"Bioavailability is the degree to which a contaminant is free for uptake (movement into or onto an organism)" (Newman and Jagoe, 1994)*

Some definitions of bioavailability further imply that the contaminant must affect the organism in order to be considered bioavailable:

> *"A metal is considered to be bioavailable when it is taken up by the organism and can react with its metabolic machinery" (Campbell et al., 1988)*

This is consistent with several pharmacological definitions:

> *"The bioavailability of an ingredient is the rate and extent to which the ingredient is adsorbed and becomes available for the site of action" (Wagner, 1979)*

The main idea behind "bioavailability", however, is that the toxic effect of a metal does not only depend on the total concentration of that metal in the surrounding environment, but rather that it depends on a complex interaction between physico-chemical and biological factors, i.e. *the same total metal concentration does not result in the same degree of toxic effect under all conditions*. In the following sections the major factors that can affect bioavailability (in terms of toxicity) will be discussed. First an introduction to metal speciation is provided, followed by a brief historical overview of physico-chemical and

biological factors affecting bioavailability and finally, the integration of the most important bioavailability factors into the generalized and integrative biotic ligand model framework is described.

1.3.2. Metal speciation and its effect on bioavailability and toxicity

The first key step in evaluating metal bioavailability is to recognize the importance of metal speciation, both physically (dissolved versus particulate metal) and chemically (free metal ions versus complexed metal forms), as some metal forms (termed 'metal species') intrinsically have different potencies to adversely affect organisms.

It is commonly accepted that the distribution of a metal between the dissolved and the particulate phase is crucial to evaluate its bioavailability as this determines the relative importance of the waterborne (dissolved) and dietary (particulate) route of metal uptake. Although most studies indicate that particulate copper does not cause acute toxicity, the potential toxicity of copper (and other metals) taken up via the diet is currently an issue of intensive debate (e.g. Clearwater et al., 2002; see chapter 5). Although the direct measurement of dissolved and particulate metal in aquatic systems is the most logical approach towards evaluating potential risks *in situ*, the distribution of metals between the dissolved and the particulate phase can nowadays also be computed by the geochemical speciation model SCAMP (Lofts and Tipping, 2003). Whereas the former approach is applicable to monitoring scenarios, the latter is probably more promising when emission scenarios need to be evaluated.

However dissolved metal concentrations have also been demonstrated to be a bad predictor of metal toxicity (Bergmann and Dorward-King, 1997; Janssen et al., 2000). Within the dissolved phase geochemists commonly discern between the free hydrated metal ion (Me^{2+}), inorganic complexes (e.g. with Cl^-, SO_4^{2-}, CO_3^{2-}, OH^-) and organic complexes (e.g. humic, fulvic and hydrophilic acids). Each of these have different potencies for affecting organisms. Based on a very large body of literature (reviewed by Campbell, 1995; Paquin et al., 2002a), the order of toxic potential is Me^{2+} > inorganic complexes > organic complexes (except some complexes with some small biomolecules; Campbell, 1995).

The crucial question to be addressed is: "How does solution chemistry translate to chemical activities of relevant toxic metal forms such as the free metal ion?" Here too, techniques are available to directly measure metal speciation (e.g. voltametry and ion-selective electrodes). Until now, however, these techniques are available for only a few metals and it is still difficult to apply them on a routine basis. Alternatively, computer models such as MINEQL (Schecher and McAvoy, 1992) and MINTEQA2 (Allison et al., 1991) allow the computation of chemical activities and concentrations of the free metal ion and metal complexes. These computations are relatively straight-forward since they are based on known (measured) physico-chemistry and on published (standardized) stability constants for inorganic complexes (e.g. Martell et al., 1997; see chapter 2, Table 2-2).

Recent research in this area has been directed towards describing metal interactions with natural organic matter (NOM). This is an immense challenge, due to the polydisperse, heterogeneous nature of NOM (MacCarthy, 2001). The Windermere Humic Aqueous Model – Model V (WHAM, Tipping, 1994) and the Non-Ideal Competitive Adsorption (NICA) model (Benedetti et al., 1995), which incorporate multiple binding sites and competition between cations, constitute some of the most significant recent advances. This is especially important for copper, since in most freshwater systems, typically over 99% of the dissolved copper is bound to NOM (Landner and Lindeström, 1999).

In the context of bioavailability modelling, WHAM-V has become an integral part of the most advanced model, i.e. the biotic ligand model (BLM, see 1.3.3), as it has been successfully calibrated to a large number of datasets including acid-base titrations and metal titrations of different types of organic matter (such as humic and fulvic acid) originating from different sources (Tipping and Hurley, 1992; Tipping, 1993; Dwane and Tipping, 1998; Bryan and Tipping, 2002**)** and under a large range of environmentally relevant conditions. The current use of WHAM V (Tipping 1994) in the BLM construct should, however, not preclude the future use of other (and/or more advanced, better calibrated) speciation models, such as WHAM 6 (Lofts and Tipping, 2002). The latter model is believed to increase the reliability of speciation calculations for lower metal concentrations (i.e. below levels for which WHAM V was originally calibrated), which may be particularly important in future modelling of chronic, low-level exposures (Paquin et al., 2002a). Within the present study WHAM V was considered sufficient for our purposes.

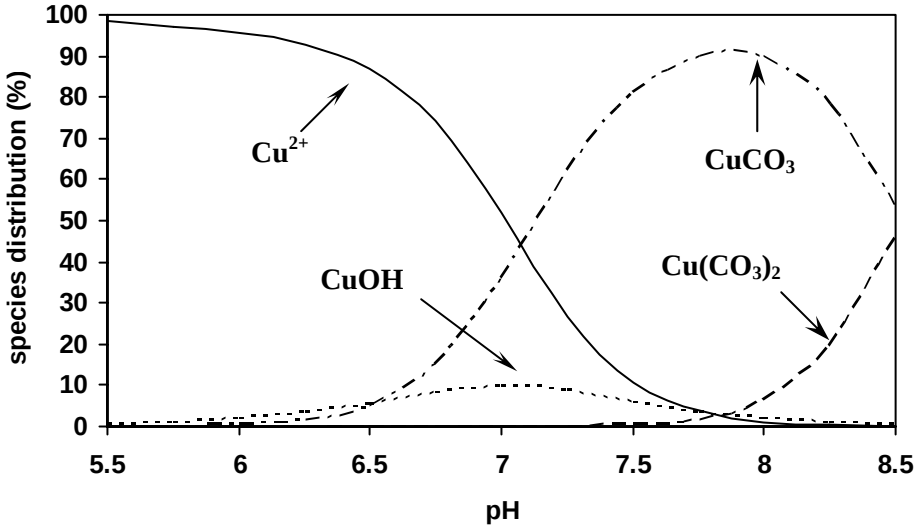

Figure 1-4 Distribution of inorganic copper species as a function of pH

Figure 1-4 presents an example of the major inorganic speciation of dissolved copper (in this case 1µM $CuCl_2$) as a function of pH levels relevant for European surface waters (5.5 to 8.5). Total alkalinity is taken as a function of pH using a relationship derived from a European river monitoring database (Heijerick et al., 2003a):

$$\text{Log (Alkalinity as Eq} \cdot L^{-1}) = -11.80 + 1.186 \cdot \text{pH}$$

Calculations were performed using Visual Minteq at a temperature of 20°C and at an ionic strength of 0.01M. Figure 1-4 clearly illustrates the sharp decrease of Cu^{2+} concentration with increasing pH. At pH > 7 $CuCO_3$ becomes the most abundant inorganic copper species. Since Cu^{2+} is generally considered the most toxic copper species, this points to a decrease of toxicity with increasing pH.

1.3.3 The Biotic Ligand Model (BLM)

The biotic ligand model (BLM) is an integrative framework to evaluate and predict bioavailability and toxicity of metals for freshwater organisms. It considers both metal speciation in the solution surrounding the organism and the interactions between metal ions and competing ions at binding sites on the organism-water interface (e.g. epithelial cells of gill tissue).

The foundations of the biotic ligand model were established in the early 1970's when researchers began to investigate the effects of water chemistry on the toxicity of metals to freshwater organisms. Zitko et al. (1973) demonstrated the protective effect of organic matter on copper toxicity to juvenile Atlantic salmon and confirmed that the toxic effect was related to the cupric ion activity. This study was probably the first in a series of similar experiments with copper (e.g. Sunda and Lewis, 1978, Pagenkopf, 1974), cadmium (Sunda et al., 1978) and zinc (Allen et al., 1980) which demonstrated that the free metal ion activity was related to the toxic effect. This has since then been confirmed by numerous studies with only a few exceptions under very particular conditions (e.g. uptake of Zn-citrates, see Campbell, 1995 for a review).

Next, Zitko and Carson (1976) identified competition of hardness cations (Ca^{2+}, Mg^{2+}) with free metal ions for binding sites at the toxic site of action, as the mechanism by which increased hardness decreased metal toxicity. This finding has ultimately led to the incorporation of a hardness based water quality criterion correction for metal in the U.S.A. (US EPA, 1985). During this same time period, researchers first began to appreciate that protons could also exert a competitive effect in much the same way as hardness cations do (e.g. Campbell and Stokes, 1985; Cusimano and Brakke, 1986).

Those early studies led to the formulation of the free-ion activity model (FIAM, Morel, 1983) and the gill surface interaction model (GSIM, Pagenkopf, 1983), whose central hypothesis was that metal toxicity was related to the amount of metal bound to toxic action sites on the organism-water interface. Although conceptually those models contained almost all the features of the BLM, the use of the former models for regulatory purposes remained limited (Paquin et al., 2002a). However, the role of these models in the development and the acceptance of the BLM should certainly be recognized. Perhaps, the reason of the current success of the BLM is that, for the first time, a model was able to integrate all state-of-the-science knowledge on both speciation and interactions at the toxic site of action into a generalized, visually attractive (Figure 1-5) and easy-to-handle computerized framework.

Another reason for the increasing acceptance of the BLM is probably the fact that since the formulation of the GSIM and the FIAM, an overwhelming body of evidence has been produced that supports the concepts formulated in the GSIM and the FIAM.

For example, for copper (but also for other metals) numerous detailed studies have demonstrated the protective effects of organic matter (e.g. Erickson et al., 1996; Winner, 1985, Meador, 1991; Ma et al., 1999) and competing cations (e.g. Winner, 1985; Erickson et al., 1996; Welsh et al., 2000) on fish, crustaceans and algae.

Concurrently, increasing physiological evidence has been reported on the protective effects of cations against metal toxicity (see section 1.2.2). The main break-through in evaluating bioavailability was, however, established in the 1990's when it was recognized that the results of toxicity studies, speciation knowledge and metal-binding studies with fish gills (the toxic action site, the biotic ligand) needed to be combined to allow accurate prediction of metal toxicity (Bergmann and Dorward-King, 1997).

In that context, Playle and co-workers were actually able to demonstrate that cations like Ca^{2+} and H^{+} decreased metal (Cu, Cd, Ag) accumulation in fish gills and they even derived (conditional) stability constants for this competitive binding (Playle et al., 1993a, 1993b; Janes and Playle, 1995) which could easily be integrated in chemical equilibrium models. It was now possible to predict metal accumulation in fish gills from solution chemistry.

A next key-step in the further BLM development was the proof that, independent of water chemistry, there is a relationship between accumulated metal in the fish gill and mortality (for Cu: MacRae et al., 1999; for Ni: Meyer et al., 1999). This finally confirmed the since-long assumed underlying hypotheses of the FIAM and the GSIM.

At last it was possible to compute, starting from dissolved copper concentration and physico-chemistry of the surrounding solution, the amount of copper accumulated at the site of toxic action (the gill) and, from that, to predict the toxic effect. *Vice versa*, starting from a critical copper accumulation level in the gill (e.g. resulting in 50% mortality), it was possible, taking into account the water chemistry, to predict the dissolved copper concentration resulting in 50% mortality.

This combined knowledge was first brought together by Di Toro and co-workers, who developed BLMs predicting acute copper toxicity to fish (Di Toro et al., 2001). Whereas toxicity varied over 2 orders of magnitude, the BLM was able to predict LC50-values within a

factor 2 of the observed values. Since then, increasingly more research has been conducted to extend the BLM-framework to other metals and other organisms (For a review see Paquin et al., 2002a)

Special attention should be given to the approach used to develop a BLM for invertebrates. The determination of gill-metal accumulation is, due to the size of the organisms, experimentally difficult to obtain. Two approaches, resulting in similar predictive performance have been used. The first approach is the one used by Di Toro and co-workers (e.g. Santore et al., 2001; Santore et al., 2002). They assume that all organisms have the same metal and competing cation affinities for their gills and assume that they only differ in their critical level of metal accumulation at the biotic ligand. In practice this critical concentration is fitted to an existing dataset and this critical concentration is then further used for toxicity predictions under all conditions.

In the second approach, which was the first objective of the present study, BLM-constants are directly derived from toxicity data (chapter 2). Not only the critical biotic ligand concentration of a metal but also all binding constants of competing cations are estimated. This approach offers the advantage that species-specific differences in toxicity modifying effects can be determined, which may be of utmost importance in understanding the importance of bioavailability factors in complex communities.

At the start of the present study, a major drawback of applying the BLM in a regulatory framework was the lack of a similar model that could predict and evaluate the bioavailability and toxicity of metals to algae. Although many reports seem to imply that similar concepts might also be applicable to algae (Heijerick et al., 2002; Franklin et al., 2000), before the start of this study, no attempts had been made to investigate the possibilities develop BLM-type models for predicting metal toxicity to algae. For this reason this was another main objective of the present study.

Finally, as a summary, Figure 1-5 represents the most important possible interactions that are currently incorporated into the BLM framework, including the findings for *D. magna* in the present study.

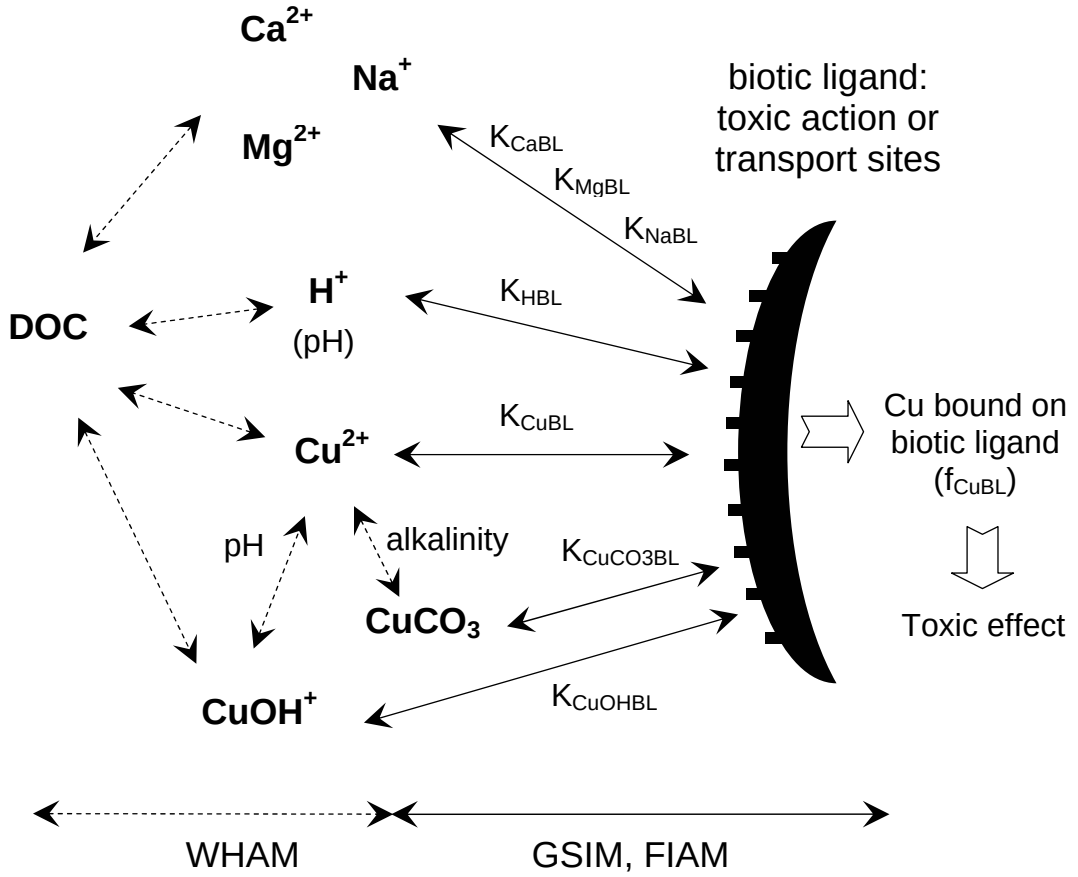

Figure 1-5 Schematic overview of the biotic ligand model of copper. Dashed lines represent speciation reactions (modelled by WHAM, Tipping, 1994); solid lines represent binding to the biotic ligand (which may be toxic action sites or transport sites, see 1.2.2). The free copper ion, i.e. Cu^{2+}, forms complexes with inorganic ligands such as OH^- and CO_3^{2-} (other inorganic ligands not shown, less important in the BLM). The concentration of OH^- and CO_3^{2-} is determined by pH and alkalinity. Cu^{2+} and $CuOH^+$ also form complexes with dissolved organic carbon (DOC) and Ca^{2+}, Mg^{2+} and H^+ (also determined by pH) competes with copper for binding sites on DOC. Cu^{2+}, but also $CuOH^+$ and $CuCO_3$ (see chapter 2 and 3) bind to the biotic ligand and the concentration of copper bound to the biotic ligand (or the fraction of biotic ligand sites occupied by copper, f_{CuBL}, see chapter 2 and 3) determines the toxic effect. The latter concentration is assumed to be constant for a given effect size (e.g. 50% mortality).The formation of complexes decreases f_{CuBL}, resulting in decrease of toxicity. Ca^{2+}, Mg^{2+}, Na^+ and H^+ can compete with copper for biotic ligand sites thus decreasing f_{CuBL} and toxicity. This concept was first formulated in the free ion activity model (FIAM, Morel, 1983) and the gill surgace interactionmodel (GSIM, Pagenkopf, 1983). Binding affinities of Cu^{2+}, $CuOH^+$, $CuCO_3$, Ca^{2+}, Mg^{2+}, Na^+ and H^+ are defined by K_{CuBL}, K_{CuOHBL}, $K_{CuCO3BL}$, K_{CaBL}, K_{MgBL}, K_{NaBL} and K_{HBL}, respectively. The chemical and mathematical equations and models that are associated with this concept are presented in detail in chapter 2 and chapter 3.

1.4. Regulatory aspects of assessing the risks of (essential) metals in freshwater environments

1.4.1. Overview of existing regulatory approaches for copper

All trace metals are ubiquitously present in the environment and essential elements have been exploited by living organisms to their own advantage. Therefore, it may be assumed that nature has conditioned itself to the fluxes and concentrations of essential trace elements that originate from natural sources (Van Tilborg, 2002). Potentially increased risk stems from anthropogenic sources that cause increases in (bioavailable) metals above critical levels that may result in adverse effects on ecosystems. Therefore, the EU regulatory agencies proposed the "added risk" approach (see 1.4.2.).

Several authorities all over the world have developed or are developing methods to derive water quality criteria and to evaluate potential risks of metals and of man-made substances. In this section we will focus on how regulatory action concerning (essential) metals requires special attention. Table 1-1 summarizes some of the WQC for copper applicable in different countries. It is recognized that this table is not complete but rather an illustration of the diversity in regulatory systems of metals in different parts of the world. For simplicity we used the term WQC in the text, although different countries use different terminologies which may have also different value in their actual implementation (refer to the cited documents). For some countries clear information is provided in official documents on how these criteria were derived, whereas for some others this information is not readily available. Where available, this information is also given in Table 1-1. Information on the possible implementation of bioavailability is given below.

Table 1-1 Example of some water quality criteria (WQC) for copper in different countries

Country	WQC (µg Cu L^{-1})	Reference	Bioavailability
U.S.A.[1]	CMC = 9; CCC = 13	US EPA, 1985a; US EPA, 1996	Hardness-correction Water effect ratios (WER)
Australia and New Zealand[2]	TV_1 = 1.0; TV_5 = 1.4; TV_{10} = 1.8	ANZECC/ARMCANZ, 2000	Hardness-correction Tiered approach
Belgium (Flanders) [3]	EQC = 40	Vlaamse Regering, 1995	Hardness correction
Netherlands[4]	MPC = 1.5	IWP, 1999	None
Germany [5]	QT = 4	Länderarbeitsgemeinschaft Wasser, 1997	None

[1] WQC as dissolved Cu; CMC = criterion maximum concentration = "never to be exceeded"; CCC = criterion continuous concentration = "maximum one exceedance of 4-day average concentration in 3 years; CMC = 5th percentile of statistical distribution of acute LC50s / 2; CCC = CMC / acute to chronic ratio; CMC and CCC in Table are for hardness = 100 mg $CaCO_3$ L^{-1}; CMC and CCC for copper can be calculated in function of hardness as follows: $CMC = 0.96 \cdot \exp\{0.9422 \cdot \ln(\text{hardness}) - 1.700\}$ and $CCC = 0.96 \cdot \exp\{0.8545 \cdot \ln(\text{hardness}) - 1.702\}$; see text for WER approach.

[2] WQC as total Cu; TV_x = trigger value = xth percentile of statistical distribution of chronic NOECs. TV_1 applies to systems with "high conservation/ecological value"; TV_5= for "slightly/moderately disturbed" systems and TV_{10} for "highly disturbed" systems; If total copper exceeds the TV, a series of different tiers may be" triggered", allowing a more realistic judgement of real bioavailability in the order: measurement of dissolved copper, speciation calculations, speciation measurements, toxicity predictions (e.g. BLM), toxicity testing; TV reported for 30 mg $CaCO_3$ L^{-1}.Hardness-modified trigger values (HMTV) at other hardness levels (H) are calculated as: $HMTV = TV \cdot (H/30)^{0.85}$

[3] EQC = Environmental Quality Criteria as dissolved Cu. EQC are valid for so-called "fish-waters", i.e. "waters in which fish species such as salmon, carp and eel live or could live". The EQG presented in the Table is for 100 mg $CaCO_3$ L^{-1}. EQC for 10, 50 and 500 mg $CaCO_3$ L^{-1} are also put forward being 5, 22 and 112 µg Cu L^{-1}, respectively. It is, however, not clear which EQC apply to hardness levels in-between. EQC should not be exceeded for 95% of the samples.

[4] MPC = maximum permissible concentration as dissolved Cu; MPC as total Cu = 3.8 µg L^{-1}; the MPC is presented as the short-term objective for 2006; it is derived as the natural background concentration + the maximum permissible addition (MPA) (i.e. the "added risk" approach). The MPA is derived as the 5th percentile of a statistical distribution of NOECs (expressed as concentrations added to the concentration in control media); The MPC refers to a standard water with 30 mg suspended solids per L; The MPC should not be exceeded for 90% of the samples. IWP = Interdepartmental Working Party on Setting Integrated Environmental Quality Standards for Substances.

[5] QT = Quality target as total Cu; since chronic NOECs of most sensitive species were close to background levels, the QT was set pragmatically at twice the upper limit of the background concentration, should not be exceeded in 90% of the monitored samples

Table 1-1 clearly illustrates the diversity of regulatory approaches of copper (and other metals) in different countries and the variety of WQC used. A common denominator of most of these approaches is that WQC are still predominantly based on total or dissolved concentrations (Bergmann and Dorward-King, 1997; Janssen et al., 2000). However, as discussed earlier, total or even dissolved concentrations of metals are no good predictors of potential harm to ecosystems. Indeed, several physico-chemical factors such as DOC, pH and hardness can modify toxicity with several orders of magnitude.

Despite this ever-increasing evidence, in most countries no bioavailability correction or only a hardness-based correction of water quality criteria (i.e. higher WQG at higher hardness) is applied. Besides the countries mentioned in Table 1-1, the latter is also the case in France, Spain, Ireland and the United Kingdom (Fraunhofer Institute, 2001). However, hardness may not always be the most important modifier of metal toxicity and this will be investigated in the present study.

The reason of the success of the hardness (and not pH or DOC) correction of metals criteria may probably be found in the U.S.A. regulatory approach for metals that was formulated in the early 1980's, a period during which a lot of literature became available on the protective effects of hardness on metal toxicity, whereas less was known about effects of DOC and pH.

A number of authorities are, however, currently recognizing this drawback. For example, in the U.S.A. the water effect ratio (WER) has been used as a bioavailability corrector for deriving site-specific WQC for controlling discharges (US EPA, 1994; US EPA, 2001). In this approach the WER is calculated as an acute LC50 in site water divided by an acute LC50 in standard laboratory water. The WQC mentioned in Table 1-1 is then multiplied by this WER to obtain the site-specific WQC. Although this was one of the first attempts to account for the integrative effects of water chemistry on metal toxicity, the approach was considered costly, inefficient and sometimes even inaccurate (US EPA, 2001; Allen and Hanssen, 1996). The US EPA is currently evaluating the possible use of the BLM for incorporating bioavailability in the WQC of copper (Paquin et al., 2002a).

In Australia and New Zealand, if the total metal concentration exceeds the trigger value (see Table 1-1), a series of successive tiers may be triggered including measurement of

dissolved concentrations, the application of a hardness correction, speciation calculations or measurements, or even ecotoxicity testing with the surface water under consideration. The approach explicitely recognizes the importance of bioavailability, with the measurement of each tier better approaching real bioavailability and more reliably predicting the metal's impact (Paquin et al., 2002a). Here too, the BLM may serve as one of the intermediate tiers between speciation calculations and ecotoxicity testing.

Although in the Netherlands no bioavailability corrections are currently applied to the existing WQC, a research project has been set up by several Dutch governmental agencies to evaluate the possibility of applying a DOC-correction to the current WQC (Kramer et al., 2001).

Table 1-1 also illustrates the different methods that were used for the derivation of WQC in the different countries. Some of these methods/concepts (e.g. "added risk" approach, statistical species sensitivity distributions) are currently adopted by the European Union (EU) for the regulation of chemical substances. This will be discussed in the following section.

1.4.2. Regulatory approach in Europe

1.4.3.1. Legal framework

Although in most countries metals and metal discharges are regulated through the establishment of WQC in one or another form (e.g. criteria, guidelines, trigger values, see Table 1-1), the EU addresses the regulation of metals (and other chemical substances) through the risk assessment approach (with the exception of metals classified as priority hazardous substances, i.e. Cd, Ni, Hg and Pb, which are currently being managed through WQC derivation via the Water Framework Directive). Nevertheless, the derivation of WQC is based on the same methodological elements as the risk assessment, as these elements have already been accepted by the different EU member states (Lepper, 2002).

The European Union Council (EEC) Regulation No. 793/93 on the evaluation and control of existing substances (European Commission, 1993a) requires the assessment of real or potential risk for man and environment of priority substances, using principles that have been described in Commission Regulation No. 1488/94 on risk assessment of existing

substances (European Commission, 1993b). The technical guidance for performing risk assessments for new and existing substances has been harmonized and published in the Technical Guidance Document (TGD) in support of Commission Directive 93/67/EEC on risk assessment for risk assessment of new substances and Commission Regulation No. 1488/94 for risk assessment of existing substances (European Commission, 2003).

Applied to the aquatic environment, the TGD describes exposure and effects assessment procedures leading to predicted environmental concentrations (PEC) and predicted no-effect concentrations (PNEC). When the PEC exceeds the PNEC for a given substance, it may be concluded that the substance poses a potential risk to the environment, and this may result in regulatory action. The objective of this study is mainly related to the effect assessment, i.e. the PNEC derivation and the incorporation of bioavailability into this part of the risk assessment.

1.4.3.2. Predicted Environmental Concentration

According to the TGD (European Commission, 2003) the PEC can be based on either measured or modelled concentrations of a substance. The former is, however, only possible for existing substances. For copper, and other metals, large monitoring databases exist, covering large parts of Europe (lakes, rivers, streams). These databases have been compiled into the Surface Water Database (SWAD, Heijerick et al., Laboratory of Environmental Toxicology and Aquatic Ecology, Ghetn University, Gent, Belgium and Euras, Zwijnaarde, Belgium).

The existence of monitoring databases, however, does not preclude the use of modelled concentrations, since these might yield insights in the effect of different emission scenarios from both point and diffuse sources. Calculating PECs is based on both emission data and data on the receiving environment and can be performed using environmental fate and distribution models described in the TGD.

Within an EU risk assessment, both a local PEC (PEC_{local}) and an regional PEC ($PEC_{regional}$) are estimated. The PEC_{local} is the PEC that is associated with release of a substance from point sources on a local scale. The $PEC_{regional}$ is associated with the concentration of a substance over a wider area resulting from both point and diffuse sources.

As the correct and relevant determination of the PEC in itself is already a very complicated task and also a matter of intensive debate, this issue will not be discussed in further detail. Moreover, the main focus of the present study is on correctly estimating the effects (PNEC) by incorporating bioavailability. The PNEC derivation is also more closely linked to WQC establishment.

1.4.3.3. Predicted No-Effect Concentration

Using the TGD procedure a PNEC for data-rich substances such as copper is derived as follows (European Commission, 2003). Chronic toxicity data for different taxonomic groups of species (fish, invertebrates, algae, higher plants, etc.) are collected. Data are screened for quality and relevance: e.g. “were good laboratory practices followed?”, “were measured metal concentrations reported?”, “which statistics were applied to the data?”, “is physico-chemistry reported?”, “were relevant endpoints chosen (e.g. mortality, growth, reproduction)?”, etc… All of these, and other criteria, are judged and data may be retained or rejected. After this selection the geometric mean of the reported NOECs (as dissolved metal) is calculated for each species and endpoint. The geometric mean of the NOECs for the most sensitive endpoint is taken as the input value for the species sensitivity distribution (SSD). In the SSD all species’ geometric NOEC-means are plotted in increasing order versus the cumulative probability. 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. The TGD proposes the log-logistic distribution (Aldenberg and Slob, 1993) but since this does not always result in significant fitting, there is an ongoing debate between academia, regulators and industry on using the best possible fitting distribution function to derive the HC5. 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. Next, to the distribution fitting issue, the size of the assessment factor is currently also a matter of very intensive debate, which is often more inspired by policy than by science.

1.4.3.4. PNEC for copper

An elaborative exercise has recently been performed by Van Sprang et al. (EURAS, Zwijnaarde, Belgium, unpublished data) to collect 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 1-6 presents the corresponding species sensitivity distribution (SSD).

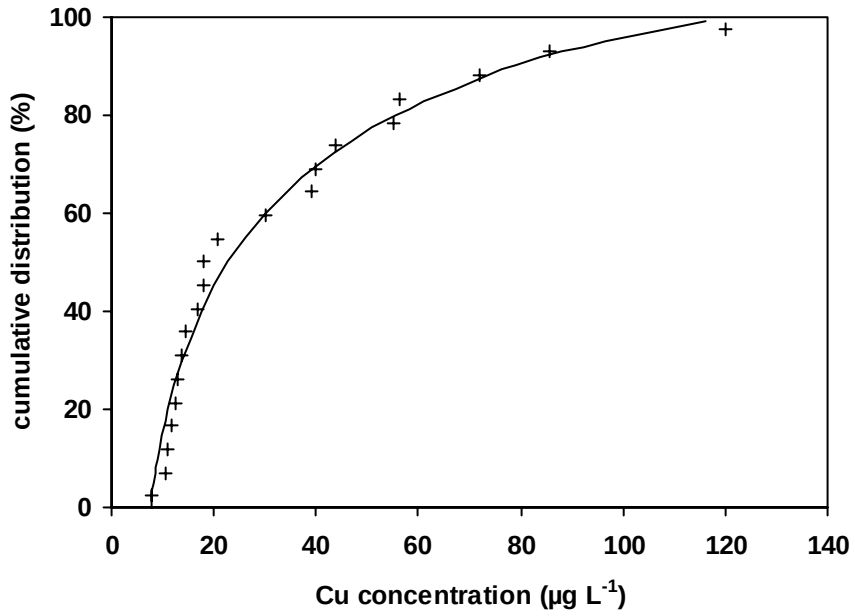

Figure 1-6 Species sensitivity distribution of copper in freshwater (Van Sprang et al., unpublished data) represented by the geometric NOEC-means of different species (n=21). The solid line represents the best fitting beta-distribution function. The HC5 is 8.3 µg Cu L^{-1}.

A beta-distribution was fitted to the SSD, resulting in an HC5 of 8.3 µg Cu L^{-1}. With an assessment factor of 1 (which may be justified given the high data quality and species richness included in the SSD, but is still a matter of debate), this would also be the PNEC and this should be compared to the PEC.

Even with an assessment factor of 1, the PEC would exceed the PNEC for an important number of monitored water bodies (PEC between 0.5 and 35 µg Cu L^{-1}, see section 1.3.2.). This would result in the conclusion that copper potentially imposes a risk to European

freshwater systems. However, a simple risk/no risk answer is both scientifically and economically not defendable.

Indeed, the data used to derive the SSD were obtained in 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 factor 100). Due to this high variability the derived PNEC may be under-protective in one type of surface water, whereas it may be under-protective in another.

In the context of sustainable development, neither overprotection nor underprotection is desirable as the former will result in increased societal costs involved with emission reduction and environmental sanitation measures, the latter may result in harm to aquatic life and biodiversity. Therefore, the best possible approach is to assess the risk of a metal on a region-specific or site-specific basis, where region-specific or site-specific PECs are compared to PNECs that are representative for a given physico-chemistry (determining the bioavailability) of that region or site.

For the latter to be established, bioavailability modelling should first be used to explain the intra-species variability observed in the SSD and caused by bioavailability relationships. This would help to reduce the level of uncertainty and would also justify the application of these models for bioavailability normalization of the PNEC.

Such an approach definitely represents the best balance between economy and ecology as no harm is done to the environment without posing too severe restrictions to a metals' production and applications. Although the possible importance of bioavailability in risk assessment of metals is recognized, it was also stated that not enough knowledge was available to quantitatively take the influence of water quality parameters into account (Lepper, 2002). The main goal of this thesis was therefore to develop tools to predict chronic bioavailability and toxicity of copper that can be used to take bioavailability into account in a quantitative manner. As such it is hoped that this study will support the risk assessment of metals and thus contribute to sustainable development.

1.5. Introduction to the test species

As discussed in section 1.4, water quality criteria and risk assessments of substances for the aquatic environment are based on toxicity data which are obtained through ecotoxicological testing. In this section, the two species that are used for the present study are introduced and their use in aquatic toxicology is discussed.

1.5.1. *Daphnia magna* (Flöβner, 2000)

1.5.1.1. Systematic classification and morphology

The diversity of crustaceans requires the use of a greater hierarchy of taxa in their classification than usually necessary for other animal groups. *Daphnia magna* belongs to the order of the cladocerans (waterfleas), which is now divided into 11 families, about 80 genera and roughly 400 species. According to Flöβner (2000) the taxonomic classification of *Daphnia magna* Straus is as follows:

Phylum	Arthropoda
Subphylum	Crustacea
Classis	Branchiopoda
Subclassis	Diplostraca
Ordo	Cladocera
Familia	Daphniidae
Subfamilia	Daphniinae
Genus	*Daphnia*
Subgenus	*Ctenodaphnia*
Species	*Daphnia magna*

The general characteristics of the daphnid morphology are presented in Figure 1-7.

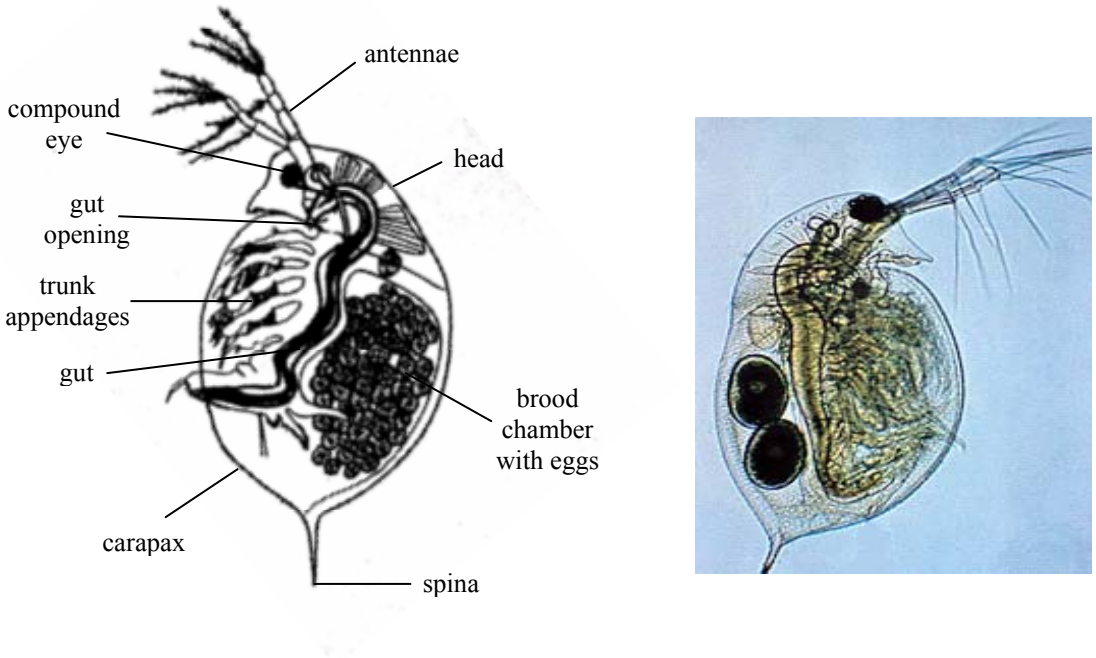

Figure 1-8 Morphology of a female individual of *Daphnia magna* carrying eggs (left) and resting eggs (right) with indication of some of the main features described in the text.

Cladocerans carry a chitinous carapax which encloses the trunk but not the head and often terminates in an apical spine. The head projects ventrally and possesses one reduced nauplius eye and a sessile compound median eye which can be rotated. Furthermore the head carries five pairs of appendages. The first pair of antennas is used as a sensory organ; the second pair is used for swimming. Movement is largely vertical. The other three pairs of appendages form the mouth parts.

All branchiopods are characterised by trunk appendages (4 - 6) that carry numerous setae and which are used for filter feeding. The thin, vasicular epipodite on each of the trunk appendages is called a gill and may perform similar functions as fish gills (Kikuchi, 1983; Kikuchi and Shiraishi, 1997).

The tip end of the trunk, commonly called the post-abdomen, is turned ventrally and forward and bears special claws and spines for cleaning the thoracic appendages and the carapax and for preventing the eggs falling out of the brood chamber.

Oviducts open into a dorsal brood chamber inside the carapax. Development in most cladocerans 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.

Most branchiopods are pale and transparent, but rose or red colours are sometimes observed (caused by the presence of haemoglobin within the body). The presence of hemoglobin frequently depends on the amount of oxygen in the water, so the animals are colourless in well-aerated water and pink in stagnant water (Barnes, 1987).

D. magna is one of the largest daphnids with adult females growing up to 6 mm. Males are smaller than females, their antennules are better developed with a flagellum and they have a claw on their first pair of trunk appendages (Flöβner, 2000).

1.5.1.2. Ecological aspects

Freshwater cladocerans are found all over the world where they inhabit all types of water bodies, from springs and groundwater to rivers and large lakes. Most species and the largest cladoceran densities are found in smaller lakes and big ponds with rich submersed and emerged vegetation. *D. magna* is a typical inhabitant of slightly polluted small water bodies, mostly without fish.

Like most branchiopods, cladocerans are mainly non-selective suspension feeders which collect food particles with fine setae arranged on the trunk appendages to form a distinct comb. The intersetular distance determines which kind of food can be consumed. The water current passes from anterior to posterior and collected particles are transferred via the food groove towards the gut, where the food is digested and assimilated. The food of cladocerans consists of microalgae (1 to 25 μm), bacteria, protozoans and detritus. Predators

among the Cladocera catch their prey (other Cladocera and Copepoda) using their trunk appendages equiped with long spines.

Reproduction in *D. magna* is cyclical parthenogenetic. Under favourable conditions females produce diploid parthenogenetic eggs which develop into clones of these females (asexual). Certain factors, however, such as a change in water temperature or the decrease of the food supply as a result of population increase, induce the appearance of males. When the males are released the female produces two larger haploid eggs, one from each ovary, which after fertilisation by the males develop into diapausing eggs. In some cladoceran species, like *Daphnia* sp., the walls of the brood chamber are transformed into a protective, saddle-like capsule (the ephippium). This is cast off at the next moult and floats, sinks to the bottom, or adheres to objects and can withstand drying and freezing and even passage through the gut of fish and of fish-eating birds and mammals. The hatching of the resting eggs and the subsequent development into females is induced when conditions become favourable again.

Juveniles of the Daphniidae undergo up to 7 moults, while in the adult phase 17 to 18 stadia follow. *Daphnia* sp. moult every 2 to 3 days. The moult itself takes only a few minutes. Organisms are able to grow in between two moults when the newly synthesised carapax is not hardened yet. The lifespan is dependent on temperature and food supply. The average lifespan of *D. magna* at 8 °C is 108 days, while at 28 °C this is only 29 days. Males have a shorter life span than females.

1.5.1.3. Use in aquatic toxicology

D. magna and *D. pulex* are the most frequently used invertebrates in standard acute and chronic bioassays. *Ceriodaphnia* sp. is used extensively in the USA, mainly because it is a more ecologically relevant test species in the USA (than *D. magna*) and because of the shorter exposure period (Mount and Norberg, 1984).

The reasons for the selection of daphnids for routine use in toxicity testing are both scientific and practical (Clesceri et al., 1998; EPS, 1990): 1) they are broadly distributed in freshwater bodies and are present throughout a wide range of habitats; 2) they are an important link in many aquatic food chains (they graze on primary producers and are food for many fish species); 3) they have a relatively short life cycle (important for reproduction tests)

and are relatively easy to culture in the laboratory; 4) they reproduce parthenogenetically, which allows testing of clones with little genetic variability and with reproducible testing results; 5) they are sensitive to a broad range of aquatic contaminants; and 6) their small size means that only small volumes of test water and little bench space are required.

Standard toxicity tests with *Daphnia* sp. can be divided into acute and chronic tests (Clesceri et al., 1998; ASTM, 1993b; ISO, 1993; OECD, 1984b; OECD, 1998; USEPA, 1993). In acute tests, neonates (< 24 h) are exposed to a range of toxicant concentrations for 24 or 48 hours, after which the concentration is determined causing 50% mortality or immobility (i.e. the 24-hour or 48-hour LC50 or EC50, respectively). In chronic tests, juveniles are introduced to the test medium and are fed. Survival and reproduction are monitored for 21 days and threshold concentrations can be derived for both of these endpoints, e.g. the no observed effect concentration (NOEC, the highest tested concentration which is not causing a statistically significant effect) or the 21-day EC50. More detailed information on the procedures used in this study can be found in chapter 2, 3 and 4.

1.5.2. *Pseudokirchneriella subcapitata*

1.5.2.1. Systematic classification and morphology

Pseudokirchneriella subcapitata (Korshikov) Hindak is a unicellular alga which belongs to the group of the green algae, the most diverse group of algae, with more than 7000 species living in a variety of habitats. *Pseudokirchneriella subcapitata* (Korshikov, 1990) was formerly known as *Selenastrum capricornutum* or *Raphidocelis subcapitata* (Nygaard et al., 1986). The classification following Korshikov (1990) is the following:

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

Their morphological appearance is presented in Figure 1-8. The green algae are characterised by their grassy green colour, mainly attributable to the pigments chlorophyll *a* and *b*. Among the algae, the Chlorophyta are the most closely related to the higher plants (Happey-Wood, 1988).

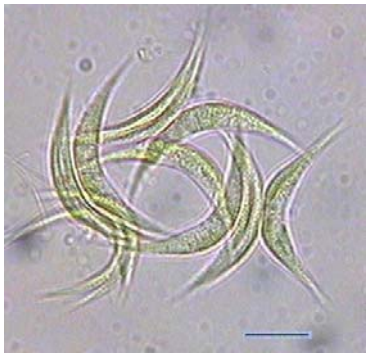

Figure 1-8 Morphological appearance of *Pseudokirchneriella. subcapitata* (5 - 7 µm)

1.5.2.2. Ecological aspects

Definitions of phytoplankton as an ecological community are many and varied in literature. Happey-Wood (1988) describes them as the community of microscopic plants, existing in suspension in aquatic environments. Green algae are almost invariably found in all freshwaters, even if present in only small numbers. Factors affecting the growth and distribution in naturally occurring communities may be considered to be of three types: 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 (e.g. grazing pressure by filter feeders).

All these processes generally result in the occurrence of population maxima through the year. These population maxima are likely to be largest in late spring prior to the high grazing pressure from herbivorous zooplankton present in summer.

1.5.2.3. Use in aquatic toxicology

Some authors have considered aquatic plants to be less sensitive than aquatic animals to chemicals. This statement is based on several studies where the acute toxicities of many chemicals were compared for fish, daphnids, several vascular plants and green algae (e.g. Blaylock et al., 1985, Versteeg et al., 1999). In contrast, other studies have shown aquatic plants to be more sensitive than invertebrates and fish to a variety of potential toxicants, including metals (e.g. Taraldsen and Norberg-King, 1990; Toussaint et al., 1995). These contradictions may probably partly be explained by differences in bioavailability. It is obvious, based on current scientific evidence, that the relative sensitivities of animals and plants to chemicals are unpredictable. Considering this and the ecological importance of freshwater plants, phyto-toxicity data are needed if the environmental risk assessment process is to be ecologically relevant (Lewis, 1993).

Algal toxicity tests have been conducted since the early 1900's but is was not until the mid-1960's that a standard methodology, the Algal Assay Procedure Bottle Test (AAP) was developed (US EPA, 1971). The AAP method was modified (Miller et al., 1978) and this version has served as the guideline for the development of methods currently used by the majority of the scientific and regulatory communities (e.g. OECD, 1984a; US EPA, 1985b; ISO, 1989; ASTM, 1993a). Most of the standard methodologies are very similar. The effect of a toxicant is determined on a rapidly growing algal population in a nutrient-enriched medium for 3 to 4 days. Biomass or cell density is monitored to determine effects on algal growth. Algal toxicity tests are chronic tests since they cover more than one generation.

Standard toxicity tests with algae species need to be applied with care for the toxicity testing of metals (Janssen and Heijerick, 2003) for the purpose of risk assessment. Common problems are pH changes (as a consequence of photosynthesis and uptake of nutrients such as NO_3^- or NH_4^+) during the test and the complexing of the metal to the metal chelator EDTA, which is present in most standard test media. Both processes are hindering the use of algal toxicity data obtained in standard test media for risk assessment purposes, as they result in erroneous, irrelevant risk estimates for these organisms. This issue is further discussed in chapter 6.

5. Conceptual framework of the study

In the above sections a number of potential short-comings in the currently used risk assessment procedures have been addressed for copper and possible solutions for incorporating bioavailability have been put forward. A major obstacle to incorporate bioavailability in the EU risk assessment framework at the start of this study was the lack of quantitative models that could predict chronic copper toxicity to two key species in standard ecotoxicity testing: *Daphnia magna* (a model invertebrate) and *Pseudokirchneriella subcapitata* (an model green alga).

At the start of this study, given the knowledge of the acute Cu-BLM for fish, it was expected that this would rapidly result in the extrapolation of the BLM concept to chronic copper toxicity to fish, and therefore this was not the initial target of the present study. Nevertheless, given the expected similarities between toxicity mechanisms in fish and daphnids, it was also envisaged that a chronic Cu-BLM for daphnids might also potentially be extrapolated to fish species.

Santore et al. (2001) demonstrated the potential of calibrating the Cu-BLM for fish to *Daphnia pulex*, by assuming the same stability constants of copper and competing cations for the biotic ligand and only adjusting the critical biotic ligand copper concentration. They stated that this was, without quantitatively appropriate measurement techniques of copper in daphnid gills, the fastest way-forward for an interim BLM for small invertebrates. However, their assumption that competition constants are similar for fish and invertebrates had never been tested.

In **chapter 2** an experimental design and a new modelling approach was developed that allows the estimation of biotic ligand model constants directly from metal toxicity data. The individual effects of Ca, Mg, Na, K and pH on acute copper toxicity to *D. magna* (48-hour immobility as endpoint) was determined and the BLM-constants were estimated and compared to the original fish constants. The method described in this chapter was also applied in the following chapters, and was also successfully used in other published papers on zinc (Heijerick et al., 2002a and 2002b). The outcome of this chapter was the first acute Cu-BLM for *D. magna*.

In **chapter 3** the developed acute Cu-BLM for *D. magna* was further refined and validated with test media that included artificial dissolved organic carbon (humic acid) and with spiked natural surface waters as the ultimate aim of bioavailability models is to predict *in situ* (field) effects.

In **chapter 4** a multi-factorial test design was used to evaluate the effects of pH, hardness and natural dissolved organic carbon (originating from 3 different sources, isolated with reverse osmosis) on the chronic toxicity of copper to *D. magna*. Next to an empirical toxicity model, attempts were made to develop a BLM-type model using similar mathematics as developed in chapter 2 and 3. These models were also validated with test data obtained with spiked natural surface waters.

Whereas the previous chapters describe the development of models that predict toxicity of waterborne copper, another potential source of copper toxicity to daphnids may be copper that is associated with food, i.e. dietary copper. In **chapter 5** the potential importance of dietary copper exposure on chronic toxicity and on the predictive capacity of the developed chronic BLM for waterborne exposure was assessed and discussed into detail.

In **chapter 6**, using a similar experimental design as in chapter 4, the effects of pH, hardness and natural DOC were investigated on the chronic toxicity of copper to the green algae *Pseudokirchneriella subcapitata* (endpoint: 72-hour growth inhibition). Empirical and semi-empirical models were developed and validated with tests using spiked field waters.

Given the great importance of DOC for copper toxicity to *D. magna* and *P. subcapitata*, in **chapter 7** the potential importance of the natural variability of copper complexing properties of DOC on toxicity was investigated and related to a simple optical property, i.e. ultraviolet (UV) light absorbance. The importance for the three developed models (acute and chronic Cu-BLM for *D. magna* and chronic model for *P. subcapitata*) was discussed.

Finally, in **chapter 8** general conclusions are drawn and future research needs are formulated. Also the potential application of the developed models with regard to risk

assessment of copper are shortly discussed, especially with respect to the incorporation of bioavailability into the existing EU risk assessment frameworks.

Chapter 2

Development of the acute Cu-BLM for *Daphnia magna*

Redrafted from:

De Schamphelaere KAC, Janssen CR. 2002. A biotic ligand model predicting acute copper toxicity for *Daphnia magna*: the effects of calcium, magnesium, sodium, potassium and pH. Environmental Science and Technology 36:48-54.

Chapter
2

Development of the acute Cu-BLM for *Daphnia magna*

Abstract - The extent to which Ca^{2+}, Mg^{2+}, Na^{+}, K^{+} ions and pH independently mitigate acute copper toxicity for the cladoceran *Daphnia magna* was examined. Higher activities of Ca^{2+}, Mg^{2+}, Na^{+} (but not K^{+}) linearly increased the 48-hour EC50 (as Cu^{2+} activity), supporting the concept of competitive binding of these ions and copper ions to toxic action or transport sites at the organism-water interface (e.g. fish gill, the biotic ligand). The increase of the EC50 (as Cu^{2+} activity) with increasing H^{+}, however, seemed to suggest co-toxicity of $CuOH^{+}$ rather than proton competition. Based on the biotic ligand model (BLM) concept, we developed a methodology to estimate stability constants for the binding of Cu^{2+}, $CuOH^{+}$, Ca^{2+}, Mg^{2+}, Na^{+} and H^{+} to the biotic ligand, solely based on toxicity data. Following values were obtained: log K_{CuBL} = 8.02, log K_{CuOHBL}= 7.45, log K_{CaBL} = 3.47, log K_{MgBL} = 3.58, log K_{NaBL} = 3.19 and log K_{HBL} ~ 5.4. Further, we calculated that on average 39% of the biotic ligand sites need to be occupied by copper to induce a 50% acute effect for *D. magna* after 48 hours of exposure. Using the estimated constants, a BLM was developed that can predict acute copper toxicity for *D. magna* as a function of water characteristics. The presented methodology can easily be applied for BLM development for other organisms and metals. After validation with laboratory and natural waters (including DOC), the developed model will support efforts to improve the ecological relevance of presently applied risk assessment procedures (chapter 3).

2.1. Introduction

Current water quality standards and risk assessment procedures of metals are predominantly based on total or dissolved metal concentrations. However, there is extensive evidence that neither total nor dissolved aqueous metal concentrations are good predictors of metal bioavailability and toxicity. Copper toxicity to freshwater organisms has been shown to be dependent on a variety of ambient water quality characteristics. For example, increases in

hardness (Pagenkopf, 1983; Erickson et al., 1996; Barata et al., 1998; Welsh et al., 2000), pH (Erickson et al., 1996; Meador, 1991; Shubauer-Berigan, 1993) and organic matter content (Erickson et al., 1996; Winner, 1985; Ma et al., 1999) have shown to result in decreased copper toxicity for fish and crustacean species. In most regulatory frameworks, however, most of these toxicity modifying factors are not taken into account. For example, when deriving site-specific water quality criteria for metals, only a hardness correction is recommended to be carried out by some instances (e.g. U.S.-Environmental Protection Agency, 1994).

A large number of studies explain part of the observed toxicity variability by attributing the toxic effect of copper to differences in the free metal ion activity in the test medium and thus support the Free Ion Activity Model (FIAM), originally formulated by Morel (1983) and critically reviewed by Campbell (1995).

However, copper toxicity does not seem to be determined by the free copper ion activity alone. Firstly, also other copper forms like hydroxy complexes (Allen and Hanssen, 1996) and complexes with low molecular weight metabolites (Campbell, 1995) have been shown to contribute to copper toxicity. Secondly, and probably more importantly, Ca^{2+}, Mg^{2+}, Na^{+} and H^{+} ions are hypothesized to compete with copper ions for binding sites at the organism-water interface (e.g. fish gill) (Pagenkopf, 1983, Zitko and Carson, 1976; Playle et al., 1992; Playle et al., 1993; Campbell and Stokes, 1985; Santore et al., 2001; Di Toro et al., 2001). Their presence consequently results in a decreased toxicity of the free copper ion (Santore et al., 2001; Di Toro et al., 2001; Playle, 1998; Meyer et al., 1999).

From the above it is clear that next to copper speciation, which determines the free copper ion activity (Allen and Hanssen, 1996), interactions at the site of toxic action also need to be considered when evaluating bioavailability (Di Toro et al., 2001). The recently developed Biotic Ligand Model (BLM) includes both and is therefore gaining increased interest in the scientific and regulatory community (Santore et al., 2001; Di Toro et al., 2001). The main assumption of the BLM is that metal toxicity occurs as the result of free metal ions reacting with binding sites at the organism-water interface (either physiologically active sites, leading to a direct biological response, or transport sites, leading to metal transport into the cell followed by a subsequent, indirect biological response), which is represented as the formation of a metal-biotic ligand complex. The concentration of this metal-biotic ligand

complex directly determines the magnitude of the toxic effect, independent of the physical-chemical water characteristics of the test medium. For fish, for example, the biotic ligand (BL) appears to be sites on the surface membrane of the gill (Pagenkopf, 1983; Di Toro et al., 2001).

In the BLM, cation-biotic ligand interactions are represented and calculated in the same way as any other reaction of a cation with an organic or inorganic ligand. To that end, the complexation capacity of the BL (analoguous to the concentration of other ligands in solution) and stability constants for the metal- and the cation-BL complexes are required and need to be incorporated in a speciation model like WHAM (Tipping, 1994) (see 2.2.6).

Initially BLMs were developed to predict copper toxicity for fish (Di Toro et al., 2001). These models combined existing datasets on both gill-copper concentration measurements and stability constants (Playle et al., 1993) and toxicity (Erickson et al., 1996; MacRae et al., 1999), and incorporated them in the metal speciation model CHESS (Santore and Driscoll, 1995), modified to include metal-DOC complexation according to WHAM (Tipping, 1994).

Unfortunately, extensive biotic ligand datasets are not available for other frequently used test organisms like *Daphnia*. Therefore, until now, the BLMs developed for fish have been calibrated to fit *Daphnia pulex* toxicity data (Santore et al., 2001). This calibration was based on the 'fish' stability constants and a modified critical biotic ligand copper concentration resulting in 50% effect. Although this 'modified fish BLM' showed promise, two questions remain unanswered: (1) are stability constants for fish and *Daphnia* really similar and (2) can these constants be derived based on toxicity data alone, i.e. without measuring BL-concentrations?

Therefore the aims of the present study were two-fold: (1) to investigate the extent to which calcium, magnesium, sodium, potassium and hydrogen ions can individually mitigate copper ion toxicity and (2) to use the obtained toxicity data to derive estimates of the parameters necessary to develop a BLM that can predict copper toxicity towards *D. magna* for a broad range of water quality characteristics. The development (this chapter) and validation (chapter 3) of such a semi-mechanistic model could support efforts to improve the ecological relevance of presently applied risk assessment procedures.

2.2. Materials and Methods

2.2.1. Experimental design

In order to distinguish between the independent effect of different cations on copper toxicity, one cation concentration at a time was varied, while keeping all other cation concentrations as low and as constant as possible. Seven sets of copper bioassays were performed: 1 Ca-set, 2 Mg-sets, 1 Na-set, 1 K-set and 2 pH-sets (Table 2-1). All sets comprised at least 4 bioassays (4 different cation concentrations) that were conducted at the same time to minimize variability. The selected cation concentrations reflected the variability occuring in natural surface waters.

2.2.2. Preparation of the test solutions

All chemicals were purchased from Vel (Leuven, Belgium) and were reagent grade. All test media were prepared by adding different volumes of stock solutions of $CaCl_2$, $MgCl_2$ or $MgSO_4$, NaCl, KCl and $NaHCO_3$ to carbon-filtered and desionized water. Except for the pH-sets, these media were adjusted to pH 6.8 by adding 0.077 mM $NaHCO_3$. pH in the pH-sets was brought to different levels by adding different amounts of $NaHCO_3$ (which then acts as a buffer through its equilibrium with the atmospheric CO_2 pressure). Sodium concentrations were kept constant through additions of NaCl. For each bioassay, the prepared test medium was then used as the dilution water to make a concentration series of copper, added as $CuCl_2$. In order to obtain near-equilibrium situations, all media were stored in the test cups at 20°C for one day before being used in the toxicity tests. The chemical characteristics of the different test-media are summarized in Table 2-1.

Table 2-1 Overview of the chemical characteristics of the test media used in the different bioassay sets and the observed 48-hour EC50s for *Daphnia magna* expressed as dissolved copper (Cu_D) and free copper ion activity (Cu^{2+}, in nM).

Bioassay Set	pH	TIC (µM)	Ca^{2+} (mM)	Mg^{2+} (mM)	Na^{+} (mM)	K^{+} (mM)	Cl^{-} (mM)	SO_4^{2-} (mM)	EC50 Cu_D (µg L^{-1})	EC50 (Cu^{2+}) (nM)
Ca	6.95	91.3	0.25	0.25	0.077	0.078	0.58	0.25	5.19	8.60
Ca	6.95	89.0	0.50	0.25	0.077	0.078	1.08	0.25	7.37	18.5
Ca	6.92	83.4	0.75	0.25	0.077	0.078	1.58	0.25	7.18	19.0
Ca	6.90	91.4	1.0	0.25	0.077	0.078	2.08	0.25	8.20	25.3
Ca	6.94	89.5	2.0	0.25	0.077	0.078	4.08	0.25	8.77	28.3
Ca	6.86	93.4	3.0	0.25	0.077	0.078	6.08	0.25	10.9	43.7
Ca	6.91	93.2	4.0	0.25	0.077	0.078	8.08	0.25	12.1	48.7
$MgSO_4$	6.77	104	0.25	0.25	0.077	0.078	0.58	0.25	3.66	6.08
$MgSO_4$	6.75	108	0.25	0.50	0.077	0.078	0.58	0.50	6.74	19.7
$MgSO_4$	6.85	96.3	0.25	1.0	0.077	0.078	0.58	1.0	4.86	10.2
$MgSO_4$	6.83	92.9	0.25	1.5	0.077	0.078	0.58	1.5	11.0	41.8
$MgSO_4$	6.83	97.5	0.25	2.0	0.077	0.078	0.58	2.0	9.47	32.4
$MgSO_4$	6.78	93.1	0.25	3.0	0.077	0.078	0.58	3.0	14.0	58.3
$MgSO_4$	6.83	97.5	0.25	5.0	0.077	0.078	0.58	5.0	17.5	70.0
$MgCl_2$	6.79	87.7	0.25	0.25	0.077	0.078	1.08	0.0	6.80	18.7
$MgCl_2$	6.85	85.3	0.25	1.0	0.077	0.078	2.58	0.0	11.1	45.4
$MgCl_2$	6.87	95.1	0.25	2.0	0.077	0.078	4.58	0.0	12.7	55.7
$MgCl_2$	6.91	92.6	0.25	4.0	0.077	0.078	8.58	0.0	14.6	64.8
Na	6.78	95.6	0.25	0.25	1.08	0.078	1.58	0.25	5.84	14.3
Na	6.78	93.1	0.25	0.25	3.09	0.078	3.59	0.25	8.83	30.3
Na	6.79	95.0	0.25	0.25	5.09	0.078	5.59	0.25	9.02	30.9
Na	6.81	91.3	0.25	0.25	7.60	0.078	8.10	0.25	12.8	52.4
Na	6.79	97.5	0.25	0.25	10.1	0.078	10.6	0.25	15.3	68.2
Na	6.81	93.8	0.25	0.25	15.1	0.078	15.6	0.25	21.2	90.0
K	6.98	97.7	0.25	0.25	0.077	0.078	0.58	0.25	6.17	10.9
K	6.96	94.2	0.25	0.25	0.077	0.500	1.08	0.25	6.86	11.5
K	6.92	85.1	0.25	0.25	0.077	1.000	1.58	0.25	8.01	14.4
K	6.89	84.3	0.25	0.25	0.077	2.000	2.58	0.25	6.93	12.4
pH1	6.44	64.0	0.25	0.25	2.16	0.078	2.70	0.25	6.74	27.4
pH1	6.75	97.5	0.25	0.25	2.16	0.078	2.66	0.25	8.83	31.2
pH1	7.28	237	0.25	0.25	2.16	0.078	2.49	0.25	11.1	22.1
pH1	7.91	1230	0.25	0.25	2.14	0.078	1.56	0.25	42.6	18.4
pH2	5.98	56.9	0.25	0.25	1.58	0.078	2.12	0.25	5.58	32.8
pH2	6.30	75.3	0.25	0.25	1.58	0.078	2.12	0.25	6.61	30.5
pH2	6.70	99.8	0.25	0.25	1.58	0.078	2.08	0.25	6.48	24.5
pH2	6.97	176	0.25	0.25	1.58	0.078	2.00	0.25	10.1	29.8
pH2	7.34	399	0.25	0.25	1.58	0.078	1.78	0.25	14.0	25.6
pH2	7.92	1740	0.25	0.25	1.54	0.078	0.58	0.25	52.4	16.9

2.2.3. Acute toxicity tests

The acute 48-hour immobilization assay with juvenile *D. magna* (<24 hour old) was performed following OECD test guideline 202 (OECD, 1984). The test organisms used originated from a healthy *D. magna* clone (K6) which has been cultured under controlled laboratory conditions in M4-medium (Elendt and Bias, 1990). For each medium an acute toxicity assay was conducted consisting of at least six treatments (control + 5 copper concentrations) with a difference of 1 log-unit or more between the lowest and highest copper concentration tested. Each treatment was performed with 3 replicates and 10 organisms per replicate in copper-free polyethylene cups (soaked for 24h in 1% HNO_3, rinsed and washed with deionized water), filled with 50 mL of test medium. The number of immobilized juveniles in each cup was counted after 24 and 48 hours.

2.2.4. Chemical measurements

The dissolved copper concentration in the test cups was determined at the start and at the end of the test using a graphite furnace atomic absorption spectrophotometer (SpectrAA800 with Zeeman background correction, Varian, Mulgrave, Australia). Water samples of 10 mL were filtered (0.45 µm, Gelman Sciences, Ann Arbor, Michigan, U.S.) and acidified with 0.14N HNO_3 (Vel, Leuven, Belgium). Calibration standards (Sigma-Aldrich, Steinheim, Germany) and a reagent blank were analysed with every ten samples.

Since earlier measurements had shown that concentrations of other cations (Ca, Mg, Na, K) and anions (Cl, SO_4) did not significantly differ from nominal values, these ions were only analysed checkwise according to standard methods (Clesceri et al., 1998).

pH (pH-meter P407, Consort, Turnhout, Belgium) was measured before and after the test. The pH glass electrode was calibrated daily using pH 4 and pH 7 buffers (Merck, Darmstadt, Germany). A maximum difference of 0.2 pH-units before and after the test was allowed.

Alkalinity was measured before and after the test according to Standard Method 2320 (Clesceri et al., 1998). Total Inorganic Carbon (TIC, $CO_3^{2-} + HCO_3^- + H_2CO_3$) was calculated from alkalinity and pH according to Stumm and Morgan (1996) and using thermodynamic stability constants from Martell et al. (1997). Dissolved Organic Carbon (DOC)

concentrations were occasionally (n=14) measured before and after the test (TOC-5000, Shimadzu, Duisburg, Germany). DOC concentrations were 271 (±54; 95% CL) µg DOC L^{-1} before the test and 280 (±68; 95% CL) µg DOC L^{-1} after the test. Appearently, juvenile daphnids, in contrast with adult daphnids (Fish and Morel, 1983), did not introduce a significant amount of dissolved organic carbon into the test medium as a result of their metabolic activity or death.

2.2.5. Data treatment and statistics

Speciation calculations were conducted using BLM version a008 (Hydroqual, 1999) which is a combination of the computation framework CHESS (Santore and Driscoll, 1995), with the specific chemical description of dissolved organic matter taken from Model V of WHAM (Tipping, 1994). Stability constants were taken from Martell et al. (1997) (See Table 2-2).

Table 2-2 Biotic Ligand Model constants (± 95% confidence limits[1]) for binding of cations to the biotic ligand of *Daphnia magna* (this study) and *Pimephales promelas* (Playle et al., 1993; Santore et al., 2001) and stability constants for inorganic copper complexes[2]

	Biotic ligand constants		Inorganic Stability constants	
	D. magna	*P. promelas*	Species	Log K
Log K_{CaBL}	3.47 (3.07-3.80)	3.6	$CuOH^+$	6.48
Log K_{MgBL}	3.58 (3.10-3.90)	(3.6) [3]	$CuOH_2$	11.78
Log K_{NaBL}	3.19 (2.92-3.48)	3.0	$CuHCO_3^+$	12.13
Log K_{HBL}	~ 5.4[4]	5.4	$CuCO_3$	6.77
Log K_{CuBL}	8.02	7.4	$Cu(CO_3)_2^{2-}$	10.2
Log K_{CuOHBL}	7.45	-	$CuCl^-$	0.4
$f_{CuBL}^{50\%}$	0.39	0.21	$CuSO_4$	2.36
$[EC50_{(Cu2+)}]_0$ (nM)	6.10 (5.45-6.74)	10.6 [5]		

[1] 95% confidence limits on stability constants were calculated by performing 1000 Monte Carlo simulations of slopes and intercept, followed by the solution of the matrix equation (11). The 0.025 and 0.975 percentiles of the calculated constants were considered being the confidence limits.
[2] taken from Martell et al. (1997).
[3] Santore (2001) did not include a Mg constant, as a first approximation K_{MgBL} could be assumed equal to K_{CaBL}
[4] K_{HBL} was assumed to be approximately the same as for fish (see text)
[5] calculated with equation (12)

Since Dwane and Tipping (1998) stated that "in general, and in the absence of other information, it is reasonable to assume for modeling purposes that 50% of 'natural' dissolved

organic matter is “active ion-binding fulvic acid”, it was assumed that (1) the average DOC content in the treatments during the test was 138 (±37; 95% CL) µg L^{-1}, (2) this DOC consisted of 100% fulvic acid and (3) this fulvic acid contained 50% C (w/w). Nominal concentrations of calcium, magnesium, sodium, potassium and chlorine, calculated inorganic carbon (cf. above) and dissolved copper and pH as measured prior to the test were used as input values (Table 2-1). Speciation calculations were performed for all experimental treatments.

48-hour EC50s expressed as dissolved copper were calculated from observed mortalities at each AAS-measured copper concentration. 48-hour EC50s expressed as free copper ion activity were calculated from observed mortalities at each calculated free copper ion activity. EC50s were calculated using the trimmed Spearman-Karber method (Hamilton et al., 1977). All linear regressions (see further) were calculated using STATISTICA® software.

2.2.6. Mathematical description of the BLM

The total number of copper binding sites on the biotic ligand (BL) is called the complexation capacity of the BL (CC_{BL}), which is analogous to a total concentration of any other ligand in the test medium. In the BLM they are treated as uniformly distributed in the exposure water, i.e. reacting with the entire water volume (Meyer, 1999). A mass balance equation on the BL can be written as

$$CC_{BL} = \left[CuBL^{+}\right] + \left[CaBL^{+}\right] + \left[MgBL^{+}\right] + \left[NaBL^{0}\right] + \left[HBL^{0}\right] + \left[BL^{-}\right] \quad (1)$$

where CC_{BL} = complexation capacity of the BL (mol · L^{-1}) ; [$CuBL^{+}$], [$CaBL^{+}$], [$MgBL^{+}$], [$NaBL^{0}$] and [HBL^{0}] = concentrations of cation-BL complexes (mol · L^{-1}), [BL^{-}] = concentration of unoccupied biotic ligand sites (mol · L^{-1}).

Equilibrium equations for the binding of cations (e.g. copper) to the BL sites can be written as (conditional) stability constant expressions of the form (Playle et al., 1993):

$$K_{CuBL} = \frac{\left[CuBL^{+}\right]}{\left(Cu^{2+}\right)\cdot\left[BL^{-}\right]} \qquad (2)$$

where K_{CuBL} = stability constant for Cu^{2+} binding to BL sites ($L \cdot mol^{-1}$), (Cu^{2+}) = activity of the free copper ion ($mol \cdot L^{-1}$). For simplicity, as in the current BLM formulation (Di Toro et al., 2001), ionic strength corrections of the stability constants that describe cation binding to the BL are not carried out. Incorporation of both the complexation capacity and the stability constants of the biotic ligand in the CHESS / WHAM framework lead to the establishment of the current acute BLM (Santore et al., 2001; Di Toro et al., 2001).

The concentration of copper bound to the BL, which according to the BLM assumptions determines the magnitude of toxic effect, can be expressed as a function of (Cu^{2+}), (Ca^{2+}), (Mg^{2+}), (Na^{+}) and (H^{+}) by combining equations (1), (2) and similar equations as (2) for all cations that compete with copper for binding sites on the BL:

$$\left[CuBL^{+}\right] = \frac{K_{CuBL}\cdot\left(Cu^{2+}\right)}{1+K_{CuBL}\cdot\left(Cu^{2+}\right)+K_{CaBL}\cdot\left(Ca^{2+}\right)+K_{MgBL}\cdot\left(Mg^{2+}\right)+K_{NaBL}\cdot\left(Na^{+}\right)+K_{HBL}\cdot\left(H^{+}\right)}\cdot CC_{BL} \qquad (3)$$

Assuming that the complexation capacity is independent of the water quality characteristics, the fraction of the total number of copper binding sites occupied by copper (f_{CuBL}) equals:

$$f_{CuBL} = \frac{\left[CuBL^{+}\right]}{CC_{BL}} = \frac{K_{CuBL}\cdot\left(Cu^{2+}\right)}{1+K_{CuBL}\cdot\left(Cu^{2+}\right)+K_{CaBL}\cdot\left(Ca^{2+}\right)+K_{MgBL}\cdot\left(Mg^{2+}\right)+K_{NaBL}\cdot\left(Na^{+}\right)+K_{HBL}\cdot\left(H^{+}\right)} \qquad (4)$$

This fraction can also be assumed, still in accordance with the BLM assumptions (cf. above), to determine the magnitude of toxic effect and, therefore, is constant at 50% effect ($f_{CuBL}^{50\%}$); i.e. independent of the water quality characteristics, as demonstrated recently for copper (Meyer et al., 1999) and first proposed as a general concept by Pagenkopf (1983). Equation (4) can be rewritten as:

$$EC50_{(Cu^{2+})} = \frac{f_{CuBL}^{50\%}}{\left(1 - f_{CuBL}^{50\%}\right) \cdot K_{CuBL}} \cdot \left\{ 1 + K_{CaBL} \cdot \left(Ca^{2+}\right) + K_{MgBL} \cdot \left(Mg^{2+}\right) + K_{NaBL} \cdot \left(Na^{+}\right) + K_{HBL} \cdot \left(H^{+}\right) \right\} \qquad (5)$$

where $EC50_{(Cu2+)}$ = free copper ion activity resulting in 50% of the juvenile *D. magna* immobilized after 48 hours of exposure. With equation (5), $EC50_{(Cu2+)}$ can be predicted when (Ca^{2+}), (Mg^{2+}), (Na^{+}) and (H^{+}) are known, provided that one can estimate the values of K_{CuBL}, K_{CaBL}, etc. (see further). Equation (5) shows that, if the BLM concept is correct, linear relationships should be observed between $EC50_{(Cu2+)}$ and the activity of one cation when other cation activities are kept constant. The absence of CC_{BL} in equation (5) indicates that measured copper concentrations on the BL are not necessarily needed to develop a BLM. Consequently, we suggest that BLM parameters can be estimated from toxicity data alone.

2.3. Results and Discussion

2.3.1. Effects of major cations on copper toxicity

Increases in Ca, Mg and Na (but not K) resulted in an elevated 48-hour EC50 (expressed as dissolved copper). In these bioassay sets ($CaCl_2$, $MgCl_2$, $MgSO_4$ and NaCl), observed EC50s ranged from about 3.7 to 21 μg L^{-1} for dissolved copper and from 6.1 to 90 nM for free copper ion activity (Table 2-1).

These observations clearly demonstrate the limitations of using free ion activity for predicting copper toxicity since 48-hour EC50s (as cupric ion activity) differed by more than a factor 10. However, a large part of these differences can be explained by the positive linear relations ($p < 0.05$) between $EC50_{(Cu2+)}$ and activities of Ca^{2+}, Mg^{2+}, Na^{+} (Figure 2-1). This supports the assumptions of the BLM concept (Eq. 5) and has often been associated with competition between metal ions and cations like calcium, sodium and hydrogen for binding on transport and toxic action sites on biological surfaces (Zitko and Carson, 1976; Pagenkopf, 1983; Campbell and Stokes, 1985; Erickson et al., 1996; Santore et al., 2001).

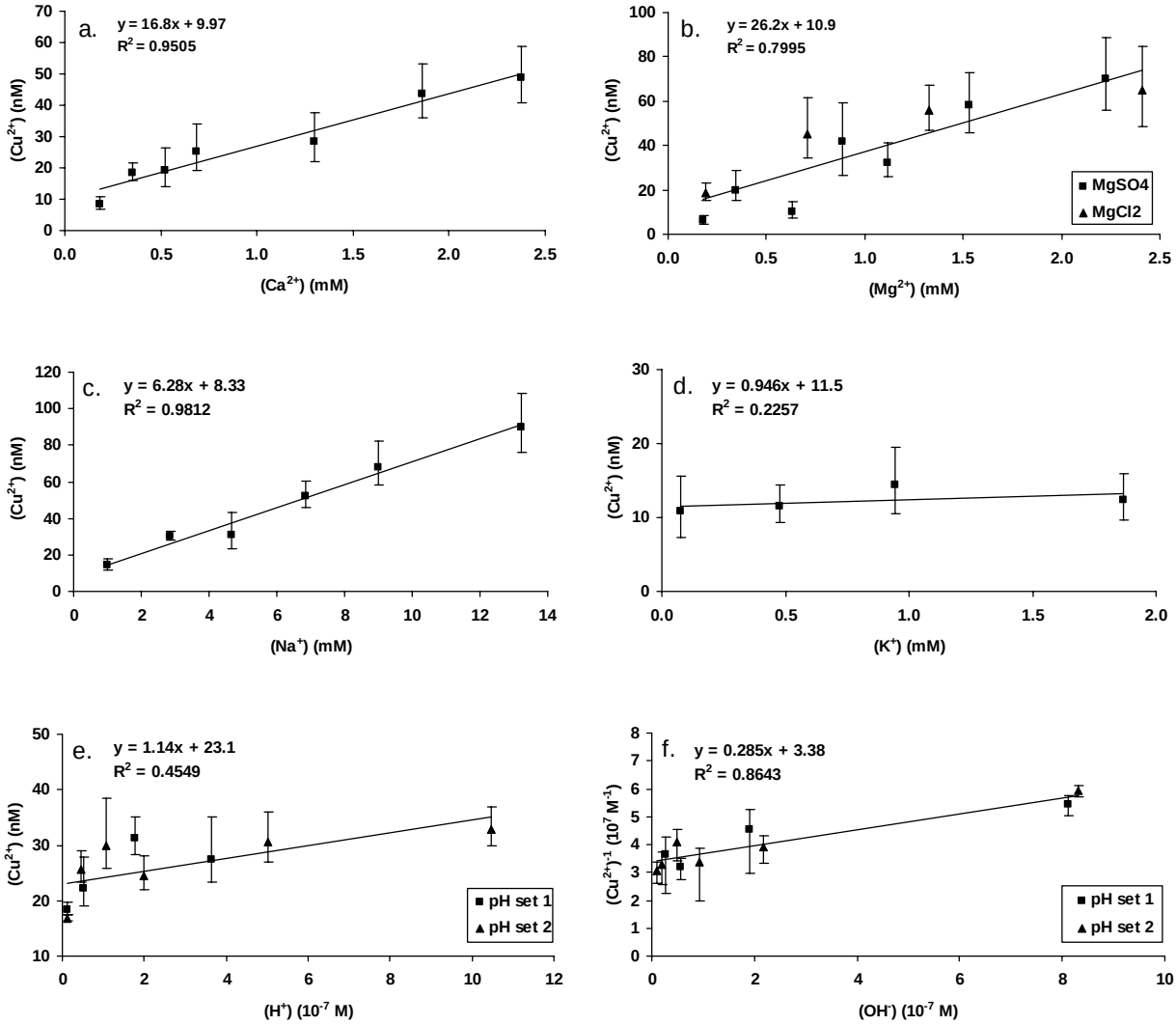

Figure 2-1 48-hour $EC50_{Cu2+}$ for *Daphnia magna* as a function of the chemical activity of Ca^{2+} (a), Mg^{2+} (b), Na^{+} (c), K^{+} (d), H^{+} (e) and OH^{-}(f). Error bars indicate 95% confidence intervals. Solid lines are the linear regression equations (R^2 and equation in figures).

Calcium and magnesium reduced copper toxicity for *D. magna* to a more or less similar extent, which is in contrast with findings in fish studies in which magnesium did not or only slightly decrease copper toxicity (Erickson et al., 1996; Welsh et al., 2000). Until now, this has been explained by the physiological mechanism of Ca (but not Mg) in modifying gill membrane permeability to metal ions (Markich and Jeffree, 1994) and by the evidence that the binding affinity of calcium to metal receptors at the cell membrane surface is superior to that of magnesium (Hille et al., 1975). The observation in this study that magnesium decreased copper toxicity for *Daphnia magna*, strongly suggests that species-specific differences might exist in the effect of modifying factors on copper bioavailability and toxicity.

Further, the fact that the effect of $MgSO_4$ and $MgCl_2$ on 48-hour EC50 were similar (Table 2-1 and Figure 2-1) suggests that neither sulphate or chloride ions have any effect on acute copper toxicity within the tested concentration range. This is supported by the observations of Erickson et al. (1996).

Finally, as mentioned above, only potassium (up to 2 mM) did not seem to affect acute copper toxicity to *D. magna* (Figure 2-1). Erickson et al. (1996), however, reported that potassium chloride concentrations of 2 and 4 mM increased acute copper toxicity to larval fathead minnow by about a third. This disagreement, however, may only be of interest in the context of copper toxicity mechanisms (e.g. Na-K-ATPase), since potassium concentrations higher than 2 mM are high compared to those found in natural waters (Erickson et al., 1996).

2.3.2. Effect of pH on copper toxicity

In the tested pH range of 5.98 to 7.92, observed 48-hour EC50s increased from about 5.6 to 52 μg L^{-1} for dissolved copper and decreased from 33 to 17 nM for free copper ion activity. This means that observed differences in (dissolved) EC50s when pH is varied, can at least partially be explained by speciation differences. If the relation between (H^+) and 48-hour $EC50_{(Cu2+)}$ is considered as being linear, this would indicate the possibility of proton competition at the biotic ligand (Figure 2-1). However, it may be suggested that a curvilinear relation with saturating EC50 at higher (H^+) would also fit the data. Two, possibly interacting, mechanisms could explain this observation. Firstly, the pH in the micro-environment (of the biotic ligand) of fish (Playle et al., 1992) and invertebrates (Gensemer and Playle, 1999) can be different from the pH in the bulk solution, leading to a misinterpretation of the interactions of copper and hydrogen ions at the organism-water interface.

Playle et al. (1992) for example, showed that the pH at the gill of fathead minnows was rendered more basic at acidic pH and more acidic at basic pH (through the release of CO_2), thus regulating the pH at the gill within a narrow range. Secondly, possible toxicity of copper hydroxide complexes (Meador, 1991; Allen and Hanssen, 1996) would imply that at a higher pH (lower H^+), less (Cu^{2+}) would be needed to exert the same toxic effect, leading to a steepening of the slope in this pH range. Within the BLM construct, the possible contribution of copper hydroxide to toxicity can best be accomodated in terms of the formation of a copper

hydroxide-biotic ligand complex, [CuOHBL]. In this case, toxicity will be determined by the total amount of copper bound to the BL, being the sum of [CuBL] and [CuOHBL]. Equations (4) and (5) can be transformed to:

$$f_{CuBL} = \frac{K_{CuBL} \cdot \left(Cu^{2+}\right) + K_{CuOHBL} \cdot \left(CuOH^{+}\right)}{\left\{1 + K_{CuBL} \cdot \left(Cu^{2+}\right) + K_{CuOHBL} \cdot \left(CuOH^{+}\right) + K_{CaBL} \cdot \left(Ca^{2+}\right) + K_{MgBL} \cdot \left(Mg^{2+}\right) + K_{NaBL} \cdot \left(Na^{+}\right) + K_{HBL} \cdot \left(H^{+}\right)\right\}} \quad (6)$$

and, given the known stability constant for the CuOH complex (K_{CuOH}, see Table 2-2), to:

$$EC50_{Cu^{2+}} = \frac{f_{CuBL}^{50\%} \cdot \left\{1 + K_{CaBL} \cdot \left(Ca^{2+}\right) + K_{MgBL} \cdot \left(Mg^{2+}\right) + K_{NaBL} \cdot \left(Na^{+}\right) + K_{HBL} \cdot \left(H^{+}\right)\right\}}{(1 - f_{CuBL}^{50\%}) \cdot \left\{K_{CuBL} + K_{CuOHBL} \cdot K_{CuOH} \cdot \left(OH^{-}\right)\right\}} \quad (7)$$

From equation (7) it can be concluded that, if $CuOH^+$ contributes to copper toxicity, a linear relation should exist between (OH^-) and $1/EC50_{(Cu2+)}$ in the pH range where there is no significant proton competition and provided that other cation activities are constant. Furthermore, the ratio K_{CuOHBL}/K_{CuBL} should be equal to the ratio slope/intercept of that linear regression, divided by K_{CuOH}. The fact that the mentioned linear relation was indeed observed and that the r^2 (0.86) was higher than the r^2 for the linear regression between (H^+) and (Cu^{2+}) (Figure 2-1, r^2=0.45) suggests that it is probably better to model the observed toxicity changes in the tested pH range in terms of copper hydroxide co-toxicity than in terms of proton competition (cf. below). K_{CuOHBL}/K_{CuBL} equaled 0.27, which, from a mechanistic point of view, means that $CuOH^+$, is less toxic than Cu^{2+}.

2.3.3. Estimation of BLM parameters

Linear regression analysis of $EC50_{(Cu2+)}$ versus cation activity (e.g. Ca^{2+}) gives an intercept and a slope, which can, according to equation (5), be expressed as (ignoring the small differences between $CuOH^+$ contribution to toxicity caused by small pH changes at pH ~ 6.8):

$$Intercept_{Ca} = \frac{f_{CuBL}^{50\%}}{\left(1 - f_{CuBL}^{50\%}\right) \cdot K_{CuBL}} \cdot \left\{1 + K_{MgBL} \cdot \left(Mg^{2+}\right)_{Ca} + K_{NaBL} \cdot \left(Na^{+}\right)_{Ca} + K_{HBL} \cdot \left(H^{+}\right)_{Ca}\right\} \quad (8)$$

and

$$Slope_{Ca} = \frac{f_{CuBL}^{50\%}}{\left(1 - f_{CuBL}^{50\%}\right) \cdot K_{CuBL}} \cdot K_{CaBL} \quad (9)$$

where $(Mg^{2+})_{Ca}$, $(Na^{+})_{Ca}$ and $(H^{+})_{Ca}$ = the mean of the calculated cation activities in all tests of the bioassay set under consideration (in this case the Ca-set). Dividing slope by intercept gives the following ratio (R_{Ca}):

$$\frac{Slope_{Ca}}{Intercept_{Ca}} = \frac{K_{CaBL}}{\left\{1 + K_{MgBL} \cdot \left(Mg^{2+}\right)_{Ca} + K_{NaBL} \cdot \left(Na^{+}\right)_{Ca} + K_{HBL}\left(H^{+}\right)_{Ca}\right\}} = R_{Ca} \quad (10)$$

Similar equations can be derived for the Mg, Na and H-sets. Rearranging these equations into a matrix form, results in:

$$\begin{pmatrix} 1 & -R_{Ca} \cdot \left(Mg^{2+}\right)_{Ca} & -R_{Ca} \cdot \left(Na^{+}\right)_{Ca} & -R_{Ca} \cdot \left(H^{+}\right)_{Ca} \\ -R_{Mg} \cdot \left(Ca^{2+}\right)_{Mg} & 1 & -R_{Mg} \cdot \left(Na^{+}\right)_{Mg} & -R_{Mg} \cdot \left(H^{+}\right)_{Mg} \\ -R_{Na} \cdot \left(Ca^{2+}\right)_{Na} & -R_{Na} \cdot \left(Mg^{2+}\right)_{Na} & 1 & -R_{Na} \cdot \left(H^{+}\right)_{Na} \\ -R_{H} \cdot \left(Ca^{2+}\right)_{H} & -R_{H} \cdot \left(Mg^{2+}\right)_{H} & -R_{H} \cdot \left(Na^{+}\right)_{H} & 1 \end{pmatrix} \cdot \begin{pmatrix} K_{CaBL} \\ K_{MgBL} \\ K_{NaBL} \\ K_{HBL} \end{pmatrix} = \begin{pmatrix} R_{Ca} \\ R_{Mg} \\ R_{Na} \\ R_{H} \end{pmatrix} \quad (11)$$

The solution of this matrix after speciation calculations and linear regression analyses (Figure 2-1) resulted in the estimation of the stability constants K_{CaBL}, K_{MgBL}, K_{NaBL} and K_{HBL} (Table 2-2). The stability constants were determined by assuming the relation between pH and $EC50_{Cu2+}$ (Figure 2-1) to be: (1) 100% due to proton competition, and (2) 100% due to copper hydroxide toxicity (by excluding the last row out of matrix equation 11). Assuming proton

competition, calculations resulted in log K_{CaBL} = 3.62, log K_{MgBL}= 3.75, log K_{NaBL} = 3.37 and log K_{HBL} = 6.52. Although stability constants for Ca^{2+} and Na^{+} were more or less similar to those obtained for fish gills and used in the original fish-BLM of Santore et al. (2001), the constant for proton competition was more than one log-unit higher than the frequently reported cell/membrane/gill surface pKa range from 4 to 5.4, as determined by surface binding analysis or estimated from low pH toxicity test results (Playle et al., 1993; Cusimano et al., 1986). This large difference again demonstrates that the relation between (H^{+}) and $EC50_{Cu2+}$ should rather be explained in terms of the earlier discussed toxicity of $CuOH^{+}$, than in terms of proton competition. Indeed, co-toxicity of $CuOH^{+}$ results in the steeper slope at higher pH (lower H^{+}) and consequently, to the high (calculated) K_{HBL}.

Therefore, it is probably better to assume log K_{HBL} ~ 5.4 (same as for fish gills). This asumption is in accordance with not allowing proton competition in the calculations, since at the lowest tested pH (5.97) proton competition would not be significant anyhow. In this case calculations resulted in log K_{CaBL} = 3.47, log K_{MgBL}= 3.58 and log K_{NaBL} = 3.19 (Table 2-2). Again log K's were in the same range as the constants reported by Playle et al. (1993), except for the Mg-constant, which has not been included in the original Cu-BLM so far (Santore et al., 2001).

Compared to the values of log K_{CaBL} and log K_{MgBL}, the apparent value for log K_{NaBL} seems unrealistically high, given the relative affinities of Na^{+}, Ca^{2+} and Mg^{2+} for typical biogenic chelating ligands (e.g. aspartate, citrate, etc., Martell et al., 1997). This discrepancy suggests that the toxicity protection provided by Na^{+} is attributable not only to its competition with Cu^{2+} for the biotic ligand, but possibly also to a direct physiological effect. It has been reported that increasing (Na^{+}) could reduce the loss of plasma electrolytes (including Na^{+}), which may be one of the copper toxicity mechanisms in fish (Grosell et al., 2002). Altough the effect of Na on copper toxicity might possibly not be a 'chemical' effect (competition), it did not significantly influence the calculated values of the ('chemical' competition) stability constants. This is demonstrated by the fact that, when calculated without taking the Na-dataset into account (by eliminating the third row from matrix equation 11), values of log K_{CaBL} and log K_{MgBL} (3.43 and 3.53, respectively), were almost identical to the values calculated with the Na-dataset included (3.47 and 3.57, respectively).

Now, for the final development of a BLM for *D. magna*, only two parameters remain to be determined: K_{CuBL} and $f_{CuBL}^{50\%}$. Since these parameters are coupled through equations (7), (8) and (9), they cannot be derived unambiguously from EC50 data alone. In practice, however, only the ratio

$$\left[EC50_{(Cu^{2+})}\right]_0 = \frac{f_{CuBL}^{50\%}}{\left(1 - f_{CuBL}^{50\%}\right) \cdot K_{CuBL}} \qquad (12)$$

is needed for the prediction of $EC50_{(Cu2+)}$ with equation (7) (since K_{CuOHBL}/K_{CuBL}= 0.27, cf. above). Mechanistically, $[EC50_{(Cu2+)}]_0$ can be interpreted as the $EC50_{(Cu2+)}$ in the hypothetical case where no competing ions are present in the test medium and where contribution of $CuOH^+$ to toxicity is unsignificant. Based on observed $EC50_{(Cu2+)}$ and ion activities for each bioassay and using the stability constants for Ca, Mg and Na and using K_{CuOHBL}/K_{CUBL} = 0.27, (cf. above), $[EC50_{(Cu2+)}]_0$ was calculated with (7) to have a mean of 6.1 nM with a standard deviation of 1.8 nM (n = 38). This value is lower than the observed intercepts (equation 8, Figure 2-1) because there was always a small amount of ions present in both Ca, Mg and Na bioassay sets. This value is also lower than the value calculated for *P. promelas* with equation (12) (10.6 nM, Table 2-2), which may reflect the higher copper sensitivity of *D. magna* to copper.

Nevertheless, good approximations of K_{CuBL} and $f_{CuBL}^{50\%}$ are important, since these provide more information on the mechanistic understanding of copper bioavailability and toxicity. Therefore, for every treatment (38 bioassays x 5 Cu-concentrations) the fraction of the BL occupied by copper was calculated with equation (6) for varying log K_{CuBL}. *A priori* it was assumed that the best approximation of the "real" K_{CuBL} would result in the best correlation between the calculated f_{CuBL} and the logit of the percent immobilized *D. magna* after 48 hours of exposure (Figure 2-2). Values for log K_{CuBL} = 8.02 (and thus log K_{CuOHBL} = 7.45) and the associated $f_{CuBL}^{50\%}$ = 0.39 resulted in the best fit (r^2 = 0.72) and were retained.

While $f_{CuBL}^{50\%}$ for *D. magna* is more or less similar to that for fish, log K_{CuBL} was found to be 0.6 log-units higher, which possibly reflects the higher copper sensivity of *D. magna* (cf. above). From the good agreement between BLM constants of Santore et al. (2001) and those presented in this study (see Table 2-2), it can be suggested - as done by Santore et al. (2001) -

that as a first approximation the daphnid biotic ligand has the same affinity for metals and cations as that of fish. This assumption would only lead to incorrect toxicity predictions at pH values where $CuOH^+$ toxicity becomes important, since Santore et al. (2001) did not take $CuOH^+$ toxicity into acount. This may be illustrated as follows: when calculated (with equation 6) with the constants derived in this study $f_{CuBL}^{50\%}$ for the bioassays of the pH set varied between 0.34 and 0.44 and between 0.09 and 0.17, when calculated with the model of Santore et al. (2001). This means that in the model of Santore et al. (2001) f_{CuBL} is not a better mortality predictor than free copper ion activity (see Table 2-1) when pH is varied, since both show a difference of about a factor 2 between the lowest and the highest value at 50% effect.

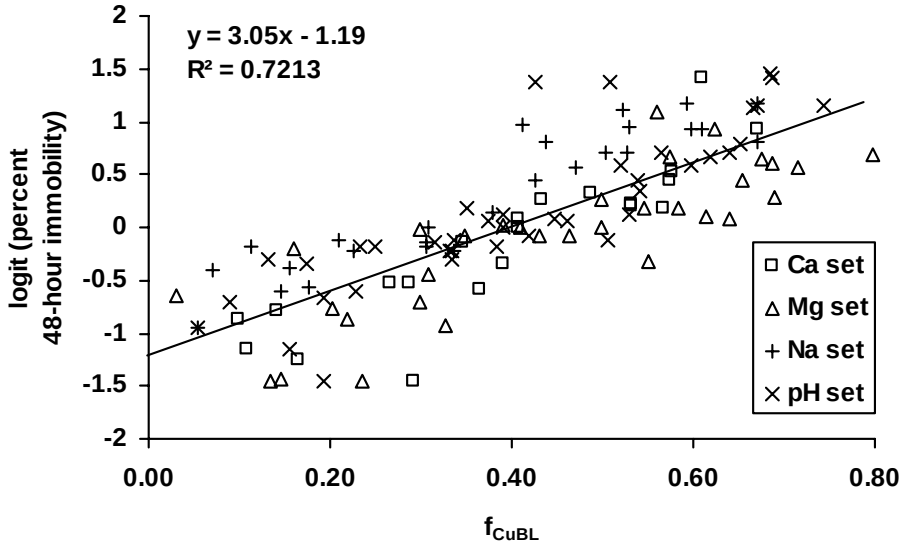

Figure 2-2 Relationship between the calculated fraction of the biotic ligand sites occupied by copper (f_{CuBL}) and the logit of the observed percent *Daphnia magna* immobilized after 48 hours of exposure. f_{CuBL} was calculated for every treatment with equation (6) using the outputs from the speciation calculations with BLM version a008 (Hydroqual, 1999) and using the stability constants obtained in this study (Table 2-2).

2.3.4. Validation and applicability of the developed BLM

Finally, all stability constants and $f_{CuBL}^{50\%}$ (Table 2-2) were included in version a008 of the BLM software (Hydroqual, 1999) to predict 48-hour EC50s (as dissolved copper) for the water quality characteristics of the bioassays conducted in this study (Table 2-1). The predicted EC50s differed from the observed EC50s with a factor of less than 1.5 (Figure 2-3). It should however be recognized that the BLM-parameters were especially estimated to fit the observed EC50 data. Although the developed *Daphnia* BLM shows promise, further validation experiments with a broader range of exposure conditions and field collected water samples (including variations of DOC concentrations) are required.

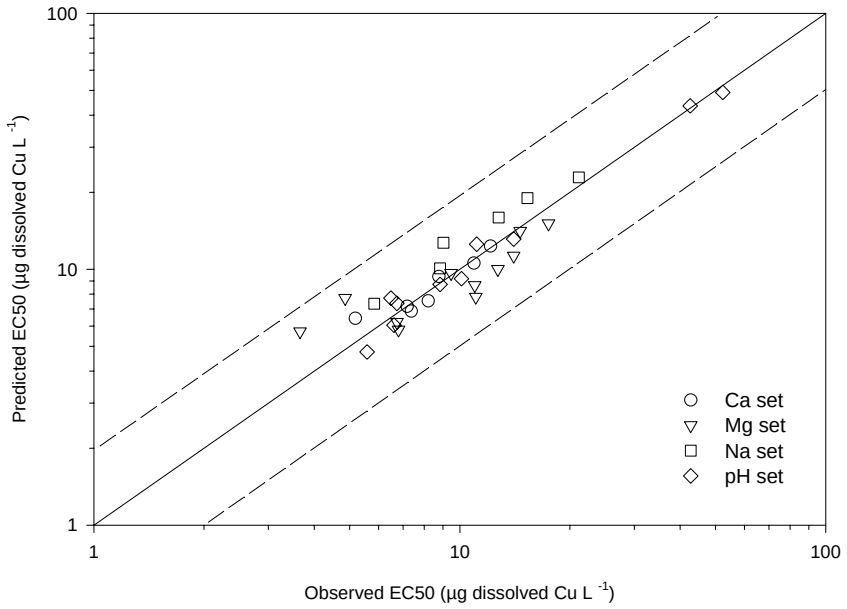

Figure 2-3 Relationship between observed and predicted 48-hour EC50s (expressed as dissolved copper, both axes are on a logarithmic scale) for *Daphnia magna* indicating the predictive capacity of the BLM developed in this study Predicted EC50s were calculated with BLM version a008 (Hydroqual, 1999) using the stability constants obtained in this study (Table 2-2). The full line indicates a perfect match between observed and predicted EC50s, the dashed lines indicate ratios of 0.5 and 2 between observed and predicted EC50s.

Indeed, in natural waters, (Cu^{2+}) (and thus copper bioavailability and toxicity) is to a large extent determined by complexation to natural organic matter. In the tests performed in this study, DOC concentrations were assumed to be constant (138 µg DOC L^{-1}) and to have the same metal complexation properties as natural organic matter (fulvic acid, cf. above). To assess the effect of this assumption on the calculations of the BLM constants, we repeated all calculations for the upper and lower 95% confidence limit of the DOC concentration, i.e. 101 and 175 µg DOC L^{-1} and the following were obtained: log K_{CuBL} = 7.75 and. 8.50 , log K_{CuOHBL}= 6.98 and 7.97, log K_{CaBL} = 3.34 and 3.82, log K_{MgBL} = 3.47 and 3.93, log K_{NaBL} = 3.04 and 3.53, $f_{CuBL}^{50\%}$ = 0.37 and 0.43, $[EC50_{(Cu2+)}]_0$ = 2.4 and 10.5 nM. It is clear that calculated constants are more (K_{CuBL}, K_{CuOHBL} and $[EC50_{(Cu2+)}]_0$) or less (K_{CaBL}, K_{MgBL}, K_{NaBL} and $f_{CuBL}^{50\%}$) sensitive to the choice of the DOC-concentration, which controls the range of the calculated (Cu^{2+}) values.

This sensitivity demonstrates that further experimental work is needed to obtain more accurate values of BLM constants and a mechanistic understanding of the BLM for *D. magna* developed in this study. This work could include both the identification of the biotic ligand for *D. magna*, the measurement of BL-concentrations of copper at different exposure conditions and the calibration of the model to toxicity data of bioassays performed with DOC additions in which EC50s are determined based on measured Cu^{2+} activities. The latter approach will be followed to refine the obtained Cu-BLM in chapter 3.

2.3.4. Conclusion

The main conclusion from this study is that, with the presented new methodology BLM constants can indeed be estimated from toxicity data alone, provided that valid metal ion activities are used as a starting point for the calculations. The developed model will be refined and validated in chapter 3 and the same methodology will also be used for the development of a chronic Cu-BLM for *D. magna* (chapter 4).

Chapter 3

Refinement and field validation of the acute Cu-BLM for *Daphnia magna*

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Chapter
3

Refinement and field validation of the acute Cu-BLM for *Daphnia magna*

Abstract – The biotic ligand model (BLM) developed in chapter 2 was validated for its capacity to predict acute 48-hour EC50s of copper to *Daphnia magna* in 25 reconstituted media with different pH and concentrations of artificial dissolved organic carbon, Ca, Mg and Na. Before the BLM validation, fitting of measured (with a copper ion selective electrode) and calculated (with the BLM) Cu^{2+}-activity was performed by adjusting the WHAM-Model V (i.e. the metal-organic speciation part of the BLM) copper-proton exchange constant to pK_{MHA}=1.9. Using this value, the observed 48-hour EC50s agreed very well with BLM-predicted EC50s for tests performed at pH < 8 but not at all for tests performed at pH > 8. Additional experiments demonstrated that this was due to toxicity of the $CuCO_3$ complex, which is the most abundant inorganic copper species at pH > 8. This was incorporated into the initial BLM by allowing the binding of $CuCO_3$ (next to Cu^{2+} and $CuOH^+$) to the biotic ligand of *D. magna* . The affinities of $CuOH^+$ and $CuCO_3$ for the biotic ligand were about 5 and 10 times lower than that of Cu^{2+}, respectively. With the refined BLM, 48-hour EC50s could be accurately predicted within a factor of 2, not only in all 25 reconstituted media but also in 19 natural waters. This validated and refined BLM could support efforts to improve the ecological relevance of presently applied risk assessment procedures.

3.1. Introduction

Current water quality standards and risk assessment procedures of metals are predominantly based on total or dissolved metal concentrations (Janssen et al., 2000). However, there is extensive evidence that neither total nor dissolved aqueous metal concentrations are good predictors of metal bioavailability and toxicity (Bergman and Dorward-King, 1997; Janssen et al., 2000).

Copper toxicity to freshwater organisms has been shown to be dependent on a variety of ambient water quality characteristics. For example, increases in Ca, Mg and Na concentrations (Pagenkopf, 1983; Erickson et al., 1996; Barata et al., 1998; De Schamphelaere and Janssen, 2002, Welsh et al., 2000), pH (Erickson et al., 1996; Meador, 1991, Shubauer-Berigan et al., 1993, De Schamphelaere and Janssen, 2002) and organic matter content (Winner, 1985; Ma et al., 1998, Erickson et al., 1996) have been shown to result in decreased copper toxicity for fish and crustacean species. Until recently only a hardness correction is provided in setting water quality criteria for copper in the United States (U.S. Environmental Protection Agency, 1994).

The Biotic Ligand Model (BLM) is gaining increased interest in the scientific and regulatory community for predicting and evaluating metal (including copper) bioavailability and toxicity, because it takes into account both metal speciation and interactions at toxic action and/or transport sites on the organism-water interface. A detailed description of the biological, chemical, mathematical and computational aspects of the BLM can be found in Di Toro et al. (2001), Santore et al. (2001) and De Schamphelaere and Janssen (2002) and in chapter 2 of this study.

In chapter 2, using a new methodology, we elaborated on the development of a BLM for predicting acute copper toxicity to *Daphnia magna* (De Schamphelaere and Janssen, 2002). Stability constants for copper and competing cations on the biotic ligand were estimated directly from ecotoxicity data. The main difference with other existing Cu-BLMs (Di Toro et al., 2001; Santore et al., 2001) was the incorporation of $CuOH^+$ bioavailability into the BLM framework. This was consistent with reports (Allen and Hanssen, 1996; Blust et al., 1991) that $CuOH^+$, next to Cu^{2+} - which is generally considered to be the most reactive and toxic copper species (Campbell, 1995; Morel, 1983) - can also be bioavailable to a certain extent.

The main drawbacks of the BLM developed by De Schamphelaere and Janssen (2002) are: 1) it is developed using results of bioassays conducted in media which did not contain naturally occuring concentrations of dissolved organic carbon (DOC), generally considered to be an important factor determining copper bioavailability and toxicity (cf. above); and 2) there is some uncertainty about the calculated (Cu^{2+}) activities in these toxicity tests, due to the uncertainty about the complexing properties of the background DOC in the exposure

media, resulting in an associated uncertainty of the derived BLM constants (De Schamphelaere and Janssen, 2002).

The aims in the present chapter were therefore: 1) to validate the initial BLM using results of toxicity tests performed with reconstituted waters containing artificial DOC; 2) to refine the BLM by performing additional experiments aimed at resolving questions arising from errors in the predicted EC50s; and 3) to validate the refined BLM for exposures in natural water samples.

3.2. Materials and Methods

3.2.1. Experimental design

Acute bioassays with *Daphnia magna* were conducted in 25 reconstituted media with different pH and DOC, Ca, Mg and Na concentrations. To each medium a level of DOC (2, 5, 10, 15 or 20 mg L^{-1}, added as Aldrich Humic Acid, AHA, Sigma-Aldrich Chemie, Steinheim, Germany), Ca (0.2, 1, 2, 3 or 4 mM, added as $CaCl_2$), Mg (0.2, 0.4, 0.6, 0.8 or 1 mM, added as $MgSO_4$), Na (1, 2, 3, 4 or 5 mM, added as NaCl) and pH (6.25, 6.75, 7.25, 7.75 or 8.25) was randomly assigned. The selected ranges of water chemistry characteristics reflect the variability occurring in natural European surface waters (Heijerick, unpublished data).

To refine the initial BLM developed by De Schamphelaere and Janssen (2002), an additional experiment was performed in which the effect of pH (5.7 to 8.5) on acute copper toxicity to *D. magna* was studied more in depth. Six bioassays were conducted in media with a different pH and containing 0.2 mM $CaCl_2$, 0.2 mM $MgSO_4$, 0.078 mM KCl and 5 mg DOC L^{-1} (AHA). pH was controlled using MOPS-buffering for pH < 7.5 (0.75 g L^{-1}) and addition of NaOH, and using $NaHCO_3$-buffering for pH > 7.5. MOPS-buffering has previously been shown not to affect copper toxicity to *D. magna* (De Schamphelaere et al., 2002). Since it has been demonstrated that sodium decreases copper toxicity to *D. magna* (De Schamphelaere and Janssen, 2002), Na concentrations were brought to the same level in all tests (3.9 mM) through additions of NaCl.

3.2.2. Preparation of the test solutions

All chemicals, except DOC (AHA, Sigma-Aldrich Chemie, Steinheim, Germany**)** were purchased from Vel (Leuven, Belgium) and were reagent grade. Stock solutions of DOC were prepared by dissolving 5 g AHA in 2L of deionised water, equilibrating the solution for 24 hours at 4°C and filtering it through a 0.45 µm filter (GelmanSciences, Ann Arbor, Michigan, U.S.). All test media were prepared by adding the correct volumes of stock solutions of $CaCl_2$, $MgSO_4$, NaCl, KCl, $NaHCO_3$, MOPS and DOC to carbon-filtered and deionised water.

3.2.3. Sampling of natural waters

Between October 2000 and May 2001, 19 natural water samples were taken at 14 different pristine sites in 5 European countries: Belgium (1), The Netherlands (9), United Kingdom (7), Sweden (1) and Germany (1). Immediately after arrival in the laboratory, samples were filtered through a 0.45 µm filter (Gelman Sciences, Ann Arbor, Michigan, U.S.) and stored at 4°C in the dark. Chemical analyses and ecotoxicity tests were performed within 2 weeks after the sampling.

3.2.4. Acute toxicity tests with copper

Acute 48-hour immobilization assays with juvenile *D. magna* (<24 hour old) were performed following OECD test guideline 202 (1984). The test organisms originated from a healthy *D. magna* clone (K6) which has been cultured under controlled laboratory conditions in M4-medium (Elendt and Bias, 1990) for several years. For each medium and for each natural water sample an acute toxicity assay was conducted consisting of at least six treatments (control + 5 copper concentrations) with a difference of 1 log-unit or more between the lowest and highest copper concentration. In order to obtain near-equilibrium situations for the copper-DOC reactions, all spiked media were stored at 20°C for two days before being used in the toxicity tests. Each treatment was performed with 3 replicates and 10 organisms per replicate in polyethylene cups containing 50 mL of test medium. The number of immobilized juveniles in each cup was counted after 48 hours.

3.2.5. Chemical measurements

Spiked natural waters and reconstituted media were analysed for their total (unfiltered) and dissolved (0.45 µm filtered, GelmanSciences, Ann Arbor, Michigan, U.S.) copper concentrations at the start and at the end of the test using a flame (quantification limit = 18 µg Cu L^{-1}) or a graphite furnace (quantification limit = 2.1 µg Cu L^{-1}) atomic absorption spectrophotometer (AAS; Varian, Mulgrave, Australia). AAS-samples were acidified with 0.14N HNO_3 (p.a., Vel, Leuven, Belgium). Calibration standards (Sigma-Aldrich Chemie, Steinheim, Germany) and a reagent blank were analysed with every ten samples.

Total Organic Carbon (TOC, not filtered), DOC (0.45 µm filtered) and Inorganic Carbon (IC) were measured only prior to the test (TOC-5000, Shimadzu, Duisburg, Germany) since previous experiments had shown that DOC concentrations do not significantly change during the course of an acute *D. magna* test (De Schamphelaere and Janssen, 2002).

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, California, USA). Since earlier measurements had shown that concentrations of these cations (Ca, Mg, Na, K) and anions (Cl, SO_4) in reconstituted media did not significantly differ from nominal values (De Schamphelaere and Janssen, 2002), these ions were only analysed in the natural water samples.

pH was measured before and after the test (pH-meter P407, Consort, Turnhout, Belgium). The pH glass electrode was calibrated before each measurement using pH 4 and pH 7 buffers (Merck, Darmstadt, Germany). A maximum difference of 0.2 pH-units before and after the test was allowed in the reconstituted media.

Cu^{2+} activities were determined before and after each bioassay 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 deionized water (DI water) and soaked in 0.01M 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+}) 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). Immediately before the initiation of each bioassay, (Cu^{2+}) was measured in each test concentration. Comparison with occasional measurements after completion of the bioassay, revealed that (Cu^{2+}) before and after the test were not significantly different (paired t-test, $\alpha = 0.05$). All values of (Cu^{2+}) mentioned in tables, figures and text refer to the value measured prior to the test.

3.2.6. Data treatment and statistics

Speciation calculations were conducted using BLM version a008 (Hydroqual, 1999) which is a combination of the geochemical computation framework CHESS (Santore and Driscoll, 1995) and the specific chemical description of metal complexation onto organic matter taken from WHAM-Model V (Tipping, 1994). For the experiments with AHA calculations were performed using 100% humic acid as input with the assumption that AHA contains 50% C (w/w). The approach for natural waters is explained in the results and discussion section. Stability constants for inorganic complexes were taken from Martell et al. (1997) (see Table 2-2 in chapter 2). EC50s were calculated using the trimmed Spearman-Karber method (Hamilton et al., 1977). EC50s were expressed as total copper, dissolved copper and (Cu^{2+}) using AAS-measured total or dissolved copper concentration or Cu-ISE-measured Cu^{2+}-activity. All regressions (see further) were calculated using STATISTICA® software.

3.2.7. Short recapitulation of the initial acute Cu-BLM for *Daphnia magna*

Although the mathematics behind the initial acute Cu-BLM for *D. magna* is extensively discussed in chapter 2, the major principles are repeated shortly hereunder. According to De Schamphelaere and Janssen (2002) the basic BLM equation, taking into account $CuOH^+$ toxicity, is (see Table 2-2 for values of constants):

$$EC50_{Cu^{2+}} = \frac{f_{CuBL}^{50\%} \cdot \left\{1 + K_{CaBL} \cdot \left(Ca^{2+}\right) + K_{MgBL} \cdot \left(Mg^{2+}\right) + K_{NaBL} \cdot \left(Na^{+}\right) + K_{HBL} \cdot \left(H^{+}\right)\right\}}{(1 - f_{CuBL}^{50\%}) \cdot \left\{K_{CuBL} + K_{CuOHBL} \cdot K_{CuOH} \cdot \left(OH^{-}\right)\right\}} \quad (1)$$

with $EC50_{(Cu2+)}$ = the 48-hour EC50 expressed as Cu^{2+}-activity, i.e. (Cu^{2+}) (unit: M); $f_{CuBL}^{50\%}$ = the fraction of the total number of copper binding sites occupied by copper at 50% effect; K_{CuBL}, K_{CuOHBL}, K_{CaBL}, K_{MgBL}, K_{NaBL} and K_{HBL} stability constants for the binding of these cations to the BL (M^{-1}); K_{CuOH} = stability constant for the formation of the $CuOH^{+}$-complex (M^{-1}); ( ) represents the activity of the mentioned ions. With this equation, 48-hour EC50s expressed as (Cu^{2+}) can be predicted, provided that cation activities and the values of the constants are known. In practice, this equation is integrated into a geochemical speciation program (WHAM, Tipping, 1994) by adding stability constants for the binding of metal species (i.e. Cu^{2+} and $CuOH^{+}$) and competing cations (calcium, magnesium, sodium and protons) onto the biotic ligand, which is considered as a third type of ligand (next to organic and inorganic ligands). Through this linkage of equation (1) and WHAM, 48-hour EC50s expressed on a dissolved (or total) basis can be predicted.

3.3. Results and Discussion

3.3.1. Bioassays in reconstituted media

Table 3-1 presents the physico-chemistry of the 25 different test media and their corresponding 48-hour EC50s, expressed as total and dissolved copper and as free copper ion activity. First, it should be noted that AHA formed precipitates at high calcium concentrations (>2 mM), resulting in important differences in TOC vs. DOC and $EC50_{total}$ vs. $EC50_{dissolved}$. This phenomenon has already been observed for this commercial humic acid by Hering and Morel (1988). However, it was also observed that the ratio DOC / TOC (i.e. the dissolved fraction of AHA) was not significantly different from the ratio dissolved copper / total copper (t-test for dependent samples, $p > 0.05$). This suggests that TOC and copper were precipitated in equal fractions. If there were major differences in the copper binding properties of aldrich humic acid between the dissolved and the particulate form, the latter would not have been the case. For example, consider the case that 50% of the AHA is in the dissolved form and the other 50% is in the particulate form. If the dissolved form of AHA had a larger copper

complexation capacity than the particulate form, then more than 50% of the copper would be associated with the dissolved AHA and the ratio dissolved copper / total copper (being higher than 0.5) would be different from the ratio DOC / TOC (being 0.5). Hence, one may assume that the particulate and the dissolved form of AHA have similar copper binding properties. Consequently, the precipitation of organically complexed copper has probably not affected the Cu^{2+}-activity or the observed copper toxicity. Therefore, all further analyses for these 25 experiments (i.e. regressions, speciation calculcations and BLM-predictions) will be based on total copper and TOC and not on dissolved copper and DOC.

In the tests with the 25 different media, the observed 48-hour $EC50_{total}$ ranged from 115 to 1455 µg L^{-1}. These results clearly illustrate that the addition of TOC to the exposure medium drastically decreases copper toxicity, as in earlier experiments in artificial media without TOC addition all 48-hour EC50 values for *D. magna* were lower (i.e. 4 to 52 µg Cu L^{-1}, De Schamphelaere and Janssen, 2002). This large variation in copper toxicity values (4 to 1500 µg L^{-1}) is in accordance with the variation found in literature (Meador, 1991; Winner, 1985; Dunbar, 1996). A linear relationship was observed between the 48-hour EC50 and the TOC concentration:

$$\text{48-hour } EC50_{total} \text{ (µg } L^{-1}) = 19.8 + 61.2 \text{ x TOC (mg } L^{-1}) \quad (n=25;\ r^2=0.882;\ p<0.001) \qquad (2)$$

This relationship explained 88% of the observed variability within the 25 tests, while the inclusion of other test variables (pH, Ca, Mg and Na) into the regression equation (multiple linear regression) only resulted in small additional increases of the explained variability (<5%). This observation seems to support that TOC is the most important factor determining copper toxicity.

Table 3-1 Results of the 25 acute *Daphnia magna* toxicity tests: composition, 'best fitting' pK_{MHA} and 48-hour EC50s of the 25 different media

Medium	pH[1]	TOC (mg L^{-1})	DOC (mg L^{-1})	pK_{MHA}[2]	IC (mM)	Concentrations of major ions					48-hour EC50[3]		
						Ca (mM)	Mg (mM)	Na (mM)	SO_4 (mM)	Cl (mM)	Cu_{total} (µg L^{-1})	$Cu_{dissolved}$ (µg L^{-1})	(Cu^{2+})[4] (nM)
1	7.59	4.776	1.532	1.85	0.765	3.0	0.6	3.0	0.6	8.23	304 (286-324)	100 (91.6-111)	26.9 (22.5-32.3)
2	7.29	17.62	3.442	1.95	0.384	4.0	0.4	2.0	0.4	9.73	1118 (1041-1200)	200 (184-223)	132 (111-157)
3	6.40	4.046	2.190	1.85	0.0798	2.0	0.6	4.0	0.6	8.00	211 (197-226)	106 (98.4-121)	111 (87.9-140)
4	7.65	4.365	3.605	1.89	0.725	1.0	0.2	1.0	0.2	2.23	350 (330-372)	276 (259-295)	37.1 (32.4-42.4)
5	6.11	8.410	6.867	1.84	0.043	1.0	0.8	4.0	0.8	5.45	353 (320-389)	292 (279-307)	52.5 (41.0-67.1)
6	6.77	1.860	1.406	1.80	0.132	1.0	1.0	1.0	1.0	2.28	115 (105-126)	92.6 (88.3-96.7)	47.2 (42.8-52.6)
7	8.39	4.648	3.788	1.83	3.19	1.0	1.0	5.0	1.0	3.15	266 (244-289)	210 (194-230)	2.52 (2.09-3.04)
8	7.67	15.38	1.723	1.88	0.764	4.0	0.8	2.0	0.8	9.23	1150 (1091-1214)	152 (141-164)	63.3 (56.2-71.3)
9	7.71	15.59	7.799	1.94	0.944	2.0	0.6	2.0	0.6	5.23	1099 (1048-1152)	526 (487-570)	55.7 (49.7-62.5)
10	8.27	17.10	14.226	1.83	2.72	1.0	0.6	5.0	0.6	3.15	979 (920-1037)	826 (786-871)	3.50 (3.01-4.07)
11	7.74	4.345	4.046	1.78	0.833	0.2	0.4	1.0	0.4	0.63	429 (404-456)	388 (345-431)	19.9 (17.3-23.0)
12	8.46	3.082	2.482	1.96	4.16	0.2	0.6	4.0	0.6	0.55	168 (159-178)	157 (144-173)	2.39 (2.03-2.83)
13	6.95	8.986	2.478	1.99	0.223	3.0	0.8	1.0	0.8	6.92	533 (493-576)	136 (122-151)	241 (198-293)
14	7.35	16.19	3.742	1.97	0.407	2.0	0.4	1.0	0.4	4.73	1030 (970-1099)	229 (203-264)	106 (89.3-126)
15	8.39	8.474	4.356	1.80	2.80	2.0	1.0	5.0	1.0	5.15	524 (494-555)	244 (220-277)	3.69 (3.14-4.34)
16	6.85	5.152	1.284	1.95	0.190	4.0	0.8	1.0	0.8	8.92	392 (370-415)	100 (91-114)	369 (318-427)
17	7.41	19.55	14.839	1.99	0.542	1.0	0.4	4.0	0.4	5.73	1455 (1394-1524)	1213 (1094-1350)	130 (107-156)
18	6.29	14.30	9.042	1.82	0.0486	2.0	0.8	4.0	0.8	7.31	661 (614-710)	421 (387-463)	90.6 (75.5-109)
19	7.43	12.90	4.551	1.99	0.563	2.0	0.8	5.0	0.8	8.73	782 (727-845)	300 (274-329)	96.5 (82.5-113)
20	7.81	12.96	4.751	1.99	1.14	2.0	0.6	2.0	0.6	5.23	833 (772-898)	289 (267-316)	55.7 (46.4-66.9)
21	6.98	7.131	1.409	1.91	0.197	4.0	0.6	3.0	0.6	10.9	534 (492-580)	117 (104-132)	243 (191-309)
22	7.00	9.430	2.211	2.00	0.260	3.0	0.6	2.0	0.6	7.92	470 (442-500)	109 (101-119)	157 (133-185)
23	6.31	17.80	8.348	1.80	0.0697	2.0	0.2	5.0	0.2	8.23	820 (768-882)	465 (423-511)	74.5 (60.3-92.0)
24	8.46	12.62	10.939	1.82	3.65	1.0	0.2	5.0	0.2	3.15	915 (869-962)	798 (745-860)	2.73 (2.38-3.12)
25	7.83	4.653	4.263	1.95	1.21	0.2	0.2	1.0	0.2	0.63	395 (370-420)	380 (343-425)	25.0 (21.5-29.2)

[1] in medium 5, 6, 18 and 23 MOPS (750 mg L^{-1}) was used as a pH buffer

[2] the 'best fitting' pK_{MHA} is the pK_{MHA} that resulted in the best match between WHAM-calculated Cu^{2+}-activity and the Cu-ISE measured Cu^{2+}-activity

[3] numbers between brackets indicate 95% confidence limits

[4] based on measured Cu^{2+} with Cu-ISE

To account for the observed variability in total copper toxicity, many authors have suggested that free copper ion activity is a better predictor of biological response (i.e. toxicity, uptake, etc.) than total copper concentration (review by Campbell et al., 1995). However, a considerable number of authors have concluded that free copper ion activity does not always accurately predict copper toxicity due to competition effects or bioavailability of inorganic and organic copper complexes (e.g. Pagenkopf, 1983; Blust et al., 1991; Campbell, 1995; De Schamphelaere and Janssen, 2002).

Our results seem to support the latter reports, since 48-hour $EC50_{(Cu2+)}$ values (EC50 expressed as free copper ion activity) for *D. magna* varied from 2.73 to 369 nM (factor 140), while $EC50_{total}$ values varied from 115 to 1455 µg L^{-1} (factor 13) This means that in the present study, (Cu^{2+}) is a worse predictor of toxicity than total copper. Multiple linear regression of $EC50_{(Cu2+)}$ versus the test variables showed that only Ca and pH significantly affected 48-hour $EC50_{(Cu2+)}$ values:

48-hour $EC50_{(Cu2+)}$ (nM) = 308 + 42.6 x Ca (mM) – 41.3 x pH ($n=25$; $R^2=0.567$; $p<0.05$) (3)

Since less than 60% of the variability was explained, only trends could be observed. Apparently, it seemed that (1) more (Cu^{2+}) was needed at higher Ca concentration and (2) less (Cu^{2+}) was needed at higher pH, to exert the same toxic effect. This is in agreement with (1) the frequently reported competition of cations with copper ions on biological membranes (i.e. the biotic ligand) and (2) the findings of De Schamphelaere and Janssen (2002), respectively. No significant effect of Mg and Na was observed, probably because of the low concentrations of these ions in the present tests compared to the earlier reported concentrations producing significant changes in $EC50_{(Cu2+)}$ (De Schamphelaere and Janssen, 2002).

Additionally, the fact that TOC did not show any correlation with $EC50_{(Cu2+)}$ suggests that AHA-complexed copper is not bioavailable to *D. magna* which corroborates with the findings of both Meador (1991) and Ma et al. (1999). They found that adding Aldrich humic acid decreased copper toxicity to *Daphnia magna* and *Ceriodaphnia dubia* when expressed as total copper, but that it remained the same when expressed as (Cu^{2+}). These observations and those of the present study, however, are in contrast with those of Erickson et al. (1996) who reported that increased Aldrich humic acid concentrations resulted in decreased $EC50_{(Cu2+)}$

values for larval fathead minnows, suggesting an apparent toxicity of organic-complexed copper. These authors proposed that this may be due to possible shifts in chemical equilibrium at the fish gill surface, causing the liberation of a fraction of the organically complexed copper. This seems to suggest that there are species-specific differences between the effect of toxicity modifying factors like DOC on copper toxicity. Species-specific differences in the copper toxicity modifying effects of major cations were also shown by De Schamphelaere and Janssen (2002).

3.3.2. Calibration of WHAM-Model V to the ISE measurements

The geochemical speciation model WHAM-Model V (Tipping, 1994) for metal-organic interactions is an integral part of the BLM computations and computer program (Hydroqual, 1999). Because AHA-Cu-complexes do not seem to be bioavailable to *D. magna*, the ability of WHAM to predict the amount of copper bound to the AHA needed to be assessed. In other words, to obtain good toxicity predictions with the BLM, it is essential that WHAM can accurately calculate the actual (measured) Cu^{2+}-activity in the exposure medium.

Speciation calculations with the default metal-binding constants (as incorporated in WHAM) demonstrated differences between the calculated and the measured (Cu^{2+}) of 1 to 2 orders of magnitude for all 25 test media. Moreover, these speciation calculations indicated that the calculated (Cu^{2+}) was always lower than the measured (Cu^{2+}), suggesting that the WHAM-Model V description of metal-organic interactions (Tipping, 1994) over-estimated copper complexation to AHA. This is possibly due to the WHAM-simplification that all humic acids contain a fixed number of binding sites and have a fixed stability constant describing proton-metal exchange at these sites. These default constants only represent the average of the values obtained from metal-titration experiments of a small number of humic acids from different origin. Moreover, these model constants exhibit a fair amount of variability across different humic acids (Tipping and Hurley, 1992; Tipping, 1993, Di Toro et al., 2001). This means that the model constants for AHA may indeed be different from the 'average' constants (i.e. the default constants in WHAM). The two WHAM-model V parameters that directly influence copper binding are the number of binding sites and the copper-proton exchange constant. The number of binding sites for most humic acids shows only little variability (Table 1 in Tipping, 1993), while the variability of the metal-proton

exchange constants is relatively large (Tables 2 and 3 in Tipping, 1993). It was decided, therefore, to fit WHAM-Model V calculations of (Cu^{2+}) to the observed (Cu^{2+}) in each exposure medium by lowering the pK_{MHA} (the metal-proton exchange constant for copper binding onto AHA) that describes the reaction

$$HA - H^+ + M^{2+} \xrightleftharpoons{K_{MHA}} HA - M^{2+} + H^+$$

where HA- represents the humic acid binding site, H^+ and M^{2+} a proton and a metal ion respectively, and K_{MHA} the metal-proton exchange constant. The larger the K_{MHA} (or the lower pK_{MHA}), the more the reaction will shift to the right resulting in a higher tendency for the metal to substitute the proton, i.e. a lower Cu^{2+}-activity.

Using least squares analyses, the pK_{MHA} that resulted in the best fit between observed and WHAM-calculated values of log(Cu^{2+}) was determined for each medium (Table 3-1). An example of this type of fitting is given for medium 1 (Figure 3-1): observed and WHAM-calculated values of (Cu^{2+}) (with default constants and with the 'best fit' pK_{MHA}) are plotted against total copper concentration.

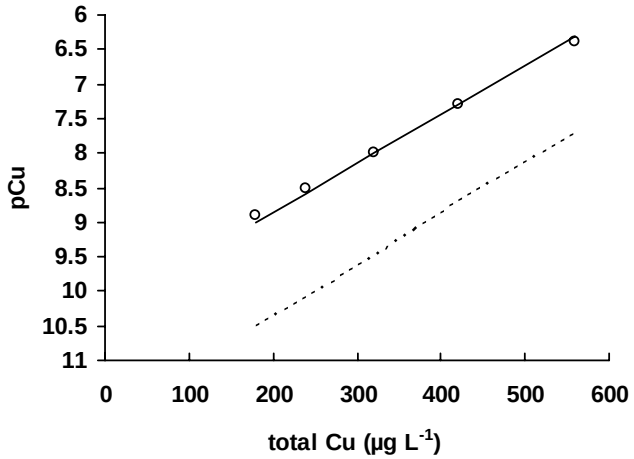

Figure 3-1 Example of determination of 'best fitting' pK_{MHA} for medium 1; pK_{MHA} was varied until the observed Cu^{2+}-activity (squares) matched best with the WHAM-calculated Cu^{2+}-activity (full and dashed lines). Calculated Cu^{2+}-activities are shown for using the default pK_{MHA} = 1.5 (dashed line) and for using the 'best fitting' pK_{MHA} = 1.85 (full line).

Performing these calculations for each test medium, the average value of pK_{MHA} resulting in the best fit was 1.90 (±0.08). The best fit values did not exhibit any correlation with one of test variables (TOC, pH, Ca, Mg and Na), meaning that other default humic acid properties (e.g. proton dissociation constant, earth alkaline metal binding) described the observations reasonably well. For further calculations (i.e. BLM-predictions) it was decided to use the adjusted pK_{MHA} value of 1.90.

This value is in close agreement with two of the three values that were used to compute the mean pK_{MHA} that is used in the default Model V thermodynamic database. Based on copper binding experiments of Stevenson (1976) and Van Dijk (1971, cited in Tipping, 1993) with different soil humic acids, Tipping derived pK_{MHA} values of 1.8 and 1.7, respectively. Fitting ModelV to the experimental data of Marinsky et al. (1982), however, resulted in pK_{MHA} = 1.1 which is far below our experimentally derived value for AHA. The fact that the value derived for AHA is higher than all three other values may be explained by variability of humic acids of different origin or by differences in experimental conditions during the titrations. Fitch *et al.* (1986), for example, demonstrated that stability constants for copper binding to soil humic acids, derived from titration experiments, increased with increasing TOC concentrations. TOC concentrations in our study (2-20 mg L^{-1}) were lower than in all other mentioned studies (up to 1000 mg L^{-1}) and this may be a possible explanation for the observed higher pK_{MHA} value.

Additionally, to investigate if the derived pK_{MHA} value is also suitable for other studies with AHA, results of our Cu^{2+}-measurements were compared to the extensive study of Ma et al. (1999) and Kim et al. (1999). They investigated the effect of AHA on total copper and copper ion toxicity to *Ceriodaphnia dubia*. The experimental 24-hour LC50 was 3.11 nM (Cu^{2+}) (based on measured Cu^{2+}-activities and according to the dose-response equation mentioned in Figure 5 in Ma et al., 1999), while we calculated (using WHAM with the adjusted pK_{MHA} = 1.9 and the physico-chemistry and the $LC50_{total}$ of each exposure medium as an input) a 24-hour LC50 of 2.6 ± 0.8 nM. This similarity between measured and calculated free copper toxicity means that the value for pK_{MHA}, derived in this study, also seems to be valid for other experiments in which the effect of AHA on copper toxicity is assessed.

3.3.3. Initial BLM predictions of observed 48-hour EC50s in reconstituted waters

After the WHAM-calibration, predictions of 48-hour EC50s for each of the tested media were carried out using the initially developed BLM (see Table 2-2 for constants) with the adjusted pK_{MHA} = 1.9 (Figure 3-2). While for media with a pH < 8 all predicted EC50s differed only by a factor of 1.5 from observed EC50s, predicted EC50s for media with a pH > 8 were 1.8 to 3.9 times higher than the observed EC50s (i.e. toxicity was under-estimated). Apparently the initial BLM, developed using toxicity data from tests performed at pH values from 6 to 8 (De Schamphelaere and Janssen, 2002), did not produce accurate toxicity predictions beyond the pH range used in its development. Since this seemed to suggest that next to Cu^{2+} and $CuOH^{+}$, inorganic copper species (e.g. $CuCO_3$ and $Cu(OH)_2$) that are abundant at pH > 8 could be bioavailable, an additional experiment was conducted to quantify their contribution to copper toxicity (next section).

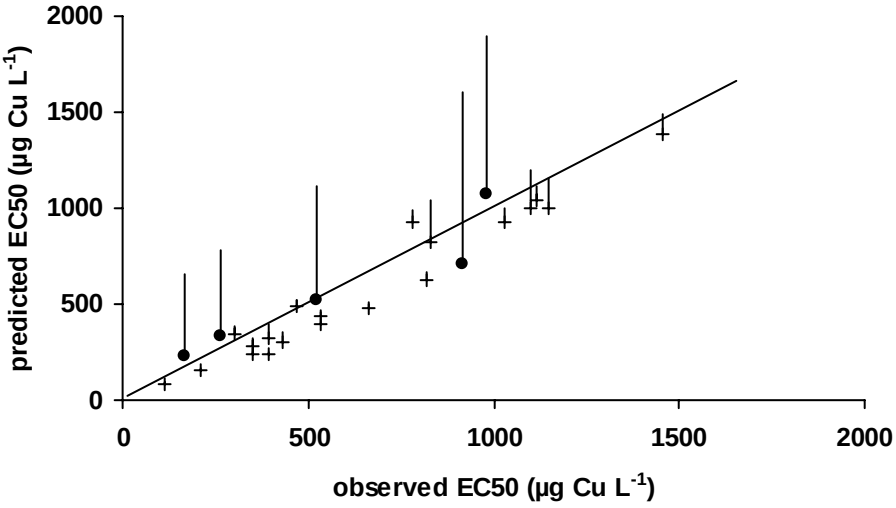

Figure 3-2 Predictive capacity of the initial BLM (De Schamphelaere and Janssen, 2002, chapter 2, Table 2-2) and the refined BLM (this chapter, Table 3-3) as shown by the relation between observed and predicted 48-hour EC50s of copper to *D. magna* in the 25 reconstituted waters (See Table 3-1) The top of the error bars represent the predictions with the initial BLM. Crosses (+) and full circles (●) denote EC50s predicted with the refined BLM. Crosses represent predictions for tests conducted at pH < 8, circles tests at pH > 8. The full line indicates a perfect match between observed and predicted EC50 values.

3.3.4. Refinement of the initial BLM - pH experiment

Since pH appeared to play a crucial role in the predictive capacity of the initial BLM, leading to overestimation of EC50 values at pH > 8, the role of pH and associated copper speciation on copper toxicity was examined more closely. Six bioassays were carried out in which the 48-hour $EC50_{(Cu2+)}$ was determined for different pH values ranging from ~ 5.7 to 8.6, i.e. the range in which the daphnid clone used in this study can survive and reproduce well. To ensure an accurate assessment of the effect of pH on copper speciation and toxicity, copper speciation needed to be kept constant throughout the test and (Cu^{2+}) needed to be measured and not calculated as in De Schamphelaere and Janssen (2002) (see chapter 2). The 6 tests were therefore performed in media that were buffered for Cu^{2+}-activities by the addition of 5 mg DOC L^{-1} (AHA). AHA was chosen because (1) it is a standardized, well characterised natural organic matter (both for speciation and toxicity effects), (2) it does not influence Cu-ISE performance (Fitch et al., 1986) and (3) copper complexes with AHA, have been demonstrated to be non-bioavailable for *D. magna* (Meador, 1991) and *Ceriodaphnia dubia* (Ma et al., 1999).

The results of the experiments are presented in Table 3-2. An increase of pH from 5.7 to 7 resulted in an increase of the 48-hour $EC50_{total}$ from 213 to 424 µg L^{-1}. A further increase in pH, however, did not result in a further significant increase of EC50.

Table 3-2 48-hour EC50 (± 95% confidence limits) at different pH levels

pH[1]	TOC (mg L^{-1})	IC (mg L^{-1})	48-hour EC50 (µg Cu L^{-1})	48-hour EC50 nM (Cu^{2+})
5.71 (5.65-5.77)	4.28	0.281	213 (201-226)	69.4 (65.3-75.2)
6.27 (6.18-6.36)	4.44	0.562	247 (235-259)	59.9 (52.9-67.8)
7.08 (7.03-7.12)	4.61	3.79	424 (408-442)	51.8 (45.8-58.4)
7.86 (7.78-7.94)	4.48	13.9	438 (418-460)	22.6 (17.5-28.7)
8.21 (8.16-8.26)	4.38	32.5	430 (411-451)	7.49 (6.81-8.26)
8.44 (8.39-8.49)	4.81	36.6	410 (398-423)	3.00 (2.57-3.68)

[1] mean pH (minimum pH-maximum pH) during bioassays

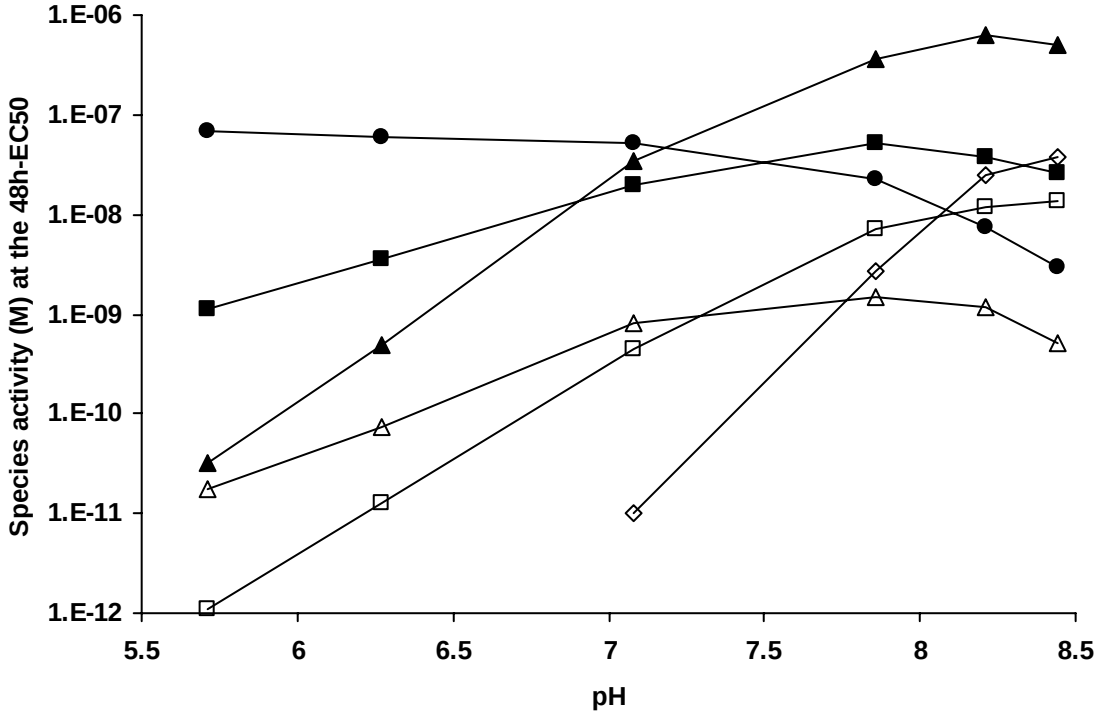

Figure 3-3 Activity of the different copper species at the 48-hour EC50 level for the different pHs used in the pH experiment (see Table 3-2). Presented copper species are Cu^{2+} (●), $CuOH^{+}$ (■), $CuCO_3$ (▲), $Cu(OH)_2$ (□), $CuHCO_3$ (Δ) and $Cu(CO3)_2$ (◊).

Expressed as (Cu^{2+}) the EC50 decreased continuously from 69 to 3 nM with pH increasing from 5.7 to 8.4 (Figure 3-3). Borgman and Ralph (1983) found a very similar decrease of $EC50_{(Cu2+)}$ for *Daphnia magna* between pH 6 and 8.4. Although many authors have suggested that this decrease is due to competition between protons and copper on the membrane (Meador, 1991; Borgmann and Ralph, 1983), it has been demonstrated in other reports (De Schamphelaere and Janssen, 2002; Brown and Markich, 2000) that such an effect requires linear relations between $EC50_{(Cu2+)}$ and H^{+}-activity. De Schamphelaere and Janssen (2002) have demonstrated that this was clearly not the case for copper toxicity to *D. magna* and that, in the pH range 6 to 8, the observed linear relation between (OH^{-}) and 1 / $EC50_{(Cu2+)}$ pointed to ($CuOH^{+}$)-bioavalability . Analysis of the additional pH experiment demonstrated that the relation between (OH^{-}) and 1 / $EC50_{(Cu2+)}$ showed an upward directed curvature above pH 8, which explains the under-estimation of toxicity in this pH region (Figure 3-4).

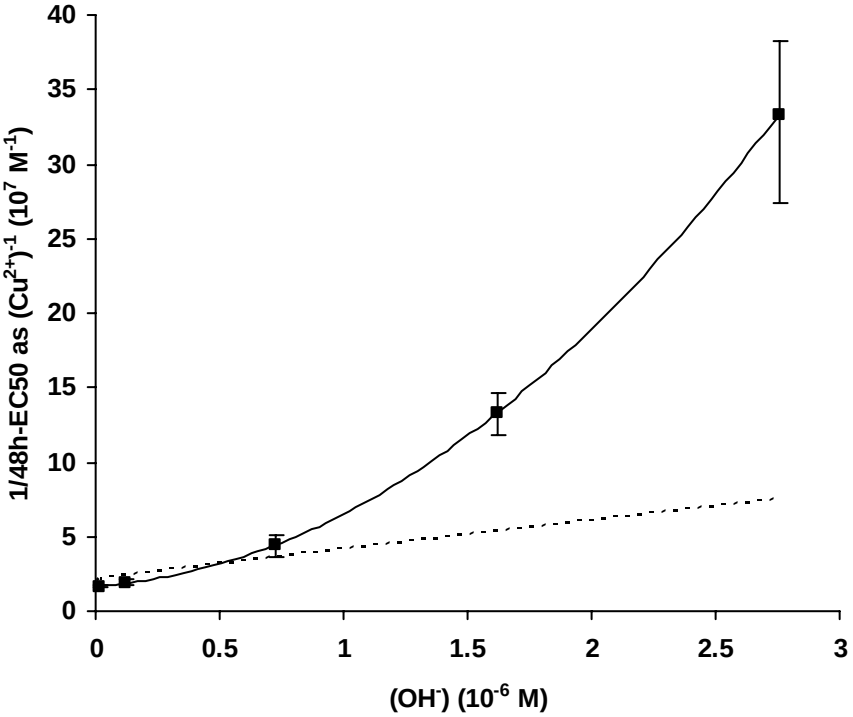

Figure 3-4 Relation between (OH^-) and the inverse of the experimentally determined 48-hour EC50 of copper expressed as (Cu^{2+}) to *D. magna* (squares). The full line represents the second order polynomial regression ($1/EC50_{(Cu2+)}$ = $3.80\ 10^{19}\ (OH^-)^2 + 1.03\ 10^{13}\ (OH^-) + 1.70\ 10^7$; $r^2 = 0.9987$) and was used for the determination of the $K_{CuCO3BL}$ constant (see text). The dotted line represents the linear relation as determined in the initial BLM (De Schamphelaere and Janssen, 2002; chapter 2). Error bars indicate 95% confidence limits.

Since the under-estimation of toxicity at high pH could be due to the toxicity of other inorganic copper complexes, activities of all inorganic Cu-species were calculated, using stability constants from Martell et al. (1997) (see Table 2-2) and based on the measured Cu^{2+}-activities in this additional pH experiment. The activity of the different copper species at the 48-hour EC50 level as a function of pH is plotted in Figure 3-3. The three most important copper species are Cu^{2+}, $CuOH^+$ and $CuCO_3$ with the latter becoming the most abundant species at high pH values. Other inorganic species (e.g. Cu(OH)2, $Cu(CO_3)_2$ and $CuHCO_3$) are less important at all pH levels, which suggests that they do not play a role in copper toxicity to *D. magna* (see below). Overall, these results indicate that at a high pH, less Cu^{2+} is needed to elicit the same toxic effect (50% immobility). Summing all activities, it is clear that more inorganic copper is needed at a high pH to produce the same toxic effect. This suggests

that species that dominate at high pH (e.g. $CuCO_3$) are probably less toxic than the free copper ion (see below).

3.3.5. Refinement of the initial BLM - Modelling

As indicated above, at higher pH levels (pH > 8) one or more inorganic copper species other than Cu^{2+} and $CuOH^+$ are required to compensate for the decrease of Cu^{2+} that is needed to elicit 50% effect. Since Cu^{2+} is generally considered to be the most toxic species (Campbell, 1995; Morel, 1983), it is reasonable to assume that the activities of other potentially bioavailable inorganic copper species in the high pH range should at least be higher than Cu^{2+} in the low pH range. For this reason, only $CuOH^+$, $Cu(OH)_2$, $CuCO_3$ and $Cu(CO_3)_2$ were considered in the further assessment of copper species contributing to the observed copper toxicity. Based on literature reports (Andrew et al., 1977; Cowan et al., 1986; French and Hunt, 1987; Allen and Hanssen, 1996), which point to the non-availability of copper-carbonate complexes, it was first assumed that next to Cu^{2+} and $CuOH^+$, only $Cu(OH)_2$ contributed to the toxicity. Therefore, within the BLM framework, next to the formation of [CuBL] and [CuOHBL] also the formation of a [$Cu(OH)_2$BL] complex was allowed. Equation (3) was extended to:

$$EC50_{Cu^{2+}} = \frac{f_{CuBL}^{50\%} \cdot \left\{1 + K_{CaBL} \cdot \left(Ca^{2+}\right) + K_{MgBL} \cdot \left(Mg^{2+}\right) + K_{NaBL} \cdot \left(Na^{+}\right) + K_{HBL} \cdot \left(H^{+}\right)\right\}}{(1 - f_{CuBL}^{50\%}) \cdot \left\{K_{CuBL} + K_{CuOHBL} \cdot K_{CuOH} \cdot \left(OH^{-}\right) + K_{Cu(OH)2BL} \cdot K_{Cu(OH)2} \cdot \left(OH^{-}\right)^{2}\right\}} \quad (4)$$

with $K_{Cu(OH)2BL}$ the stability constant for the reaction [$Cu(OH)_2$] + [BL] < = > [$Cu(OH)_2$BL] and $K_{Cu(OH)2}$ the stability constant for the second order copper hydroxide complex. This equation suggests that, if next to Cu^{2+} and $CuOH^+$ also $Cu(OH)_2$ adds to copper toxicity, a second order polynomial regression ($y = ax^2 + bx + c$) should fit the relation between (OH^-) and $1/EC50_{(Cu2+)}$ (provided that ion activities of Ca, Mg, Na and H are constant, which was the case in this experiment). Figure 3-4 shows that such a relation is indeed observed and according to equation (4) and using the estimated regression coefficients ($a = 3.80\ 10^{19}$, $b = 1.03\ 10^{13}$, $c = 1.70\ 10^{7}$; $r^2 = 0.9987$; see Figure 3-4), the relative ratios between K_{CuBL}, K_{CuOHBL} and $K_{CU(OH)2BL}$ can be calculated:

$$\frac{K_{CuOHBL}}{K_{CuBL}} = \frac{b}{K_{CuOH} \cdot c} = 0.20 \pm 0.04 \quad (5)$$

and

$$\frac{K_{Cu(OH)_2 BL}}{K_{CuBL}} = \frac{a}{K_{Cu(OH)_2} \cdot c} = 3.72 \pm 0.23 \quad (6)$$

This means that the affinity of $CuOH^+$ for the BL is about 5 times lower than the affinity of Cu^{2+}, which is a value very similar to the previously reported value (De Schamphelaere and Janssen, 2002; chapter 2). The estimated affinity of $Cu(OH)_2$, however, was about 4 times higher than the affinity of Cu^{2+}, which means that $Cu(OH)_2$ would be more toxic than Cu^{2+}. This is in contradiction with reports on Cu-hydroxide toxicity and uptake (Cowan et al., 1984; Blust et al., 1991) and in clear disagreement with classic theory of metal coordination chemistry. According to this theory the more a metal becomes co-ordinated by ligands (in this case hydroxide), the less it is able to form stable complexes with additional ligands (in this case the biotic ligand). Therefore, another plausible explanation needed to be found for the observed relation in Figure 3-4.

A possible solution for this problem is that in open systems (i.e. free CO_2 exchange) total carbonate concentration is directly related to the pH in the system through the reactions (Stumm and Morgan, 1996):

$$CO_2(g) + H_2O \xleftrightarrow{K_H} H_2CO_3 \xleftrightarrow{K_1} H^+ + HCO_3^- \xleftrightarrow{K_2} 2H^+ + CO_3^{2-} \quad (7)$$

with K_H = gas-water partitioning constants (Henry constant) for $CO_2 = 10^{-1.5}$ M atm^{-1} and K_1 and K_2 acidity constants for the protolysis of H_2CO_3 and HCO_3^-, respectively ($K_1 = 10^{-6.35}$ M and $K_2 = 10^{-10.33}$ M). This means that CO_3^{2-}-activity can be written as follows:

$$\left(CO_3^{2-}\right) = \frac{K_1 \cdot K_2 \cdot K_H \cdot p_{CO_2}}{\left(H^+\right)^2} = \frac{K_1 \cdot K_2 \cdot K_H \cdot p_{CO_2}}{K_w^{\ 2}} \cdot \left(OH^-\right)^2 \quad (8)$$

with P_{CO2} = partial pressure of CO_2 (atm) and K_w = the ion product of water (M^2). Equation (8) indicates that CO_3^{2}-activity is directly proportional to $(OH^-)^2$. In this study, based on the experimental results of the 6 pH experiments (Table 3-2), it was derived that:

$$\left(CO_3^{2-}\right) = 3.88 \cdot 10^6 \cdot \left(OH^-\right)^2 \quad (r^2=0.97) \quad (9)$$

which after calculation with equation (8) corresponds to a log P_{CO2} = -3.57, which is close to the earth's atmospheric log P_{CO2} = -3.5 (Stumm and Morgan, 1996).

The derived relation between (CO_3^{2-}) and (OH^-) leads to the possibility of substituting $(OH^-)^2$ in equation (4) by (CO_3^{2-}) / (3.88 10^6), which would involve an inverse relation between (CO_3^{2-}) and $EC50_{(Cu2+)}$. According to Brown and Markich (2000), this would correspond to $CuCO_3$ being bioavailable and contributing to toxicity. The corresponding BLM equation in that case would be:

$$EC50_{Cu^{2+}} = \frac{f_{CuBL}^{50\%} \cdot \left\{1 + K_{CaBL} \cdot \left(Ca^{2+}\right) + K_{MgBL} \cdot \left(Mg^{2+}\right) + K_{NaBL} \cdot \left(Na^+\right) + K_{HBL} \cdot \left(H^+\right)\right\}}{(1 - f_{CuBL}^{50\%}) \cdot \left\{K_{CuBL} + K_{CuOHBL} \cdot K_{CuOH} \cdot \left(OH^-\right) + K_{CuCO3BL} \cdot K_{CuCO3} \cdot \left(CO_3^{2-}\right)\right\}} \quad (10)$$

Taking into account both the relation between (CO_3^{2-}) and $(OH^-)^2$ and the values of the regression coefficients a and c (cf. above) for the relation $1/EC50_{(Cu2+)}$ versus (OH^-), it can de deduced that:

$$\frac{K_{CuCO_3BL}}{K_{CuBL}} = \frac{a}{K_{CuCO_3} \cdot 3.88 \cdot 10^6 \cdot c} = 0.098 \pm 0.006 \quad (11)$$

This means that, under the BLM assumptions, $CuCO_3$ is less bioavailable than Cu^{2+}. At first sight, the finding that $CuCO_3$ is slightly bioavailable to *D. magna*, seems to contradict other reports (French and Hunt, 1987, Andrew et al., 1977, Cowan, 1984). However, in all of these studies, as outlined by Erickson et al. (1996), the effects of copper complexation to

carbonate were confounded by other factors such as co-linearity between alkalinity and major ion concentrations (e.g. by $NaHCO_3$ addition to increase alkalinity, Andrew et al., 1977). Based on their own results, Erickson et al. (1996) stated that "the apparent non-toxicity of carbonate complexes is not definitive" and this suggestion is supported by an early study of Shaw and Brown (1974). The strength of the pH experiment in this study was that it was designed to minimize confounding factors (e.g. by equal Na-concentrations in each exposure medium), thus ensuring that the observed effects were only pH effects and that analysis of the observations could unambiguously be interpreted as $CuCO_3$ bioavailability.

Summarizing, since it seems more logical that $CuCO_3$ is less bioavailable than Cu^{2+} than that $Cu(OH)_2$ is more bioavailable than Cu^{2+}, the addition of a $K_{CuCO3BL}$ to the BLM was favoured over the addition of a $K_{Cu(OH)2BL}$. A summary of all retained constants for use in the refined BLM is given in Table 3-3. Using all stability constants and observed $EC50_{(Cu2+)}$, for each exposure medium used in the pH experiments, $f_{CuBL}^{50\%}$ was calculated with (10) and was found to be 0.465 ± 0.019. This value is slightly higher than that found during the initial BLM-development (i.e. 0.39; De Schamphelaere and Janssen, 2002; chapter 2, Table 2-2). As the $f_{CuBL}^{50\%}$ estimation in the present study is based on measured Cu^{2+}-activities, the value of 0.465 will be retained for all further calculations.

In summary, the refinement of the BLM was performed by the incorporation of $CuCO_3$-toxicity to *D. magna*. It should be noted that incorporating the toxicity of $CuOH^+$ and $CuCO_3$ by allowing direct binding of these complexes to the BL-sites (i.e. transport or toxic action sites), may be an over-simplification of the real mechanisms taking place at the organism-water interface. Frequently reported examples of other possible mechanisms are the lability of metal complexes (Tessier et al., 1994) and the differences between physico-chemistry of the bulk solution and the organism's micro-environment (Playle et al., 1992; Gensemer and Playle, 1999). However, as long as these suggestions are only indicative of possible processes, bioavailability models will always tend to over-simplify. Therefore, the derived stability constants should not be regarded as classical stability constants, but rather as parameters that summarize the processes reflected in the observed relation between pH and copper toxicity.

Table 3-3 Model constants used in different Biotic Ligand Models for predicting copper toxicity. Values between brackets indicate 95% confidence limits.

	D. magna		*P. promelas*
	Initial BLM[1]	Refined BLM[2]	
Log K_{CaBL}	3.47	3.47	3.6[4]
Log K_{MgBL}	3.58	3.58	(3.6)[5]
Log K_{NaBL}	3.19	3.19	3.0[4]
Log K_{HBL}	5.40	5.40	5.4[3]
Log K_{CuBL}	8.02	8.02	7.4[3]
Log K_{CuOHBL}	7.45	7.32 (7.22-7.39)	-
Log $K_{CuCO3BL}$	-	7.01 (6.98-7.04)	-
$f_{CuBL}^{50\%}$	0.39	0.47 (0.45-0.49)	0.21[4,5]

[1] Constants from the 'initial' Copper-BLM for *D. magna* (De Schamphelaere and Janssen, 2002; chapter 2, Table 2-2)

[2] Constants from the present chapter, see Results and Discussion section

[3] Gill binding constants for *Pimephales promelas* (Playle et al., 1993)

[4] Constants from Santore et al. (2002)

[5] Santore et al. (2002) did not include a Mg constant, as a first approximation K_{MgBL} could be assumed equal to K_{CaBL}

[6] The original constant of 0.33 (Di Toro et al., 2001) was adjusted to 0.21 by Santore et al. (2001)

3.3.6. Validation of refined BLM – Reconstituted media

Predicted 48-hour EC50s were re-calculated using the refined BLM. All predicted EC50s were within a factor of 1.5 from the observed value, including all EC50s in tests performed at pH >8 (Figure 3-2). Higher experimental pHs resulted in larger differences between the initial BLM and the refined BLM prediction, as indicated by the length of the error bars in Figure 3-2. This indicates the advantage of using the refined BLM for EC50 predictions as compared to using the initial BLM. In general, the BLM predictions were better than predictions based on the empirical TOC-EC50 relation (equation 1), as reflected by a lower sum of squared residuals.

3.3.7. Validation of refined BLM – Natural waters

Using 19 copper-spiked European natural surface waters, 48-hour EC50s for *D. magna* varied between 35.2 and 792 µg Cu L^{-1} (expressed as dissolved copper) (Table 3-4). When performing predictions with the refined BLM, an important difference between reconstituted media and natural waters regarding the DOC composition needs to be adressed. While the artificial DOC (AHA) in the reconstituted media used in this study consisted of 100% humic acid, natural waters have DOCs of variable composition (i.e. a variable humic acid / fulvic acid ratio). Thus, before performing EC50 predictions, the following question needs to be addressed: which DOC-composition should be used in the BLM computations? Dwane and Tipping (1998) found that in copper titration experiments of several natural waters, measured and WHAM-calculated free copper ion activities corresponded best when DOC was assumed to consist of 40 to 80% of 'active fulvic acid' with the remaining DOC-fraction being inert for metal complexation. Supported by studies of Cabaniss and Shuman (1988) and McKnight et al. (1983) they also suggested that if nothing is *a priori* known about the DOC characteristics the best overall estimate of copper complexation and speciation is obtained when assuming that 50% of the DOC is 'active fulvic acid'. Using this assumption in our calculations it seemed that all observed 48-hour EC50s were accurately predicted within a factor of 2 for all surface waters tested (Figure 3-5).

It is not clear, however, if the remaining prediction error is due to the variability of metal complexing properties of the DOCs of the different waters (i.e. variable percentage of active fulvic acid) or due to other factors (e.g. statistical errors on the biotic ligand stability constants, statistical errors on the calculated EC50s). In chapter 7, we will investigate the importance of metal complexation behaviour of natural DOC for copper toxicity in natural waters. This type of research will allow to determine if it is necessary to incorporate DOC-variability into existing BLMs and, if so, how this can result in a further improvement of their predictive capacity.

Table 3-4 Physico-chemical characteristics of the natural surface waters and corresponding 48-hour EC50s of copper for *Daphnia magna*

Date (dd/mm/yy)	Site ID – Country Code	pH[1]	DOC (mg L^{-1})	IC (mM)	Ca (mM)	Mg (mM)	Na (mM)	K (mM)	SO_4 (mM)	Cl (mM)	EC50 as $Cu_{dissolved}$[2] (µg L^{-1})
17/10/00	Ankeveen A – NL	7.35	22.8	0.380	1.19	0.306	0.646	0.092	0.978	0.697	792 (701-894)
24/10/00	Ankeveen A - NL	7.30	17.8	0.337	1.30	0.353	0.513	0.021	1.14	0.570	686 (667-706)
22/05/01	Ankeveen A - NL	7.50	20.4	0.586	1.08	0.253	1.160	0.024	0.898	0.191	648 (612-689)
17/10/00	Ankeveen B - NL	7.85	11.8	1.22	0.871	0.255	2.880	0.138	0.357	1.60	332 (316-349)
30/10/00	Arun – UK	7.02	10.1	0.234	0.424	0.103	0.492	0.107	0.193	0.606	295 (259-334)
03/03/01	Bihain – B	6.35	4.87	0.071	0.142	0.039	0.192	0.026	0.042	0.240	40.9 (37.5-44.6)
13/03/01	Bleeke Meer – NL	7.60	18.4	0.795	1.000	0.250	0.830	0.540	0.427	1.04	529 (490-571)
31/10/00	Clywydog – UK	6.31	2.72	0.061	0.055	0.046	0.178	0.013	0.050	0.197	37.9 (33.2-43.3)
31/05/01	Clywydog – UK	6.10	2.34	0.041	0.077	0.047	0.190	0.018	0.048	0.221	33.8 (31.4-36.6)
30/10/00	Great Stour –UK	7.72	7.88	0.956	0.973	0.107	0.565	0.113	0.242	0.711	366 (324-418)
17/10/00	Laarder Wash - NL	7.93	6.60	1.22	1.20	0.156	2.710	0.050	0.350	1.32	276 (259-295)
17/10/00	Maarsseveen - NL	7.87	14.3	1.30	1.020	0.145	0.423	0.050	0.061	0.496	399 (367-433)
24/10/00	Markermeer - NL	8.26	6.42	2.49	1.52	0.671	3.59	0.266	1.11	3.75	188 (165-211)
22/05/01	Markermeer - NL	8.30	8.24	2.69	1.66	0.702	3.34	0.242	1.12	3.58	257 (231-286)
30/10/00	Medway -UK	7.59	7.81	0.695	0.574	0.122	0.655	0.113	0.257	0.598	281 (258-306)
30/10/00	Mole – UK	7.70	10.0	0.797	0.798	0.121	0.670	0.148	0.249	0.592	484 (446-526)
29/05/01	Mole – UK	7.55	6.13	0.644	1.060	0.256	1.160	0.090	0.500	0.930	175 (165-185)
09/05/01	Rhine - G	8.06	1.98	1.70	1.51	0.390	1.09	0.083	0.40	1.17	119 (106-133)
30/10/00	Skarsjön – S	5.52	10.3	0.013	0.060	0.020	0.343	0.159	0.029	0.068	35.2 (30.6-40.6)

[1] pH values refer to the pH at the start of the test

[2] values between brackets indicate 95% confidence limits

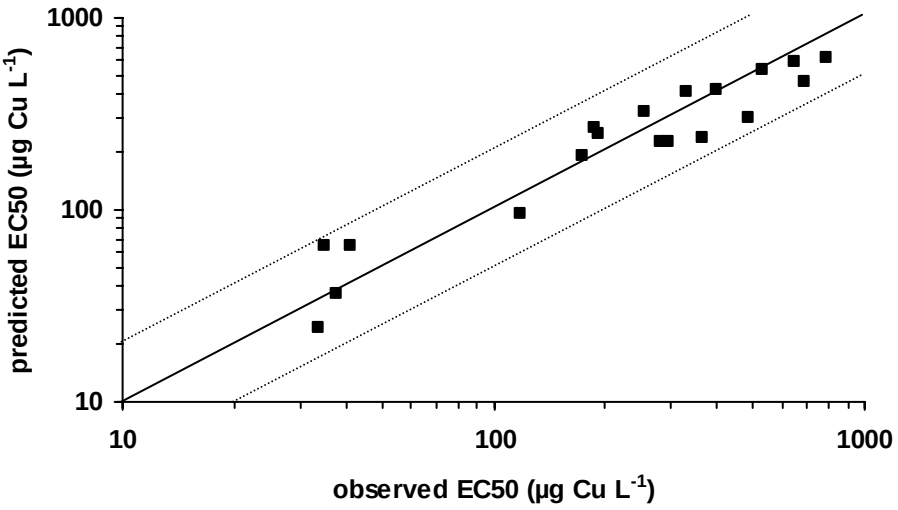

Figure 3-5 Predictive capacity of the refined BLM as shown by the relation between observed and predicted 48-hour EC50 of copper to *D. magna* tested in 19 European surface waters. The full line indicates a perfect match between observed and predicted EC50 values. The dotted line indicates a factor of 2 error between observed and predicted EC50s.

Until that time, the current refined BLM as developed and validated in this study can be used to relatively accurately predict 48-hour EC50s to *D. magna* in natural surface waters. However, to set relevant water quality criteria that are protective of aquatic ecosystems, one rather needs a model that can predict chronic copper toxicity. Therefore, in chapter 4 it will be investigated how the modelling concepts of the acute *Daphnia* BLM can be translated to chronic exposures.

3.4. Conclusions

Validation experiments showed that the BLM developed in chapter 2 for predicting acute copper toxicity to *D. magna* failed in correctly predicting copper toxicity in test media with pH > 8. The model was refined using new experimental data. The refined BLM takes into account the bioavailability of $CuOH^+$ and $CuCO_3$, next to Cu^{2+}. Using this BLM, predictions of 48-hour EC50s of copper to *D. magna* in 25 reconstituted waters and 19

European natural surface waters were very accurate (factor 2). Since the results of the validation dataset were not used to develop the model (i.e. it was not an auto-validation), the refined BLM will be applicable to exposures of *D. magna* to copper in other surface waters also. It may thus be concluded that the refined BLM could support efforts to improve the ecological relevance of presently applied risk assessment procedures.

Chapter 4

Development and field validation of a chronic Cu-BLM for *Daphnia magna*

Redrafted from:

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Chapter
4

Development and field validation of a chronic Cu-BLM for *Daphnia magna*

Abstract – The effects of pH (5.3 to 8.7), water hardness (25 to 500 mg $CaCO_3$ L^{-1}) and dissolved organic carbon (DOC) concentration (1.6 to18.4 mg L^{-1}) and source on the chronic toxicity of copper to *Daphnia magna* were investigated using a multi-factorial, central composite test design. Natural dissolved organic matter (DOM) was collected at three sites in Belgium and the Netherlands using reverse osmosis. For a total number of 35 toxicity tests performed, 21-day NOECs (no observed effect concentrations) based on reproduction ranged from 29.4 to 228 µg Cu L^{-1} and 21-day EC50s (concentration causing 50% reduction of reproduction) ranged from 41.5 to 316 µg Cu L^{-1}. Statistical analysis revealed that DOC concentration and pH had a significant effect on copper toxicity but hardness (at the levels tested) did not. In general, an increase in pH or DOC resulted in a linear increase of 21-day NOEC and EC50 values. All DOMs (originating from three different sources) reduced copper toxicity to the same extent. Statistical analysis revealed that DOC concentration is the most important factor for chronic toxicity of copper to *D. magna*, explaining about 60% of the observed variability, whereas pH only explained about 15% of the observed variability. Next to empirical regression models, the obtained toxicity data were also used to investigate the possibility of BLM modelling of chronic toxicity. BLM constants were derived and compared with those obtained for the acute BLM (chapter 2 and 3): 1) The effect of Ca and Mg was not important for chronic toxicity (both on the basis of dissolved and free copper), 2) the competitive effect of Na was similar and 3) proton competition and the bioavailability of $CuOH^+$ and $CuCO_3$ was more important in chronic exposures. Some evidence was also found for toxicity of complexes of copper with two of three tested types of dissolved organic matter. As the latter was only a minor effect, this factor was not included in the chronic Cu-BLM. Both the empirical regression model and the BLM exhibited a similar predictive capacity with regard to the 21-day EC50s and NOECs in natural water samples: most of the toxicity threshold values were predicted within a factor 2 of the observed values. It is clear, however, that more research is needed to provide information on the exact mechanisms that have resulted in different BLM constants for chronic exposures (as opposed to acute exposures). Both models can contribute to the improvement of risk assessment procedures of copper.

4.1. Introduction

Like all materials, metals may present risks to man and the environment and are being managed through the establishment of environmental quality criteria and standards. Recently it has been recognized by both regulators, industry and academic scientists that standard procedures for deriving environmental quality criteria for metals are inadequate to accurately assess the potential impact of metals on the ecological quality of ecosystems (Bergman and Dorward-King, 1997; Janssen et al., 2000). This is because current water quality standards and risk assessment procedures of metals are predominantly based on total or dissolved metal concentrations. However, there is extensive evidence that neither total nor dissolved aqueous metal concentrations are good predictors of metal bioavailability and toxicity.

In literature many reports can be found that describe the effects of water characteristics like pH, hardness and organic carbon content on the acute (short-term) toxicity of copper (Meador, 1991; Erickson et al., 1996; De Schamphelaere and Janssen, 2002; De Schamphelaere et al., 2002a). Studies examining these effects on the chronic toxicity of copper, especially using invertebrates, are rare.

Belanger and Cherry (1990) concluded that pH had negligible effects on reproductive toxicity of copper to *Ceriodaphnia dubia*. These authors did, however, observe a decrease of acute copper toxicity (48-hour mortality) with increasing pH, thus pointing to a difference in how acute and chronic toxicity can be affected by the physico-chemistry of the test medium.

Winner (1985) studied the interactive effects of hardness and humic acid on the chronic (42-day survival) toxicity of copper to *Daphnia pulex*. While hardness only had small effects, increased humic acid concentrations resulted in a significant decrease of copper toxicity, due to the formation of organic copper complexes.

Bossuyt et al. (unpublished data, see results and discussion) found that chronic copper toxicity threshold values (21-day reproduction) to *Daphnia magna* in natural waters may vary up to a factor of about 15. They also found a linear relationship (explaining 65% of the observed variability) between the 21-day EC50 and the dissolved organic carbon (DOC) concentration. Possible effects of other factors such as pH and water hardness, however, could not explain the remaining variability.

The two latter studies clearly demonstrate that a hardness-based correction of water quality criteria (e.g. US EPA, 1994) may not be the most appropriate approach for copper. In general, however, too little is known on how water chemistry parameters affect chronic copper toxicity to cladocerans to be of use in a regulatory context.

Therefore the first aim of this study was to investigate the individual and interactive effects of DOC concentration, pH and hardness on chronic copper toxicity to *D. magna* (21-day reproduction). The effect of natural dissolved organic matter (DOM) source was investigated by using DOM originating from three different sources. For this study, a total of 35 chronic reproduction experiments were carried out using a multi-variate test design.

Recently, biotic ligand models (BLM) are gaining increased interest by both scientific and regulatory instances. These models try to integrate all effects of physico-chemistry on metal toxicity and exhibited a high capacity to correctly predict metal toxicity. However, despite the initial efforts towards a Zn-BLM for an algal species (Heijerick et al., 2002), only BLMs that predict short-term (acute) metal toxicity have been developed (Di Toro et al., 2001, Santore et al., 2001; De Schamphelaere and Janssen, 2002; De Schamphelaere et al., 2002; Heijerick et al., 2002). However, in the context of risk assessment and EQC setting procedures in the European Union, only chronic toxicity data are taken into account. Thus, in order to incorporate bioavailability in these procedures, a chronic BLM would be needed.

Hence, the second aim of this paper was to investigate the possibilities of extrapolating the acute BLM concepts to chronic exposures and to develop and validate an initial BLM that that predicts chronic copper toxicity to *Daphnia magna*.

4.2. Materials and Methods

4.2.1. DOM sampling, treatment and characterization of natural DOM

Three sampling sites were selected based on geographical, physico-chemical and environmental variability encountered in European surface waters. The characteristics of the three sites are summarized in Table 4-1; photographs of these sites are given in Figure 4-1.

Bihain Ossenkolk Ankeveen

Figure 4-1 Photographs of the three sampling sites in this study

Both Bihain and Ossenkolk waters have low pH (5.7 and 5.6, respectively) and low hardness (18 and 2.5 mg $CaCO_3$ L^{-1}), but differ in their geomorphological characteristics. While Bihain is a small creek coming from and running through the highland peat area 'Hoge Venen' (Bihain, Belgium), Ossenkolk is a small lake in a mixed, temperate forest in the nature reserve 'Hoge Veluwe' (Nunspeet, The Netherlands). At the time of the sampling, the third site, Ankeveen had high pH (7.9 to 8.2) and medium hardness (130 mg $CaCO_3$ L^{-1}), although pH at this site may vary from 6.5 to 8.4 depending on the season and the time of the day (unpublished data). This body of water is a narrow side arm of a larger lake system called the 'Ankeveensche Plassen' (Nederhorst den Berg, The Netherlands) and is part of a nature reserve located in a lowland peat area.

Table 4-1 Characteristics of the sampling locations where dissolved organic matter was collected

Site ID	Bihain	Osssenkolk	Ankeveen
Name	Ruisseau de St. Martin	Ossenkolk meer	Ankeveensche plas
Location	Bihain (Belgium)	Nunspeet (Netherlands)	Nederhorst den Berg (Netherlands)
Water Type	Creek	Small lake	Lake system
Ecosystem type	highland peat	mixed forest	lowland peat
Sampling Date	7-8 March 2001	16 March 2001	22 May 2001
Temperature (°C)	3.0	6.0	22.0
DOC (mg L^{-1})	8.3	18.4	20.4
pH	5.7	5.6	7.9 - 8.2
Hardness (mg $CaCO_3$ L^{-1})	18	2.5	134
Conductivity (µS cm^{-1})	90	59	269
Ca (mg L^{-1})	5.68	0.38	43.4
Mg (mg L^{-1})	0.59	0.59	6.15
Na (mg L^{-1})	2.9	5.5	26.6
K (mg L^{-1})	<1	1.00	0.93
SO4 (mg L^{-1})	3.43	3.09	86.3
Cl (mg L^{-1})	6.48	32.8	6.77
Cu (µg L^{-1})	0.62	0.54	3.26

DOM was concentrated in the field using a portable Reverse Osmosis (RO) system (PROS/2) as described by Serkiz and Perdue (1990) and Sun et al. (1995) (Figure 4-2).

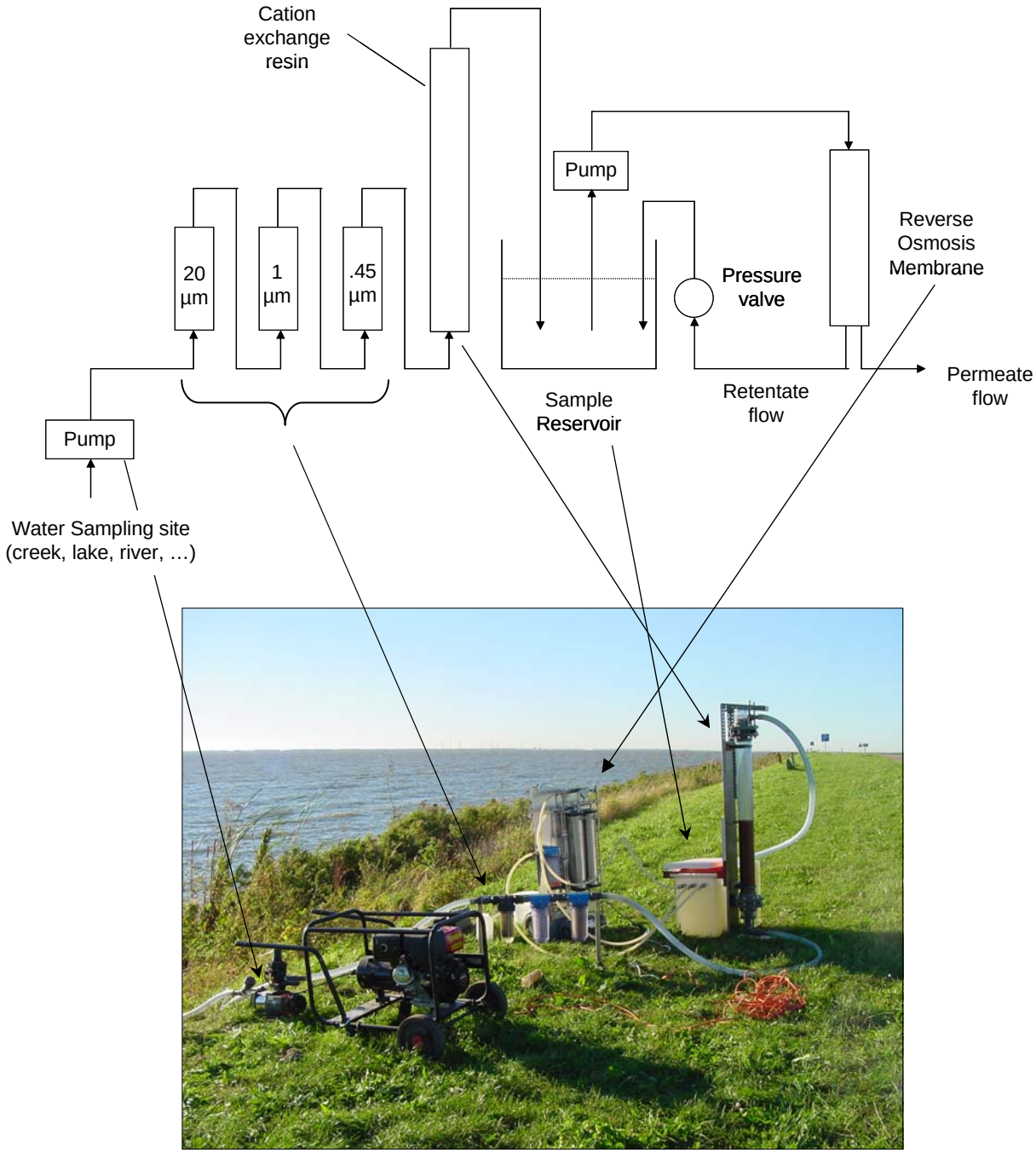

Figure 4-1 Schematic representation of the set-up for sampling with the reverse osmosis device (top, see text for more detail), illustrated with photograph in-the-field (bottom).

Water from the sampling site was pumped through three cartridge filters (pore diameters of 20 µm, 1 µm and 0.45 µm respectively) and subsequently through a sodium-type of cation exchanger to remove cations (e.g. Ca^{2+} and Mg^{2+}) that might cause precipitation of DOM on the RO membranes. The filtered water sample was collected in a 60L reservoir and was pumped through an RO membrane with the aid of a high-pressure pump (Filmtec FT30, Dow Chemical, Dalton, GE, USA; consisting of a 0.2 µm thick, highly crosslinked aromatic polyamide skin on a 35 µm polysulphone support). The permeate solution was discarded and the concentrate solution was recycled into the sample reservoir and mixed with additional water from the sampling site. This continuous process caused a gradual increase of the DOM concentration in the reservoir.

After transfer to the laboratory, concentrated DOM samples were immediately treated to remove as many metals as possible from the stock solution. The treatment is based on the procedure reported in Ma et al. (2001) and consisted of the fractionation of the DOM into a humic acid fraction and a fulvic + hydrophilic acid fraction. DOM samples were acidified with HCl to pH ~ 1, followed by the percolation of the latter fraction through a proton exchange column (Dowex 50WX8, Fluka Chemical Co., Milwaukee, WI, USA). Recombination of both fractions yielded the 'metal free' DOM stock solution (Ma et al., 2001). Finally, stock solutions were adjusted to pH = 7 with NaOH to avoid hydrolysis and stored in complete darkness at 4°C. Subsamples of these DOM stock solutions were shipped to the University of Delaware for chemical characterisation: DOC-concentration (Tekmar-Dohrmann DC-190 TOC, Rosemount Analytical, Dohrmann Division, Cincinnati, OH, USA), major cation and trace metal concentrations (ICP, Spectro Analytical Instruments, Kleve, Germany) and chloride and sulphate concentrations (DIONEX2000i/SP, Dionex, Sunnyvale, CA, USA).

4.2.2. Experimental design

Daphnid toxicity tests were conducted in 9 (using Bihain and Ossenkolk DOM) or 17 different media (using Ankeveen DOM) in which DOC concentration (1.6 to 18.4 mg/L), pH (5.3 to 8.7) and hardness (0 to 500 mg $CaCO_3$ L^{-1}; Ca:Mg = 4:1) were varied. The characteristics of all experimental test media were generated using the central composite design (CCD). This kind of test design was first used for optimizing the yield of a chemical process (Box et al., 1978) and has recently been successfully applied in the field of metal ecotoxicology (Lock et al., 2000; Heijerick et al., 2002). Most test designs are generated in such a way that one factor is varied while the others are kept constant. The disadvantage of this type of design is that possible

interactions between the different factors cannot be assessed. The advantage of the CCD is that it can generate a maximum of information on the direct effect of test variables and their interactions while testing a minimum number of combinations. A complete second-order CCD, with three independent variables (i.e. DOC, pH and hardness) requires 17 combinations to be tested. The CCD design is summarized in Table 2 in which the theoretical (nominal) levels of DOC, pH and hardness are presented. This CCD was developed using the statistical software package STATISTICA (Statsoft, Tulsa, OK, USA).

Table 4-2 Theoretical combinations of dissolved organic carbon (DOC) concentration, pH and hardness as calculated with the central composite test design. The exact composition of all test media is given in Table 4-4. See text for meaning of Cube, Centre and Star.

Medium No.	DOC ($mg\ L^{-1}$)	pH	hardness ($mg\ CaCO_3\ L^{-1}$)
1 (Cube)	5	6	100
2 (Cube)	15	6	100
3 (Cube)	5	8	400
4 (Star)	1.6	7	250
5 (Cube)	15	8	400
6 (Center)	10	7	250
7 (Star)	10	7	500
8 (Star)	10	8.7	250
9 (Star)	18.4	7	250
10 (Cube)	5	6	400
11 (Cube)	15	6	400
12 (Cube)	5	8	100
13 (Cube)	15	8	100
14 (Star)	10	5.3	250
15 (Star)	10	7	0[a]
16 (Center)	10	7	250
17 (Center)	10	7	250

[a] the test at a theoretical hardness of 0 $mg\ CaCO_3\ L^{-1}$ was conducted at 25 $mg\ CaCO_3\ L^{-1}$ (nominal)

The 17 test media can be divided into three groups: "cube points", "center points" and "star points". Each "point" represents a combination of DOC, pH and hardness (the independent variables or factors) that was used in a toxicity test with copper resulting in an EC50 or EC10 (the dependent variables). The rationale behind this nomenclature and the exact choice of combinations is beyond the scope of this paper but details can be found in the Statistica manual and in Box and Draper (1987). Once all toxicity test results are available, the central composite analysis module of STATISTICA allows estimating the significance of linear, quadratic and interaction components of the relationship between the dependent and the independent variables. In the text we will refer to linear, quadratic and interaction effects if any of these components are significant ($p<0.05$).

Additonal to the test media of the CCD, tests were also conducted to assess the individual effects of Na on chronic toxicity of copper to *Daphnia magna*, chronic toxicity test were conducted in reconstituted test media with 3 different Na concentrations. Four mg DOC L^{-1} was added to these test media to buffer copper speciation. Aldrich humic acid was used as the DOC source since the WHAM V speciation model (Tipping, 1994) has been calibrated to this commercial humic acid in a previous study (De Schamphelaere et al., 2002; chapter 3); pH was adjusted to 6.8 with NaOH.

4.2.3. Daphnia magna chronic toxicity testing

Exposure media were prepared by adding the appropriate amounts of stock solutions of $CaCl_2$, $MgSO_4$, DOC to a 0.078 mM KCl solution. MOPS (3-N morpholino propane sulfonic acid, 750 mg L^{-1}) was added as a pH buffer in all experiments except for medium 8 (pH ~ 8.5, $NaHCO_3$-buffering). MOPS was chosen because it is completely non-complexing for metals (Kandegedara and Rorabacher, 1999). It is also recommended by US-EPA (1991) since it does not change the toxicity of effluents toxic and sediment pore waters. Moreover, (unpublished) experiments at our laboratory demonstrated that the addition of 750 mg MOPS L^{-1} does not alter copper or zinc toxicity to *P. subcapitata* and *D. magna*.

The test media were then adjusted to the desired pH level with NaOH or HCl and spiked with copper. The difference between the lowest and highest copper concentration was 1 order of magnitude or less. The spiked media were stored at 4°C in complete darkness throughout the

experiment. 48 hours prior to use (test-renewal) appropriate volumes of these media were equilibrated at 20°C.

Chronic toxicity tests were performed according to the Organization for Economic Cooperation and Development (OECD), test guideline 211 (OECD, 1998). Test organisms originated from a healthy *D. magna* clone (K6) which has been cultured under standardized conditions in M4 medium (Elendt and Bias, 1990) for several years. At the start of each test, 10 juvenile animals (<24 hours old) per concentration were transfered individually to polyethylene cups containing 50 mL of the test medium (i.e. 10 replicates per concentration). Animals were fed every day with an algal mix of *P. subcapitata* and *Chlamydomonas reinhardtii* in a 3:1 ratio. Each organism received 8.10^6 cells per day in the first week, 12.10^6 cells per day in the second week and 16.10^6 cells per day in the third week of exposure, which coincides with food rations between 100 to 300 µg organic carbon per daphnid per day depending on the age of the daphnid. Every other day, the medium was renewed and parent mortality and the number of produced juveniles was noted.

4.2.4. Chemical analyses

Dissolved copper concentrations (0.45 µm filter, Gelman Sciences, Ann Arbor, Michigan, USA) were determined at the beginning and at the end of the tests using a flame-atomic absorption spectrophotometer (SpectrAA100, Varian, Mulgrave, Australia). Calibration standards (Sigma-Aldrich, Steinheim, Germany) and a reagent blank were analyzed with every ten samples. DOC (0.45 µm filtered) and inorganic carbon (IC) were measured before each test (TOC-5000, Shimadzu, Duisburg, Germany). Concentrations of major cations (Na, K, Ca, Mg) and anions (Cl, SO_4) were calculated as the sum of (1) ions added along with the DOC, (2) NaOH or HCl additions for bringing pH to the desired level and (3) $CaCl_2$ and $MgSO_4$ additions. For all of these ions concentrations were measured occasionally and these were always within 10% of the reported concentrations. pH-measurements were performed with pH-meter P407 (Consort, Turnhout, Belgium). The pH glass electrode was calibrated before each use using pH 4 and pH 7 buffers (Merck, Darmstadt, Germany).

4.2.5. Data treatment and analysis

According to the OECD test protocol (1998) the net reproductive rate (R_0, unit: total number of offspring per parent animal) was calculated for each copper concentration after 21 days of exposure. 21-day NOECs (no observed effect concentration) and 21-day LOECs (lowest observed effect concentration) were derived (based on R_0) by comparing reproduction in the copper treatments with that in the control using the Mann-Whitney U test ($\alpha = 0.05$). This non-parametric test was used because homogeneity of variances was often not observed, not even after log-transformation of the data (Bartlett test, $\alpha = 0.05$).

In addition to NOECs and LOECs, 21-day EC50s were calculated (based on R_0). However, due to the very steep slope of the concentration response curve (i.e. in many tests less than 10% reproduction reduction in one exposure concentration, followed by over 90% reproduction reduction in the next tested exposure concentration) common probit, logit or even hormesis (Van Ewijk and Hoekstra, 1993) models could not be significantly fitted to the toxicity data for 22 out 35 toxicity tests. As an alternative, the EC50 was estimated by a linear relation between the % reproduction reduction and the logarithm of the copper concentration. For each concentration response curve only the two data points that were most close to the 50% effect level were used (one lower than 50% effect and one higher than 50% effect). Although, the authors are aware of the fact that this method is rather uncommon, it is the only method that yields EC50 values for all tests. Moreover, in cases where a common probit, logit or hormesis model could indeed be fitted to the data, the derived EC50s were always within 10% of those derived with the above described method. For reasons of uniformity, and to avoid possible artefacts in the subsequent statistical analyses, all reported EC50 values were calculated with the above described method.

Analysis of linear, quadratic and interaction effects of DOC, pH and hardness on the calculated 21-day NOEC and EC50 values was carried out with STATISTICA (Statsoft, Tulsa, OK, USA) using a central composite analysis (Box and Draper, 1978; Box et al., 1978) using the toxicity data obtained with Ankeveen DOM. Next to this analysis, a forward regression analysis technique (Neter et al., 1989) was applied to the toxicity data obtained with Bihain, Ossenkolk and Ankeveen DOM (STATISTICA, Tulsa, OK, USA). This technique adds terms (linear, quadratic and interaction terms) to a regression equation one by one, starting with the most significant term, until no further significant improvement of the overall equation is obtained.

Other statistics are explained in detail in the results and discussion section. All statistical analyses were performed taking into account at a significance level $\alpha = 0.05$.

4.3. Results and discussion

4.3.1. Chemical composition of the DOM samples

The chemical composition (major ions and metals) of the DOM stock solutions that were used for the preparation of the test media is reported in Table 4-3. Iron and aluminum are the major trace metals in all DOM samples. The main reason for this is the high chemical affinity of Fe^{3+} and Al^{3+} ions for DOM (Tipping, 1994; Bryan and Tipping, 2002). The high concentration of Na and Cl in all of the samples is due to the metal removal treatment (i.e. the DOM clean-up process) that requires acidification with HCl to pH = 1 to precipitate the humic acids and the use of NaOH to neutralize the pH of the final stock solution. As a consequence of these high concentrations of ions in the DOM stock solutions, considerable amounts of these ions may be added to the reconstituted ecotoxicity test media and this might influence the toxicity test results. Therefore, the maximum concentrations of ions added to the ecotoxicological test media, through DOC addition, was calculated (Table 4-3). Except for iron, relatively low trace metal concentrations were present in all test media.

Also relatively high concentrations of sodium and chloride, originating from the concentrated DOM stocks, were added to the test medium (up to 17.4 mM Na and 20.3 mM Cl). Chloride has shown not to affect copper toxicity to fish (Erickson et al., 1996) and daphnids (De Schamphelaere et al., 2002; chapter 2). Sodium on the contrary is known to decrease copper toxicity to fish (Erickson et al., 1996) and daphnids (De Schamphelaere et al., 2002; chapter 2). However, the possible effect of these increased Na concentrations will be taken into account using the results of the experiment in which the individual effect of Na was investigated (see further). The chemical composition of the DOM stocks is also taken into account in calculating the chemical composition of the test media.

The humic acid fraction of the DOC was 11.7% for Bihain, 24.1% for Ossenkolk and 4.2% for Ankeveen, which is comparable to humic acid levels reported for a number of North American isolated organic matter samples (i.e. between 13% and 29%; Ma et al., 2001).

Table 4-3 Composition of the three DOM stocks and theoretical maximum ion concentrations added (between brackets), along with the DOM stock solutions, to the ecotoxicological test media. This maximum concentration occurs in test medium 9 (highest theoretical DOC-concentration, i.e. 18.4 mg L^{-1}) and was calculated as: maximum metal concentration added to test medium = (metal concentration in DOM stock solution) x (18.4 / DOC concentration in DOM stock solution). See Table 1 for information about the three sampling locations.

	Bihain	Ossenkolk	Ankeveen
DOC (mg L^{-1})	145.5	400	921
% humic acid	11.7	24.1	4.17
Ca (mM)	0.012 (0.002)	0.015 (<0.001)	0.039 (<0.001)
Mg (mM)	BDL	BDL	0.001 (<0.001)
Na (mM)	138 (17.4)	96.3 (4.43)	232 (4.63)
Cl (mM)	161 (20.3)	104 (4.76)	199 (3.98)
SO_4 (mM)	0.043 (0.005)	0.016 (<0.001)	25.5 (0.51)
Cu (µg L^{-1})	20.9 (2.64)	31.3 (1.44)	29.8 (0.59)
Zn (µg L^{-1})	62.5 (7.90)	86.6 (3.98)	201 (4.01)
Mn (µg L^{-1})	BDL	BDL	10.1 (0.20)
Al (µg L^{-1})	109 (13.8)	422 (19.4)	188 (3.76)
Fe (µg L^{-1})	499 (63.1)	3,880 (179)	449 (8.97)
Cd (µg L^{-1})	9.34 (1.18)	9.90 (0.46)	10.2 (0.20)
Ni (µg L^{-1})	8.61 (1.09)	15.7 (0.72)	39.5 (0.79)
Cr (µg L^{-1})	13.5 (1.70)	21.1 (0.97)	38.1 (0.76)
Pb (µg L^{-1})	BDL	BDL	BDL

DOM = dissolved organic matter; DOC = dissolved organic carbon

BDL = below detection limit (Mg = 0.00025 mM, Mn=0.18 µg/L and Pb=2.2µg/L)

4.3.2. Ecotoxicological test data

All reported effect concentrations are based on dissolved copper concentrations measured at the beginning of the experiment. The largest decrease of dissolved copper was observed in the third week of the 21-day tests, i.e. when the largest number of algal cells (food) is added to the test medium. This possibly indicates that the loss of dissolved copper is, at least partially, due to direct adsorption and/or internalization of copper (Crist et al., 1988; Xue and Sigg, 1990) or to adsorption of organically complexed copper (Campbell et al., 1997) to algal cell walls. On average (± 1 standard deviation) the decrease of dissolved copper during the two days before each renewal was 9.12% ± 8.83% in the experiments with Bihain DOM, 13.9 % ± 10.2% for Ossenkolk DOM and 7.84% ± 4.32% for Ankeveen DOM. No significant differences were observed between the percentage of dissolved copper decrease in the tests with the different DOMs (non-parametric median test; decrease of dissolved copper did not follow the normal distribution for any of the DOMs; Shapiro-Wilkinson test; variances were not homogeneous in every case, Levene's test). From the current study, no conclusions can be drawn on the possible contribution of particulate copper to the observed chronic toxicity relationships via a dietary pathway.

The issue of dietary copper toxicity will be investigated and discussed in further detail in chapter 5. In this study, the effects of water chemistry on chronic copper toxicity will be considered to be effects on the waterborne toxicity of copper; but this premise will also be critically discussed in chapter 5, with regard to the validity of the observed toxicity relations and derived bioavailability models for other exposure conditions (e.g. other feeding regime).

The 21-day NOECs, LOECs and EC50s that were calculated for the 35 test media are presented in Table 4-4. NOECs ranged from 29.4 to 228 µg Cu L^{-1} (factor 8) and EC50s from 41.5 to 316 µg Cu L^{-1} (factor 8). Bossuyt et al. (unpublished data) also found large differences in the toxicity of copper to *D. magna* in tests performed with 10 natural surface waters. In these studies 21-day NOECs ranged from 20 to 300 µg Cu L^{-1} (factor 17) and 21-day EC50s from 23 to 367 µg Cu L^{-1} (factor 16). Using media containing between 0 and 1.5 mg humic acid per liter (~ 0.75 mg DOC L^{-1}), Winner (1985) reported 42-day NOECs for *Daphnia pulex* between 4 and 40 µg Cu L^{-1} (factor 10). Our results and those of the cited authors clearly point to the importance of incorporating copper bioavailability into risk assessment and water quality criteria setting procedures.

Table 4-4 Physico-chemistry [a] of *Daphnia magna* test media and corresponding chronic (21-day) effect concentrations (as dissolved copper, µg L^{-1}). Dissolved organic carbon (DOC) from three locations was added to the test media (see Table 4-1 for information about sampling locations).

Medium	DOC (mg L^{-1})	pH	Ca (mM)	Mg (mM)	Na (mM)	Cl (mM)	SO_4 (mM)	CO_3 (mM)	21-day NOEC	21-day LOEC	21-day EC50
Bihain											
1	5.59	6.09	0.8	0.2	5.68	7.85	0.202	0.0401	30.3	57.1	45.8
2	16.9	6.08	0.8	0.2	16.4	20.4	0.205	0.0393	79.7	90.0	99.4
3	6.27	7.88	3.2	0.8	9.24	13.4	0.802	1.20	81.2	153	115
4	2.13	6.99	2.0	0.5	3.61	6.43	0.501	0.223	40.8	55.9	48.3
5	18.1	7.94	3.2	0.8	20.4	26.5	0.805	1.35	121	166	195
6	9.84	7.05	2.0	0.5	10.9	15.0	0.503	0.249	112	158	144
7	9.98	7.06	4.0	1.0	11.0	19.1	1.00	0.253	111	203	152
8	10.2	8.42	2.0	0.5	17.4	15.4	0.503	3.36	121	146	152
9	21.6	7.05	2.0	0.5	22.0	27.9	0.506	0.247	228	279	316
Ossenkolk											
1	6.19	6.09	0.8	0.2	1.89	3.28	0.200	0.0396	31.8	47.5	49
2	16.9	6.06	0.8	0.2	4.47	6.05	0.201	0.0376	84.7	109	122
3	5.69	7.88	3.2	0.8	4.69	7.95	0.800	1.21	78.6	154	108
4	2.32	7.02	2.0	0.5	2.16	4.68	0.500	0.233	31.8	42.4	34.6
5	17.8	7.91	3.2	0.8	7.62	11.1	0.801	1.28	145	183	195
6	12.8	6.96	2.0	0.5	4.69	7.40	0.500	0.208	162.5	200	192
7	11.9	7.00	4.0	1.0	4.46	11.2	1.00	0.226	114	163	156
8	11.2	8.35	2.0	0.5	10.4	6.97	0.500	2.95	135	180	157
9	20.1	7.01	2.0	0.5	6.45	9.29	0.501	0.230	190	249	250
Ankeveen											
1	5.04	6.15	0.8	0.2	1.73	2.77	0.340	0.0445	29.4	48.4	41.5
2	14.5	6.16	0.8	0.2	4.10	4.81	0.600	0.0458	89.2	157	152
3	5.15	7.83	3.2	0.8	4.79	7.59	0.942	1.10	68.8	111	84.8
4	1.74	7.16	2.0	0.5	2.60	4.45	0.548	0.307	41.9	47.3	49.7
5	15.6	7.85	3.2	0.8	7.25	9.85	1.23	1.14	153.1	247	216
6	10.2	7.15	2.0	0.5	4.57	6.29	0.783	0.300	90.2	160	144
7	10.1	7.15	4	1.0	4.54	10.3	1.28	0.299	85.9	161	182
8	12.3	8.32	2.0	0.5	10.3	6.30	0.785	2.79	120.3	179	145
9	16.1	7.08	2.0	0.5	6.21	7.56	0.946	0.265	213	265	263
10	4.81	6.05	3.2	0.8	1.60	7.52	0.933	0.0371	46.7	57.8	57.0
11	13.2	6.06	3.2	0.8	3.71	9.34	1.17	0.0373	93.1	140	122
12	4.81	7.88	0.8	0.2	4.53	2.72	0.333	1.22	76.7	111	154
13	13.5	7.89	0.8	0.2	6.78	4.60	0.574	1.24	196	259	276
14	9.02	5.62	2.0	0.5	2.40	6.03	0.750	0.0162	56.8	81.4	70.6
15	9.11	7.05	0.2	0.05	4.61	2.45	0.302	0.25	101	145	166
16	10.0	7.07	2.0	0.5	4.52	6.25	0.778	0.258	93.5	164	145
17	10.3	7.06	2.0	0.5	4.58	6.29	0.784	0.253	87.4	141	138

[a] The concentration of K = 0.078 mM in all tests

NOEC = no observed effect concentration

LOEC = lowest observed effect concentration

EC50 = concentration resulting in 50% reduction of reproduction relative to the control

4.3.3. Statistical analysis and interpretation of the ecotoxicological data

Central composite analysis of the ecotoxicological test data obtained with Ankeveen DOM demonstrated that interactive effects of the different experimental factors (pH, DOC, hardness) were not significant for chronic copper toxicity to *D. magna*. Individual effects of hardness (at the levels tested) were also not significant (neither linear nor quadratic effects, α = 0.05). For example, for the tests with Ankeveen DOM, one can easily see that the EC50 at the lowest hardness (test medium 15, hardness = 25 mg $CaCO_3$ L^{-1}, EC50 = 166 µg Cu L^{-1}) is very similar to the EC50 at medium hardness (test medium 6, 16 and 17; hardness = 250 mg $CaCO_3$ L^{-1}; EC50 = 138 to 145 µg Cu L^{-1}) and at the highest hardness (test medium 7, hardness = 500 mg $CaCO_3$ L^{-1}; EC50 = 181.7 µg Cu L^{-1}). Hardness was therefore not included as a factor in any of the subsequent regression analyses.

This observation may seem to contradict the general thought that increased hardness decreases copper toxicity (i.e. through "competition", e.g. Pagenkopf, 1983; Erickson et al., 1996; De Schamphelaere and Janssen, 2002). However, all of the latter studies based their conclusions on short-term toxicity studies. Our observations, however, do agree with those of the chronic copper toxicity reported by Winner (1985). In this study with *D. pulex*, this author even observed a small increase of copper toxicity with increasing hardness in the presence of humic acid. He argued that although Ca and Mg may not have a direct effect on chronic copper toxicity, they may have a (small) indirect effect through competition with copper for binding sites on the organic matter.

Contrary to water hardness, pH and DOC did have a significant effect on chronic copper toxicity to *D. magna* (central composite analysis and regression analysis). Overall, copper toxicity was lower at higher DOC concentrations and at higher pH levels. For example, 21-day EC50s in test medium 9 (highest DOC, 16.1 to 21.6 mg/L) were between 250 and 316 µg Cu L^{-1}, about 5 to 7 times higher than in test medium 4 (lowest DOC, 1.7 to 2.3 mg/L) with 21-day EC50s between 34.6 and 49.7 µg Cu L^{-1}. With Ankeveen DOM, the 21-day EC50 in test medium 8 (highest pH, i.e. 8.3) was 145 µg Cu L^{-1}, about 2 times higher than in test medium 14 (lowest pH, i.e. 5.6) with an EC50 of 70.6 µg Cu L^{-1}. The percentage of the experimental variability explained by DOC and pH is reported in Table 4-5.

Table 4-5 Percentage of experimental variance on 21-day NOEC and 21-day EC50 explained by linear effects of dissolved organic carbon (DOC) and pH and by the quadratic effect of pH (pH^2). Numbers indicate percentage as calculated by regression analysis; numbers between brackets are calculated using central composite analysis. See Table 1 for information on dissolved organic matter (DOM) sources.

DOM source	Endpoint	DOC [a]	pH [a]	pH^2 [b]	Total % variance explained
Bihain	NOEC	61.5	9.2	10.2	80.9
Bihain	EC50	65.9	10.2	11.2	87.3
Ossenkolk	NOEC	64.1	15.0	6.9	86.0
Ossenkolk	EC50	65.9	11.0	6.9	91.8
Ankeveen	NOEC	62.2 (61.5)	18.0 (20.3)	0.2 (2.5) [c]	80.2 (81.8)
Ankeveen	EC50	60.6 (60.3)	14.6 (22.7)	3.1 (4.8) [c]	81.2 (83.0)

NOEC = no observed effect concentration

EC50 = concentration resulting in 50% reduction of reproduction relative to the control

[a] linear effect

[b] quadratic effect

[c] effects not significant (p>0.05)

For all three DOMs, the linear effect of DOC concentration explained between 60.6 and 65.9% of the observed variability of effect concentrations (NOECs and EC50s). The linear effect of pH explained between 9.2 and 18 % observed variability. The only different trend between the different DOMs seemed to be the importance of the pH^2 effect (quadratic effect of pH). In the specific case of this study, this quadratic effect can be regarded as an inverse U-shaped relation between NOEC or EC50 and pH. This quadratic effect explained an additional variance of 6.9 to 11.2% for Bihain and Ossenkolk DOM but was not significant for Ankeveen DOM. One might argue that this observation points to a difference between how the three DOMs alter copper toxicity at higher pH levels. However, when performing regression analysis on the toxicity data obtained with Ankeveen test media 1 to 9, it becomes apparent that the pH^2 effect is significant and explains approximately 8% of the variability. This finding suggests that the pH^2 effect might only be a statistical artefact, originating from the fact that only 9 data are used to fit 4 regression parameters.

The possible effect of DOM source was investigated by comparing NOEC-values and EC50-values obtained with test media 1 to 9 with different DOM sources. For both NOECs and

EC50s, 3 t-tests for dependent samples were carried out: (1) comparing Bihain DOM with Ossenkolk DOM, (2) comparing Bihain DOM with Ankeveen DOM, and (3) comparing Ossenkolk DOM with Ankeveen DOM. Paired differences followed the normal distribution (Shapiro-Wilkinson test) in all cases, which allows the use of a t-test for dependent samples. No significant differences were observed between EC50s (and NOECs) obtained with test media containing the three different DOMs. This finding further supports the idea that all three DOMs affect chronic copper toxicity to *D. magna* in a very similar way.

This finding contradicts the observation that for the same three DOMs, DOM-source did affect copper toxicity to the green alga *P. subcapitata* (see chapter 6). It was also shown that the latter was due to differences in complexation capacities. This may suggest species-specific differences of the effect of DOM-source on copper toxicity. A possible process that could result in a levelling-off of the effect of natural DOM-source on chronic copper toxicity to *D. magna* (i.e. all DOMs reducing chronic toxicity to the same extent, without having the same copper complexing capacity) is the fact that organically complexed copper may be bioavailable to some extent in chronic exposures. Clearly more research is needed to clarify the importance of DOM-source on copper toxicity to a number of organisms.

Since all three DOMs seemed to reduce chronic copper toxicity to *D. magna* to the same extent, a regression analysis was performed on the pooled results of all 35 toxicity tests. DOC concentration was again the most important parameter, explaining 61.5% and 62.4% of the variability for NOECs and EC50s, respectively. The pH effect was also significant explaining 14.4% and 14.1% of the variability for NOECs and EC50s, respectively. The following DOM-source independent regression equations were obtained:

$$\text{21-day NOEC} = -160.5 + 7.652 \cdot \text{DOC} + 25.50 \cdot \text{pH} \quad (r^2 = 0.76,\ p<0.001) \qquad (1)$$

and

$$\text{21-day EC50} = -212.4 + 10.41 \cdot \text{DOC} + 34.36 \cdot \text{pH} \quad (r^2 = 0.77,\ p<0.001) \qquad (2)$$

with NOEC and EC50 in µg Cu L^{-1} and DOC in mg L^{-1}. Similarly, Heijerick et al. (2002) found that linear effects of DOC (humic acid) and pH were the most important in determining chronic zinc toxicity to *D. magna*, together explaining 78.4% of the variability around the 21-day EC50s.

Further on, the developed regression models will be validated using toxicity data with spiked natural surface waters. However, following sections will first investigate the possibility of applying the BLM framework to the observed toxicity test data.

4.3.4. Copper speciation –general approach

To develop a mechanistic copper toxicity model one must be able to correctly predict copper speciation from physico-chemical water characteristics. Inorganic speciation calculation is the most straightforward of the computation because the ligands (such as OH^- and CO_3^{2-}) are well characterized and their binding constants are known (Di Toro et al., 2001). In this paper all subsequent speciation calculations will be performed using stability constants for inorganic copper complexes taken from Martell et al. (1997, see Table 2-2). Modelling the complexation of copper to organic molecules is more difficult. In the framework of the develoment of BLMs, WHAM-Model V (Tipping, 1994) has become an integral part of the computations because the model has been calibrated to multiple datasets of titrations of isolated organic matter with acid, base and a number of metals (Tipping and Hurley, 1992; Tipping, 1993). However, WHAM V tends to overestimate copper binding to DOM under natural conditions (De Schamphelaere et al., 2002, chapter 3; Dwane and Tipping, 1998; Bryan and Tipping, 2002). In other words, the WHAM V-calculated Cu^{2+}-activity under natural conditions is generally lower than the measured Cu^{2+}-activity. For natural waters, Dwane and Tipping (1998) observed that the best match between WHAM-calculated and measured Cu^{2+}-activity was obtained when 40 to 80% of the DOM was considered to be "active fulvic acid" (active FA), and when the rest was considered to be inert for copper complexation. Supported by the findings of Cabaniss and Shuman (1988), they also suggested that, if nothing is *a priori* known about the copper complexation characteristics of the DOM, 50% active FA should be used as the input for the speciation calculations. Using that assumption in a BLM, accurate predictions were made of acute (48-hour) toxicity of copper to *D. magna* (De Schamphelaere et al., 2002, chapter 3) and chronic (72-hour) toxicity to the alga *P. subcapitata* (De Schamphelaere et al., 2003, chapter 6) in 19 and 13 natural surface waters, respectively.

Table 4-6 21-day NOEC and EC50 values for Daphnia magna expressed as Cu^{2+} activity (in M) in 35 exposure media containing natural dissolved organic carbon (DOC) from Bihain, Ossenkolk and Ankeveen. Ion activities that are used in the equations and for model development (see text) are also reported. Cu^{2+} activities were calculated using BLM version 1.0.0 (Hydroqual, 2002) with dissolved 21-day NOECs and EC50s and the physico-chemistry of the test media as input (Table 4-4). Stability constants for inorganic copper complexes were taken from Martell et al. (1997), constants for organic complexes with fulvic acid were taken from Tipping (1994).

			Ion activities (M)						50% FA		Optimal % FA	
Medium	DOC (mg/L)	pH	H^+	OH^-	CO_3^{2-}	Ca^{2+}	Mg^{2+}	Na^+	NOEC	EC50	NOEC	EC50
Bihain												
1	5.585	6.09	8.13E-07	8.32E-09	6.13E-10	4.85E-04	1.21E-04	5.13E-03	1.29E-08	2.30E-08	9.24E-09	1.58E-08
2	16.935	6.08	8.32E-07	8.13E-09	5.75E-10	4.19E-04	1.05E-04	1.41E-02	1.25E-08	1.67E-08	8.94E-09	1.18E-08
3	6.267	7.88	1.32E-08	5.13E-07	2.81E-06	1.62E-03	4.05E-04	7.98E-03	1.35E-09	2.90E-09	8.25E-10	1.61E-09
4	2.129	6.99	1.02E-07	6.61E-08	5.94E-08	1.15E-03	2.88E-04	3.23E-03	2.05E-08	3.22E-08	1.11E-08	1.69E-08
5	18.105	7.94	1.15E-08	5.89E-07	3.52E-06	1.47E-03	3.67E-04	1.70E-02	3.87E-10	8.63E-10	2.63E-10	5.44E-10
6	9.844	7.05	8.91E-08	7.59E-08	7.68E-08	1.05E-03	2.63E-04	9.47E-03	6.34E-09	1.13E-08	3.90E-09	6.33E-09
7	9.98	7.06	8.71E-08	7.76E-08	7.77E-08	1.94E-03	4.84E-04	9.36E-03	6.91E-09	1.41E-08	4.24E-09	7.72E-09
8	10.213	8.42	3.80E-09	1.78E-06	2.73E-05	9.65E-04	2.43E-04	1.49E-02	2.38E-10	3.71E-10	1.51E-10	2.25E-10
9	21.56	7.05	8.91E-08	7.59E-08	7.42E-08	9.49E-04	2.37E-04	1.85E-02	5.88E-09	1.22E-08	3.63E-09	6.71E-09
Ossenkolk												
1	6.189	6.09	8.13E-07	8.32E-09	6.00E-10	5.17E-04	1.29E-04	1.75E-03	1.12E-08	2.05E-08	8.09E-09	1.43E-08
2	16.908	6.06	8.71E-07	7.76E-09	5.13E-10	4.88E-04	1.22E-04	4.06E-03	1.26E-08	2.10E-08	9.09E-09	1.47E-08
3	5.685	7.88	1.32E-08	5.13E-07	2.86E-06	1.70E-03	4.25E-04	4.12E-03	1.50E-09	3.05E-09	9.31E-10	1.74E-09
4	2.324	7.02	9.55E-08	7.08E-08	6.74E-08	1.18E-03	2.94E-04	1.95E-03	8.63E-09	1.03E-08	5.21E-09	6.06E-09
5	17.846	7.91	1.23E-08	5.50E-07	3.23E-06	1.64E-03	4.10E-04	6.62E-03	5.49E-10	9.19E-10	3.72E-10	5.93E-10
6	12.837	6.96	1.10E-07	6.17E-08	5.10E-08	1.13E-03	2.82E-04	4.18E-03	9.27E-09	1.38E-08	5.53E-09	7.70E-09
7	11.895	7.00	1.00E-07	6.76E-08	6.01E-08	2.07E-03	5.14E-04	3.87E-03	5.76E-09	1.10E-08	3.75E-09	6.55E-09
8	11.17	8.35	4.47E-09	1.51E-06	2.09E-05	1.04E-03	2.62E-04	9.13E-03	3.02E-10	4.05E-10	1.95E-10	2.54E-10
9	20.12	7.01	9.77E-08	6.92E-08	6.45E-08	1.10E-03	2.75E-04	5.70E-03	4.67E-09	8.17E-09	3.03E-09	4.88E-09

Table 4-6 (continued)

			Ion activities (M)						50% FA		Optimal % FA	
Medium	DOC (mg/L)	pH	H^+	OH^-	CO_3^{2-}	Ca^{2+}	Mg^{2+}	Na^+	NOEC	EC50	NOEC	EC50
Ankeveen												
1	5.04	6.15	7.08E-07	9.55E-09	8.42E-10	5.12E-04	1.28E-04	1.60E-03	1.12E-08	1.85E-08	1.49E-08	2.56E-08
2	14.46	6.16	6.92E-07	9.77E-09	9.07E-10	4.73E-04	1.18E-04	3.73E-03	1.29E-08	3.02E-08	1.76E-08	4.51E-08
3	5.15	7.83	1.48E-08	4.57E-07	2.32E-06	1.69E-03	4.22E-04	4.21E-03	1.56E-09	2.43E-09	2.44E-09	4.03E-09
4	1.74	7.16	6.92E-08	9.77E-08	1.28E-07	1.17E-03	2.92E-04	2.35E-03	2.52E-08	3.95E-08	4.09E-08	6.17E-08
5	15.57	7.85	1.41E-08	4.79E-07	2.50E-06	1.62E-03	4.02E-04	6.30E-03	8.69E-10	1.70E-09	1.31E-09	2.79E-09
6	10.21	7.15	7.08E-08	9.55E-08	1.22E-07	1.11E-03	2.78E-04	4.08E-03	2.95E-09	7.74E-09	4.44E-09	1.35E-08
7	10.12	7.15	7.08E-08	9.55E-08	1.18E-07	2.04E-03	5.07E-04	3.94E-03	3.34E-09	1.72E-08	4.95E-09	3.28E-08
8	12.29	8.32	4.79E-09	1.41E-06	1.72E-05	8.73E-04	2.25E-04	8.89E-03	3.07E-10	4.21E-10	4.19E-10	5.79E-10
9	16.10	7.08	8.32E-08	8.13E-08	8.96E-08	1.08E-03	2.69E-04	5.50E-03	7.92E-09	1.34E-08	1.37E-08	2.52E-08
10	4.81	6.05	8.91E-07	7.59E-09	4.80E-10	1.76E-03	4.38E-04	1.42E-03	4.06E-08	5.74E-08	5.54E-08	7.96E-08
11	13.22	6.06	8.71E-07	7.76E-09	5.01E-10	1.69E-03	4.20E-04	3.26E-03	2.74E-08	4.17E-08	3.78E-08	5.99E-08
12[a]	4.81	7.88	1.32E-08	5.13E-07	3.02E-06	4.82E-04	1.21E-04	4.14E-03	1.40E-09	1.06E-08	2.35E-09	1.86E-08
13	13.53	7.89	1.29E-08	5.25E-07	3.13E-06	4.51E-04	1.13E-04	6.11E-03	1.18E-09	2.86E-09	2.00E-09	5.74E-09
14	9.02	5.62	2.40E-06	2.82E-09	3.75E-11	1.16E-03	2.88E-04	2.16E-03	5.72E-08	7.64E-08	7.28E-08	9.82E-08
15	9.11	7.05	8.91E-08	7.59E-08	8.04E-08	1.24E-04	3.08E-05	4.27E-03	3.14E-09	1.06E-08	5.05E-09	2.02E-08
16	10.04	7.07	8.51E-08	7.94E-08	8.51E-08	1.12E-03	2.78E-04	4.03E-03	3.86E-09	9.73E-09	5.88E-09	1.72E-08
17	10.25	7.06	8.71E-08	7.76E-08	8.13E-08	1.12E-03	2.78E-04	4.09E-03	3.40E-09	8.43E-09	5.07E-09	1.45E-08

[a] the 21-day EC50 value calculated for this test medium is an extrapolated value as the highest tested concentration only resulted in 28% reproduction reduction, therefore this data point is not included in the modelling efforts

In the latter study (see chapter 6), it was demonstrated that the DOMs, the same three as used in the present study, had different copper complexation properties. The 'optimal % active FA' (i.e. the % active FA that resulted in an optimal fit between measured and calculated Cu^{2+}-activity) were 65.2%, 64.8% and 41.4% for Bihain, Ossenkolk and Ankeveen DOM, respectively. Although this difference resulted in a lower protective effect of Ankeveen DOM with respect to copper toxicity to *P. subcapitata*, this was not the case in the chronic daphnid experiments, as all three DOMs seemed to reduce copper toxicity to the same extent.

Therefore, it was decided to start the speciation computations with assuming 50% active FA for all three DOMs, as this reflects the similarity in protective effect between the three DOMs. This assumption is a good starting point to unravel possible mechanisms of chronic copper toxicity, to derive first estimates of BLM constants and, possibly, to provide a possible explanation for the different effect of DOM type in tests with algae and daphnids. However, the assumption of an optimal % of active FA will also be used throughout the modelling efforts.

It has to be kept in mind, however, that the calculated Cu^{2+}-activities are only estimations representing the (pseudo)-equilibrium situation at the moment daphnids are transferred to fresh medium and that these activities slightly decreased between two renewals. The latter is probably the result of excretion of the often-reported organic copper-complexing agents by algae and/or daphnids (e.g. Xue and Sigg, 1990; Fish and Morel, 1993). However, previous measurements have shown that decreases of Cu^{2+}-activity between two renewals were smaller than a factor of 2 (De Schamphelaere, unpublished data). Based on this observation and since no complete dataset of measured Cu^{2+}-activities was available for the present toxicity test dataset, changes in copper speciation will not be accounted for in the modelling efforts. Therefore, the developed model will only be applicable to situations in which a near-equilibrium copper speciation exists.

4.3.5. Interpretation of toxicity data expressed as Cu^{2+}-activity

In Table 4-6 (previous pages), the physico-chemical speciation and the 21-day NOECs and EC50s expressed as free copper ion activities for the 35 different test media are summarized. Reported Cu^{2+}-activities were calculated using both the assumption of 50% active FA and the optimal % active FA for each DOM type (cf. above). When calculated using the assumption of 50% active FA, NOECs ranged from 0.238 to 57.2 nM (Cu^{2+}) (factor 240) and EC50s from 0.342 to 76.4 nM (Cu^{2+}) (factor 220). When calculated using the assumption of an optimal % active FA

for each DOM, NOECs ranged from 0.151 to 72.8 nM (Cu^{2+}) (factor 480) and EC50s from 0.225 to 98.2 nM (Cu^{2+}) (factor 440).

In general, these calculations seem to indicate that Cu^{2+} activity is a worse predictor of copper toxicity than dissolved copper, since effect concentrations (expressed as dissolved copper) (for the same 35 test media) only differed by a factor of less than 10 (see Table 4-4). Additionally, it seems that when using the previously derived optimal % of active FA (i.e. taking into account the different copper complexation characteristics of the different DOMs as derived from Cu^{2+}-measurements during algal growth inhibition tests), a larger variability of effect concentrations (expressed as Cu^{2+}-activity) is obtained. With this assumption, more Cu^{2+} is needed in the experiments with Ankeveen DOM to exert the same degree of toxic effect (paired t-test, $p<0.05$) as in the tests with Bihain and Ossenkolk DOM. In the following sections detailed data analysis will be performed to find a suitable explanation for this observation.

In the modelling we will first focus on calculations based on the assumption that all three DOMs consist of 50% active FA, as this assures the least variability in calculated copper speciation across tests with different DOM types. Figure 4-3 shows the calculated copper speciation (three most abundant inorganic species) at the 21-day EC50 level as a function of pH (50% active FA assumption). The higher the pH, the less free copper ions are needed to exert the same degree of toxic effect (i.e. 50% reduction in reproduction). This has already been observed for acute copper toxicity to *D. magna* (chapter 2 and 3, Meador, 1991) and was also observed for chronic copper toxicity to green algae (chapter 6).

Very significant linear relations ($p<0.01$) were found between log(21-day $EC50_{Cu2+}$) and log(21-day $NOEC_{Cu2+}$) versus pH:

$$Log(NOEC_{Cu2+}) \text{ (nM)} = - 0.747 \text{ pH} – 3.087 \ (r^2 = 0.82) \quad (3)$$

and

$$Log(EC50_{Cu2+}) \text{ (nM)} = - 0.705 \text{ pH} – 3.097 \ (r^2 = 0.77) \quad (4)$$

By linking these regressions to the speciation model WHAM V (Tipping, 1994), a semi-mechanistic toxicity model would be obtained that is similar in structure as that developed for predicting copper toxicity to algae (see chapter 6). However, with respect to the current interest

of both the regulatory and the scientific community in biotic ligand models (BLM) for modelling metal toxicity, the possibilities and limitations of a BLM approach for predicting chronic copper toxicity will be investigated.

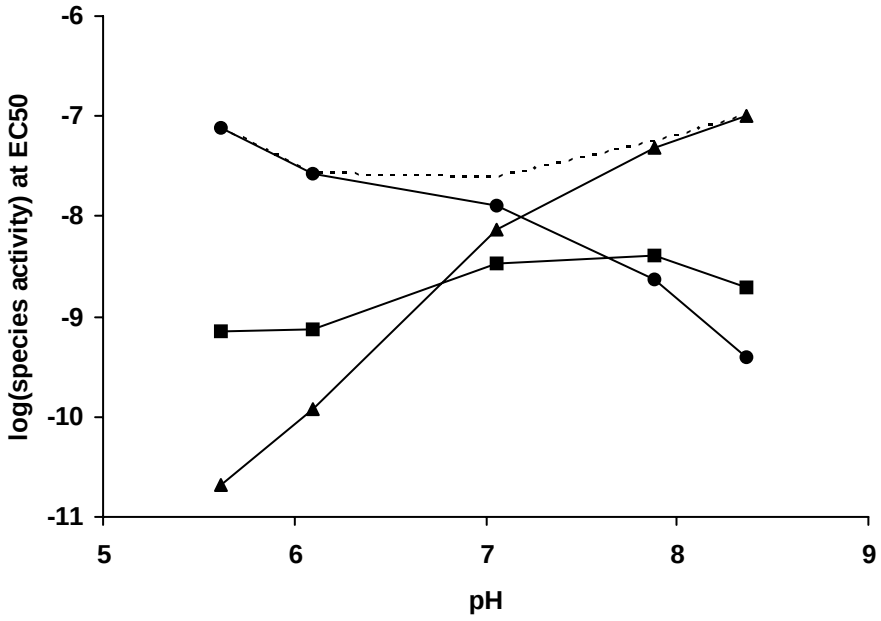

Figure 4-3 Copper speciation at the 21-day EC50-level as a function of pH. The average of the logarithm of the calculated activity of Cu^{2+} (●), $CuOH^{+}$ (■) and $CuCO_3$ (▲) at each pH-level (average pH) are presented. Speciation was calculated with BLM for windows version 1.0.0 (Hydroqual, 2002) using physico-chemistry of the test media, assuming 50% active fulvic acid (see text), and 21-day EC50 levels of copper. The dashed line represents the sum of the activities of the inorganic copper species.

4.3.6. Initial BLM-development (chronic Cu-BLM-1)

First, the possibility of simply extrapolating the acute copper BLM for *D. magna* (chapter 3) to chronic exposures was assessed. The same approach as used by Santore et al. (2001) to calibrate the acute Cu-BLM for fathead minnow to *Daphnia pulex* was used in this study too. These authors indicated that the simplest means of dealing with differences in sensitivity between

species is to maintain all stability constants for cation binding to the biotic ligand at the original values and only to adjust the critical copper concentration on the biotic ligand.

Using the acute copper BLM constants derived in previous studies (De Schamphelaere et al., 2002; chapter 3) and the physico-chemistry of the test media, and assuming all three DOMs to consist of 50% active FA, it was calculated that the percentage of the (acute) biotic ligand sites occupied by copper at the 21-day NOEC and EC50 level, varied from 0.4 to 41% (factor ~ 100) and from 1 to 48% (factor ~ 50), respectively.

Moreover, there seemed to be a correlation of this critical site occupation with the pH level (correlation coefficients: r = - 0.66 for the EC50 level and r = - 0.68 for the NOEC level), with less copper at the biotic ligand needed at higher pH values. It thus seems that, using the acute BLM constants less copper needs to be bound to the biotic ligand for the same effect at a higher pH. This is in clear contrast with the BLM assumption that the effect is related to the amount of copper bound to the biotic ligand, independent of water characteristics (Di Toro et al., 2001). This means that the acute BLM cannot simply be translated to a chronic (reproduction) BLM by simply adjusting the critical biotic ligand site occupation. It thus seems that an adaptation of at least some of the BLM constants is required to fit chronic toxicity data. The above mentioned pH dependency of the calculated chronic ciritical biotic ligand Cu-concentrations, points to the possibility of different pH-related BLM-constants as compared to acute exposures, i.e K_{HBL} (proton competition), K_{CuOHBL} and/or $K_{CuCO3BL}$ (toxicity of $CuOH^+$ and $CuCO_3$ complexes).

In what follows we will explore which combination of BLM-constant values best fits the observations. For clarity, a short outline on the mathematical background of the estimation of these constants is given first. The basic BLM equation is (see chapter 3, example for 21-day EC50 level):

$$21d-EC50_{Cu^{2+}} = \frac{f_{CuBL}^{50\%}}{(1-f_{CuBL}^{50\%})} \cdot \frac{1+K_{CaBL}\cdot\left(Ca^{2+}\right)+K_{MgBL}\cdot\left(Mg^{2+}\right)+K_{NaBL}\cdot\left(Na^{+}\right)+K_{HBL}\cdot\left(H^{+}\right)}{K_{CuBL}+K_{CuOHBL}\cdot K_{CuOH}\cdot\left(OH^{-}\right)+K_{CuCO3BL}\cdot K_{CuCO3}\cdot\left(CO_3^{2-}\right)} \qquad (5)$$

with $EC50_{(Cu2+)}$ = the 21-day EC50 expressed as Cu^{2+}-activity, i.e. (Cu^{2+}) (unit:M); $f_{CuBL}^{50\%}$ = the fraction of the total number of copper binding sites occupied by copper at 50% effect; K_{CuBL},

K_{CuOHBL}, K_{CaBL}, K_{MgBL}, K_{NaBL} and K_{HBL} stability constants for the binding of these cations to the BL (M^{-1}); K_{CuOH} and K_{CuCO3} = stability constant for the formation of the $CuOH^+$ and $CuCO_3$ complex, respectively (M^{-1}); ( ) represents the activity of the mentioned ions.

It is clear that, according to Eqn. 5, increasing (Ca^{2+}), (Mg^{2+}) and/or (Na^+) results in a higher $EC50_{Cu2+}$. Increasing (H^+) (lower pH) results in a higher $EC50_{Cu2+}$, not only because of the increased proton competition but also due to the associated decrease of (OH^-) and (CO_3^{2-}).

With regard to the earlier mentioned pH dependency of the chronic effect of copper, a simplified approach will be used first to estimate initial values for the pH-related constants. Possible effects of Ca, Mg and Na can then be investigated based on $EC50_{Cu2+}$ values corrected for these pH effects. Equation 5 is simplified to (pH effects only):

$$21d - EC50_{Cu^{2+}} = \frac{f_{CuBL}^{50\%}}{(1 - f_{CuBL}^{50\%})} \cdot \frac{1 + K_{HBL} \cdot (H^+)}{K_{CuBL} + K_{CuOHBL} \cdot K_{CuOH} \cdot (OH^-) + K_{CuCO3BL} \cdot K_{CuCO3} \cdot (CO_3^{2-})} \quad (6)$$

This equation can be rewritten as:

$$21d - EC50_{Cu^{2+}} = \left[EC50_{Cu^{2+}} \right]_0 \cdot \frac{1 + K_{HBL} \cdot (H^+)}{1 + R_{CuOHBL} \cdot K_{CuOH} \cdot (OH^-) + R_{CuCO3BL} \cdot K_{CuCO3} \cdot (CO_3^{2-})} \quad (7)$$

with R_{CuOH} = K_{CuOHBL} / K_{CuBL}, $R_{CuCO3BL}$ = $K_{CuCO3BL}$ / K_{CuBL} and $[EC50_{Cu2+}]_0$ = $\frac{f_{CuBL}^{50\%}}{(1 - f_{CuBL}^{50\%}) \cdot K_{CuBL}}$ (representing the EC50 value expressed as Cu^{2+} in the hypothetical situation without proton competition and in the absence of $CuOH^+$ and $CuCO_3$ in the solution). The latter model parameters were estimated by a non-linear regression using STATISTICA (Statsoft, Tulsa, OK, USA). A weighted regression was performed such that each pH level and each DOM type were equally accounted for. The best fit was defined as that which yielded the lowest sum of squares of the log-transformed $EC50_{Cu2+}$ values. Rosenbrock pattern search combined with the quasi-Newton method was used as the iterative regression procedure. Two additional conditions were taken into account for the regression analysis: $R_{CuOH} < 1$ and $R_{CuCO3BL} < 1$, meaning that $CuOH^+$ and $CuCO_3$ are assumed not to be more toxic than Cu^{2+}, a reasonable assumption based on numerous literature reports [e.g. chapter 2 and 3, Campbell, 1995; Erickson et al., 1996).

The best-fit parameters (constants) are presented in Table 4-7 and this model will be referred to as c-CuBLM-1. As compared to the acute Cu-BLM for *Daphnia magna*, K_{CuOHBL}, $K_{CuCO3BL}$ and log K_{HBL} are higher for chronic toxicity.

Table 4-7 Summary of constants of the acute CuBLM (a-CuBLM; chapter 3) and the different developmental stages of the chronic CuBLM (c-CuBLM, this chapter). c-CuBLM-1 and c-CuBLM 2 were developed using 50% active FA as an assumption for all three DOM types and without or with Na competition, respectively. c-CuBLM-3 was developed from toxicity test data with Ankeveen DOM only and using the optimal % active FA (i.e. 41.4%) and with Na competition. c-CuBLM-3 was retained as the best predictive model for copper toxicity in natural waters. All constants were derived using the regression equations and methods described in the text.

	a-CuBLM	c-CuBLM-1	c-CuBLM-2	c-CuBLM-3
Regression Parameters				
$[EC50_{Cu2+}]_0$ (nM)	8.47	13.5	2.78	6.17
R_{CuOH}	0.20	0.84	1.00	1.00
R_{CuCO3}	0.10	0.22	0.26	0.26
Copper constants				
log K_{CuBL}[a]	8.02	8.02	8.02	8.02
log K_{CuOHBL}[a]	7.32	7.95	8.02	8.02
log $K_{CUCO3BL}$[a]	7.01	7.36	7.44	7.44
$f^{50\%}_{CuBL}$[a, b]	0.47 ± 0.02	0.585 ± 0.038	0.226 ± 0.019	0.393 ± 0.081
f^{NOEC}_{CuBL}[a, b]	-	0.409 ± 0.043	0.090 ± 0.013	0.260 ± 0.077
Competition constants				
log K_{HBL}	5.40	6.14	6.68	6.67
log K_{CaBL}	3.47	-	-	-
log K_{MgBL}	3.58	-	-	-
log K_{NaBL}	3.19	-	2.91	2.91

[a] For estimating these constants log K_{CuBL} was assumed to be the same both in acute as in chronic exposures

[b] 95% percent intervals are given for the fraction of BL sites occupied by copper at the 21-day EC50 and at the 21-day NOEC level. These intervals were determined *a posteriori* by using the given stability constants to calculate the f-values for each test medium. These intervals account for the uncertainty of the different models and are an integrative measure of the errors on all constants.

c-CuBLM-1 was now used for correcting 21-day EC50 levels for the pH effect in order to assess effects of other physico-chemical parameters. To that end, the ratio (observed $EC50_{Cu2+}$) / (predicted $EC50_{Cu2+}$) was calculated (further denoted "O/P"). The "observed" $EC50_{Cu2+}$ is the $EC50_{Cu2+}$ as represented in the 11th column of Table 4-6 (50% FA assumption), the "predicted" $EC50_{Cu2+}$ is calculated with Eqn. 7, using the physico-chemistry from Table 4-6 and the constant values of c-CuBLM-1 reported in Table 4-7. Subsequently, the correlation (r) was determined between O/P and (1) the sum of Ca^{2+} and Mg^{2+} activity (factor water hardness), (2) Na^{+} activity and, (3) DOC concentration. Ca^{2+} and Mg^{2+} were evaluated together because their activites were correlated as a consequence of the experimental design (Ca:Mg = 4:1) and since they had similar competition strength with regard to acute copper toxicity to *D. magna* (De Schamphelaere and Janssen, 2002). Both hardness (r=0.20) and Na (r = -0.13) effects were not significant ($\alpha = 0.05$).

Recent (unpublished) work at our laboratory seems to confirm that increased Ca^{2+} or Mg^{2+} activities do not decrease chronic copper toxicity (expressed as Cu^{2+} activity). With regard to Na, however, it is important to note that even if Na did have a protective effect on chronic copper toxicity, this effect may have been masked by the pH effect in these experiments, because Na activity was positively correlated to pH in the experiments ($r = 0.40$, $p<0.01$).

The DOC effect, on the contrary, was significant ($r = -0.49$, $p<0.01$) with O/P > 1 at low DOC concentrations and O/P < 1 at high DOC concentrations (for clarity: this results in over-estimations of toxicity at low DOC-levels and for under-estimations at high DOC-levels, see also model validation section for more information). This suggests that less free copper ions are needed at high DOC levels to exert the same toxic effect, or alternatively, that Cu-DOC complexes may be bioavailable to a certain extent. The importance of this DOC effect might, however, not have been fully appreciated since it might have been partially masked by the presence of the previously mentioned Na effect. Indeed, Na activity was positively correlated with DOC concentration ($r = 0.56$, $p<0.001$). In summary, it seems that (1) water hardness (Ca+Mg) does not significantly affect chronic copper toxicity, (2) Cu-DOC complexes may be bioavailable to a certain extent, and (3) the correlation of Na concentrations with both pH and DOC concentration might have resulted in the masking of a possible Na effect, an underestimation of the strength of the pH-effect, and an underestimation of the bioavailability of Cu-DOC complexes. Another point of interest is that the estimated $\left[21d-EC50_{Cu^{2+}}\right]_0$ was 13.5 nM, whereas the $\left[48h-EC50_{Cu^{2+}}\right]_0$ (based on 48-hour immobilization assays) was estimated to be

significantly lower (t-test, $p<0.01$), i.e. 8.47 nM (De Schamphelaere et al., 2002, chapter 3). This does not agree with ones intuition, since this means that, after correction for effects of physico-chemistry, *D. magna* seems more sensitive in acute than in chronic exposures. Again, this might point to the non-inclusion of a protective Na effect in c-CuBLM-1. Indeed, adding a term for Na-competition to Eqn. 7 would result in a lower estimate of the $\left[21d - EC50_{Cu^{2+}}\right]_0$, possibly to a value below the $\left[48h - EC50_{Cu^{2+}}\right]_0$, which would seem more logical.

4.3.7. BLM development: incorporation of Na effect (chronic Cu-BLM-2)

Summarizing all previous remarks, it seems that additional testing for the individual Na effect was required. To that end, additional chronic toxicity tests were performed in 3 test media with different Na concentrations. The organic matter used in these tests was Aldrich humic acid, as copper complexation properties of this DOM are well characterized (De Schamphelaere et al., 2002, chapter 3). The physico-chemistry of these test media and the corresponding 21-day NOECs and 21-day EC50s are given in Table 4-8. At increased Na concentrations, increased NOECs and EC50s were observed. Calculated $NOEC_{Cu2+}$ and $EC50_{Cu2+}$ values increased with increased Na^+ activity. This points to a protective effect of Na on chronic copper toxicity.

Table 4-8 Physico-chemistry (ion concentrations, TOC = 4.28 mg L-1; pH = 6.8) and 21-day NOEC and EC50 values for *Daphnia magna* for three exposure media containing different Na concentrations and Aldrich humic acid as organic carbon. Cu^{2+} activities were calculated using BLM version 1.0.0 (Hydroqual, 2002) with dissolved 21-day NOECs and EC50s and the physico-chemistry of the test media as input (Table 4-4). Stability constants for inorganic copper complexes were taken from Martell et al. (1997, see Table 2-2), the stability constant for proton-copper exchange (pK_{MHA} = 1.9) for Aldrich humic acid was taken from De Schamphelaere et al. (2002, chapter 3).

Ca (mM)	Mg (mM)	Na (mM)	K (mM)	Cl (mM)	SO_4 (mM)	CO_3 (mM)	NOEC (μg L^{-1})	EC50 (μg L^{-1})	NOEC (nM Cu^{2+})	EC50 (nM Cu^{2+})
2.0	0.5	1.57	0.078	4.08	0.5	0.154	62.6	70.6	8.91	10.6
2.0	0.5	6.57	0.078	9.08	0.5	0.157	89.2	104.6	15.0	19.3
2.0	0.5	11.6	0.078	14.1	0.5	0.146	125.6	144.3	26.5	34.0

Until now, this protective effect of Na had only been described for acute copper toxicity to both *Daphnia magna* (De Schamphelaere et al., 2002; chapter 2) and juvenile fathead minnows (Erickson et al., 1996). Taking into account this protective effect, the chronic Cu-BLM equation now becomes:

$$21d-EC50_{Cu^{2+}} = \frac{f_{CuBL}^{50\%}}{(1-f_{CuBL}^{50\%})} \cdot \frac{1+K_{NaBL}\cdot\left(Na^{+}\right)+K_{HBL}\cdot\left(H^{+}\right)}{K_{CuBL}+K_{CuOHBL}\cdot K_{CuOH}\cdot\left(OH^{-}\right)+K_{CuCO3BL}\cdot K_{CuCO3}\cdot\left(CO_3^{2-}\right)} \quad (8)$$

In order to derive a stability constant to incorporate this effect in the chronic BLM, a linear regression was performed between Na^+ activity and 21-day $EC50_{Cu2+}$ (Figure 4-4). According to De Schamphelaere and Janssen (2002, chapter 2) the ratio slope / intercept of this regression equals (since Ca^{2+} and Mg^{2+} have shown not to affect chronic copper toxicity):

$$\frac{Slope}{Intercept} = \frac{K_{NaBL}}{1+K_{HBL}\cdot\left(H^{+}\right)_{Na-test}} = 471 \quad (9)$$

With $\left(H^{+}\right)_{Na-test}$ = the H^+-activity in the Na-test (see Table 4-8) this results in:

$$K_{NaBL} = 471\cdot\left\{1+K_{HBL}\cdot 10^{-6.8}\right\} \quad (10)$$

Since the exact value of K_{NaBL} is thus dependent on the value for K_{HBL}, Eqn 10 should be integrated into Eqn. 8:

$$21d-EC50_{Cu^{2+}} = \frac{f_{CuBL}^{50\%}}{(1-f_{CuBL}^{50\%})} \cdot \frac{1+471\cdot\left\{1+K_{HBL}\cdot 10^{-6.8}\right\}\cdot\left(Na^{+}\right)+K_{HBL}\cdot\left(H^{+}\right)}{K_{CuBL}+K_{CuOHBL}\cdot K_{CuOH}\cdot\left(OH^{-}\right)+K_{CuCO3BL}\cdot K_{CuCO3}\cdot\left(CO_3^{2-}\right)} \quad (11)$$

and a new non-linear regression analysis should be performed using:

$$21d-EC50_{Cu^{2+}} = \left[EC50_{Cu^{2+}}\right]_0 \cdot \frac{1+471\cdot\left\{1+K_{HBL}\cdot 10^{-6.8}\right\}\cdot\left(Na^{+}\right)+K_{HBL}\cdot\left(H^{+}\right)}{1+R_{CuOHBL}\cdot K_{CuOH}\cdot\left(OH^{-}\right)+R_{CuCO3BL}\cdot K_{CuCO3}\cdot\left(CO_3^{2-}\right)} \quad (12)$$

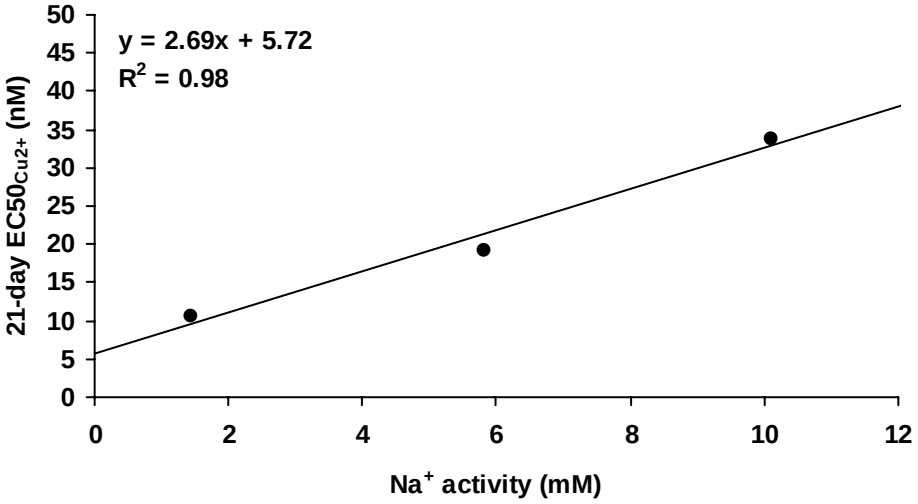

Figure 4-4 The effect of increased Na^+-activity on the chronic toxicity of copper (expressed as Cu^{2+}-activity) to Daphnia magna. Speciation was calculated with BLM for windows version 1.0.0 (Hydroqual, 2002). The solid line represents the linear relation that was used to estimate log K_{NaBL}. This constant incorporates the Na effect in the chronic Cu-BLM (see text, Eqn. 8-10).

The regression was performed as described previously. The derived regression parameters and BLM constants are given in Table 4-7. This model will be referred to as c-CuBLM-2. The new constants seem more reliable as $\left[21d-EC50_{Cu^{2+}}\right]_0$ = 2.78 nM is lower than the acute $\left[48h-EC50_{Cu^{2+}}\right]_0$ = 8.47. Other stability constants are similar to the values obtained without Na competition (c-CuBLM-1) included in the model (except log K_{HBL}, which was about about 0.5 log unit higher). Log K_{NaBL} (2.91) is similar to the competition constant derived for acute (48-hour) exposures (log K_{NaBL} = 3.19).

Together with the observation that the competition of calcium and magnesium with copper were not significant with regard to effects on reproduction, this suggests an increased importance of a physiological component of the chronic model. Indeed, some authors suggest the Na^+ "competition" effect to be a physiological one related to the mode of toxic action of copper (Wood, 1992; Grosell et al., 2002a). Mortality induced by copper is believed to occur as a result of the net loss of plasma electrolytes from body fluids (including Na^+). Increased Na^+ levels in the exposure water may, however, prevent the net loss of plasma electrolytes from body fluids

(including Na^+) induced by copper exposure (Wood, 1992; Grosell et al., 2002a) and thus prevent mortality. The observed effect of Na on chronic toxicity indeed seems to be a mainly mortality-driven effect: elevated Na concentrations resulted in a decrease of the copper-induced 21-day mortality but not in a decrease of the copper-induced reproduction reduction of the surviving animals (data not shown).

Calcium and magnesium do not share common uptake pathways with copper and thus the competition by these ions at lethal copper concentrations is probably mostly of a chemical nature. Possibly, the importance of chemical competition decreases with both increasing exposure duration and decreasing (sublethal) copper concentrations. This hypothesis is supported by the fact that calcium and magnesium very effectively compete with zinc to reduce both acute and chronic zinc toxicity to *Daphnia magna* whereas the competition with Na is much less effective (Heijerick et al., 2002b). Indeed, calcium and magnesium do share common uptake pathways with zinc, whereas Na does not.

4.3.8. BLM development: the effect of DOM type (chronic Cu-BLM-3)

c-CuBLM-2 was also used to correct $EC50_{Cu2+}$ values for the combined effect of pH and Na in a similar manner as described above (calculation of O/P). The correlation of O/P with DOC concentration was significant ($r = -0.58$, $p<0.001$, $n = 34$), thus pointing again to the possible bioavailability of Cu-DOC complexes. In order to investigate this in more detail, the correlation coefficient between O/P and the DOC concentration was calculated for each DOM type. For Bihain, Ossenkolk and Ankeveen DOM, r-values were -0.70, -0.82 and -0.60, respectively and all relations were significant ($p<0.001$). This seems to suggest that Cu-complexes with all three DOM types are toxic to *D. magna*. However, it should be noted that for Ankeveen DOM, the statistical significance of this relation was due to the very high O/P for test medium 4 (O/P = 6.6). Thus, in test medium 4, the observed $EC50_{Cu2+}$ was much higher than the predicted $EC50_{Cu2+}$. Since test medium 4 was the test medium with the lowest DOC concentration, it is most susceptible to copper speciation changes, which may occur between two renewals caused by DOC excretion by both daphnids and algae. Therefore r-values were re-calculated excluding test medium 4. Now the r-values were -0.89, -0.77 and -0.34 for Bihain, Ossenkolk and Ankeveen DOM, respectively. Comparison with the previously derived r-values showed that whereas r-values for Bihain and Ossenkolk DOM remained similar and significant, the r-value for Ankeveen DOM was now much lower and the correlation was not significant anymore.

This finding may explain the fact that, although the three DOMs have different copper complexation capacities, they all protect against chronic copper toxicity to the same degree (on a dissolved basis). Bihain and Ossenkolk DOM have a higher complexation capacity than Ankeveen DOM, but copper complexes with Bihain and Ossenkolk DOM seem to be bioavailable to some extent whereas this is not the case for Ankeveen DOM. Although most studies report the non-bioavailability of copper complexes with organic matter (De Schamphelaere et al., 2002, chapter 3; Meador, 1991; Sunda and Lewis, 1978) some documented mechanisms may explain the bioavailability of DOC-complexed copper observed in this study.

For example, Tao et al. (2000) demonstrated the apparent uptake of fulvic acid complexed copper in the gills of *Paracheirdon innesi*. They suggested that the complex first adhered to the mucus of the epithelial cell surface after which kinetically controlled dissociation of the (labile) complex resulted in uptake of the released free copper ion. Erickson et al. (1996) suggested a similar mechanism to explain the slight bioavailability of copper complexed to humic acid. With the present data, however, no definite answers can be given regarding to the toxicity of Cu-DOM complexes, especially since it might be dependent on DOM-type (this study), exposure duration (acute versus chronic exposures), species (the alga *P. subcapitata* vs. *D. magna*, see chapter 6) and/or experimental set-up (flow-through vs. semi-static, Erickson et al., 1996). Clearly more research is needed to elucidate these toxicity mechanisms.

From a modelling point of view, the bioavailability of Cu-DOC complexes is not incorporated in one of the above-mentioned BLM equations. Consequently these equations may be considered incorrect to some degree. However, these equations do apply for DOM types whose copper complexes are not bioavailable (i.e. Ankeveen DOM). Therefore, a new regression was performed with Eqn. 12 on the Ankeveen toxicity data only, resulting in BLM constants that are not biased by the possible DOC effect observed for Bihain and Ossenkolk DOM. For this regression, $EC50_{Cu2+}$ values were used that were calculated using the optimal % FA assumption. These values provide a better estimate of real copper speciation since this optimal %FA was calibrated to a large dataset using measured Cu^{2+} activities (see further, chapter 6). These constants are presented in Table 4-7 and the model based on these constants will be referred to as c-CuBLM-3.

As these new stability constants are similar to those established earlier, it could be concluded that these estimates and the resulting model are independent of the DOC-assumptions made. The main difference between c-CuBLM-2 and c-CuBLM-3 is in the estimated sensitivity of *D. magna*: c-CuBLM-3 is calibrated to higher Cu^{2+}-activities and thus generally predicts somewhat higher toxicity threshold values.

4.3.9. Model validation

To demonstrate the advantages and/or disadvantages of using c-CuBLM-3 versus c-CuBLM-2, the predictive capacity of both models was evaluated (Figure 4-5). For c-CuBLM-2 the 50% FA assumption was used, for c-CuBLM-3 the optimal% FA was used. With c-BLM-2 the errors on the predicted effect concentrations seem to be dependent on the effect concentration resulting in over-predictions at high observed effect concentrations and under-predictions at low observed effect concentrations. Since DOC is the main factor determining the NOEC and the EC50 this is due to the fact that the bioavailability of Cu-complexes with Bihain and Ossenkolk DOM was not accounted for when deriving the c-CuBLM-2 constants.

Although c-CuBLM-2 predicts 86% of the effects concentration with an error of less than factor 2, this kind of dependency usually designates a model as being incorrect. c-BLM-3, developed with data from tests with Ankeveen DOM only, does not show this kind of dependency of prediction errors. Over-predictions and under-predictions occur equally along the whole range NOEC and EC50s values. All NOECs and EC50s of Ankeveen toxicity data were predicted within a factor 2 of observed values. Naturally, c-BLM-3, overpredicts NOECs and EC50s obtained with the tests with Bihain and Ossenkolk DOM. Again, this is logic, since the bioavailability of Cu-complexes with the latter DOMs was neglected. However, from a viewpoint of protecting the environment, the model does seem to correctly predict toxicity in the most critical region, i.e. where NOEC-values are lowest. Anyhow, it should be concluded that c-CuBLM-3 will correctly predict toxicity independently of DOC-concentration in the cases where DOM does not form significantly bioavailable complexes with copper.

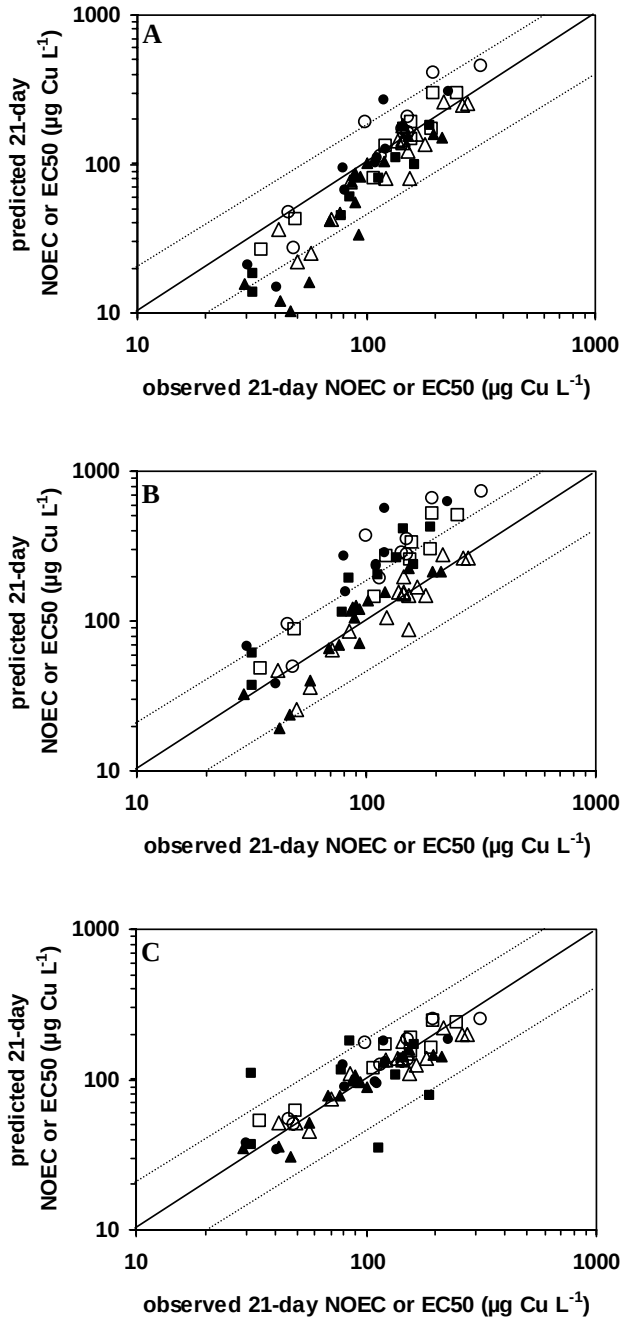

Figure 4-5 Predictive capacity of c-CuBLM-2 (A) and c-CuBLM-3 (B) and the empirical regression equations 1 and 2 (C) as shown by observed versus predicted 21-day NOECs and 21-day EC50s. DOC-assumption: 50% active fulvic acid (A) and optimal % fulvic acid (B). Symbols: Bihain NOECs (●), Bihain EC50s (○), Ossenkolk NOECs (■), Ossenkolk EC50s (□), Ankeveen NOECs (▲), Ankeveen EC50s (Δ). The full line indicates a perfect match between observed and predicted values; the dashed lines indicates a factor of 2 error between observed and predicted values.

In order to test this hypothesis, NOECs and EC50s were predicted for another chronic copper toxicity dataset (Bossuyt et al., unpublished data). These authors performed 21-day reproduction tests with *D. magna* in 10 natural surface water samples of varying water chemistry. Figure 4-6 shows the predictive capacity of both c-BLM-2 and c-BLM-3. Since the characteristics of the DOM in these surface waters are not known, the 50% active FA assumption was used for both models. The same assumption was successfully used to correctly predict 48-hour EC50s of copper to *D. magna* in natural surface water samples (De Schamphelaere et al., 2002). For one surface water, both c-BLM-2 and c-BLM-3 yielded predictions that were up to 8 times higher than the observed values. This large overprediction may be due to the large amounts of iron and aluminum in this sample (data not shown) preventing copper to complex with the DOM . Indeed, because of their higher valency (i.e. 3), Fe(III) and Al (III) ions are known to form very strong complexes with organic matter (thermodynamic database in Tipping, 1994), especially at the low pH of this sample (pH = 5.5).

However, except for this sample, c-BLM-3 generally performs better than c-CuBLM-2. Predictions with c-CuBLM-3 are evenly distributed around the 1:1 reference line whereas c-CuBLM-2 generally underpredicts EC50s and NOECs. This seems to indicate that the bioavailabity of copper complexed to DOM (in these natural surface waters) seemed to be limited. If this would not have been the case, c-BLM-3 would have over-predicted NOECs and EC50s, as observed in tests with isolated DOM.

It can be suggested that Cu-DOM complexes are only biovailable to some extent when using (some) DOM types that have undergone harsh isolation conditions. It remains unclear, however, why this seems only to be the case for Bihain and Ossenkolk DOM and not for Ankeveen DOM. Another possible explanation is that Cu-DOM toxicity might be positively correlated to the complexing capacity, i.e. the higher the complexing capacity, the higher the biovailability of Cu-DOM complexes. Both mechanisms may counteract each other and thus result in no significant effect of DOM-source on chronic copper toxicity. Indeed, when ignoring the complexing properties of the three DOM types and using 50% FA as assumption, 90% of the predicted NOECs and EC50s with c-CuBLM-3 for all three DOM types are within a factor of 2 of the observed values (data not shown).

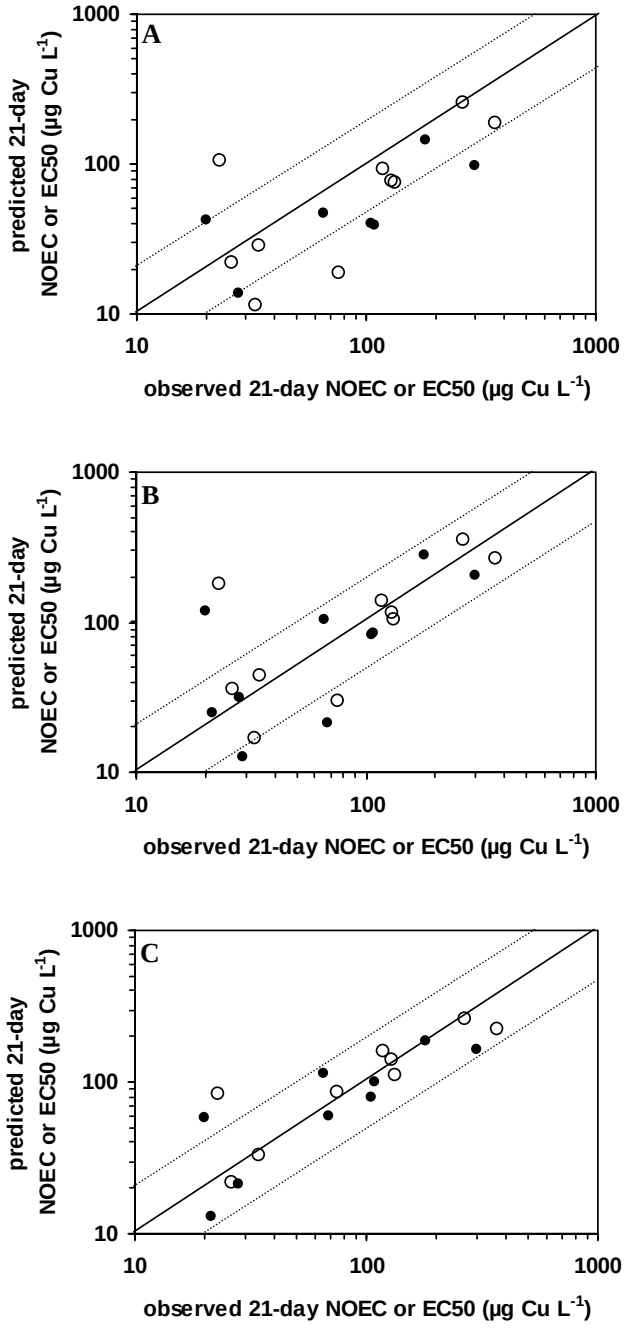

Figure 4-6 Predictive capacity of c-CuBLM-2 (A), c-CuBLM-3 (B) and of empirical regression models (equation 1 and 2) as shown by observed versus predicted 21-day NOECs (●) and 21-day EC50s (○) for tests in natural waters (data from Bossuyt et al., unpublished). The full line is the 1:1 reference line indicating a perfect match between observed and predicted values; the dashed lines indicate a factor of 2 error between observed and predicted values.

Finally, when looking at the predictive capacity of the empirical regression equations 1 and 2, it seems that these simple regression models perform better than the BLMs for the tests in reconstituted media (Figure 4-5) and perform more or less equally well in natural waters as c-CuBLM-3. This does not mean, however, that the empirical models are *in se* better than the biotic ligand models. Although they may be relatively accurate within the ranges of DOC and pH for which they were calibrated, one of the disadvantages (as compared to the BLMs) of the empirical regression equations is that they predict meaningless negative NOECs and EC50s outside these ranges of DOC and pH (i.e. low DOC – low pH combination). Since waters with low pH and DOC are the most sensitive for adverse copper effects to *D. magna*, these are the DOC and pH ranges were good model predictions are most critical. This problem is not encountered with BLM modelling. Moreover, another disadvantage of the empirical regression is that they do not provide profound insights in the bioavailability relationships, whereas with the initial efforts to developed BLM-type models, areas for future research can be identified (see further).

4.3.10. Relevance of BLM-constants

Both $CuOH^+$ and $CuCO_3$ bioavailability and proton competition were shown to be higher in chronic exposures than in acute exposures (See Table 4-7). Although a number of hypotheses can be put forward for this observation, it should be underlined that the set of derived BLM constants for chronic copper toxicity to *D. magna* cannot be interpreted as one that exactly describes the mechanisms underlying the observed relations. Instead, the derivation of these constants is aimed at integrating all mechanisms in such a way to maximize the correlation between observed and predicted toxicity. In other words, the increased $CuOH^+$ and $CuCO_3$ bioavailability and proton competition observed in the chronic tests might be the result of a number of processes.

The most important difference between acute and chronic exposures is probably the fact that physiological components of copper toxicity might become more important with increasing exposure duration, since this allows for acclimation responses to be established. For example, for fish it is known that copper speciation in the gill-micro-environment can be substantially different from speciation in the bulk solution because of the existence of either a mucus-layer or a pH/alkalinity regulation (Playle et al., 1992; Tao et al., 2000). Perhaps, this mechanism may be more important in acclimated organisms (long term exposures) than in non-acclimated organisms

(short term exposures), resulting in higher calculated values of K_{HBL}, K_{CuOHBL} and $K_{CuCO3BL}$ in chronic exposures.

Exposed to sub-lethal Cu-concentrations, organisms can also acclimate to copper resulting in reduced toxicity (Bossuyt et al, 2001). In all BLMs developed up to now, stability constants have been assumed independent of water characteristics and exposure duration. However, it has been demonstrated that in juvenile rainbow trout acclimated to sublethal copper concentrations, Cu-gill binding constants were lower and binding site densities were higher than in non-acclimated rainbow trout (Taylore et al., 2000). If similar processes occur in daphnids and if these processes are pH dependent, this might also explain the observed difference between the acute and chronic Cu-BLM.

Finally, decreased reproduction in chronic exposures may be the result of more than ionoregulatory disturbance alone. It may also be the consequence of increased energy requirements for maintenance or of decreased food assimilation rates (Kooijman, 2000) and dietary copper exposure may also be involved (see chapter 5). However, the fact that the BLM concept did not completely fail in predicting chronic toxicity may point to an important role of an ionoregulatory dependence of toxicity responses. The excat mechanisms of chronic copper exposure on mortality and reproductive success in *D. magna*, however, remains to be determined, and this should be one of the future research topics in BLM modelling of chronic copper toxicity.

4.4. Conclusion

This study demonstrates that DOC concentration is the most important factor for chronic copper toxicity to *D. magna* (explaining about 60% of the observed variability) and that pH has also a significant effect (explaining about 15% of the variability). Water hardness (at the levels tested) and DOM source, however, did not exert significant effects. Interactive effects between the DOC concentration, pH and hardness were also not significant. Empirical regression equations were derived to predict NOECs and EC50s. Additionally, based on the ecotoxicological test results and speciation calculations, a chronic copper BLM was developed. Important differences with the acute CuBLM are increased bioavailability of $CuOH^+$ and $CuCO_3$, increased proton competition and the absence of a hardness effect. The Na effect seemed to be similar in chronic and in acute exposures, indicating an increased importance of physiological

processes in chronic exposures. Although limited toxicity of complexes of copper with a few types of (isolated) DOM was observed, this was not clearly supported by validation efforts for tests in natural water samples. Hence, this factor was not included in the chronic BLM. Although clearly more research is needed to fully understand the mechanisms underpinning the bioavailability relationships observed in this study, the developed BLM has a good predictive capacity and may contribute to the improvement of the ecological relevance of current risk assessment procedures of copper.

Chapter 5

Effects of chronic dietary copper exposure on growth, reproduction and copper accumulation of *Daphnia magna*

Redrafted from:

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Chapter

5

Effects of chronic dietary copper exposure on growth, reproduction and copper accumulation of *Daphnia magna*

Abstract - An important issue and a current matter of intensive debate with regard to metal toxicity is the potential toxicity of dietary metals such as metals associated with food particles. Recently developed biotic ligand models (BLM) typically predict the toxicity of waterborne metals and these models may not be valid if the dietary exposure route contributed to metal toxicity. The present study is to our knowledge the first that investigates the potential toxicity of dietary copper to a freshwater invertebrate (i.e. *Daphnia magna*) feeding on a live diet (i.e. cells of the green alga *Pseudokircheneriella subcapitata*). Algae were first exposed for 3 days to different copper concentrations and this resulted in copper burdens of the algae between about $6 \cdot 10^{-16}$ and $200 \cdot 10^{-16}$ g Cu $cell^{-1}$. These algae were then used as food in chronic 21-day *D. magna* toxicity tests in which growth, reproduction and copper accumulation was assessed. Test media for both *D. magna* and *P. subcapitata* had the same physico-chemistry, i.e. dissolved organic carbon = 10 mg L^{-1}, pH ~ 6.8 and hardness ~250 mg $CaCO_3$ L^{-1}. Three exposure scenarios were tested: a waterborne exposure, a dietary exposure and a waterborne + dietary exposure. First, it was demonstrated that the 21-day NOEC and EC50 for reproduction were nearly identical in the waterborne and in the waterborne + dietary exposure, being 95 and 110 µg Cu L^{-1} (as dissolved copper) respectively. This indicated that the contribution of dietary copper to toxicity was unimportant and did not interfere with BLM-predicted chronic copper toxicity. Although the exposure to dietary copper resulted in an increased copper body burden of the adult daphnids, no toxicity was observed. On the contrary, the addition of a dietary exposure route resulted up to a 75% increase in growth and reproduction at the highest dietary exposure levels. In order to better understand this stimulatory effect, a model was developed for copper adsorption, uptake and elimination in *P. subcapitata* to allow to estimate the possible variation of algal copper burdens and copper ingestion rates of the daphnids in all the *D. magna* exposure scenarios. As such the average level of dietary exposure throughout the test could be estimated and it was demonstrated that the stimulation of growth and reproduction were indeed significantly related to these dietary exposure levels (p<0.05). To our knowledge this is the first evidence that dietary copper exposure of a fresh water invertebrate feeding on a live diet resulted in a beneficial effect. Further research is, however, needed to elucidate the ecological importance of this process.

5.1. Introduction

During the last decade regulators, industry, and academic scientists have started to recognize that the effects of bioavailability mitigating parameters on metal toxicity need to be taken into account in deriving environmental quality criteria (Janssen et al., 2000; Bergmann and Dorward-King, 1997). In that context, the biotic ligand model (BLM) has shown to be a promising and integrative concept/tool to evaluate and predict the effects of physico-chemical parameters (such as dissolved organic carbon, pH, water hardness, sodium, alkalinity) on acute (lethal) metal toxicity to several freshwater species (De Schamphelaere and Janssen, 2002, chapter 2; De Schamphelaere et al., 2002, chapter 3; Di Toro et al., 2001; Santore et al., 2001; Heijerick et al., 2002b, Santore et al., 2002).

The BLM-concept was originally proposed for predicting the toxicity of metals in the dissolved phase (waterborne exposure) and does not (yet) include the possible impact of metals associated with the particulate phase (e.g. food particles = dietary exposure). A relatively large number of studies on dietary metal uptake and accumulation is available (e.g. Wang and Fisher, 1999) and these have generally demonstrated that metals accumulated from the dissolved phase are more likely to deposit in gill tissues (generally considered to be the main target site of lethal toxic action) whereas metals taken up from food are mainly deposited in internal tissues. Due this differential distribution it is likely that dietary metals could exert toxicity via different mechanisms than dissolved metals (Hook and Fisher, 2001b). However, uptake and bioaccumulation of metals are not consistently related to toxicological effects and hence increased uptake and/or bioaccumulation through the dietary pathway cannot be considered sufficient evidence of dietary metal toxicity (Brix and DeForest, 1999). Still, the number of studies investigating toxicity of dietborne metals is rather limited.

Clearwater et al. (2002) reviewed the literature on the toxicity of dietary copper and zinc to several fish species and concluded that it is highly dependent on and/or influenced by factors such as species, life stage, feeding rations and daily doses, diet composition, nutritional value of the food, feeding with laboratory prepared (i.e. artificial) versus live diets (i.e. biologically incorporated metals). With regard to the latter feeding with biologically incorporated metals can either result in higher (e.g. when metals are bound to readily

absorbable methionine or proteinate complexes, Paripatananont and Lovel, 1995, 1997) or lower toxicity (e.g. when metals are complexed in insoluble granules; Bryan and Gibbs, 1983) as compared to laboratory-prepared diets. The lack of studies on biologically incorporated dietary copper makes it difficult to assess the comparative bioavailability of Cu in natural diets versus in laboratory-prepared diets (Clearwater et al., 2002). Although invertebrates such as crustacean zooplankton are important component of pelagic food webs, dietary metal toxicity studies with these animals are very scarce.

Hook and Fisher have demonstrated in short-term feeding experiments the potentially reduced reproductive output upon dietary exposure to Hg, Cd, Ag, Mn and Zn in marine copepods feeding on diatoms (Hook and Fisher, 2001a, 2002) and to dietary Ag in freshwater cladocerans feeding on a green alga (Hook and Fisher, 2001b). The fact that prolonged feeding with Zn-contaminated diatoms did not longer result in toxicity to the copepods (Hook and Fisher, 2001a), adds to the complexity of understanding and interpreting dietary metal toxicity.

The issue of dietary metal toxicity to filter-feeding organisms such as cladocerans is extremely important. Indeed, standard chronic toxicity testing protocols with these organisms (e.g. OECD, 1998), which are the basis of regulatory assessments, are performed in test media to which sufficient food (mostly green algae) is added to ensure sufficient reproduction. Hence, dietary exposure may occur. Indeed, the food-algae can take up dissolved copper from the exposure solution before being ingested by the daphnids. Since the BLM concept is designed to predict toxicity of metals in the dissolved phase (i.e. waterborne exposure), this model can only be valid if the dietary copper exposure in these tests do not contribute to the overall toxicity effects. De Schamphelaere and Janssen (2003b, chapter 4) have developed a BLM to predict chronic waterborne copper toxicity to *Daphnia magna* assuming that there was no contribution of dietary copper to toxicity in the tests that were used for this BLM development. However, if this assumption is wrong, the developed model would mechanistically be incorrect and could possibly not be applicable to other conditions (food type, food ration). Contrary, if the dietary exposure route in these tests did not contribute to the overall toxic effect then the developed BLM would be applicable to all other situations in which the dietary toxicity is negligible.

The major aim of this study was to test the hypothesis if dietary exposure affects the (dissolved) effect concentrations of copper in (standard) chronic toxicity tests with *D. magna*. To that end, a series of carefully designed experiments was conducted that allowed to discriminate between effects of waterborne and dietary copper exposure on growth and reproduction in a standard chronic toxicity test. Great care was taken to ensure the best possible level of ecological relevance (e.g. same test media for exposing algal food and *D. magna*) and to monitor dissolved copper concentrations and feeding rates of the daphnids. Finally a preliminary model was developed that allows to simulate the potential variations of dietary copper exposure in these types of tests, which allowed to mechanistically understand the observed effects of chronic dietary copper exposure of *D. magna*.

5.2. Materials and Methods

5.2.1. Experimental design and terminology

Batches of the green alga *Pseudokirchneriella subcapitata* (one of the two algae species used as food for *D. magna* in chronic toxicity tests in our laboratory and also in the tests that were used to develop the chronic Cu-BLM, De Schamphelaere and Janssen, 2003b, chapter 4) were cultured for 3 days in 6 different copper concentrations and a control. As such, at the end of this exposure period, control algae (with only a background copper burden) and algae with 6 different elevated copper burdens were harvested. These algae were subsequently used as the food in standard 21-day chronic toxicity tests with *D. magna*. Three types of chronic exposure of the daphnids to copper were simultaneously performed: (1) waterborne exposure (2) dietary exposure and (3) waterborne + dietary exposure. In the waterborne exposures copper (the same 6 Cu concentrations as used in the algae cultures) was spiked into the exposure solution and daphnids were fed with control algae. This type of exposure is representative of the standard toxicity test procedure in which (initially) there is only background dietary exposure. In the dietary exposure daphnids were exposed to control solution and were fed with algae with increased copper burdens (initially only background dissolved copper in the exposure solution). In the waterborne + dietary exposure daphnids were exposed to dissolved copper in solution (same 6 concentrations) and were fed algae that had been cultured in the same copper concentration. Next to these exposures, one control exposure was also conducted in which no copper was added to the dissolved phase and control algae were used as food.

Dissolved copper concentrations, survival, reproduction and feeding rates of the daphnids were monitored throughout the 21-day exposure. At the end of the tests dry weight (growth) and copper body burdens of the daphnids were determined.

5.2.2. Test medium

Exposures of *Pseudokirchneriella subcapitata* and *D. magna* were performed in a reconstituted test medium (deionized water as basis with addition of reagent grade salts) containing natural dissolved organic carbon that had been collected by reverse osmosis from the Ankeveensche Plassen, a large lake system in a natural reserve in the Netherlands (for a detailed description, see chapter 4, section 4.2.1.). The composition of the test media is given in Table 5-1. The test medium is similar in composition as the center point test medium in a previous study (average pH, hardness and DOC concentration compared to the range for which the chronic BLM has been developed, chapter 4, center point in Table 4-2). In addition to the here-reported concentrations, the alga test medium also contained nutrients in the concentrations described in OECD test guideline 201 (OECD, 1984a). The strong metal-chelator ethylene diamine tetra acetic acid (EDTA), normally added in standard OECD medium to keep Fe in solution, was not added. Instead, natural organic carbon was used as this has been demonstrated to perform this task equally well (Heijerick et al., 2002a). In order to buffer pH, 750 mg L^{-1} MOPS (3-N morpholino propane sulfonic acid, Sigma-Aldrich, Steinheim, Germany) was added to the test media. MOPS was chosen because it is completely non-complexing for metals (Kandegedara and Rorabacher, 1999), it does not change the toxicity of toxic effluents and sediment pore waters (US EPA, 1991) and it does not alter copper or zinc toxicity to *P. subcapitata* and *Daphnia magna* (De Schamphelaere et al., 2003b) The test medium was adjusted to a pH level of approximately 6.8 with NaOH or HCl and was spiked with different copper concentrations ($CuCl_2$). No copper was added to the control culture of algae and to the control exposure of the daphnids.

Table 5-1 Major (dissolved) composition of test media for exposure of the alga *Pseudokirchneriella subcapitata* and *Daphnia magna*.

	pH [a]	DOC [b]	Ca [c]	Mg [c]	Na [c]	K [c]	SO_4 [c]	Cl [c]	IC [d]	Fe [e]	Al [e]
P; subcapitata	6.80 (6.76-6.91)	10	2.0	0.5	1.8	0.08	0.50	4.1	0.15	4.9	2.0
D. magna	6.87 (6.81-6.97)	10	2.0	0.5	1.8	0.08	0.50	4.1	0.17	4.9	2.0

[a] mean (minimum – maximum)

[b] dissolved organic carbon (mg / L)

[c] mM

[d] inorganic carbon (mM)

[e] µg/L

5.2.3. Algae exposure

P. subcapitata Printz starter cultures were obtained from the Culture Collection of Algae and Protozoa (CCAP 278/4, Ambleside, UK). Stock cultures of algae have been maintained for several years in aerated Ghent city tap water enriched with nutrients. These stock cultures are kept at 20 ± 1°C under continuous light (5000 lux) and with continuous aeration. Log-phase algae were used for starting the exposures. Copper concentrations tested were 0, 35, 50, 70, 100, 140 and 200 µg L^{-1}. For each copper concentration, 10 L of test medium was prepared, spiked with copper and transferred into a 12L poly-ethylene bag. The spiked media were equilibrated in complete darkness for 48 hours at 4°C and 48 hours at 20°C before the exposure. At the start of the exposure, each bag was inoculated with $4 \cdot 10^5$ cells mL^{-1}. Exposures were carried out under continuous illumination (5000 lux). The temperature in the media during the exposure period was 20°C ± 1°C. The cell density was recorded in triplicate after 48 and after 72 hours with the aid of an electronic particle counter (Model DN, Harpenden, Herts, UK) and the specific growth rate µ (d^{-1}) was determined according to OECD guideline 201 (OECD, 1984a). pH was adjusted twice daily to 6.8 with dilute NaOH or HCl.

After 72 hours cell suspensions were centrifuged to harvest the algae, the supernatans was carefully pipetted off and the obtained pellets were suspended in a specified volume of supernatant (i.e. the same solution chemistry and copper content as they were exposed to) to yield a cell density of $8 \cdot 10^7$ cells mL^{-1}. The latter was done to minimize leaching of copper out of the algae during storage of the suspensions and to ensure a constant Cu burden of the algae during the period in which they were used as food in the chronic toxicity tests with *D. magna*. Finally, suspensions of the cells were stored in darkness at 4°C throughout the whole daphnid testing period.

5.2.4. Copper burdens of the algae

After 2 days of exposure and at the start and at the 21st day of the daphnid exposure three sub-samples of 625 µL ($5 \cdot 10^7$ cells) of each cell suspension were transferred into 50 mL metal-free polyethylene tubes for the determination of internal and external copper burdens of the algae. The algae were first washed with 50 mL of of a 0.01 M KNO_3 + 5 mM Na_2EDTA solution (pH = 6.8) to remove external (surface-bound, adsorbed) copper. This was

accomplished by shaking the algal suspensions for 10 minutes and subsequently centrifuging them at 500 g for 10 minutes. One-hundred µL of 14N $HN0_3$ was added to 9.9 mL of filtered (0.45 µm) supernatant. The Cu concentrations of these samples were immediately analyzed and this resulted in the external copper burden of the algal cells. Next the pellet was digested in 500 µL of 14N HNO_3. After digestion, 4.5 mL of double deionized water was added and the Cu content of the samples was determined, resulting in the internal copper burden of the algae.

Copper analyses for both internal and external copper burdens of the algae were performed with graphite furnace atomic absorption spectrophotometry (SpectrAA800 with Zeeman background correction, Varian, Mulgrave, Australia). A reference plankton sample (BCR-414, Institute for Reference Materials and Measurements, Geel, Belgium) was analyzed using the same method and measured copper levels were always within 10% of the certified value.

Metal concentrations were determined and reported as g Cu $cell^{-1}$ and can be recalculated to µg Cu (g dry weight)$^{-1}$ using an average (± SEM, standard error of mean, n = 21) cell dry weight of $1.47\pm0.04 \cdot 10^{-11}$ g $cell^{-1}$. Average cell weight of the algae was determined in triplicate for all tested Cu concentrations and was observed not to be significantly different between treatments (one-way analysis of variance, $p>0.05$). In parallel, the organic carbon:dry weight ratio was determined using a total organic carbon analyzer (TOC-5000, Shimadzu, Duisburg, Germany). This ratio was 0.40±0.04 and was also not significantly different across different copper exposures.

5.2.5. Chronic exposures of daphnids

Four days before the start of the chronic bioassays (tests were started 6 days after the harvesting of the algal cells) test media were spiked with nominal copper concentrations of 0, 35, 50, 70, 100, 140 and 200 µg L^{-1}. Throughout the test period, test media were stored in darkness at 4°C. Forty eight hours prior to use (for each test medium renewal) appropriate volumes of these media were transferred to and equilibrated at 20°C. Next to a control treatment (control copper in solution and daphnids fed with control algae), 18 different treatments were tested (see also under experimental design): 6 treatments with copper in solution only (waterborne exposure), 6 treatments in control solution and with copper-laden

food (dietary exposure), and 6 treatments with copper in both solution and in food (waterborne + dietary exposure).

For all of these treatments chronic bioassays were performed according to OECD guideline 211 (OECD, 1998). Test organisms originated from a healthy *D. magna* clone (K6) which has been cultured in the laboratory under standardized conditions in M4 medium (Elendt and Bias, 1990) for several years. At the start of each test, 10 juvenile animals (<24 hours old, 8.0±0.2 µg dry weight, mean ± SEM, n=3 samples of 20 organisms each) per concentration were transferred individually to polyethylene cups containing 50 mL of the test medium (i.e. 10 replicates of one organism per concentration). Every day the organisms were fed with $8 \cdot 10^6$ cells per day from day 0 to day 6, with $12 \cdot 10^6$ cells per day from day 7 to day 8 and with $16 \cdot 10^6$ cells per day from day 9 to day 20, corresponding with food rations of 120, 180 and 240 µg dry weight (DW) per daphnid per day or 47, 70 and 93 µg total organic carbon (TOC) per daphnid per day depending on the age of the daphnid. On day 2, 5, 7, 9, 12, 14, 16 and 19 the medium was renewed and parent mortality and the number of juveniles was noted. At the end of the 21 day exposure period, mortality and the number juveniles was noted for the last time and parent daphnids were collected for weight and copper body burden determination.

The latter was accomplished by washing the daphnids in control test medium for 10 minutes (to remove adhered particles) and subsequently in a 5 mM Na_2EDTA solution for 20 minutes (to remove metal externally bound to the exoskeleton). Daphnids were then dried individually at 40°C (until no further decrease in weight was observed) and were weighed to the nearest 1 µg. Daphnids from each treatment were then pooled and digested in 500 µL of 14N HNO_3. To the digest 4.5 mL of double deionized water was added and the Cu content of the sample was analyzed using graphite furnace AAS. The Cu content of the samples was used to calculate internal body burdens of the daphnids, expressed as µg Cu $(g\ DW)^{-1}$.

5.2.6. Algal ingestion rates by daphnids

To estimate copper ingestion rates of the daphnids, it was deemed necessary to have measurements on cell ingestion rates throughout the exposure. To that end, immediately before each test media renewal algal concentrations remaining in the test vessels (i.e. uneaten cells) were measured in pooled samples of all replicates (n=10). The algal concentration

remaining in the test vessels was between about 50,000 and 750,000 cells mL^{-1} (average ~ 350,000 cells mL^{-1}). This corresponds to organic carbon levels between 0.28 and 4.0 mg C L^{-1} with an average of 1.7 mg C L^{-1}. Since these levels were always higher than the incipient limiting level reported for *Daphnia magna* feeding on another green alga *Chlamydomonas* (0.2 mg C L^{-1}) and for most other *Daphnia* species feeding on various green algae (Lampert, 1987), it is reasonable to assume for our further calculations that the ingestion rate in-between two test media renewals was near maximal (note: the incipient limiting level is the food concentration above which ingestion rate is assumed to be maximal). Possible interference by algal ingestion by new-born juveniles was considered very minimal. Average cell ingestion rate of the adults was estimated from the difference between added cells and remaining cells just before test media renewal.

5.2.7. Chemical analyses

Copper concentrations were determined using a flame-atomic absorption spectrophotometer (Cu > 20 µg L^{-1}, SpectrAA100, Varian, Mulgrave, Australia) or a graphite furnace atomic absorption spectrophotometer (Cu < 20 µg L^{-1}, SpectrAA800 with Zeeman background correction, Varian, Mulgrave, Australia). Calibration standards (Sigma-Aldrich, Steinheim, Germany) and a reagent blank were analysed with every ten samples. In the algae exposures total copper was measured at the start of the exposure to check if total concentrations were comparable to nominal concentrations. Dissolved copper (filter through 0.45 µm, Gelman Sciences, Ann Arbor, Michigan, U.S.) was measured daily in the algal tests and just before each medium renewal in the *D. magna* tests.

DOC (0.45 µm filtered) and inorganic carbon (IC) in the test media was measured before the tests in all treatments (TOC-5000, Shimadzu, Duisburg, Germany). DOC was for all the samples within 5% of the nominal value of 10 mg L^{-1}. Reported concentrations of major cations and anions (Table 1) are nominal values. Previous studies have shown that these were always within 10% of the nominal concentrations (De Schamphelaere et al., 2003a). pH-measurements were performed daily in the algal tests and with each renewal in the *D. magna* tests (pH-meter P407, Consort, Turnhout, Belgium). The pH glass electrode was calibrated before each use using pH 4 and pH 7 buffers (Merck, Darmstadt, Germany).

5.2.8. Speciation calculations

Speciation calculations were performed using the BLM software (HydroQual, 2002) using all inorganic parameters (pH, Ca, Mg, Na, K, SO_4, Cl and IC), the DOC concentration and the dissolved Cu concentration as input values. The organic matter used in this study was assumed to consist of 41.4 % active fulvic acid and 58.6 % of inert organic matter, as determined by De Schamphelaere et al. (2003a, chapter 6) based on a large number of Cu^{2+} ion-selective electrode measurements over a large range of copper concentrations (~ 10 to 1000 µg Cu L^{-1}) and for a large range of water characteristics (including DOC concentration).

5.2.8. Statistics

Data are reported as mean ± standard error of mean (SEM), unless noted otherwise. Growth rates in the algae exposures were compared to the control growth rates using two-sided Student *t*-tests ($p<0.05$). Normality assumptions (Shapiro-Wilkinson, $p<0.05$) and homogeneity of variances assumptions (Bartlett, $p<0.05$) were fulfilled. Since the latter was not the case for many growth and reproduction data of *D. magna*, data were first log-transformed before performing *t*-tests. More specified statistical analyses are explained in the results and discussion section. All statistics were carried out using Statistica software (Statsoft, Tulsa, Ok, U.S.A.)

5.3. Results and Discussion

5.3.1. Growth and algal copper burdens during exposure

Total copper in the control at the start of the exposure was 2.4 µg Cu L^{-1}. Dissolved copper in the control varied between 1.1 and 2.8 µg Cu L^{-1} (with a mean ± SEM of 2.1± 0.4 µg Cu L^{-1}, n=4) but no time-dependent trend was observed. For the other copper concentrations, at the start of the exposures dissolved copper represented > 97% of the total copper in all exposures and a time-dependent decrease of dissolved copper was observed, which was related to the adsorption and uptake of copper by the growing algae (Figure 5-1).

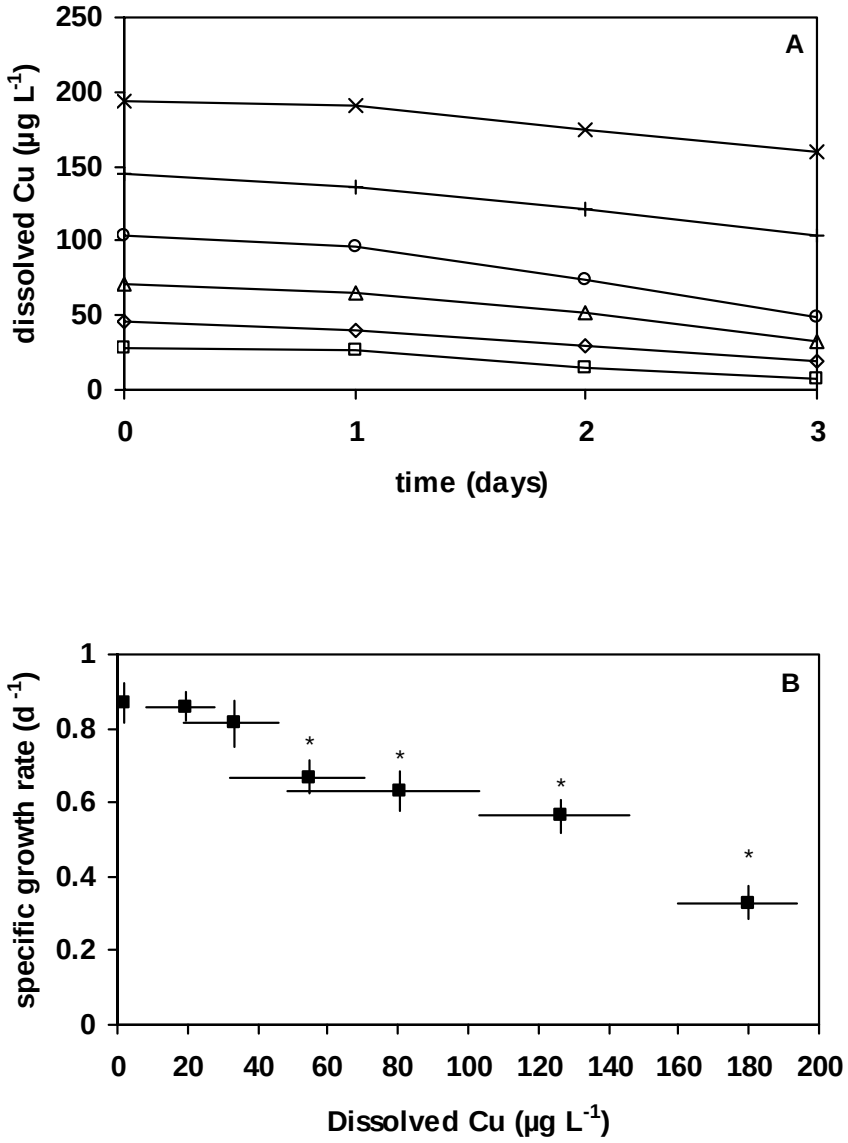

Figure 5-1 Change of measured dissolved copper during 3-day exposure of *Pseudokirchneriella subcapitata* to nominal Cu concentrations of 35 (□), 50 (◊), 70 (Δ), 100 (○), 140 (+) and 200 (x) µg Cu L^{-1} (A) and specific growth rate of this algae as a function of the average dissolved copper concentration in the exposure (B); horizontal error bars indicate minimal (day 3) and maximal (start of exposure) dissolved copper; vertical error bars represent standard error of mean (n=3), * indicates significantly different from control growth (two-sided Student *t*-test, $p<0.05$).

The effect of copper on the growth rate of the algae is also given in Figure 5-1. The no observed effect concentration (NOEC) after 72 hours was 50 µg Cu L^{-1} (nominal; measured dissolved copper was 46 µg Cu L^{-1} at the start of the test; 19 µg Cu L^{-1} after 72 hours), a value very similar to that obtained for this test medium by De Schamphelaere et al. (2003a, chapter 6).

The decrease of dissolved copper in the exposure solutions did not affect the growth rate of the algae: growth rates determined after two days of exposure (not shown) were identical to those determined after three days of exposure. This can be explained by the fact that effects on algal growth are determined by internal copper concentrations (Franklin et al., 2002) and these were observed to remain nearly constant between day 2 and 3. The latter is shown by the mass balance exercise summarized in Table 2. After two days of exposure the mass balance indicates that the sum of the copper on and inside the algal cells (i.e. algal cell density · algal copper burden) and the dissolved copper represents more than 96% of the dissolved copper at the start of the exposures. The total algal burden on day 3 was also calculated using the remaining dissolved copper and the cell density at that time. The estimated copper burdens of the algae were very similar to those obtained after two days, demonstrating that a near equilibrium copper burden is reached within 3 days of exposure.

5.3.2. Algal copper burdens during storage

Internal, external and total algal copper burdens during storage are also presented in Table 5-2. The average copper burden of the control food-algae was $6.17 \cdot 10^{-16}$ g Cu $cell^{-1}$ or about 42 µg Cu g DW^{-1}. This is about factor 6 higher than the algal mix that was used in chronic toxicity tests (i.e. about 7 µg g DW^{-1}) performed to develop the chronic Cu-BLM (De Schamphelaere and Janssen, 2003b, chapter 4). This difference is probably due to the low copper concentration (~ 1.5 µg Cu L^{-1}) and the high EDTA-concentration in the stock culture of the latter algae, resulting in a very low Cu availability. However it is safe to assume that, when the latter algae were introduced as food in *D. magna* toxicity tests with similar characteristics as tested in the present study, their copper burdens would soon have reached very similar copper burdens as those observed in the present study, due to the rapid adsorption and uptake kinetics of the algae (see further).

Table 5-2 Algal copper burdens (10^{-16} g Cu $cell^{-1}$, mean ± standard error of mean, n=3) during the exposure and during the storage. * indicates significant differences between storage and exposure, ° indicates significant differences between day 0 and day 21 of the exposure (two-sided Student t-tests on log-transformed values, $p<0.05$, n=3). Values between brackets are total copper burdens at day 2 and day 3 estimated from a mass balance of copper, calculated as (dissolved Cu at start – dissolved copper at day 2 or day 3) / algal cell density at day 2 or day 3. Exposures sharing the same letter in the first column resulted in not-significantly different total and internal copper burdens during the storage (two-sided Student t-tests on log-transformed values, $p<0.05$, n=6, values from day 0 and day 21 pooled)

	During exposure				During storage as food for *Daphnia magna*					
Cu ($\mu g\ L^{-1}$)	Day 2 Internal	Day 2 External	Day 2 Total	Day 3 Total	Day 0 Internal	Day 0 External	Day 0 Total	Day 21 Internal	Day 21 External	Day 21 Total
0 [a]	2.30±0.12	2.80±0.25	5.11±0.34	ND	4.14±0.50	3.53±0.24	7.67±0.61	2.54±0.46	2.13±0.45	4.67±0.91
35 [b]	45.0±3.1	5.36±0.35	50.4±3.4 (50.8)	(39.3)	45.9±2.8	4.16±0.72	50.1±2.3	50.1±3.4	4.57±0.35	55.7±3.0
50 [c]	68.2±4.7	8.36±0.76	76.6±5.4 (69.2)	(60.6)	70.0±4.9	4.99±0.78	75.0±5.7	61.1±4.29	7.23±0.88	68.3±4.4
70 [d]	103±6.5	20.9±1.8	124±8 (129)	(127)	103±6	21.1±1.9	124±6	98.3±5.1	18.6±1.8	117±3
100 [e]	158±4.7	27.7±2.6	186±7 (209)	(204)	178±9	18.5±2.8	197±10	140±11	30.0±3.2	170±13
140 [ef]	137±7	46.2±5.3	183±12 (191)	(198)	233±10 *	19.3±3.0 *	252±13 *	182±9 *°	27.4±3.1 *	209±12°
200 [f]	147±7	76.4±4.3	223±10 (276)	(310)	191±4 *	34.6±3.2 *	226±6	208±8 *	57.8±3.0 *°	266±11*°

ND = not determined as it could not be calculated due to high variability of dissolved copper in control solution

Copper burdens of the algae gradually increased with increased exposure concentrations and during the storage they were all significantly different from each other, up to the exposure to 70 µg Cu L^{-1}. Those exposed to 100 µg Cu L^{-1} had similar burdens as those exposed to 140 µg Cu L^{-1}, who in turn had similar burdens as those exposed to 200 µg Cu L^{-1} (see letter indications in column 1 of Table 5-2). This indicates that some overlap of the magnitude of dietary copper exposure during the daphnid bioassays may have occurred (see also further).

During storage, copper burdens of the algae remained constant up to exposure concentrations of 100 µg Cu L^{-1}. At these concentrations, copper burdens during storage were also not significantly different from those measured during the exposure. Only at the two highest exposure concentrations, changes in algal copper burdens were observed during storage, and also some differences with those measured during the exposure were noted. The changes in internal copper burdens (the most important with regard to dietary effects, see further) during storage were however smaller than 25%. This indicates that the storage conditions were sufficient to ensure a relatively constant copper burden of the food algae, which is crucial for long-term dietary toxicity studies with live diets (Clearwater et al., 2002).

5.3.3. Surface binding, uptake and elimination kinetics of copper by the algae

As the copper burden of algae, which are used as food during chronic *D. magna* toxicity tests, is the result of a dynamic process a kinetic uptake/elimination model is needed to assess possible changes of the algal copper burdens during the time-course of these exposures. This approach allows estimating the range of dietary copper to which the daphnids were exposed during the different exposure scenarios. Indeed, before the algae are consumed, they are subjected to the copper concentration present in the daphnid exposure medium and the algae will strive towards a new equilibrium copper burden. In the waterborne exposure, control algae (with low copper burden) will adsorb and take up copper from the daphnid exposure medium and as such the copper algal burden will increase. In the dietary exposure, copper-laden algae are introduced into a control medium and as such their copper burden may decrease. The algal copper burden in the waterborne + dietary exposure will be the least subjective to change.

In accordance with numerous other papers describing the adsorption and uptake processes of metals in algae (e.g. Wolterbeek et al., 1995; Knauer et al., 1997), it was decided to use a simplified two-compartment model, with a kinetically fast adsorption to the cell surface (external copper) and a much slower uptake into the cell (internal copper). Although it is most likely that these two compartments are linked, the experiments performed in the present study do not support the establishment of such a linked model. Moreover, for the purpose of this paper, i.e. understanding the importance of dietary copper exposure for *D. magna*, such mechanistic model is not really needed. Instead, adsorption and uptake will be treated separately and will both be related directly to the free cupric ion activity in the exposure solution, as this is the key copper species in uptake and adsorption of copper to algae (Xue et al., 1988; Knauer et al., 1997).

The external copper ([Cu-ext], mol $cell^{-1}$) can be related to the free copper ion activity in the exposure medium using following one-site binding model:

$$[Cu-Ext] = \frac{K_{Cu-Ext} \cdot \left(Cu^{2+}\right)}{1 + K_{Cu-Ext} \cdot \left(Cu^{2+}\right)} \cdot [Cu-Ext]_{\max} \qquad (1)$$

With $K_{Cu\text{-}Ext}$ = conditional binding constant of copper to the cell surface (L mol^{-1}), (Cu^{2+}) = the free cupric ion activity (mol L^{-1}) and $[Cu\text{-}Ext]_{max}$ = copper adsorption capacity of the cell surface (g Cu $cell^{-1}$). $K_{Cu\text{-}Ext}$ and $[Cu\text{-}Ext]_{max}$ were determined based on measured adsorbed copper concentrations on day 2 of the algal exposure. Figure 5-2 shows the relation between adsorbed copper and calculated Cu^{2+}-activity. Linearization of Eq. 1 yielded log $K_{Cu\text{-}Ads}$ = 7.81 L mol^{-1} and $[Cu\text{-}Ext]_{max} = 9.88 \cdot 10^{-15}$ g Cu $Cell^{-1}$. The latter corresponds to 10.6 µmol Cu g DW^{-1}, a value very similar to that found by Knauer et al. (1997) for the closely related and similar-sized green alga *Scenedesmus subspicatus* (i.e. 9 µmol g DW^{-1}). Their conditional log $K_{Cu\text{-}Ext}$ of 11 was higher but was obtained at a pH = 7.9, which usually results in higher conditional binding constants for algal surfaces (Xue et al., 1988). Xue et al. (1988) derived a log $K_{Cu\text{-}Ext}$ = 7.6, conditional at pH = 7 for *Chlamydomonas reinhardtii*.

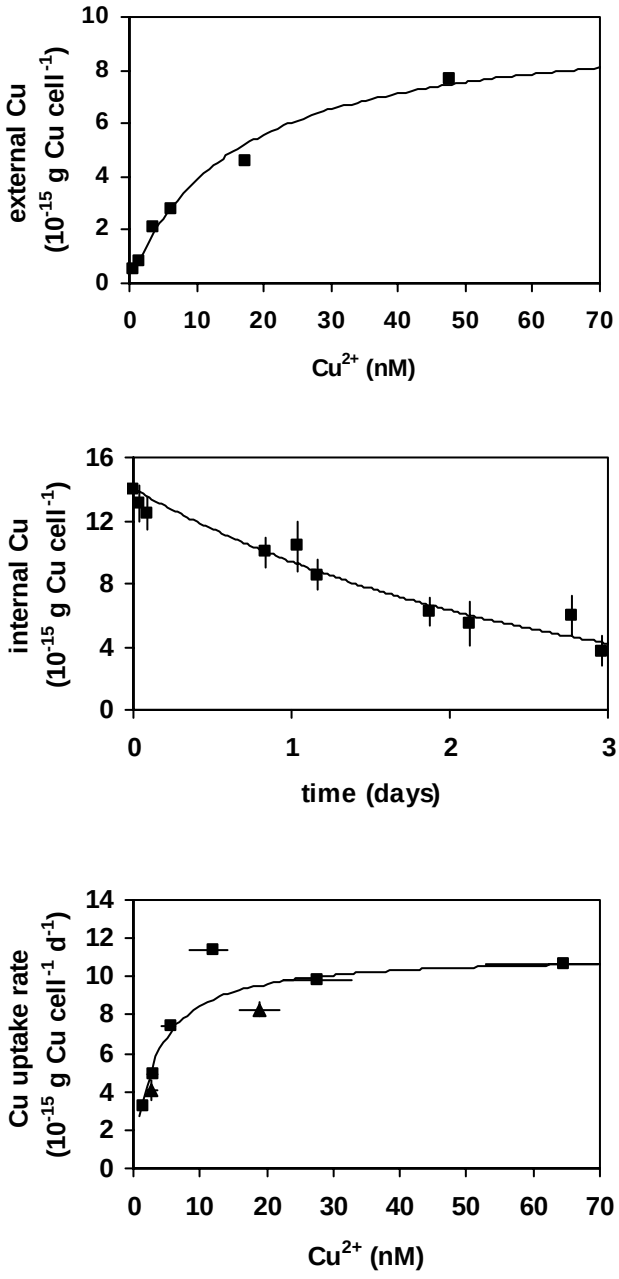

Figure 5-2 Adsorption (A), elimination (B) and uptake (C) of copper in *Pseudokirchneriella subcapitata*; (A) external (adsorbed, surface-bound Cu) as a function of Cu^{2+}-activity; (B) internal copper as a function of time in an elimination experiment, error bars indicate standard error of mean (n=3); (C) uptake rate (of internal copper) as a function of Cu^{2+}-activity. Squares are estimations from the 3-day algal exposure with growth (algal test medium and conditions, see Figure 5-1), triangles are estimations from separate experiments with no growth (*D. magna* test media and conditions). Lines represent fitted curves using the models and model parameters described in the text.

The kinetic uptake and elimination processes can be summarized by the following differential equation:

$$\frac{\partial[Cu-Int]}{\partial t} = k_u - k_e \cdot [Cu-Int] \quad (2)$$

With [Cu-Int] = internal copper burden of algae (g Cu cell^{-1}), k_u = the uptake rate (g Cu cell^{-1} d^{-1}) and k_e = the elimination rate constant (d^{-1}).

The elimination rate is a first-order function of the internal copper burden and k_e was determined from a 3-day kinetic elimination experiment using the algae cultured at 100 µg Cu L^{-1} (nominal) and stored during the full 21 days of the daphnid test, with a copper burden of $1.40 \cdot 10^{-14}$ g Cu Cell^{-1} (internal) and $0.30 \cdot 10^{-14}$ g Cu Cell^{-1} (external). The algae were transferred into 3 replicates of 100 mL of control solution (same physico-chemistry as exposures, containing 1.6 ± 0.2 µg Cu L^{-1}) at a concentration of $3.9 \cdot 10^5$ cells mL^{-1}. After 10 minutes, the adsorbed copper was reduced to background (~ $2.7 \pm 0.2 \cdot 10^{-16}$ g Cu Cell^{-1}) as indicated by the increase of dissolved copper in the solution to 2.6 ± 0.2 µg Cu L^{-1}. Knauer et al. (1997) also observed complete desorption of surface-bound copper from *S. subspicatus* within 10 minutes. From that time onward, the increase of dissolved copper in the medium was monitored at regular intervals (n=10) and this was used to estimate the decrease of the algal internal copper burden. Using an exponential decay regression, an elimination rate k_e = 0.40 ± 0.07 d^{-1} was obtained (mean ± SEM, n=3) (Figure 5-2). To our knowledge, no other reports are available on elimination rates of copper in green algae, but using a similar technique Wolterbeek et al. (1995) found a very similar k_e of 0.5 d^{-1} for Zn in *P. subcapitata*.

For determining uptake rate, Eq. 2 was solved as follows:

$$[Cu-Int]_t = \frac{k_u + \left(k_e \cdot [Cu-Int]_0 - k_u\right) \cdot e^{-k_e \cdot t}}{k_e} \quad (3)$$

With $[Cu\text{-}Int]_t$ and $[Cu\text{-}Int]_0$ the internal copper burdens of the algae after t days of exposure and at the start of the exposure, respectively. The uptake rate k_u for the different copper concentrations of the algae exposure was fitted to the measured internal copper burden at day

2 of the exposure (t=2) and using k_e = 0.4 d^{-1} and $[Cu\text{-}Int]_0$ = 1.91 ± 0.22 · 10^{-16} g Cu $Cell^{-1}$ (mean ± 1 SEM, n=3). The latter is the internal copper burden of the algae taken from the stock cultures immediately before the start of the exposures.

To investigate if the cell growth and/or the specific conditions of the algal exposure did not affect the uptake rate estimates an additional 3-day uptake experiment was carried out in which no algal growth occurred (same physico-chemistry as in algal exposure, but without nutrient addition and with light conditions as in *D. magna* test). Cells exposed for 3 days in the control medium and then stored during the whole 21-day *D. magna* exposure, containing 2.54 · 10^{-16} g Cu $Cell^{-1}$ (internal) and 2.13 · 10^{-16} g Cu $Cell^{-1}$ (adsorbed) were transferred for 3 days into 3 replicates of 100 mL solutions spiked with 53.1 and 133.2 µg Cu L^{-1} at a cell density of 2.1 · 10^6 Cells mL^{-1}. After the initial fast adsorption (10 minutes), the decrease of dissolved copper from the solutions was monitored at regular intervals (n=10) and these were used to calculate the uptake rates k_u (by fitting of Eq. 3 to the data; raw data not shown). Estimated uptake rates (k_u) (mean ± 1 SEM, n=3) were 4.1 ± 0.5 · 10^{-15} g Cu $Cell^{-1}$ d^{-1} and 8.3 ± 0.4 · 10^{-15} g Cu $Cell^{-1}$ d^{-1} for the 53.1 and 133.2 µg L^{-1} exposure, respectively. These uptake rates are very similar to those observed in the exposures (Figure 5-2).

Uptake rates were then plotted against the time-averaged mean of the calculated Cu^{2+}-activity and a Michaelis-Menten curve was fitted to the data (Figure 5-2):

$$k_u = \frac{\left(Cu^{2+}\right)}{K_M + \left(Cu^{2+}\right)} \cdot k_{u,\max} \qquad (4)$$

With K_M = the half-saturation constant = 3.2 · 10^{-9} mol L^{-1}, and $k_{u,max}$ = maximum uptake rate = 1.1 · 10^{-14} g Cu $cell^{-1}$ d^{-1} (or 8.1· 10^{-9} mol Cu g DW^{-1} min^{-1}). In a similar range of cupric ion activities as in the present study (about 10^{-9} to about 5 ·10^{-8}) Knauer et al. (1997) observed very similar $k_{u,max}$ ~ 8 · 10^{-9} mol Cu g DW^{-1} min^{-1} and K_M ~ 9 mol $L^{-1}$for the green alga *Scenedesmus subspicatus* (see their Figure 2). The above information will further be applied to the different *D. magna* exposure scenarios taking into account the specific feeding regime.

5.3.4. Monitoring of copper concentrations in D. magna tests

Table 5-3 represents the dissolved copper concentrations and the associated Cu^{2+}-activities observed in all exposures in the *D. magna* tests. Dissolved copper in the control solution was between 1.0 and 3.4 µg Cu L^{-1} (mean ± SEM of 2.2 ± 0.4 Cu L^{-1}) and was not significantly different from the copper in the control algal exposure (Student's two-tailed *t*-test, $p>0.05$). Copper concentrations in the waterborne and in the waterborne + dietary exposure were close to nominal concentrations, did not show any time-dependent increase or decrease (correlation analysis of dissolved copper versus time, $N=10$, $p>0.05$) and were not significantly different from each other in the corresponding exposures (Student's two-tailed *t*-test, $p>0.05$). Dissolved copper concentrations in all dietary exposures steadily increased with time (significant positive correlations, $p<0.05$). This is the result of desorption and elimination of copper from the algae, non-assimilation of part of the ingested copper by the daphnids and elimination of accumulated copper by the daphnids.

Table 5-3 Dissolved copper concentrations (mean ± standard error of mean) and Cu^{2+}-acitivties in the waterborne (W), waterborne + dietary (W+D) and dietary (D) exposures. Cu^{2+}-acitivties were calculated with BLM-software (Hydroqual, 2002) based on average dissolved copper concentrations and water characteristics (see Table 1).

Nominal Cu (µg Cu L^{-1})	Dissolved Cu (µg L^{-1})			Cu^{2+}-activity (nM)	
	W	W+D	D	W	W+D
0 [1]	2.2±0.4	2.2±0.4	2.2±0.4	NC	NC
35	26.2±1.5	29.5±1.7	3.8±0.5	1.20	1.39
50	43.3±1.7	43.5±1.5	6.2±1.7	2.30	2.31
75	62.3±2.0	64.2±2.1	7.8±1.3	3.96	4.15
100	92.5±2.3	96.2±2.1	9.3±1.8	8.09	8.78
140	130±3	134±9	13.7±2.5	17.8	19.1
200	179±4	183±6	14.5±2.6	44.3	47.3

[1] The control exposure serves as control for W, W+D and D

[2] NC = not calculated since the organic matter complexation characteristics were not fitted to such low concentrations

Although undesirable, this could not be prevented in the experimental setup used in this study (semi-static, pulse feeding). Although other experimental designs may be suggested to prevent this (flow-through exposure, continuous feeding), we have chosen for the same design as the one used for developing the chronic copper BLM for *D. magna* (De Schamphelaere and Janssen, 2003b, chapter 4) as this would allow to extrapolate the findings of the current study to this model. To prevent this, Hook and Fisher (2001a, 2002) have used short-term exposures (4 hours) of invertebrates to dietborne metals. The rationale for this design was that it may be representative of pulse loadings of metals into the aquatic environment. Although this may be a good approach when one wants to protect organisms living near point sources, the approach of continuous dietary exposure is certainly more representative of aquatic systems in which increased copper concentrations persist for longer periods.

Nevertheless, average dissolved copper concentrations in the dietary exposure remained between 2 and 15 µg Cu L^{-1}, concentrations which have been shown to lie in the concentration range (1 to 35 µg Cu L^{-1}) in which copper burdens are regulated and reproductive performance is optimal (Bossuyt and Janssen, 2003b). Hence, it seems reasonable to assume that the effects observed in the dietary exposure are caused by the actual dietary exposure and not by the dissolved copper leached from the algae.

5.3.5. Effect concentrations of copper in waterborne and waterborne + dietary exposure

No mortality occurred in any of the dietary exposures. In the waterborne and waterborne + dietary exposures mortality did not occur up to copper concentrations of 100 µg Cu L^{-1}. In the waterborne exposure to 140 µg Cu L^{-1}, only 1 out of 10 organisms survived the waterborne exposure, whereas none survived the waterborne + dietary exposure at this cocnentration. The surviving organism, however, remained very small throughout the whole test and did not produce offspring. At 140 µg Cu L^{-1} and higher, 100% mortality occurred after only two days of exposure. Effect concentrations of dissolved copper for the waterborne and the waterborne + dietary exposure are summarized in Table 5-4.

It is clear that an increased dietary exposure did not result in a change of the dissolved copper toxicity, since 21-day NOEC and EC50 values were nearly identical. The BLM-predicted 21-day EC50s and NOECs for this medium are 120 and 90 µg Cu L^{-1}, respectively

(calculated with the chronic Cu-BLM, De Schamphelaere and Janssen, 2003b, chapter 4). These values are very close to the observed values, indicating that the predictive capacity of the chronic Cu-BLM is not affected by increasing the dietary exposure.

Table 4 Effect concentrations of copper (as µg dissolved Cu L^{-1}) in the waterborne and the waterborne + dietary copper exposure.

Exposure	21d-NOEC[a]	21d-EC50[b]
Waterborne	92.5	113
Waterborne + dietary	96.2	121

[a] two-sided Student t-test (p<0.05) of log-transformed number of produced juveniles

[b] method explained in De Schamphelaere and Janssen (2003a, chapter 4)

5.3.6. Effects on growth and reproduction

Figure 5-3 presents the growth and reproduction of *D. magna* in the different exposures. Overall, if significant effects of exposures were observed, effects on reproduction were always more significant than effects on growth. Since a significant correlation between growth (dry weight at end of test) and reproduction was observed (r = 0.9; N=16, p<0.001), the latter is probably not the result of different resource allocation to growth and reproduction between different exposures, but rather due to statistical reasons. Indeed, the variation coefficients for reproduction (average = 0.14) were smaller than the variation coefficient for growth (average = 0.23).

The waterborne exposures to copper did not significantly affect growth and only the 70 µg Cu L^{-1} treatment resulted in an increased reproduction. For the waterborne + dietary exposure effects on growth were also only significant for the 70 µg Cu L^{-1} exposure. Reproduction, on the other hand was significantly higher in all waterborne + dietary exposures.

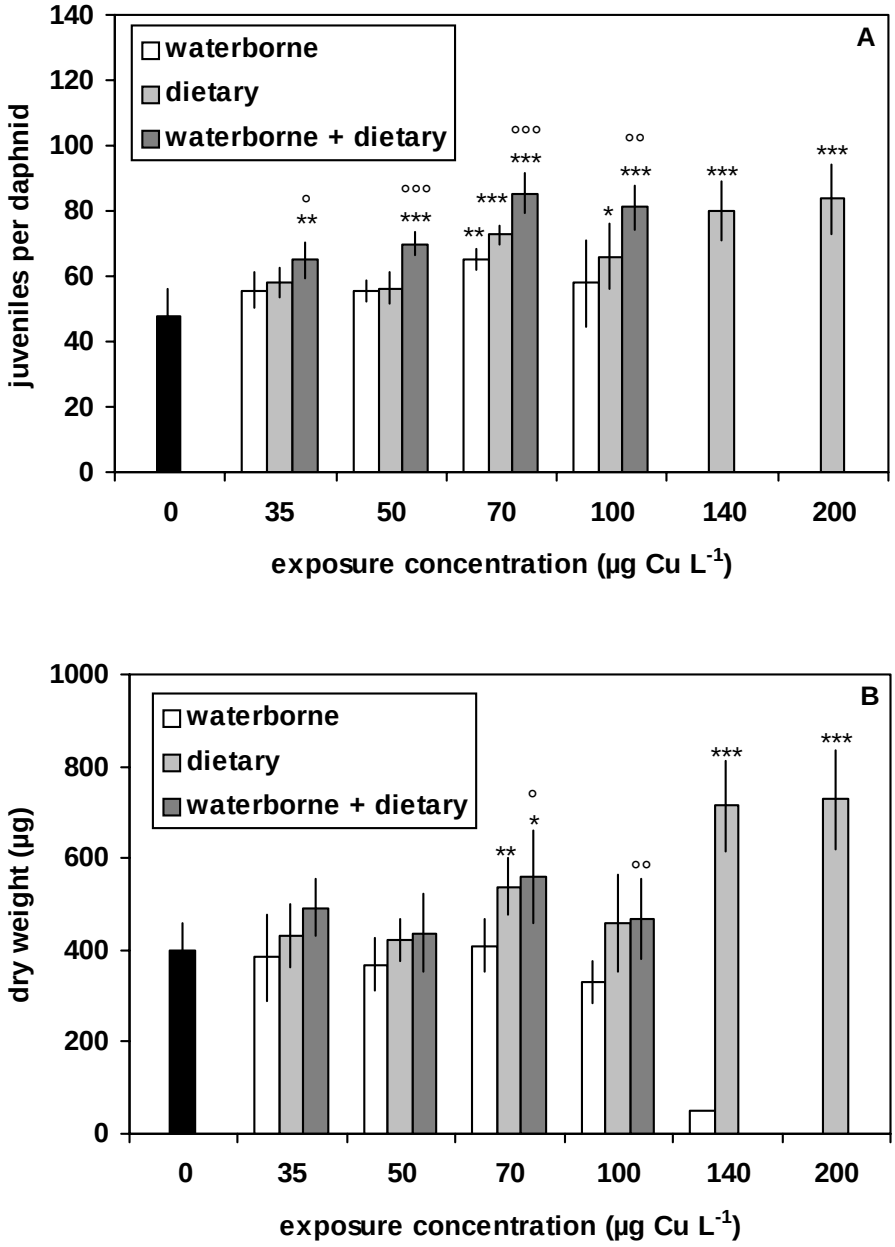

Figure 5-3 Reproduction (juveniles produced per daphnid) (A) and growth (µg dry weight at end of test) (B) of *Daphnia magna* after 21 days of exposure to waterborne, dietary and waterborne + dietary exposure. The black bar represents the control for all exposures. Error bars indicate 95% confidence limits (n=10). The exposure concentration refers to the nominal dissolved concentration in the *D. magna* exposure (for waterborne and waterborne + dietary exposure) and to the concentration at which the algae were pre-exposed for 3 days before being used as food in the dietary and the waterborne + dietary exposure. Significant differences with control are indicated with * (p<0.05), ** (p<0.0.1) or *** (p<0.001). Significant differences between waterborne + dietary and waterborne are indicated with ° (p<0.05), °° (p<0.01) or °°° (p<0.001).

In the dietary exposure growth and reproduction demonstrated an increasing trend with increasing dietary copper. Reproduction and growth in daphnids fed with algae cultured in copper concentrations ≥ 70 µg Cu L^{-1} was significantly higher than in the daphnids fed with control algae (p<0.05). A steady increase of the reproduction with increasing dietary copper is observed.

However, since average dissolved copper concentrations in the dietary only exposures increased from about 2 µg Cu L^{-1} (in the control) up to about 15 µg Cu L^{-1} (See Table 3), one may suggest that the increased reproduction and/or growth at higher dietary copper levels, may have been the result of these higher copper levels (since copper is an essential element). Bossuyt et al. (2003a, 2003b), however, demonstrated the existence of an optimum dissolved copper concentration range between 1 and 35 µg Cu L^{-1}, in which reproduction levels are generally between 10 to 15 % of each other. It thus seems very unlikely that increased dissolved copper levels during the test are explanatory of the increases of reproduction (as compared to the control reproduction) observed in this study (up to 75%).

Further evidence for increased performance of daphnids fed with algae cultured at higher copper concentrations is provided by comparing waterborne exposures with waterborne + dietary exposures with identical average dissolved copper concentrations. Whereas growth is significantly higher in the waterborne + dietary exposures at concentrations > 70 µg Cu L^{-1}, reproduction is significantly higher in all waterborne + dietary exposures (p<0.05).

It is clear that the feeding of the daphnids with algae exposed to increased copper concentrations did not result in toxic effects (i.e. decreased reproduction or growth) at all tested levels. On the contrary, the only effects that were observed seemed to be stimulatory effects of increased dietary exposure. At first sight the observed trends across the different exposure scenarios seems rather complex. In a further section we will use an integrative modelling approach combining algal copper burdens, daphnid body burdens and algal and copper ingestion rates of the daphnids, to explain both the non-toxicity and the stimulatory effect of dietary copper.

5.3.7. Algal ingestion rates by D. magna

Cell ingestion rates were the same in all exposures, except the waterborne exposure to 140 μg Cu L^{-1} where it was lower (Sign-test, p>0.05, non-parametric test for dependent samples). The average (± SEM) cell ingestion rate (except the 140 μg Cu L^{-1} waterborne exposure) was 5.02 ± 0.11 · 10^5 cells d^{-1} (N=15) in the first two days of the assays and it linearly increased up to a plateau of 1.31±0.02 · 10^7 cells d^{-1} (N=60) between day 12 and day 21. This indicates that the daphnids have probably reached their near-maximal length at around day 12 of the exposure, which is very common for well-fed daphnids (Kooijman, 2000). For the waterborne 140 μg Cu L^{-1} exposure the ingestion rate of the single surviving daphnid was 4.7 · 10^4 cells d^{-1} in the first two days of exposure and reached a plateau of 6.24±0.24 · 10^6 cells d^{-1} between day 12 and day 21. Increased copper exposure has previously been demonstrated to result in decreased feeding of *D. magna* and has been suggested as one possible mechanism for reduction of growth and reproduction upon copper exposure (Flickinger et al., 1982; Ferrando and Andreu, 1993). Measured cell ingestion rates are used further on in the simulation exercises. For simplicity ingestion rates were assumed to be constant in-between two test media renewals.

5.3.8. Development of an integrative modelling approach to discriminate between waterborne and dietary exposure

Both the uptake and elimination kinetics of copper in the algae and the algal ingestion rates determine changes in algal copper burdens (i.e. dietary copper) during chronic exposures of *D. magna*. Here we describe a relatively simple model that applies to the specific feeding regime of *D. magna* in the present study, but which, with slight modifications, can be extended to any feeding regime.

The feeding in the chronic *D. magna* assays was discontinuous, i.e. each day a given number of cells was transferred into the test solutions and the interval between test media renewal was 2 to 3 days (see materials and methods). Taking this into account, a maximum of three "types" of algal cells can be present in the test medium: cells that are present in the test medium for 1 day, for 2 days and for 3 days. Since uptake and elimination of copper are kinetic processes the copper burdens of these three types of cells may be different at any given time. The algal copper burdens are indeed dependent on both their initial copper burden

at the time of introduction into the test medium, the copper concentration in the test medium and the time passed since their introduction. Hence, it is also obvious that in order to determine the average copper burden of the algae present in the daphnid test medium, the copper burdens and the fractional abundances of the different "types" of algae need to be known.

The following equation describes, at any given time point *t* the change of the number of algae (ΔN_i) of each "type" of cell within a time step (Δt):

$$\frac{\Delta N_i}{\Delta t} = f_i \cdot IR(t) + N_{i,added}(t) \qquad (5)$$

With N_i = the number of algae of type *i* present in the test solution, and $f_i = \frac{N_i}{N_{total}} =$ the fraction of cells of type i; N_{total} = total number of cells (all types); $IR(t)$ = the daphnids' cell ingestion rate at time t (cells day^{-1}); $N_{i,added}$ (t) = the number of algae of type i added at time t (see materials and methods for these numbers).

For each "type" of algae the external and the internal copper burdens at any time need to be known. The external copper (Cu-Ext) is calculated for each exposure concentration with Eq. 1, using the average calculated Cu^{2+}-activity for the whole exposure period (Table 5-3). In accordance with the fast adsorption/desorption kinetics of copper with algal cell walls (Knauer et al., 1997) this amount is assumed to be immediately in equilibrium with the test medium and to be constant throughout the whole exposure. Hence, for the dietary exposure, it is assumed that only background external copper is present (Table 2). The internal copper burden of the algae (Cu-Int) at each time point is calculated using Eq. 3 and Eq. 4 (with $Cu\text{-}Int_0$ = the average of Cu-Int at day 0 and at day 21 of the exposure, see Table 5-2; and with t = time passed since the introduction of the algae in the test medium, which is different for different "types" of algae). The average internal and external copper burden of the algae in the daphnid exposure at each time point is:

$$\overline{Cu-Int} = \sum_{i=1}^{3} \left\{ f_i \cdot \left[Cu-Int \right]_i \right\} \qquad (6)$$

and

$$\overline{Cu-Ext}=\sum_{i=1}^{3}\left\{ f_i \cdot \left[Cu-Ext\right]_i \right\} \quad (7)$$

And the copper ingestion rate (IR_{Cu}, g Cu d^{-1}) by the daphnids at each time-point t is:

$$IR_{Cu}=\left(\overline{Cu-Int}+\overline{Cu-Ads}\right)\cdot IR\left(t\right) \quad (8)$$

Equations 6 to 8 are used to simulate the variations of algal copper burdens and copper ingestion rates of the daphnids throughout the whole *D. magna* exposure. The model simulations are carried out with time steps Δt = 1 hour.

5.3.9. Applications of the model - actual algal copper burdens

In Figure 5-4, the simulated time-dependent variation of total copper burdens of the food-algae during the daphnid exposure is given for the exposure concentration of 100 µg Cu L^{-1}, both for waterborne, dietary and waterborne + dietary exposure. Similar time-dependent patterns are observed for other exposure concentrations.

In the waterborne exposure the algal copper burden at each test media renewal starts with the external burden (relation with Cu^{2+}-activity, constant throughout test) and gradually increases upon copper internalization from the medium until new (control) algae are added, at which point a drop in average algal burden is noticed. After that the average algal burden increases again and so on. In the dietary exposure the reverse is observed. After introduction of the algae, the external copper is immediately lost (not shown on graph) and slower copper elimination occurs after this, resulting in a decrease of the average algal burden until new algae are added. In the waterborne + dietary exposure the simulated variation is much smaller, and only a small change of the copper burden is noticed with each addition of algae. The latter is due to the fact that dissolved copper concentrations in the daphnid exposure were only slightly different from the copper concentration in the algal stock suspensions (i.e. copper at day 3 of the algal exposure).

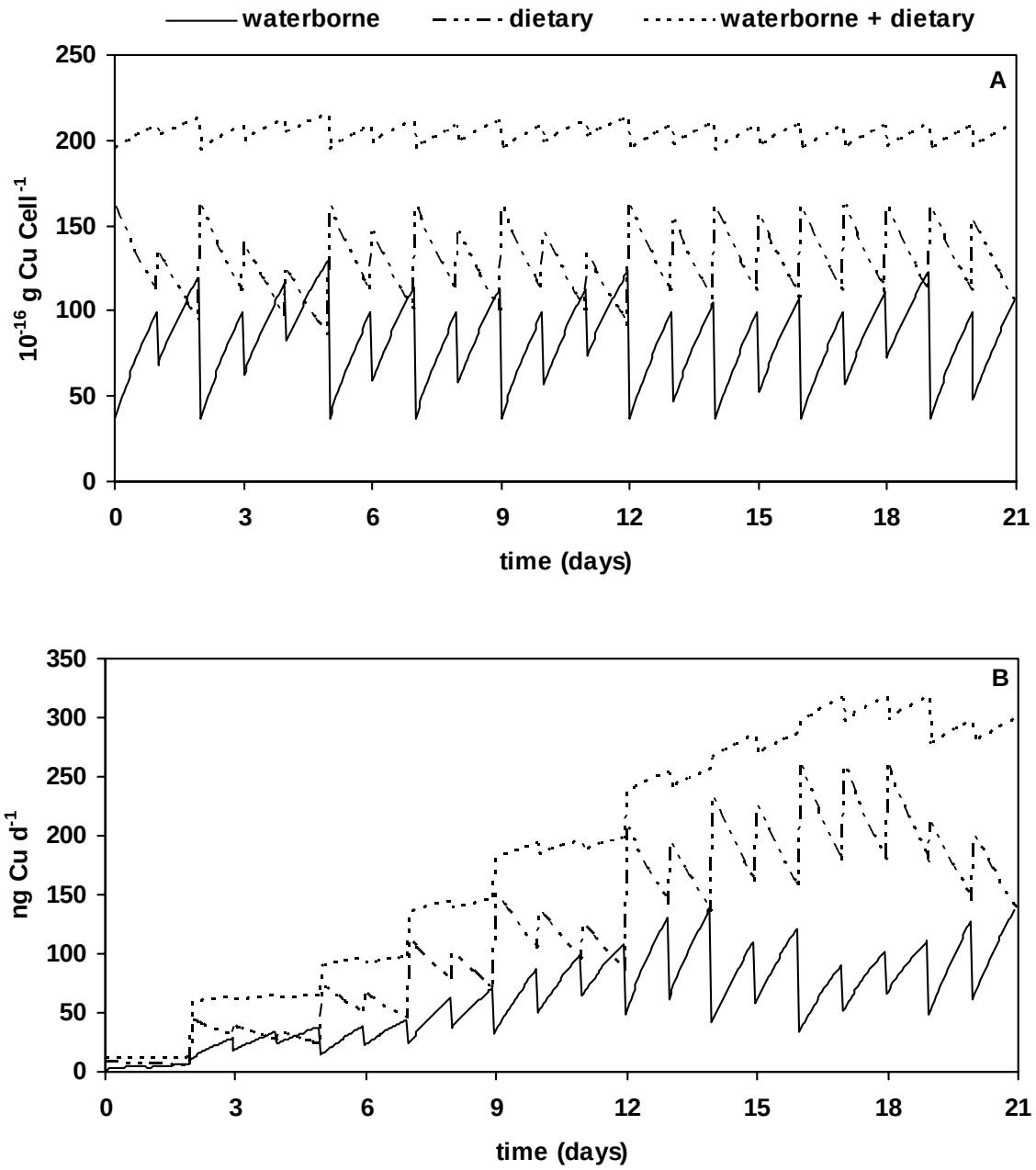

Figure 4 Simulated variations of total copper burdens of the algal food (A) and the copper ingestion rates of *Daphnia magna* (B) during the chronic exposures to 100 µg Cu L^{-1}. Simulations were carried out as described in text. Waterborne exposure refers to daphnids exposed at 100 µg Cu L^{-1} dissolved copper and fed with control algae. Dietary exposure refers to daphnids exposed in control solution and fed with algae cultured for three days at 100 µg Cu L^{-1}. Waterborne + dietary refers to daphnids exposed to 100 µg Cu L^{-1} dissolved copper and fed with algae cultured for three days at 100 µg Cu L^{-1}. For further explanation see text.

These simulations allowed to derive the average total and internal copper burdens of the algae in all daphnid exposures and the simulated range of these burdens (for the whole exposure period) (Figure 5-5). It is quite striking that algal copper burdens in the so-called waterborne exposure can reach relatively high levels, even higher than in some dietary exposures (e.g. compare the simulated algal copper burdens in the waterborne exposure at 140 µg Cu L-1 with that of the dietary exposure at 35 µg Cu L^{-1}). This clearly demonstrates that current standard chronic toxicity testing procedures with daphnids fed with live algae also have a dietary metal component included.

Second, the variation of total algal copper burdens (indicated by the top and the bottom of the error bars in Figure 5-5) during the daphnid exposure could be relatively large. Variations of about factor 3 to 4 for waterborne exposure, about factor 2 for dietary exposure, and less than factor 1.5 for the waterborne + dietary exposure were simulated. Similar variations were simulated for the internal algal copper burden for the dietary and the waterborne + dietary exposure; but for the waterborne-only exposures simulated variations of internal copper burden were much larger (as these started only at a background level). To our knowledge this study is the first to report on the potential quantitative importance of these variations.

When comparing corresponding exposures (same copper exposure concentrations on horizontal axis of Figure 5), simulated algal copper burdens are consistently highest in the waterborne + dietary copper exposures. The total and internal algal copper burdens are on average about factor 2.5 to 3 higher in the waterborne + dietary exposure than in the waterborne only exposure, with no or very little overlap with the waterborne and with the dietary copper exposure (compare bottom and top of error bars in Figure 5). This is very important as it actually allows qualifying the differences in growth, reproduction and body burdens between waterborne + dietary and waterborne only exposures, as the result of a higher level of dietary copper exposure. This has already been exploited above for reproduction effects and will be used below with regard to body burdens of the daphnids.

Finally, the quite large ranges of algal copper burdens in the different exposure scenarios and the overlap of these across different scenarios, help to explain why, at first sight, the observed trends in growth and reproduction effects are so difficult to interpret (see Figure 5-4), as discussed above. Such difficulties may always be expected in cases where live

diets are used for investigating dietary toxicity when variations of the dietary exposure level during a test are not taken into account.

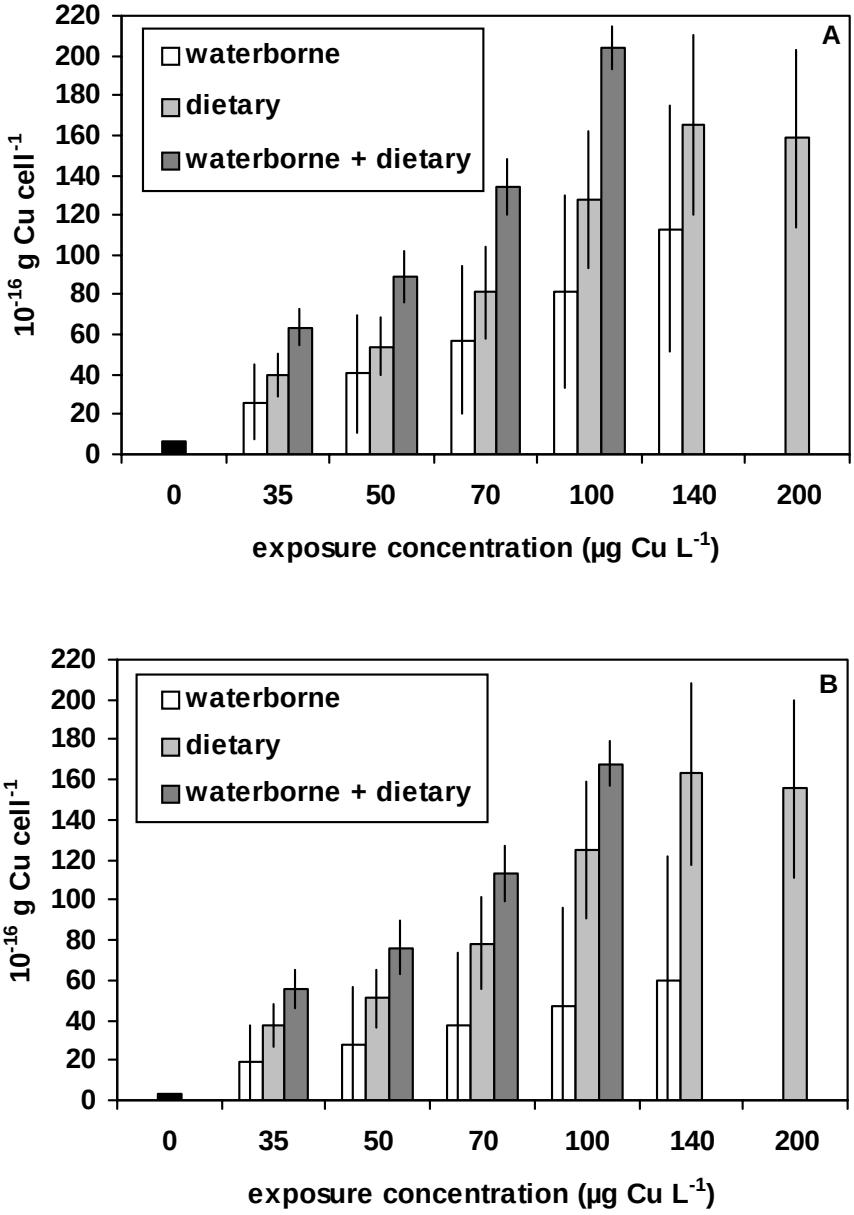

Figure 5 Simulated total (A) and internal (B) copper burdens of food algae in chronic *Daphnia magna* tests as calculated with the model described in the text. See Legend of Figure 3 for explanation on meaning of exposure concentrations. Error bars indicate minimal and maximal values of copper burdens that were calculated for the whole exposure period.

5.3.10. Applications of the model - copper ingestion rates

The oscillatory pattern of the algal copper burden is also reflected in the copper ingestion rates of the daphnids (Figure 5-4) and these clearly follow the same trend as the cell ingestion rates. Indeed, like the cell ingestion rate, the copper ingestion rate is low at the initial stage of the exposures and increases up to around day 12, where a plateau is reached.

As pointed out by Clearwater et al. (2002), if any toxicity is expected from dietary exposure to metals, it is rather the mass-specific copper ingestion rate ($IR_{M,Cu}$; g Cu g daphnid^{-1} d^{-1}) than the copper ingestion rate per daphnid (g Cu d^{-1}) that would determine the toxic effect. An increase of the copper ingestion rate is proportional to an increased of the cell ingestion rate which in turn is proportional to the squared daphnids' length (Kooijman, 2000) or proportional to its weight $^{2/3}$. Since $IR_{M,Cu}$ equals IR_{Cu} / weight, $IR_{M,Cu}$ is proportional to weight $^{2/3}$ / weight or to weight $^{-1/3}$ or to length^{-1}. In other words the mass specific copper ingestion rate is inversely proportional to the organisms' length. This indicates that smaller organisms will have a larger mass specific copper ingestion rate and hence earlier life stages will inherently be more susceptible to dietary copper uptake and toxicity.

Table 5-5 presents the simulated average of the mass specific copper ingestion rates of total copper for juveniles (day 0 to day 2, using their average dry weight at the start of the test, i.e. 8 µg) and 21-day old adults (day 19 to day 21, using their average dry weight at the end of the test, see Figure 3). To allow comparison with the results of Clearwater et al. (2002), mass specific ingestion rates are calculated and reported as g Cu (g wet weight)$^{-1}$ d^{-1} using a dry weight : wet weight ratio for *D. magna* of 0.085±0.003 (mean ± SEM, *N*=30 *D. magna* between 1 and 21 days old).

Mass specific copper ingestion rates of juveniles were roughly about factor 2 to 3 higher than those of adults, with the exception of the waterborne exposure to 140 µg Cu L^{-1}, for which it was about 25 times higher at the end of the test than at the start of the test. In the latter exposure, however, these estimates are for the single surviving organism only. These numbers, however, suggest that other organisms in this exposure most probably died as a consequence of the real waterborne part of the exposure.

Table 5-5 Estimated average mass specific ingestion rates of total algal copper (µg Cu per g wet weight per day) for juvenile and adult *D. magna* in the waterborne (W), dietary (D) and waterborne + dietary (D) exposures.

Nominal Cu (µg Cu L^{-1})	juveniles			adults		
	W	D	W+D	W	D	W+D
0 [1]	3.5			1.9		
35	15	20	31	7.1	11	15
50	23	27	49	11	16	24
75	36	40	73	15	18	28
100	48	67	124	25	32	53
140	5.6	76	NC	120	28	NC
200	NC	78	NC	NC	26	NC

[1] The control exposure serves as control for W, W+D and D

NC = not calculated, no cell ingestion rates were available since all organisms died within two days

Indeed, the mass specific copper ingestion rates in this exposure were between 5.6 µg Cu g^{-1} d^{-1} (start) and 120 µg Cu g^{-1} d^{-1}, which are both lower than the highest simulated copper ingestion rate of 124 µg Cu g^{-1} d^{-1}, which did not result in any mortality or adverse effects on growth and reproduction. Hence, it is not likely that the dietary part of this exposure contributed to the very low growth and the non-reproductivity of this single organism. Most likely this organism used most available energy-resources for fighting the copper stress resulting from the waterborne exposure. The high total copper body burden (127 µg Cu g DW^{-1}, see further) of this daphnid may perhaps be considered as a level which exceeds the capacity of the daphnid to store the excess metal in non-toxic forms (such as metallothionein or copper granules) and therefore results in very significant toxicity, perhaps through copper-mediated oxy-radical production (Mason and Jenkins, 1995).

Except for this rather exceptional exposure, total copper ingestion rates were estimated between 3.5 and 124 µg Cu g^{-1} d^{-1} for juveniles and between 1.9 and 53 µg Cu g^{-1} d^{-1} for adults. Ingestion rates of internal algal copper were between 1.9 and 102 µg Cu g^{-1} d^{-1} for juveniles and between 1.4 and 47 µg Cu g^{-1} d^{-1} for adults. Neither of these copper ingestion

rates resulted in adverse effects on growth and reproduction (see Figure 4). According to an extensive literature review, dietary copper toxicity occurred at daily doses between 1 to 15 µg Cu g^{-1} d^{-1} for Atlantic salmon (*Salmo salar*) and between 35 and 45 µg Cu g^{-1} d^{-1} for rainbow trout (*Oncorhynchus mykiss*) (Clearwater et al., 2002). Our analysis demonstrates that *D. magna* does not show any adverse affects up to daily dietary doses up to 124 µg Cu g^{-1} d^{-1}. *D. magna* can thus be considered as being less susceptible to dietary Cu-toxicity than those fish species. However, the Clearwater et al. (2002) analysis with fish only included laboratory-prepared diets. Studies with fish fed live diets could not unambiguously demonstrate adverse effects caused by dietary copper doses tested for rainbow trout fed *Artemia* (21-48 µg g^{-1} d^{-1}; Mount et al., 1994) and for gurnard fed on *Nereis diversicolor* worms (20 to 40 µg g^{-1} d^{-1}, Bryan and Gibbs, 1983). In the former study, a 30% mortality of rainbow trout fed with 40 and 48 µg Cu g^{-1} d^{-1} was observed, but the effect was at least partially attributed to Cu leaching from the diet resulting in toxic waterborne exposure. Based on existing fish literature data it is thus impossible to determine toxicity thresholds for dietary copper incorporated in live diets. To our knowledge, no other reports are available which directly link toxic effects with dietary copper exposure (either laboratory-prepared or live diets) to aquatic invertebrates. Dietary toxicity studies with live diets are also very scarce for other metals.

Short-term (24 hours) dietary exposure of *Daphnia magna* to the non-essential cadmium has been suggested to significantly reduce its algal ingestion rate to a considerable extent (Taylor et al., 1998). Although Cd also seems to decrease the digestive enzyme activity in short-term exposures (48 hours), longer exposures (96 hours) seemed to result in increased digestive enzyme activities (De Coen and Janssen, 1998). These authors suggested that, through this mechanism, daphnids may try to compensate the Cd-induced decrease of the ingestion rate, thus improving the food assimilation efficiency, which may result in less-than-expected adverse effects on growth and reproduction.

Hook and Fisher (2001b) reported decreased reproduction in two freshwater cladocerans (*Ceriodaphnia dubia* and *Simocephalus vetulus*) after 4 hours of feeding on Ag-contaminated algae. Ag is not an essential element and is generally known to much more toxic than copper. Similar reduced egg production was observed in marine copepods fed for 4 hours with diatoms contaminated with the non-essential metals Ag, Hg, Mn, Cd, but also with the essential element Zn (Hook and Fisher, 2001a). However, in comparing the impact of Zn accumulated over a 1-week period to that acquired over a 4-hour period, no effects on egg

production following longer Zn exposures were seen, even if the body burden of Zn increased to the same degree in both exposure scenarios. They suggested, however, that in the short-term exposures, the copepods did not have sufficient time for the induction of detoxicification mechanisms, allowing the Zn to remain accumulated in the sensitive ovarian tissue during their further life.

To conclude, none of the above-mentioned studies are in disagreement with the observed non-toxicity of dietary copper for *D. magna* in our study. However, not only was no toxicity observed in this study; but for the first time a clear 'beneficial' dietary threshold for an invertebrate species chronically exposed to Cu was established. Based on the dietary exposure, it may be suggested that daily dietary doses between 40 and 124 μg g^{-1} d^{-1} for juveniles and between 18 and 53 μg Cu g^{-1} d^{-1} for adults are beneficial for *D. magna* under the conditions tested (see effects shown in Figure 5-3 and dietary copper ingestion rates in Table 5-5).

5.3.11. Copper body burdens in D. magna

Body burdens of copper in *D. magna* for all exposures are reported in Figure 5-6. Given the fact that in the waterborne exposure also a considerable amount of dietary copper seems to be ingested by the daphnids, it would be inappropriate to try to assess the importance of, and the interaction between, waterborne and dietary metal uptake based on copper body burdens alone. The main goal of this section is, however, to demonstrate that dietary copper was effectively taken up by *D. magna* and that this resulted in an increased body burden. Three observations seem to support this hypothesis.

First, in the dietary exposure copper body burdens exhibit an increasing trend with increasing dietary copper exposure (from 14.4 μg g DW^{-1} in the control to about a constant level of 40 μg Cu g DW^{-1} in the three highest exposures). The copper burden in the control daphnids after 21 days of exposure is nearly identical to the copper burden of the juvenile daphnids at the start of the test (14.0±1.1; mean ± SEM, 3 replicates of 50 pooled juveniles < 24 hours old, from stock culture). This copper burden probably represents the optimal metabolic requirement in daphnids.

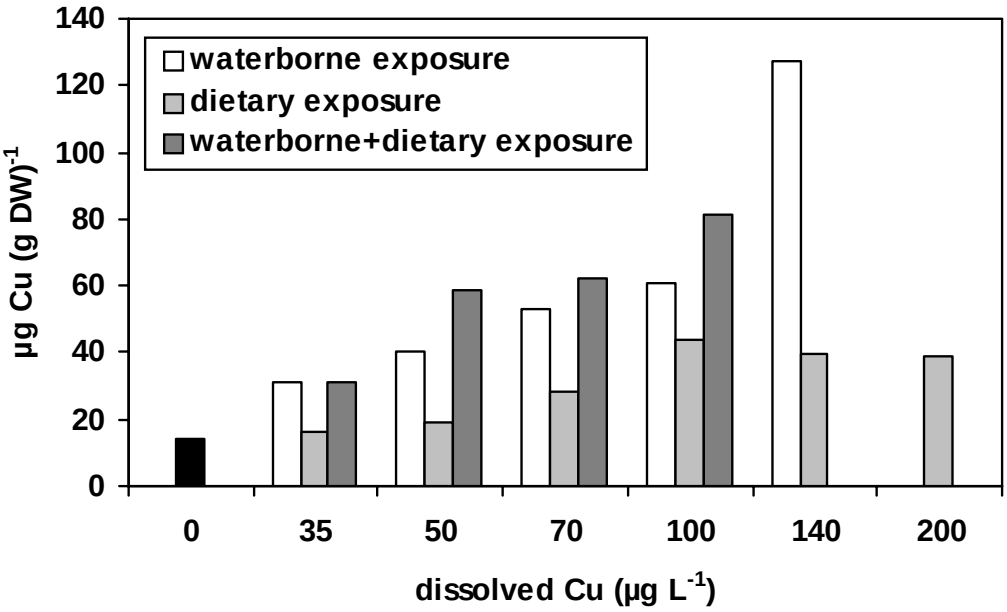

Figure 5-6 Copper body burdens in *Daphnia magna* after 21 days of exposure to different combinations of waterborne and dietary copper. See legend of Figure 3 for meaning of exposure concentration.

This hypothesis is supported by the fact that *D. magna* seems to be able to rapidly (within one life-cycle) regulate their body burden between about 12 and 20 µg Cu g DW^{-1} for a copper concentration range of 1 to 12 µg Cu L^{-1} (Bossuyt and Janssen, 2003a). Since the highest average dissolved copper concentrations that was leached from by the algae (in a dissolved form) into the daphnids' test medium was only 14.5 µg L^{-1}, this can probably not explain the increased daphnids' body burden at higher dietary exposure levels.

Second, copper body burdens of the daphnids in the waterborne exposures were compared with those in the waterborne + dietary exposure (comparing the 35, 50, 75 and 100 µg Cu L^{-1} exposures). The dietary algal copper burdens to which the daphnids were exposed were always higher in the waterborne + dietary exposure (factor 2.5 to 3, see Figure 5). Here too, copper burdens of the daphnids in the waterborne + dietary exposure were always higher than in the waterborne exposure.

Third, a highly significant linear relation between simulated average total copper ingestion rates of 21-day old daphnids (Figure 6) and the added copper body burden (body burden minus the metabolically required background body burden of 14.4 µg Cu g^{-1}) for the

dietary exposure was observed (r = 0.97, p<0.001). This linear relation corroborates the results of Kamunde et al. (2002) and established dynamic models for accumulation of metals from food (Wang and Fisher, 1999) and hence provides additional evidence that copper was indeed taken up via the food. Given the limited knowledge on parameter values for these models (assimilation efficiency, elimination rate, etc.) for *D. magna* (or other freshwater invertebrates) for copper and given the demonstrated interactions between dietary and waterborne copper uptake routes in rainbow trout (Kamunde et al., 2002), no further evaluation of the body burden – exposure relationships was possible.

The fact that increased copper body burdens, above the metabolically required quantity, did not result in toxic effects, may possibly be explained by the storage of the excess copper in a non-toxic form (e.g. bound to metallothionein or stored in copper granules; Mason and Jenkins, 1995).

Future research is clearly needed enhance our understanding of copper accumulation routes in daphnids. To that end, the advantageous use of tracer methods (i.e. radio-isotopes or stable isotopes) is highly recommended for discerning between dietary and waterborne uptake routes (and/or their in interactions). This is the subject of currently ongoing research at our laboratory.

5.3.12. Relation between exposure routes and enhanced performance

Although no adverse effects of increased dietary exposure to copper have been observed, it would nevertheless be interesting to find a mechanistic explanation for the stimulatory effect observed in the copper exposures (compared to the control exposures). To that end, a correlation analysis was conducted between the effect parameters (growth and reproduction) on the one hand, and dissolved copper, Cu^{2+}-activity, copper body burden of the 21-day old daphnids, simulated average algal copper burdens (total, internal and external) during the test and simulated mass-specific copper-ingestion rates of juveniles and adults (total, external and internal algal copper) on the other hand. For this analysis, the waterborne exposure to 140 µg Cu L^{-1} was not taken into account, given its rather exceptional outcome (cf. before).Table 5-6 summarizes the results of this analysis.

Table 5-6 Correlation coefficients (*r*) of different exposure measures with effects on reproduction and growth on *Daphnia magna* their corresponding probabilities (*p*). Significance levels are highlighted with * ($p<0.05$), ** ($p<0.01$) or *** ($p<0.001$).

	Reproduction (Figure 5-3)		Growth (Figure 5-3)	
Exposure measure	*r*	*p*	*r*	*p*
Dissolved Cu (Table 5-3)	0.15	0.617	-0.36	0.208
Cu^{2+}-activity (Table 5-3)	0.36	0.378	-0.10	0.812
Body burden of *Daphnia magna* (Figure 5-6)	0.47	0.087	-0.06	0.830
Juvenile mass specific Cu ingestion rate				
Total (Table 5-5)	0.81	<0.001 ***	0.50	0.068
Internal (Data not shown)	0.85	<0.001 ***	0.64	0.013 *
External (Data not shown)	0.12	0.694	-0.39	0.163
Adult mass specific Cu ingestion rate				
Total (Table 5-5)	0.68	0.007 **	0.32	0.272
Internal (Data not shown)	0.71	0.004 **	0.41	0.150
External (Data not shown)	0.15	0.602	-0.37	0.192
Average algal Cu burden				
Total (Figure 5-5)	0.86	<0.001 ***	0.65	0.012 *
Internal (Figure 5-5)	0.87	<0.001 ***	0.76	0.002 **
External (Data not shown)	0.12	0.675	-0.39	0.172

Correlations with dissolved copper and Cu^{2+}-acitivity were not significant. This seems to suggest that the enhanced reproduction and growth were not due to the waterborne exposure. Increased copper body burdens were also not significantly correlated with increased growth or reproduction, indicating that body burdens are no good predictors of effects (cf. Brix and DeForest, 1999). Although the increased performance at higher copper exposures may be suggested to be related to the concentration of copper in a certain tissue, this cannot be confirmed as only total body burdens were available.

Overall, correlations with effects on growth were lower and less significant than correlations with reproduction, most likely a consequence of the larger errors associated with growth (cf. before). Summarizing, for the measures describing dietary copper exposure,

correlations with growth and reproduction were decreasing in the following order: algal copper burdens > juvenile mass specific copper ingestion rates > adult mass specific copper ingestion rates. Correlations with internal copper burdens of the algae were always slightly better than with total algal burdens and this is confirmed by the non-significant relations with external algal burdens or with ingestion rates of external algal copper. The latter probably points to the lesser importance of externally bound algal copper in stimulatory effects of copper. Although Taylor et al. (1998) suggested that Cd, externally bound to algal cells after a 20-minute incubation period in increased Cd concentrations, resulted in reduced ingestion rates of *D. magna* in a 24-hour exposure, no measurements of the corresponding internal Cd were performed that could confirm their hypothesis. Moreover, external copper on algal cells has been demonstrated to be strongly bound, in a covalent way to different functional groups of the polymeric cell-wall molecules (Crist et al., 1988), which perhaps makes this copper fraction less available within the daphnids' gut than cytosolic copper. The best correlations, highly significant ($p<0.01$) with both growth and reproduction were obtained with the average internal algal copper burdens. With the current dataset we cannot explain why correlations of effects with copper burdens of the algae are (slightly) better than with mass specific copper ingestion rates.

It is clear that the increased dietary exposure (indicated both by increased algal copper burdens and increased copper ingestion rates by the daphnids) can enhance the daphnids' performance. Given the fact that similar feeding rates are observed for all exposures it seems, however, that increased performance is the result of increased food assimilation efficiency.

One could argue that algae pre-exposed to higher copper may have had a different nutritional value for the daphnids and that this may have resulted in the daphnids' increased assimilation efficiency and increased performance. Unfortunately diet quality was not monitored during the experiments. Although possible diet quality effects cannot be ruled out *a priori*, some reports seem to indicate that this is definitely not the most likely explanation for the increased performance. For example, McLarnon-Riches et al. (1998) did not observe a change in total lipid content (which constitutes the largest pool of energy reserves in *D. magna*, De Coen and Janssen, 1997) or lipid composition even after a 7-day exposure of *P. subcapitata* to copper. Perrein-Ettajani et al. (1999) demonstrated for a marine alga that its content of polysaccharides, proteins and lipids remained unchanged even when growth was affected by copper exposure. All this indicates that the effects observed are most likely due to

the exposure to dietary copper itself, but research towards diet quality effects on dietary copper effects are warranted.

Both increased copper ingestion rates and algal copper burdens are probably directly linked to the eventual soluble copper concentration that will be established in the gut fluid. By titrating gut fluids of marine benthic invertebrates with copper, Chen et al. (2002) demonstrated that Cu initially produced an increase in protease activities before reaching a threshold level above which enzyme activity was inhibited. Furthermore, these authors observed a nearly ubiquitous enhancement of activities of other digestive enzyme in 32 out of 34 tested species. The same type of metal-enhanced activities has been observed in pigs (Kirchgessner et al., 1976) and it is suggested that, at least for proteases, this enhancement may involve conformational optimization of proteases or activitation of apo-proteases (dormant forms of proteases) after copper binding. The fact that the enhancement of protease activities for some species amounted up to a factor of 66 gives reason to belief that a similar type of enhancement in *D. magna* is plausible and could easily have resulted in the factor 2 increase in reproduction and growth, as observed in the present study. The complex nature of this mechanism certainly deserves further attention in freshwater invertebrates as it may be of large importance in evaluating results of dietary toxicity studies.

5.4. Conclusions and future perspectives

In the present study it was demonstrated that dietary copper is indeed taken up by *D. magna* but that it does not result in toxic effects. Although not demonstrated here, this may have been a consequence of accumulation of copper in non-toxic forms. A clear link was established between dietary copper exposure and increased performance of the daphnids. Although not examined in this study, this may be due to copper-enhanced digestive enzyme activities in the gut. We also developed a model which allows the simulation of how dietary copper exposure varies during a standard chronic *D. magna* test as a consequence of kinetic Cu adsorption, uptake and elimination processes in the algae. It was shown that so-called "waterborne only" exposures, in which daphnids were fed with live algae, also contained an important dietary component and this should certainly be acknowledged when evaluating the results of such toxicity tests (also with other metals). The fact that uptake (and perhaps also elimination kinetics) of algae are affected by water characteristics such as pH, suggests that

investigation of the dietary exposure route in different water types may be required to fully understand the ecological importance of this process. Finally, it was demonstrated that the dietary exposure component did not affect the effect concentrations (expressed as dissolved, waterborne copper) or the predictive capacity of the chronic Cu-BLM.

Chapter 6

Development and field validation of a copper toxicity model for the green alga *Pseudokirchneriella subcapitata*

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Chapter
6

Development and field validation of a copper toxicity model for the green alga *Pseudokirchneriella subcapitata*

Abstract – In this study, the combined effects of pH, water hardness and dissolved organic carbon (DOC) concentration and type on the chronic (72-hour) effect of copper on growth inhibition of the green alga *Pseudokirchneriella subcapitata* were investigated. Natural dissolved organic matter (DOM) was collected at three sites in Belgium and The Netherlands using reverse osmosis. A full central composite test design was used for one DOM and a subset of the full design for the two other DOMs. For a total number of 35 toxicity tests performed, 72-hour E_bC10s (concentration resulting in 10% growth inhibition) ranged from 14.2 to 175.9 µg Cu L^{-1} (factor 12) and 72-hour E_bC50s from 26.9 to 506.8 µg Cu L^{-1} (factor 20). Statistical analysis demonstrated that DOC concentration, DOM type and pH had a significant effect on copper toxicity; hardness did not affect toxicity at the levels tested. In general, an increase in pH resulted in increased toxicity whereas an increase of the DOC concentration resulted in decreased copper toxicity. When expressed as dissolved copper, significant differences of toxicity reduction capacity was noted across the three DOM types tested (up to factor 2.5). When expressed as Cu^{2+} activity, effect levels were only significantly affected by pH; linear relationships were observed between pH and the logarithm of the effect concentrations expressed as free copper ion activity, i.e. log (E_bC50_{Cu2+}) and log (E_bC10_{Cu2+}): (1) Log (E_bC50_{Cu2+}) = - 1.431 pH + 2.050 (r^2 = 0.95), and (2) Log (E_bC10_{Cu2+}) = -1.140 pH - 0.812 (r^2 = 0.91). A copper toxicity model was developed by linking of these equations to the WHAM V geochemical speciation model. This model predicted 97% of the $E_bC50_{dissolved}$ and $E_bC10_{dissolved}$ values within a factor of 2 of the observed values. Further validation using toxicity test results that were obtained previously with copper-spiked European surface waters demonstrated that for 81% of tested waters, effect concentrations were predicted within a factor of 2 of the observed. The developed model is considered to be an important step forward in accounting for copper bioavailability in a regulatory context.

6.1. Introduction

Like many chemicals, metals may present risks to man and the environment. Recently, however, it has been recognised by regulators, industry, and academic scientists that standard procedures for deriving environmental quality criteria (EQC) for (organic) chemicals are inadequate to protect, in an scientifically defendable manner, the integrity of terrestrial and aquatic ecosystems against possible metal contamination (Bergmann and Dorward-King, 1997; Janssen et al., 2000). Because of their key position as primary producers in ecological systems, algae are important test species for deriving EQC. However, algae toxicity test results for EQC derivation of metals are mostly obtained using international standard test procedures such as those prescribed by the Organisation of Economical Cooperation and Development (OECD, 1984) and the International Standardisation Organisation (ISO, 1989). All these tests are performed in reconstituted standard test media with a relatively high pH (>7.5), low concentrations of major cations and a very low (background) dissolved organic carbon (DOC) concentration. Consequently, the variability of physico-chemical characteristics in natural surface waters is neglected and thus, the use of metal toxicity data generated using these standard media may not be representative for assessing in situ metal toxicity (Janssen and Heijerick, 2003).

The importance of physico-chemical parameters such as pH, water hardness and complexing compounds on copper toxicity to aquatic species has been recognized for a long time, not only for invertebrate and fish species [e.g. Campbell, 1995; Erickson et al., 1996; Jannsen et al., 2000; De Schamphelaere et al., 2002; Heijerick et al., 2002), but also for a number of algae species.

Heijerick et al. (unpublished data), for example, have demonstrated that an increase of Ca and Mg concentrations in the test media from 0.25 to 2.5 mM resulted in a 2-fold increase of the 72-hour E_bC50 (the concentration causing 50% growth inhibition after 72 hours of exposure) of copper for *Pseudokirchneriella subcapitata*. The authors attributed this to a competition effect of hardness cations and copper at the cell surface. Similarly, an increase in DOC concentration in the test media has been reported to result in a decreased copper toxicity to the algae species *Monochrysis lutheri* (Sunda and Lewis, 1978) and *Oocystis pusilla* (Meador et al., 1998).

Reduced copper toxicity with decreasing pH has been reported for *Chlorella* sp. (Franklin et al., 2000), *Chlamydomonas reinhardtii* (Macfie et al., 1994) and three *Scenedesmus* strains

(Nalejawko et al., 1997). These observations clearly contrast with the usually observed effect of pH on copper toxicity to invertebrates and fish species for which a decrease of pH generally results in an increased copper toxicity (Meador, 1991; Erickson et al., 1996; De Schamphelaere and Janssen, 2002, chapter 2; De Schamphelaere et al., 2002, chapter 3).

In most of these types of studies, the effect of a single factor (water hardness, DOC concentration or pH) on copper toxicity was investigated. No research has been performed examining the combined effects of these factors on copper toxicity to algae. The aim of this study was thus to investigate the combined effects of DOC concentration, pH and hardness on copper toxicity to the green alga *P. subcapitata*. Additionally, the importance of the effect of natural dissolved organic matter (DOM) source was investigated by carrying out similar experiments (equal DOC concentration, pH and hardness) for DOM originating from three different sites. Finally, the observed toxicity data and measurements of Cu^{2+} activity during the toxicity tests were used to build a semi-mechanistic model predicting copper speciation in the presence of natural DOM and copper toxicity to *P. subcapitata.*

6.2. Materials and methods

6.2.1. Sampling sites; DOM sampling, treatment and characterisation; experimental design

The same sampling sites and DOM was used for this study as the ones presented in chapter 4 (see section 4.2.1.). A detailed description of the procedures followed is given in chapter 4. The experimental design for this study was exactly the same as that for the chronic *D. magna* tests. This design also is extensively explained in chapter 4 (see section 4.2.2.).

6.2.2. Algal toxicity testing

All tests with *Pseudokirchneriella subcapitata* were conducted in accordance with OECD Guideline No. 201 (OECD, 1984). *P. subcapitata* Printz starter cultures were obtained from the Culture Collection of Algae and Protozoa (CCAP 278/4, Windermere, UK). Algae are continuously cultured in aerated Ghent city tap water enriched with nutrients. Cultures were kept at 20 ± 1°C under continuous light (5000 lux) and with continuous aeration. Before testing algae were pre-acclimated for 5 days (8 to 10 generations) to standard OECD-medium under the same conditions. Only log-phase algae were used for starting toxicity tests.

Exposure media were prepared by adding the correct amounts of stock solutions of $CaCl_2$, $MgSO_4$ and DOC to standard OECD medium. EDTA (ethylene diamine tetra acetic acid), which is normally present in standard OECD medium to keep Fe in solution, was omitted since it could have dominated the complexation of Cu, especially at low concentrations of natural DOM. Moreover, the natural DOM in the test media performed this task equally well (data not shown). 750 mg L^{-1} MOPS (3-N morpholino propane sulfonic acid, Sigma-Aldrich, Steinheim, Germany) was added as a pH buffer in all experiments except for medium 8 (pH ~ 8.5, $NaHCO_3$-buffering). MOPS was chosen because it is completely non-complexing for metals (Kandegedara et al., 1999) and because it is recommended by US-Environmental Protection Agency (1991) as it does not change the toxicity of effluents toxic and sediment pore waters. Additionally, De Schamphelaere et al. (2003) have demonstrated that addition of 750 mg MOPS L^{-1} does not alter copper or zinc toxicity to *P. subcapitata* and *Daphnia magna*. The medium was adjusted to the desired pH level with NaOH or HCl and was spiked with different copper concentrations ($CuCl_2$). The difference between the lowest and highest copper concentration was one order of magnitude.

Tests were performed in 100 mL glass erlenmeyer flasks containing 50 mL of test medium. Each test consisted of six control replicates and three replicates for each of the five copper concentrations tested. The spiked media were equilibrated in complete darkness for 48 hours at 4°C and 48 hours at 25°C before testing. At the beginning of each test, each flask was inoculated with 10^4 cells mL^{-1}. Tests were carried out under continuous uniform illumination (4000 lux at the surface of the exposure medium). The temperature in the media during the test period was 25°C ± 1°C. The cell density was measured after 24, 48 and 72 hours with the aid of an electronic particle counter (Model DN, Harpenden, Herts, UK). Concurrently, the pH of the medium was measured and, if required adjusted to the initial pH with NaOH or HCl.

6.2.3. Chemical analysis during the ecotoxicological experiments

Dissolved copper concentrations (filtered through a 0.45µm filter, Gelman Sciences, Ann Arbor, Michigan, USA) were determined at the beginning and at the end of the test using a flame-atomic absorption spectrophotometer (SpectroAA100, Varian, Mulgrave, Australia). Calibration standards (Sigma-aldrich, Steinheim, Germany) and a reagent blank were analysed before each series of ten samples. Dissolved organic carbon (0.45 µm filtered) and Inorganic Carbon (IC) were measured before each test (TOC-5000, Shimadzu, Duisburg, Germany).

Concentrations of major cations (Na, K, Ca, Mg) and anions (Cl, SO_4) were calculated as the sum of (1) ions added along with the DOC, (2) NaOH or HCl additions for bringing pH to the desired level and (3) $CaCl_2$ and $MgSO_4$ additions. For all of these ions concentrations were measured occasionally and these were always within 10% of the reported concentrations. pH-measurements were performed with pH-meter P407 (Consort, Turnhout, Belgium). The pH glass electrode was calibrated before each use using pH 4 and pH 7 buffers (Merck, Darmstadt, Germany).

Before and after the ecotoxicological experiments, the activity of the free copper ion was determined using a cupric ion selective electrode (Cu-ISE, model 94-29, Orion Research, Boston, MA, USA) and a double junction Ag/AgCl reference electrode (Model 90-02, Orion Research). The Cu-ISE was calibrated before each use with a Cu-ethylenediamine buffer over the pCu range of 5 to 11. A detailed description of the exact preparation and use of the Cu-ISE is given in De Schamphelaere et al. (2002, chapter 3).

6.2.4. Data treatment and analysis

The algae growth inhibition percentages compared to the control were calculated according to the 'area under the curve' method prescribed by the OECD (1984). Seventy-two hour E_bC50 and E_bC10 values (effect concentrations resulting in 50% and 10% growth inhibition, respectively) and their 95% confidence intervals were calculated using the probit method. The NOE_bC (no observed effect concentration with respect to growth inhibition) for each test medium was determined using the post-hoc test of Dunnet ($p<0.05$). Effect concentrations were calculated based on dissolved copper concentrations and free copper ion activities.

Analysis of linear, quadratic and interaction effects of DOC, pH and hardness on the calculated E_bC50s and E_bC10s was carried out with the central composite analysis module of STATISTICA (Statsoft, Tulsa, OK, USA) and the regression analysis module of SIGMAPLOT (SPSS Inc., Chicago**,** IL, USA). A central composite analysis was performed on the ecotoxicological data obtained with Ankeveen DOM, with DOC, pH and hardness as independent variables and 72-hour E_bC50 and 72-hour E_bC10 as dependent variables. It was not possible to perform the same kind of statistical analysis with the Bihain and Ossenkolk DOM test

results since only 9 out of the 17 required exposure media were tested. On these results second-order polynomial regression analysis was applied to assess the extent to which the different effects (linear, quadratic and interaction) explained variability. Second-order polynomial regression analysis was also performed for the results obtained with Ankeveen DOM, additional to the central composite analysis. To that end, the forward regression analysis technique in Statistica was applied. This technique adds terms to a regression equation one by one, starting with the most significant term, until no further significant improvement of the overall equation is obtained.

6.3. Results and discussion

6.3.1. Chemical composition of the DOM stocks

The chemical composition of the DOM stocks is already discussed in chapter 4 and this is not repeated here (see Table 4-3 and section 4.3.1.). With respect to the relatively high concentrations of sodium (up to 17.4 mM), that were added to the test media along with the DOM, following should be noted. Sodium is known to decrease copper toxicity to fish (Erickson et al., 1996) and daphnids (De Scahmphelaere et al., 2002, chapter 2). However , iIt is suggested that this apparent “competition” effect in these organisms is related to copper disturbing the sodium balance resulting in death (Grosell et al., 2002). However, this mechanism of copper toxicity has not been demonstrated for algal cells and initial experiments at our laboratory indicate that increased Na concentrations (up to 20 mM) result in only small decreases in copper toxicity (factor 1.5). Therefore it is not very likely that the high Na concentrations have affected the observed toxicity relationships with pH and DOC (as these induced larger variations in bioavailability, see further).

6.3.2. Ecotoxicological data

All reported effect concentrations are based on dissolved Cu concentrations measured at the start of the experiment. Dissolved copper concentrations were also monitored during the assays. It was noted that with time dissolved copper decreased, which is probably due to adsorption of copper at the algal surface and/or uptake by the algae. Dissolved copper decreased on average 17.7%, 25.7% and 3.6% during the 72-hour time course of the experiments with

Bihain, Ossenkolk and Ankeveen DOM, respectively. These average decreases were all significantly different from one another (*t*-tests, $p<0.01$). The reason for this decrease is adsorption of copper to the algal surface during the experiment either directly, through adsorption / internalization of inorganic copper species, or indirectly, through adsorption of organically complexed copper (Campbell et al., 1997). Whereas the former reaction might be directly related to the degree of the toxic response (see further), the latter reaction is believed not to have an effect on copper toxicity. Campbell et al. (1997) state that although the accumulation of DOM at the phytoplankton cell surface would tend to slow down the kinetics of metal diffusion to uptake sites, this type of interaction is probably unimportant for metals with fast ligand-exchange kinetics such as copper. In this context, it is noteworthy that the average decrease of dissolved copper was positively related to the humic acid content of the DOM used.

Next to these mechanistic considerations, one could argue that, if there is an important fraction of inorganic copper that is directly adsorbed by the algal surface, free copper ion activity in the test medium should decrease during the time-course of the experiment (resulting in a decreased toxicity). Occasional measurements demonstrated, however, that free copper ion activity in the algal test media did not significantly change during the toxicity tests. Based on this, it might be suggested that the major fraction of algae-bound copper is organically complexed copper. Since this reaction does not alter the ratio organic matter/copper in the bulk solution, one would indeed not expect free copper ion activity in the test medium to change. It can be concluded that the decrease of dissolved copper during the experiments does not have major implications for the conclusions drawn from this study.

Table 6-1 represents the chemical characteristics of and the 72-hour E_bC50s, E_bC10s and NOE_bCs obtained with the 35 different test media. For some tests, no NOE_bCs could be derived, as even at the lowest tested copper concentration a significantly adverse effect was noted. As a surrogate for the NOE_bC the E_bC10 was calculated. This is justified in this study: in those cases where both the NOE_bC and the E_bC10 were calculated, no significant differences between both endpoints were noted (t-test for dependent samples, $p>0.05$).

72-hour E_bC10s ranged from 14.2 to 175.9 µg Cu L^{-1} and E_bC50s from 26.9 to 506.8 µg Cu L^{-1}. These observations agree well with copper toxicity values obtained with *P. subcapitata* tested in natural waters: 72-hour NOE_bCs ranged from 4.2 to 164.1 µg Cu L^{-1} and E_bC50s from 32.0 to 245 µg Cu L^{-1} (Heijerick et al., unpublished data).

Table 6-1. Physico-chemistry of *Pseudokirchneriella subcapitata* test media and corresponding chronic (72-hour) effect concentrations for copper expressed as dissolved copper and Cu^{2+} activity (See text for meaning of NOE_bC, E_bC10 and E_bC50). Dissolved organic carbon (DOC) from three locations was added to the test media (see Table 1 for information about sampling locations). Active fulvic acid (FA) is calculated as explained in text. Figures between brackets indicate the magnitude of one side of the 95% confidence interval.

											Dissolved (µg Cu/L)			Cu^{2+} activity (nM)		
Medium	DOC (mg/L)	active FA (%)	pH	Ca (mM)	Mg (mM)	Na (mM)	K (mM)	Cl (mM)	SO_4 (mM)	IC (mM)	72-hour NOE_bC	72-hour E_bC10	72-hour E_bC50	72-hour NOE_bC	72-hour E_bC10	72-hour E_bC50
Bihain																
1	5.23	83.0	6.19	0.8	0.2	5.59	0.01	7.51	0.142	0.0482	63.9	61.8 (35.5)	178.4 (45.0)	10.6	13.0 (13.2)	103.5 (50.2)
2	15.8	69.5	6.22	0.8	0.2	15.6	0.01	19.2	0.145	0.0505	110.6	175.9 (13.8)	506.8 (15.2)	3.90	13.2 (2.8)	143.7 (7.2)
3	5.99	64.9	7.92	3.2	0.8	9.45	0.01	13.2	0.742	1.30	-	25.4 (5.9)	66.1 (5.4)	-	0.084 (0.049)	0.357 (0.118)
4	2.21	64.0	7.05	2	0.5	4.19	0.01	6.58	0.441	0.248	-	15.4 (3.9)	29.1 (3.5)	-	0.989 (0.644)	3.3 (1.29)
5	15.1	67.2	8.01	3.2	0.8	18.1	0.01	23.2	0.744	1.55	-	93.7 (17.5)	234 (10.0)	-	0.124 (0.381)	0.385 (0.099)
6	9.99	56.5	7.09	2	0.5	11.5	0.01	15.2	0.443	0.265	57.5	55.1 (20.7)	127.6 (25.7)	0.643	0.678 (0.489)	4.684 (1.990)
7	9.89	58.7	7.04	4	1	11.5	0.01	19.1	0.943	0.241	59.1	55.8 (14.2)	168.1 (16.7)	0.802	0.610(0.364)	9.891 (3.360)
8	10.3	74.8	8.37	2	0.5	21.1	0.01	15.5	0.443	3.05	-	42.5 (24.7)	156.6 (32.7)	-	0.0081(0.0026)	0.082 (0.011)
9	18.5	48.4	7.17	2	0.5	19.8	0.01	24.5	0.445	0.312	-	75.2 (38.3)	285.1 (63.2)	-	1.062 (0.868)	11.3 (4.4)
Ossenkolk																
1	5.31	76.7	6.20	0.8	0.2	1.92	0.01	3.10	0.140	0.0491	111.2	118.5 (46.3)	171.9 (34.6)	3.20	9.8 (17.5)	105.2 (125.5)
2	15.6	57.9	6.20	0.8	0.2	4.41	0.01	5.78	0.141	0.0491	112.8	134.7 (52.6)	490.3 (119.8)	6.84	10.9 (10.4)	231.7 (136.9)
3	5.75	58.5	8.07	3.2	0.8	5.28	0.01	8.02	0.740	1.72	49.4	47.4 (32.0)	88.2 (30.6)	0.247	0.171 (0.947)	0.574 (0.432)
4	2.06	67.2	7.08	2	0.5	2.59	0.01	4.66	0.440	0.260	19.4	16.6 (5.4)	30.5 (4.3)	0.899	1.23 (0.81)	3.268 (1.849)
5	16.1	68.8	8.05	3.2	0.8	7.77	0.01	10.7	0.741	1.66	174.0	146.3 (85.0)	316.7 (109.6)	0.221	0.167 (0.126)	0.418 (0.322)
6	11.1	51.6	7.09	2	0.5	4.76	0.01	6.99	0.440	0.265	53.7	43.3 (44.8)	130.3 (49.1)	0.653	0.477 (0.944)	5.137 (4.612)
7	10.5	55.1	7.12	4	1	4.63	0.01	10.9	0.940	0.284	67.7	89.7 (41.8)	198.3 (40.6)	0.896	1.7 (2.1)	13.6 (8.2)
8	10.3	89.5	8.25	2	0.5	13.9	0.01	6.80	0.440	2.42	-	42.5 (36.2)	127.7 (55.9)	-	0.0234 (0.0096)	0.0404 (0.008)
9	19.9	58.2	7.11	2	0.5	6.88	0.01	9.27	0.441	0.278	170.8	148.5 (126.3)	453.6 (230)	0.648	1.074 (1.871)	16.1 (19.3)

Table 6-1 (continued)

Medium	DOC (mg/L)	active FA (%)	pH	Ca (mM)	Mg (mM)	Na (mM)	K (mM)	Cl (mM)	SO_4 (mM)	IC (mM)	Dissolved (µg Cu/L)			Cu^{2+} activity (nM)		
											72-hour NOE_bC	72-hour E_bC10	72-hour E_bC50	72-hour NOE_bC	72-hour E_bC10	72-hour E_bC50
Ankeveen																
1	5.07	26.9	6.18	0.8	0.2	1.87	0.01	2.83	0.280	0.0468	40.8	53.4 (24.2)	134.6 (10.9)	27.1	52.7 (32.1)	427.8 (218.0)
2	14.9	39.2	6.17	0.8	0.2	4.34	0.01	4.95	0.552	0.0459	89.2	94.0 (18.7)	341.0 (56.3)	16.1	16.8 (8.6)	300.9 (148.7)
3	5.46	42.7	7.92	3.2	0.8	4.97	0.01	7.71	0.891	1.29	-	25.0 (5.6)	65.6 (3.1)	-	0.328 (0.200)	1.256 (0.067)
4	1.95	44.7	7.02	2	0.5	2.58	0.01	4.55	0.494	0.232	-	14.2 (5.3)	26.9 (5.2)	-	1.98 (2.48)	10.66 (2.48)
5	15.2	49.8	7.78	3.2	0.8	7.73	0.01	9.83	1.162	0.989	97.2	96.5 (16.0)	138.3 (8.9)	0.176	0.181 (0.162)	0.787 (8.9)
6	10.1	39.7	7.04	2	0.5	4.64	0.01	6.32	0.721	0.244	-	52.0 (10.0)	105.0 (16.8)	-	3.38 (1.25)	8.90 (16.8)
7	10.4	32.5	7.02	4	1	4.70	0.01	10.4	1.226	0.232	60.2	59.9 (9.5)	104.5 (11.4)	2.1	2.42 (1.71)	15.7 (11.4)
8	10.5	37.2	8.58	2	0.5	15.2	0.01	6.39	0.730	4.55	37.6	33.6 (4.0)	60.7 (3.3)	0.045	0.044 (0.003)	0.058 (3.3)
9	18.2	30.9	6.98	2	0.5	6.16	0.01	8.06	0.943	0.215	91.3	91.6 (28.3)	179.2 (25.8)	2.96	3.12 (2.68)	15.8 (25.8)
10	5.64	62.9	5.99	3.2	0.8	2.31	0.01	7.75	0.896	0.0326	-	46.3 (3.4)	142.4 (14.9)	-	17.1 (3.0)	182.8 (14.9)
11	15.3	45.1	5.99	3.2	0.8	4.70	0.01	9.85	1.165	0.0326	-	92.7 (16.1)	356.0 (30.4)	-	25.1 (11.1)	402.5 (30.4)
12	5.42	23.1	8.02	0.8	0.2	4.96	0.01	2.90	0.290	1.58	-	18.4 (3.7)	44.0 (5.2)	-	0.198 (0.062)	0.779 (5.2)
13	15.3	44.3	8.05	0.8	0.2	8.10	0.01	5.05	0.564	1.67	53.3	68.4 (11.3)	154.5 (18.2)	0.036	0.102 (0.037)	0.437(18.2)
14	9.84	67.2	5.68	2	0.5	3.07	0.01	6.26	0.712	0.0181	54.6	66.2 (10.9)	311.3 (71.1)	-	16.2 (14.3)	419.2 (71.1)
15	10.4	38.1	7.19	0.13	0.13	5.56	0.01	2.62	0.348	0.324	49.2	53.4 (5.4)	121.2 (8.2)	0.0896	0.849 (0.323)	4.79 (8.2)
16	9.98	44.6	7.03	2	0.5	4.60	0.01	6.29	0.716	0.239	-	43.9 (11.0)	90.0 (14.4)	-	2.39 (0.88)	7.06 (14.4)
17	10.2	34.4	7.01	2	0.5	4.67	0.01	6.34	0.723	0.230	-	56.9 (14.2)	105.0 (16.8)	-	3.54 (1.31)	8.88 (16.8)

This large variability in toxicity test results observed in our and other studies demonstrates the need for incorporating bioavailability into risk assessment and water quality criteria (WQC) setting procedures for copper. However, before this type of procedure can be established it is important (1) to know which physico-chemical factors affect copper toxicity and (2) to develop mathematical tools that can calculate threshold concentrations as a function of water characteristics. This will be investigated in the following sections.

6.3.3. Statistical analysis and interpretation of the ecotoxicological data

Table 6-2 summarizes how much of the experimental variance of the observed E_bC10s and E_bC50s is explained by the different experimental factors (linear, quadratic and interaction effects). Linear effects refer to a linear relation between an experimental factor and the observed E_bC10s and E_bC50s, a quadratic effect points to a curvature in that relation, and an interaction effect can most easily be interpreted as a change of the slope of the effect of one factor when another factor is varied.

Table 5. Percentage of experimental variance on E_bC10 and E_bC50 explained by the different experimental factors (linear effects, quadratic effects and interaction effects) (see text for meaning of NOE_bC, E_bC10 and E_bC50) for the tests with DOM from three locations (see Table 4-1 for information about sampling locations). For Bihain and Ossenkolk DOM, only terms including pH and DOC were considered. Numbers indicate percentage as calculated by regression analysis; numbers between brackets are calculated using central composite analysis (not taking into account differences between theoretical and experimental values of factors). Underlined figures indicate significant effects (p<0.05).

		DOC [a]	pH [a]	pH [2 b]	DOC · pH [c]	Cumulative % variance explained
Bihain	E_bC10	<u>49.2</u>	<u>23.2</u>	<u>12.3</u>	<u>3.1</u>	87.6
Bihain	E_bC50	<u>59.5</u>	<u>21.4</u>	<u>12.3</u>	<u>3.7</u>	96.8
Ossenkolk	E_bC10	<u>55.2</u>	<u>11.6</u>	<u>6.5</u>	<u>9.0</u>	82.3
Ossenkolk	E_bC50	<u>77.0</u>	<u>12.9</u>	<u>3.3</u>	0.8	94.0
Ankeveen	E_bC10	<u>77.4 (77.5)</u>	<u>13.7 (11.9)</u>	0.2 (2.9)	1.0 (1.0)	93.3 (93.3)
Ankeveen	E_bC50	<u>34.3 (34.2)</u>	<u>47.4 (45.7)</u>	<u>8.3 (9.2)</u>	<u>4.3 (4.4)</u>	94.3 (93.5)

[a] linear effects ; [b] quadratic effect; [c] interaction effect

The effects of hardness are not included in Table 6-2, since central composite analysis of the test data with Ankeveen DOM indicated that hardness did not have a significant effect on the 72-hour E_bC10 and E_bC50 values. This observation was extrapolated to the Bihain and Ossenkolk data. Hence, hardness was not included in the regression equations for these DOMs either. At first sight, not taking into account hardness effects, contrasts with the general idea that increased hardness decreases copper toxicity (i.e. through competition, [e.g., Pagenkopf, 1983; Erickson et al., 1996; De Schamphelaere and Janssen, 2002, chapter 2) and with findings of Heijerick et al. (unpublished data). This will be discussed in more detail below.

pH and DOC did have significant effects on copper toxicity to *P. subcapitata*. For all three DOMs tested, the two most important effects were the linear effects of DOC and pH. Together they explained between 72.4 and 91.1% of the variability around the E_bC10 and between 66.8 and 81.7% of the variability around the E_bC50. In chapter 4, it was described that linear protective effects of DOC were also the most significant for chronic copper toxicity to *D. magna*. Heijerick et al. (2003) also found that linear effects of DOC (added as humic acid) and pH were the most important factors determining chronic zinc toxicity to *Daphnia magna*, explaining 78.4% of the variability around the 21-day EC50s.

Overall, the results of this study indicate a decreased copper toxicity with increased DOC concentration and an increased copper toxicity with increased pH. This is in agreement with other previously mentioned studies with algae (Sunda and Lewis, 1978; Meador et al., 1998; Franklin and Stauber, 2000; Macfie et al., 1994; Nalejawko et al., 1997). However, a number of important differences across the three different DOMs were noted.

First, the protective effect of DOC against copper toxicity was more pronounced for Bihain and Ossenkolk DOM than for Ankeveen DOM. T-tests for dependent samples (for test media 1 to 9) indicated that experiments with DOM from Bihain and Ossenkolk yielded significantly ($p<0.05$) higher E_bC10s and E_bC50s than those with DOM from Ankeveen. This difference can possibly be attributed to different copper complexation capacities of DOMs from different sources. A difference of factor 5 in copper complexation capacities of natural DOM from different sources has been reported (Abbt-Braun and Frimmel, 1999; Frimmel and Abbt-Braun, 1999). The difference in copper toxicity in tests with DOM from different sources, however, only amounted to a maximum of about factor 2.5 (See Table 6-1).

Second, the extent to which linear, quadratic and interaction effects explained experimental variability seems to be dependent on both the type of DOM and the endpoint (E_bC50 or E_bC10) used (Table 6-2). These differences are also reflected in different forms of the regression equations (Table 6-3) and are probably the result of the enormous complexity of natural organic matter (MacCarthy, 2001).

Table 6. Non-linear regression analysis of the form $y = a + b \cdot DOC + c \cdot pH + d \cdot pH^2 + e \cdot pH \cdot DOC$ for copper toxicity experiments with *Pseudokirchneriella subcapitata* using dissolved organic carbon (DOC) from three locations (see Table 1 for information about sampling locations). Numbers between brackets indicate the standard errors on the coefficients. See text for meaning of NOE_bC, E_bC10 and E_bC50.

	y	a	b	c	d	e	r^2	p
Bihain	E_bC10	1620	25.23	-464.6	31.95	-2.683	0.876	0.043
		(857)	(19.18)	(233.7)	(16.03)	(2.713)		
Bihain	E_bC50	4914	81.95	-1376	95.37	-8.710	0.968	0.0031
		(1297)	(29.01)	(353)	(24.52)	(4.104)		
Ossenkolk	E_bC10	1925	- 25.31	-463.3	27.18	4.506	0.823	0.032
		(1170)	(22.58)	(320.7)	(22.01)	(3.165)		
Ossenkolk	E_bC50	3600	24.97	-940.6	59.80	-	0.932	0.0024
		(2002)	(3.28)	(558.1)	(38.58)			
Ankeveen	E_bC10	88.93	5.061	-11.81	-	-	0.921	<0.0001
		(18.30)	(0.465)	(2.50)				
Ankeveen	E_bC50	2263	56.87	-627.2	43.25	-6.292	0.943	<0.0001
		(570)	(15.94)	(158.6)	(11.13)	(2.262)		

A few remarks can be made concerning the use of these regression models for regulatory purposes. First, extrapolation of the regression models to the high pH – low DOC range results in physically meaningless negative predicted values of 72-hour E_bC50s and E_bC10s (see also chapter 4). Second, the regression models are different for the different DOMs investigated, which means that one should choose one of the models for predicting toxicity in field waters without *a priori* knowing what model would be the best predictor for a given field water. Hence, it is obvious that the use of this kind of empirical regression equations for predicting copper effects in field waters should be avoided. Instead, it is probably more advisable to use the

experimental data to develop a more mechanistic model which can account for differences in DOM characteristics and which can predict both copper speciation and toxicity. Before trying to model toxicity in a more relevant manner, the following section will investigate to which level of detail the variability of the three DOMs should be taken into account in these models, especially with respect to different complexation capacities.

6.3.4. Copper speciation – general approach

Central to the development of a mechanistic copper toxicity model (such as the Biotic Ligand Model, BLM) is the ability to correctly predict copper speciation as a function of physico-chemical water characteristics. Di Toro et al. (2001) stated that the inorganic speciation is the most straightforward of the computation because the ligands (such as OH^- and CO_3^{2-}) are well characterized and their binding constants have been determined. All subsequent speciation calculations will be performed with stability constants for inorganic copper complexes taken from Martell et al. (1997, Table 2-2). The complexation of copper to organic molecules is however more difficult. In the framework of the develoment of BLMs, WHAM-Model V (Tipping, 1994) has become an integral part of the computations because the model has been calibrated to multiple datasets of titrations of isolated organic matter with metals and acid/base (Tipping and Hurley, 1992; Tipping, 1993; Dwane and Tipping, 1998). However, WHAM tends to overestimate copper binding onto DOM under natural conditions (Dwane and Tipping, 1998). Indeed, the WHAM-calculated Cu^{2+}-activity under natural conditions is usually lower than the measured Cu^{2+}-activity. Based on their own experimental results and those of Cabaniss and Shuman (1988), Dwane and Tipping (1998) suggested that, if nothing is *a priori* known about the chemical characteristics of a specific DOM, the DOM should be considered to consist of 50% active fulvic acid (active FA), with the rest being inert for copper complexation. Using that assumption in a BLM approach, De Schamphelaere et al. (2002; chapter 3) were able to accurately predict acute 48-hour EC50s of copper to *D. magna* in 19 natural surface waters with unknown DOM characteristics.

In the present study we used the percentage of active FA as the sole calibration parameter for each DOM: DOM was considered to consist of no humic acid, a certain percentage of active FA and an inert fraction. This simplified approach allows testing if adjusting only one parameter would result in reasonable fits between measured and calculated Cu^{2+}-activities for the whole range of tested physico-chemical conditions.

6.3.4. Calibration of WHAM to the observed free Cu^{2+} measurements

For each exposure medium, the percentage of active FA was determined that resulted in the best fit between observed and WHAM-calculated free copper ion activity (as determined by least squares analysis of observed and calculated pCu = -log (Cu^{2+}) for the 5 exposure concentrations). This procedure is explained in detail in De Schamphelaere et al. (2002, chapter 3). Percentages of active FA are presented in Table 4. The average percentages of active FA were 65.2% (Standard deviation, SD=10.2%, *n*=9) for Bihain DOM, 64.8% (SD=12.1%, *n*=9) for Ossenkolk DOM and 41.4% (SD=11.4%, *n*=17) for Ankeveen DOM. These values correspond to the range found by Dwane and Tipping (1998) for a number of UK water samples (i.e. 40-80%). The percentage active FA of Ankeveen was significantly lower than that of Bihain and Osenkolk DOM (*t*-tests, $p<0.05$). Since a lower percentage of active FA means that less copper can bind to the DOM under consideration, the latter matches with the observation that Ankeveen DOM reduced copper toxicity to *P. subcapitata* to a lesser extent than Bihain and Ossenkolk DOM.

The percentages of active FA for each DOM were not related in any way to the concentration of DOC, pH and hardness. Moreover, Figure 6-1 shows that both observed E_bC50_{Cu2+} and E_bC10_{Cu2+} values can be accurately calculated from the physico-chemistry of the test media and the dissolved E_bC50s and E_bC10s by assuming the average percentage of active FA for each exposure medium. Over 90% of the E_bC50_{Cu2+} and E_bC10_{Cu2+} values are predicted within a factor of 2 difference of the observed E_bC50_{Cu2+} and E_bC10_{Cu2+} values. The latter values were calculated with the probit method based on growth inhibition percentages at each measured free copper ion activity (Table 6-1).

It can be concluded that by varying only one parameter in the input of the speciation calculation, copper speciation can accurately be predicted using the WHAM V speciation model. In other words, it is not necessary to know the exact values of all DOM chemical parameters (i.e. binding constants for copper and competing cations, protons, binding site densities, etc.) to be able to make reasonably accurate predictions of copper speciation and thus to form a basis for further modelling of copper toxicity.

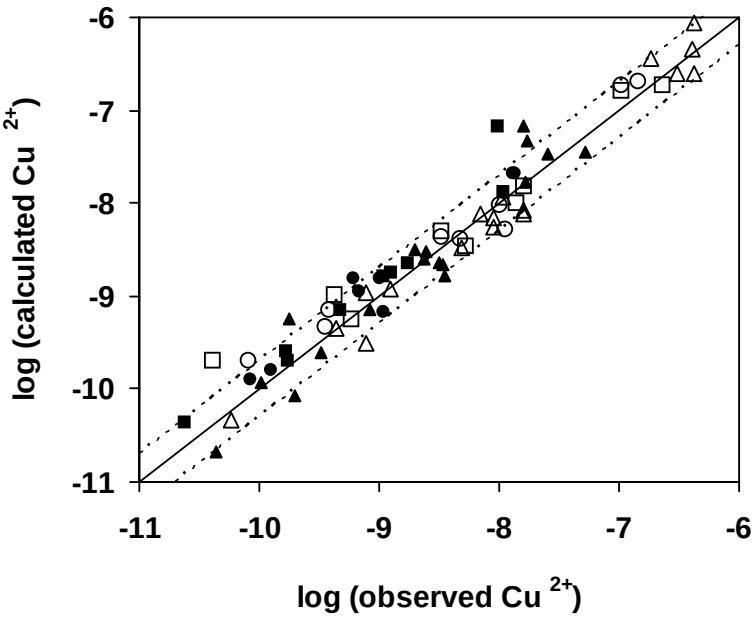


Figure 6-1 Observed versus calculated Cu^{2+}-acitivity at the E_bC50 (open symbols) and E_bC10 (closed symbols) level. "Observed Cu^{2+}" refers to the E_bC50 and E_bC10 values expressed as Cu^{2+}-activity and were calculated for each test medium by probit analysis of the growth inhibition at each measured Cu^{2+}-activity (see text for meaning of E_bC50 and E_bC10). "Calculated Cu^{2+}" refers to the Cu^{2+}-acitivity calculated with Biotic Ligand Model version a008 (Hydroqual, 1999) using the physico-chemistry of the exposure media, assuming the average percentage of active fulvic acid for the dissolved organic matter from each site: 65.2% for Bihain (circles), 64.8% for Ossenkolk (squares) and 41.4% for Ankeveen (triangles). See Table 1 for information about sampling sites.

6.3.5. Statistical analysis of toxicity expressed as free copper ion activity

Table 6-1 also presents the 72-hour NOE_bCs, E_bC10s and E_bC50s expressed as free copper ion activities for the 35 different test media. E_bC10s ranged from 0.0081 to 52.7 nM (Cu^{2+}) (~ factor 6,500) and E_bC50s ranged from 0.0404 to 427.8 nM (Cu^{2+}) (~ factor 10,000). This seems to indicate that free copper ion activity is a worse predictor of toxicity than dissolved copper, since effect concentrations expressed as dissolved copper for the same 35 media only differed by a factor of about 20.

To overcome the problems associated with statistical analysis on values that vary over several orders of magnitude, central composite analysis of the Ankeveen toxicity data was performed on log-transformed E_bC10_{Cu2+} and E_bC50_{Cu2+} values. From this analysis it appeared that the linear effect of pH on log(E_bC10_{Cu2+}) and log(E_bC50_{Cu2+}) was the only significant one, explaining 91.2% and 89.3% of the variability, respectively. A similar trend was observed for regression analysis of the toxicity data with Bihain and Ossenkolk DOM. No significant differences of the log(E_bC50_{Cu2+}) and the log(E_bC10_{Cu2+}) values between the experiments with the different DOMs were found (t-tests for dependent samples for test media 1 to 9, $p>0.05$). These findings support the idea that when copper toxicity is expressed as free copper ion activity, the type and the amount of organic matter used is not important for predicting copper toxicity to *P. subcapitata*.

6.3.6. Modelling copper toxicity

Highly significant linear regression equations between pH and log(E_bC50_{Cu2+}) or log(E_bC10_{Cu2+}) were derived ($p<0.001$):

$$\text{Log}(E_bC50_{Cu2+}) = - 1.431\ \text{pH} + 2.050\ (r^2 = 0.95) \quad (1)$$

and

$$\text{Log}(E_bC10_{Cu2+}) = -1.140\ \text{pH} - 0.812\ (r^2 = 0.91) \quad (2)$$

Seventy two percent of the observed E_bC50_{Cu2+} and E_bC10_{Cu2+} values deviated less than 0.3 log units (factor 2) from the regression line (the predicted E_bC50_{Cu2+} and E_bC10_{Cu2+} values), while 88% deviated less than 0.5 log units (factor 3). This means that these regressions form a solid basis for predicting copper toxicity.

It was also observed that at higher pH less free copper is needed to exert the same toxic effect. This has already been observed for *Chlorella* sp. (Franklin and Stauber, 2000), *C. reinhardtii* (Macfie et al., 1994) and for three *Scenedesmus* strains (Nalejawko et al., 1997). Before discussing the different kinds of chemical and biological processes that might result in the observed strong pH effect, a summary of how the mechanism of metal toxicity to algae is given (Campbell, 1995; see also chapter 1, section 1.2.2.4.).

On approaching the surface of an algal cell, a metal ion will normally first encounter a protective polysaccharide or glycoprotein layer (the cell wall). The macromolecules making up this external layer contain a variety of functional groups, many of which become ionised when pH is increased. This results in a matrix of negatively charged sites at which the metal can accumulate and through which the metal must migrate before eventually meeting the plasma membrane. At this membrane the metal can then bind to physiologically active sites, resulting in direct or indirect toxicity (see section 1.2.2.4.). It is clear that the more negatively charged the protective layer (cell wall) becomes, the more positively charged cations (e.g. Cu^{2+}) can 'collect' in this layer, and eventually, the more Cu can enter the alga cell, and eventually the more Cu can bind to physiologically active sites on the plasma-membrane or in the cytoplasm, resulting in a larger toxic response. An overview of these possible 'sites' of toxic action is given in Stauber and Davies (2000).

To our knowledge, however, not one study has attempted to demonstrate that the amount of adsorbed metal ions is directly related to a toxicity response, independent of the physico-chemical characteristics of the test media. In the light of the current interest for biotic ligand models, the latter is an absolute requirement if one would like to consider the cell wall as the biotic ligand for algae.

Accepting this assumption and the above-described mechanism, a number of authors have described and suggested processes that indeed may result in the steep decrease of E_bC50_{Cu2+} and E_bC10_{Cu2+} with increasing pH (not discussed in order of importance).

First, the two most abundant inorganic copper species at higher pH levels, i.e. $CuOH^+$ and $CuCO_3$, may also be toxic, in addition to Cu^{2+}. To date, this has only been shown to play a role in copper toxicity to fish and daphnid species (Erickson et al., 1996; De Schamphelaere and Janssen, 2002, chapter 2; De Schamphelaere et al., 2002, chapter 3). Whereas in these organisms the toxicity of $CuOH^+$ is believed to occur mainly through direct binding to the biotic ligand, the toxicity of $CuCO_3$ is suggested to be the result of pH and alkalinity differences between the bulk solution and the microenvironment of the biotic ligand (Playle et al., 1992). To our knowledge, the latter mechanism has not been demonstrated for algae.

Second, a commonly used interpretation for the observed decreased toxicity at lower pH levels is competition of protons and copper for binding sites on the algal surface (Franklin and Stauber, 2000, Macfie et al., 1994; Nalejawko et al., 1997). However, whereas biotic ligand

models for fish and invertebrates typically consider only one type of site, with one characteristic pKa value for proton binding (Di Toro et al, 2001; De Schamphelaere and Janssen, 2002, chapter 2; De Schamphelaere et al., 2002, chapter 3; Santore et al., 2001), this may not necessarily be valid for algae.

If proton competition at a single site would be the only mechanism responsible for the observed pH-toxicity relationship then, according to De Schamphelaere and Janssen (2002, chapter 2) and Brown and Markich (2000), a linear relation between H^+-activity and the E_bC50_{Cu2+} should be observed. However, we observed a highly significant linear relation of the form $\log(E_bC50_{Cu2+}) = a + b \cdot pH$ (See equations 1 and 2) which points to the mathematical incorrectness of trying to fit a linear relation between H^+-activity and the E_bC50_{Cu2+}. Indeed, rearranging this relation results in the power function: $E_bC50_{Cu2+} = 10^a \cdot (H^+)^{-b}$. This non-linearity indicates that the validity of the proton competition hypothesis at a single site should be questioned. It thus seems that, when a single site is assumed, proton competition alone cannot account for the observed 4 orders of magnitude decrease of E_bC50_{Cu2+} over the tested pH range.

In *P. subcapitata* growth inhibition experiments with zinc, Heijerick et al. (2002) also did not observe a linear relation between H^+ activity and the E_bC50_{Zn2+}. Their data suggest that multiple binding sites for zinc to the algal cell wall may exist. This seems to be supported by Xue and Sigg (1990) and by Crist et al. (1988). Based on alkalimetric titrations of suspensions of *C. reinhardtii*, Xue and Sigg (1990) demonstrated the existence of at least two distinct types of proton dissociation groups. Using an ingenious acid titration technique on algal suspensions of *Vaucheria* sp. and *Spirogyra* sp., Crist et al. (1990) observed a linear decrease of pH with added HCl equivalents in the pH range 4 to 7 and they attributed this to the existence of acidic groups (carboxylate groups) having several pKa values, along with a pKa dependency on surface charge. While metal reactions with algal surfaces are considered to be mainly electrostatic bonds with carboxylic groups (with O-donors) (Campbell, 1995), copper has been shown to form also more covalent type of bonds with N- and S-donors of amino and sulfhydryl groups (Crist et al., 1990; Van Cutsem and Gillet, 1981), which usually have pKa values > 8 (Stumm and Morgan, 1996).

All together this would result in a continuous increase of deprotonated binding sites for copper on the algal surface with increasing pH, an increase of copper bound to the algal surface and an increased toxicity with increasing pH. Additionally, it should also be kept in mind that Cu^{2+} ions may also be able to form divalent bonds with algal cell wall functionalities (Crist et al.,

1990), a mechanism that is very commonly accepted for humic and fulvic substances [e.g., Tipping, 1994). By performing copper titrations with algal suspensions, Crist et al. (1990) demonstrated that copper ions were able to replace two protons from algal cell wall functional groups, indicating the strong covalent nature of copper ion binding and thus favouring a divalent type of complex formation.

In summary, a large number of mechanisms may be explanatory of the observed pH-toxicity relationship. Given our current limited knowledge about both copper and proton binding to algal cell walls and the mechanisms of copper toxicity to algae, no mechanistic toxicity model can be developed yet. Our data, together with these of Heijerick et al. (2002), however, seem to illustrate that the conventional BLM approach for metal toxicity as used for fish and invertebrates may not be applicable to algae. Thus, as long as further detailed knowledge is lacking, it is advised to use the empirical relation between E_bC50_{Cu2+} and E_bC10_{Cu2+} and pH, as established in this study, to predict threshold concentrations for copper. Additonally, it would be desirable to experimentally determine if the pattern observed in this study can be applied across a broad spectrum of algal species from different major taxa.

6.3.7. Incorporation of possible hardness effects into the copper toxicity model

The possible effects of increased hardness ion (calcium and magnesium) concentrations on copper toxicity will be discussed in more detail. Increased concentrations of these ions have been shown to decrease the toxicity of copper (and metals in general) to a number of species [e.g., Erickson et al., 1996; De Schamphelaere et al., 2002, chapter 3). Heijerick et al. (unpublished data) reported a decrease of copper toxicity to *P. subcapitata* with a factor of about 2 when calcium and magnesium concentrations were increased from 0.25 mM to 2.5 mM (i.e. hardness from 25 to 250 mg $CaCO_3$ L^{-1}). Recent experiments at our laboratory however seem to indicate that a further increase of calcium and magnesium concentrations (up to 5 mM or 500 mg $CaCO_3$ L^{-1}) did not result in a further reduction of copper toxicity. On the contrary copper toxicity seemed to increase again (Heijerick et al., unpublished data). This suggests that competition of hardness ions may only be important at relatively low hardness. In our study, Ca and Mg concentrations were correlated (Ca:Mg = 4:1) and their sum also varied from 0.25 to 5 mM. However, we did not observe an effect of hardness ions on E_bC50_{Cu2+} values, neither at levels below 250 mg $CaCO_3$ L^{-1} nor at levels above 250 mg $CaCO_3$ L^{-1}. This apparent contradiction may be due to the fact that the toxicity tests of Heijerick et al. (unpublished data)

were conducted at only one pH level (i.e. ~ 7.5). Our data indicate that, perhaps, the hardness effect observed by these authors cannot be generalized to other pH levels. Until the effects of hardness are better understood, we feel that this (minor) aspect (as compared to the pH-effect) of copper toxicity to algae cannot be incorporated in a predictive model.

6.3.8. Predictive capacity of the developed copper toxicity model

By coupling equations 1 and 2 to the geochemical speciation model WHAM-Model V (Tipping, 1994) a toxicity model that can predict $E_bC50_{dissolved}$ and $E_bC10_{dissolved}$ as a function of the physico-chemistry of the test medium was developed. The computational procedure is similar to that in the original BLM software (Hydroqual, 1999) and can be compared to a titration (Di toro et al., 2001): (1) compute Cu^{2+}-activity for increasing concentrations of $Cu_{dissolved}$, taking into account the physico-chemistry of the exposure water and (2) the $Cu_{dissolved}$ concentration that results in a Cu^{2+}-activity equal to the predicted E_bC50_{Cu2+} or E_bC10_{Cu2+} at the specific pH of the exposure water (equations 1 and 2) is the predicted $E_bC50_{dissolved}$ or $E_bC10_{dissolved}$, respectively.

First, the developed model was used to predict $E_bC10_{dissolved}$ and $E_bC50_{dissolved}$ values for the 35 experiments conducted in this study. The physico-chemical characteristics presented in Table 6-1 were used as input, and the DOM from the different sites was assumed to consist of the average optimal percent active FA (i.e. 65.2% for Bihain DOM, 64.8% for Ossenkolk DOM and 41.4% for Ankeveen DOM). Figure 6-2 demonstrates the predictive capacity of the developed toxicity model. Ninety-one percent of the $E_bC50_{dissolved}$ values were predicted within a factor of 1.5 of the observed $E_bC50_{dissolved}$ values and 100% within a factor of 2; 77% of the $E_bC10_{dissolved}$ values were predicted within a factor of 1.5 and 97% within a factor of 2. No pH, DOC or hardness-dependency of the prediction accuracy was observed. Although of course one would expect that a model will make good predictions for the experiments from which the model was developed, the success of this validation demonstrates that the developed model is solid enough to predict copper toxicity, despite of the fact that only one parameter accounting for chemical variability of the different DOMs was taken into account, i.e. the percentage active FA, describing the difference in copper complexing capacity.

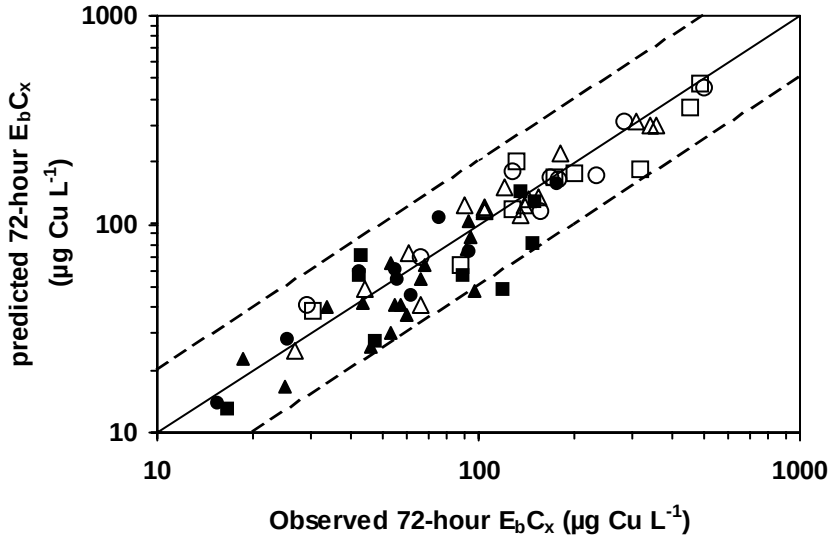
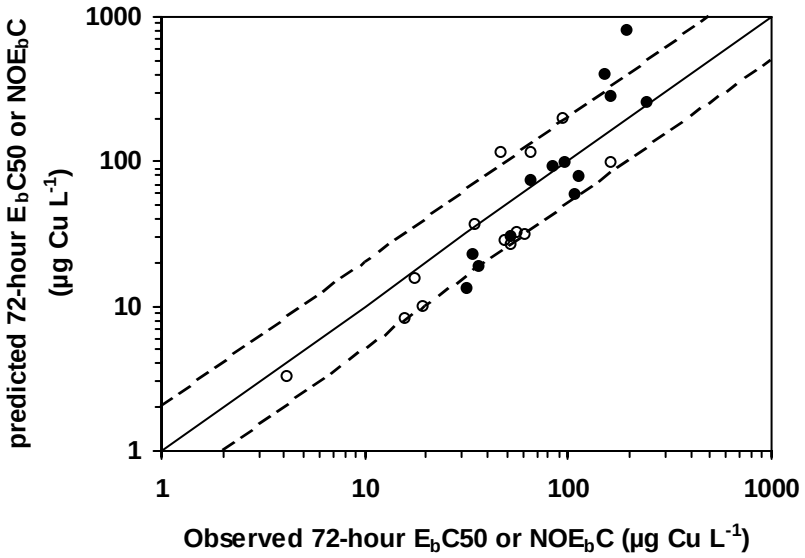


Figure 6-2 Relationship between observed and predicted 72-hour $E_bC50_{dissolved}$ (open symbols) and $E_bC10_{dissolved}$ (closed symbols) of copper to *Pseudokirchneriella subcapitata* for experiments with Bihain (circles), Ossenkolk (squares) and Ankeveen DOM (triangles) (see Table 6-1 for information about sampling locations). Predictions were performed as explained in the text using equations 1 and 2 coupled to the speciation model WHAM-Model V (Tipping, 1994). Computations were performed using Biotic Ligand Model version a008 (Hydroqual, 1999). The solid line indicates a perfect match between observed and predicted E_bC_x. The dashed lines indicate a factor of two difference between observed and predicted E_bC_x. See text for meaning of $E_bC50_{dissolved}$ and $E_bC10_{dissolved}$.

In the next validation phase, the ability of the developed model to predict 72-hour E_bC50s and NOE_bCs of copper to *P. subcapitata* in natural waters was investigated using the ecotoxicological dataset of Heijerick et al. (unpublished data). The physico-chemical characteristics of the natural waters were used as an input to the model, assuming 50% of the DOC being active fulvic acid. Observed 72-hour NOE_bCs were compared with predicted 72-hour E_bC10s as in our study it was shown that NOE_bCs and E_bC10s were comparable (see above). Figure 6-3 shows the predictive capacity of the developed copper toxicity model.



Relationship between observed and predicted 72-hour $E_bC50_{dissolved}$ (closed symbols) and $NOE_bC_{dissolved}$ (open symbols) of copper for *Pseudokirchneriella subcapitata* in natural waters (Heijerick et al., unpublished data). Predictions were performed as explained in the text using equations 1 and 2 coupled to the speciation model WHAM-Model V (Tipping, 1994). Computations were performed using BLM version a008 (Hydroqual, 1999). Predicted E_bC10-values were used as a surrogate for predicted NOE_bC-values (see text). The solid line indicates a perfect match between observations and predictions. The dashed lines indicate a factor of two difference between observations and predcitions. See text for meaning of $E_bC50_{dissolved}$ and $NOE_bC_{dissolved}$.

Eighty-one percent of the predictions of 72-hour $E_bC10_{dissolved}$ and 72-hour $E_bC50_{dissolved}$ for toxicity tests performed with 13 surface waters, differed less than a factor of two with the observed 72-hour $NOE_bC_{dissolved}$ and 72-hour $E_bC50_{dissolved}$ values. For two surface waters, both predicted $E_bC10_{dissolved}$ and $E_bC50_{dissolved}$ were more than a factor of 2 (up to factor 4) higher than the observed $NOEC_{dissolved}$ and $E_bC50_{dissolved}$. Both surface waters were however, characterized by low pH (<6) and high Fe and Al contents (Heijerick, personal communication). A possible explanation for the weaker performance of the model in these cases may be that at such low pH values, Fe and Al may be present in the dissolved phase and thus compete with copper for binding sites on the DOM, thus making copper more bioavailable than predicted. Indeed, because of their higher valence, Fe(III) and Al (III) ions are known to form very strong complexes with organic matter

(Tipping, 1994). The same overestimation of NOECs and EC50s was observed in chronic exposure of *D. magna* in the same surface water (chapter 4).

6.4. Conclusion

Based on extensive ecotoxicological testing and on measurements of free copper ion activity in all toxicity tests, a copper toxicity model was developed for the green alga *P. subcapitata*. Although more research is needed to mechanistically understand the relationships observed in this study, the developed model has a high predictive capacity and will help to improve the ecological relevance of current risk assessment procedures. This is especially important, because such a model could be used in combination with existing Biotic Ligand Models that predict copper toxicity to fish and daphnid to predict environmentally safe concentrations for three important groups of organisms.

Chapter 7

The effect of dissolved organic matter source on copper toxicity

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Chapter 7

The effect of dissolved organic matter source on copper toxicity

Abstract – The protective effect of dissolved organic matter (DOM) on metal toxicity to aquatic organisms has been reported by numerous authors. Bioavailability models such as the biotic ligand model (BLM) thus account for this factor - in addition to other bioavailability parameters such as pH, alkalinity and concentrations of major ions – to predict metal toxicity to aquatic organisms. Until now, however, few attempts have been made to assess the importance of the factor DOM quality for BLM-predictions. A large number of studies demonstrate the variability of natural DOM across different sources with respect to its effect on both metal speciation and toxicity. Most of these studies have focussed on the chemical variability of these natural DOM sources, and not on the biologically and toxicologically relevant differences in metal binding properties. In the present study, we investigated the effect of DOM source on copper toxicity to the freshwater cladoceran *Daphnia magna*. Acute toxicity tests were carried out (1) in artifical test water enriched with DOMs isolated from 6 locations in Europe and North America and (2) in 7 natural European surface waters containing different concentrations of DOM and exhibiting a different physico-chemical composition. The acute Cu-BLM for *D. magna* was then used to estimate the copper complexing capacity of each DOM (expressed as % active fulvic acid, %AFA). A factor of 6 difference was observed between the lowest and the highest copper complexing capacity, which is similar to differences found in other (mostly chemical) studies. A significant linear relationship was observed between the UV-absorbance coefficient at 350 nm (ε_{350}) and the %AFA. Linking this relationship to the acute Cu-BLM, resulted in a significant improvement of the predictive capacity of this BLM. Without accounting for DOM quality 90% of the predicted 48-hour EC50s were within a factor of 2 of the observed EC50s; taking DOM quality into account, 90% of the EC50s were predicted with an error of less than factor 1.3. The present study and other studies seem to indicate that UV-absorbance may be a good measure of biologically and toxicologically relevant differences in copper binding behaviour of DOM.

7.1. Introduction

Dissolved organic matter (DOM) has been shown to decrease metal uptake and/or toxicity to a number of freshwater organisms (De Schamphelaere et al., 2002, chapter 3; De Schamphelaere et al., 2003, chapter 6; Heijerick et al., 2003; Ma et al., 1999; Winner, 1985; Meador, 1991; Erickson et al., 1996). For practical reasons, most of these studies have examined the effects of one particular source of organic matter: the commercially available, soil-derived Aldrich humic acid. However, Malcolm and MacCarthy (1986) have clearly demonstrated that this humic acid bears little structural resemblance to true aquatic humic acids. Moreover, the humic acid fraction is usually less than 30% of the organic matter in natural freshwater (Ma et al., 2001).

These considerations have, more recently, resulted in investigations aimed at increasing our understanding of the effects of natural aquatic DOM on metal bioavailability and toxicity (De Schamphelaere et al., 2003a; chapter 6; De Schamphelaere et al., 2003b, chapter 6, Pempkowiak et al., 1999; Richards et al., 2001). Such studies provide more relevance to the field of metal bioavailability as they recognize the variability and the complexity of DOM (MacCarthy, 2001).

Given the fact that DOM decreases metal bioavailability by complexation – and thus by decreasing the chemical activity of the most toxic metal species in solution (i.e. the free metal ion, Campbell, 1995) - it seems appropriate to relate the variability of metal bioavailability effects across different DOMs to the variability of metal complexation properties. This type of variability has been demonstrated in a large number of chemical studies. Several studies with copper indicate that important features such as binding strength (or affinity) and complexation capacity (or site density) may vary up to one order of magnitude (Tipping and Hurley, 1992; Tipping, 1993; Ma et al., 1999; Dwane and Tipping, 1998; Abbt-Braun and Frimmel, 1999; Frimmel and Abbt-Braun, 1999; Bryan and Tipping, 2002).

To our knowledge, however, only a limited number of studies are available that have investigated the importance of this chemical variability for metal toxicity and/or uptake to freshwater organisms. Based on copper uptake experiments with blue mussels (*Mytillus trossulus*), Pempkowiak et al. (1999) suggested that reduction of copper uptake was related to

a higher aromaticity of organic matter. Richards et al. (2001) examined the effect of DOM source on metal uptake and mortality of rainbow trout during exposure to a metal mixture containing Pb, Hg, Cd, Cu, Ag and Co. They demonstrated that relatively simple optical characteristics of DOM such as UV-absorbance and absorbance-to-fluorescence ratios may adequetely reflect the biologically relevant differences in organic matter quality. Although they stressed that more DOM-sources should be investigated, these authors suggested that a larger reduction of metal toxicity and uptake was related to both higher UV-absorbance and absorbance-to-fluorescence ratios (both indicative of higher aromaticity). They also suggested that these DOM quality measures might be one way to incorporate DOM variability into bioavailability models such as the Biotic Ligand Model (BLM).

The BLM concept, originally developed by Di Toro et al. (2001) and further refined by other researchers (De Schamphelaere et al., 2002, chapter 3, Heijerick et al., 2002; Santore et al., 2002), has recently gained increased attention from both academic scientists and regulators and is now considered to be the state-of-the-science metal bioavailability model/concept, as it incorporates both speciation and interaction of metals and other cations at the organism-water interface.

The aims of this study were (1) to investigate to what extent DOMs from different sources differ in their ability to decrease acute copper toxicity to the cladoceran *Daphnia magna* and (2) to evaluate if UV-absorbance measurements may be a simple and effective method to incorporate DOM variability into the acute Cu-BLM for *D. magna*. Finally, the applicability of the observations to *Pseudokirchneriella subcapitata* and to chronic toxicity to *D. magna* will be investigated and discussed.

7.2. Materials and methods

7.2.1. Short description of DOM sampling

Dissolved organic matter was collected between summer 1997 and spring 2001 at six sites: 3 in North America and 3 in Europe. The main physico-chemical characteristics of the sampling sites are given in Table 7-1. The three European sampling sites were the same as discussed in chapter 4. Bihain is a small creek located in the highland peat area 'Hoge

Venen' (Belgium). Ossenkolk is a small lake (<0.1 km^2) in a mixed, temperate forest in the natural park 'Hoge Veluwe' (The Netherlands). Ankeveen is a narrow side arm of a larger lake system called the 'Ankeveensche plassen' and is located in a lowland peat area in the Netherlands. Bihain and Ossenkolk both represent relatively acidic/low conductivity waters in Europe; Ankeveen is typical for high pH/high conductivity surface waters. Big Moose Lake is a fairly large lake (~ 5 km^2) in the Adirondack National Park (NY, US) and is slightly acidic. Newport River (NC, USA) and Suwannee River (GA, USA) are both very acidic, dilute freshwater rivers containing very high concentrations of DOM. The International Humic Substances Society (IHSS) used the Suwannee River as the source of their standard aquatic humic and fulvic acids. A detailed description of the reverse osmosis sampling procedure and the characterisation and analysis of the chemical composition of the DOM samples is given in chapter 4 (section 4.2.2.).

Table 7-1 General information and physico-chemical characteristics of the sampling sites

Site ID	Big Moose	Newport	Suwannee	Bihain	Osssenkolk	Ankeveen
Name	Big Moose Lake	Newport River	Suwannee River	Ruisseau de St. Martin	Ossenkolk meer	Ankeveensche plas
Location	Big Moose NY, USA	Beaufort NC, USA	Okefenokee Swamp GA, USA	Bihain Belgium	Nunspeet Netherlands	Nederhorst den Berg Netherlands
Sampling Date	23-27 may 2000	21 may 1998	7-8 june 1997	7-8 march 2001	16 march 2001	22 may 2001
DOC (mg L^{-1})	~ 10	38.6	34.3-48.7	8.3	18.4	20.4
pH	6.2-6.5	4.3	3.9-4.0	5.7	5.6	7.9 - 8.2
Conductivity (µs cm^{-1})	ND	ND	< 45	90	59	269

ND = not determined

7.2.2. Daphnia magna toxicity testing with isolated DOMs and natural waters

Using each of the six collected DOMs, 48-hour toxicity tests with *Daphnia magna* were conducted in copper-spiked test media with three different DOC concentrations. Natural

DOM was added to reconstituted water containing 2 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.77 mM $NaHCO_3$ and 0.078 mM KCl. To buffer the pH of the test solutions, 750 mg L^{-1} MOPS was added and pH was adjusted to 7 with NaOH. MOPS has been shown not to affect copper toxicity to *D. magna* (De Schamphelaere et al., 2003c).

For each bioassay, the prepared test medium was then used as the dilution water to make a concentration series of copper, added as $CuCl_2$. In order to obtain near-equilibrium situations, all solutions were stored in the test cups at 20°C for two days before being used in the toxicity tests (Ma et al., 1999). The acute 48-hour immobilization assay with juvenile *D. magna* (<24 hour old) was performed following OECD test guideline 202 (OECD, 1984). The test organisms used originated from a healthy *D. magna* clone (K6), which has been cultured under controlled laboratory conditions in M4-medium (Elendt and Bias, 1990). For each medium an acute toxicity assay was conducted consisting of six treatments (control + 5 copper concentrations) with a difference of 1 log-unit between the lowest and highest copper concentration tested. Each treatment was performed in triplicate with 10 organisms per replicate in polyethylene cups containing 50 mL of test solution. The number of immobilized juveniles in each cup was counted after 48 hours. In addition to toxicity tests in reconstituted media with DOM additions, toxicity tests were also performed in 6 natural water samples from Belgium, France and The Netherlands. Toxicity tests in these natural waters were conducted as described above and in De Schamphelaere et al. (2002; chapter 3).

7.2.3. Chemical analysis during the ecotoxicological experiments

Dissolved copper concentrations (filtered through a 0.45 µm filter, Gelman Sciences, Ann Arbor, MI, USA) were determined at the beginning and at the end of the test using a flame-atomic absorption spectrophotometer (SpectroAA100, Varian, Mulgrave, Australia). Calibration standards (Sigma-Aldrich, Steinheim, Germany) and a reagent blank were analysed for each series of ten samples. Dissolved organic carbon (0.45 µm filtered) and Inorganic Carbon (IC) were measured before each test (TOC-5000, Shimadzu, Duisburg, Germany). Reported concentrations of major cations (Ca, Mg, Na, K) and anions (Cl, SO_4) are nominal as previous measurements had shown that measured concentrations were always within 10% of the nominal concentrations. pH-measurements were performed before and after each test with pH-meter P407 (Consort, Turnhout, Belgium). The pH glass electrode was calibrated before each use using pH 4 and pH 7 buffers (Merck, Darmstadt, Germany). Since

values of all measured parameters were essentially the same at the beginning and at the end of the tests, all reported results refer to values at the start of the test.

7.2.4. Absorbance measurements

UV-absorbance at 350 nm was measured (Perkin Elmer, Lambda/Bio, Ueberlingen, Germany) for both RO isolates and natural water samples according to OECD guideline 101 (OECD, 1981). Measurements were performed at 25°C, with a path-length of 1 cm, with carbon-filtered, deionised water as a blank and with $K_2Cr_2O_7$ as the reference substance. RO isolates were first diluted with carbon-filtered, deionised water to a concentration of 20 mg DOC L^{-1}. Since it has been demonstrated that UV-absorbance of natural organic matter is pH-dependent (Abbt-Braun and Frimmel, 1999), these solutions were adjusted to pH 7 prior to measurement using dilute NaOH. Similarly, natural water samples were also adjusted to pH 7 with dilute HCl or NaOH. Absorbance coefficients (ε, L mg^{-1} cm^{-1}) were calculated as:

$$\varepsilon_{350} = \frac{A_{350}}{d \cdot [DOC]} \quad (1)$$

with A_{350} = the absorbance (optical density) at 350 nm; d = the path length (cm) and [DOC] = DOC concentration (mg C L^{-1}). To allow a comparison with the data of Richards et al. (2001), specific absorption coefficients (SAC, cm^2 mg^{-1}) were computed according to Curtis and Schindler (1997):

$$SAC = \frac{(2.303 \cdot A_{350})/d}{[DOC]/1000} \quad (2)$$

7.2.5. Data treatment and analysis

48-hour EC50s expressed as dissolved copper were calculated from observed mortalities at each measured copper concentration using the trimmed Spearman-Karber method (Hamilton et al., 1977). All copper toxicity predictions were carried out using the acute Cu-BLM for *D. magna* described in De Schamphelaere et al. (2002, chapter 3) and using the BLM software (Windows Version 1.0.0., HydroQual, 2002). Stability constants for

inorganic complexes were taken from Martell et al. (1997, Table 2-2). Statistics and regressions were performed using Statistica software (Statsoft, Tulsa, OK, USA). The type of statistics used is reported in the results and discussion section.

7.3. Results and discussion

7.3.1. Chemical composition of the DOM stock solutions

The composition of the DOM stocks is given in Table 7-2. The high Cl and/or Na concentrations in these stocks are the result of the clean-up procedure (see chapter 4, section 4.3.1.) The low chloride concentration in DOM samples from the United States as compared to the European ones is due to the fact that during the clean-up procedure of the American samples HNO_3 was used to precipitate the humic acid fraction as compared to HCl, which was used for the European samples. However, the differences in composition of the DOM stocks are accounted for in calculating the ionic composition of the test media (See Table 7-3). This is especially important with regard to Na, since it is known that increased concentrations of this ion decrease copper toxicity to *D. magna* (De Schamphelaere and Janssen, 2002, chapter 2).

Table 7-2 Major ion composition of the dissolved organic matter stocks (isolated using reverse osmosis and after cation exchange through H^+-resin)

Sample	Big Moose	Newport	Suwannee	Bihain	Ossenkolk	Ankeveen
DOC (mg L^{-1})	319	931	1103	146	400	921
Ca (µM)	230	12.1	0.175	12.2	15.3	39.4
Mg (µM)	191	9.5	BDL	BDL	BDL	0.885
Na (µM)	12,100	160,000	65,200	138,000	96,400	232,000
K (µM)	ND	5.91	29.9	ND	ND	ND
Cl (µM)	8,520	5,670	13,500	161,000	104,000	199,000
SO_4 (µM)	20.8	1,070	82.2	42.8	15.5	25,800

BDL = below detection limit for Mg (0.25 µM)

ND = not determined

7.3.2. Effect of DOM on acute copper toxicity to *Daphnia magna*

Table 7-3 summarizes the composition of all test solutions and the corresponding 48-hour EC50 values of copper for *D. magna*. For the isolated DOMs, DOC concentrations were between about 2 and 18 mg L^{-1}, 48-hour EC50s ranged from 51 to 638 µg Cu L^{-1} (factor 12). For natural waters, 48-hour EC50s ranged from 34 to 1086 µg Cu L^{-1} (factor 30). This large variation indicates the importance of explicitly considering bioavailability in risk assessments and water quality criteria derivation procedures for copper. The BLM was developed to account for these differences in copper toxicity caused by factors that affect copper toxicity, i.e. DOC, pH, alkalinity, Ca, Mg and Na.

The acute Cu-BLM for *D. magna* developed by De Schamphelaere et al. (2002, chapter 3) has been validated for 19 natural surface waters. This BLM could predict 48-hour EC50s of all surface waters within a factor of 2 of the observed 48-hour EC50s. This predictive capacity was achieved by assuming that organic matter from each site had similar complexing properties. For these predictions, it was assumed that, according to Dwane and Tipping (1998), with regard to copper speciation organic matter in natural waters behaved as 50% active fulvic acid (AFA) if organic copper complexing was calculated using WHAM-Model V (Tipping, 1994). The %AFA can be interpreted as a measure of complexation capacity of binding site density of DOM.

The application of the concept "%AFA" in the BLM-software (Hydroqual, 2002) is contained in the equation:

$$DOC_{input} = (\%AFA/100) \cdot DOC_{test} \quad (3)$$

With DOC_{input} the DOC-concentration that needs to be put into the BLM software and DOC_{test} the DOC concentration in the test solution. This can be illustrated with following example: if a test water contains 10 mg DOC L^{-1} and if 50% AFA is assumed, the input for the BLM-software would be 5 mg DOC L^{-1} with all of the organic matter being fulvic acid; the remaining 5 mg DOC L^{-1} is treated as "inert".

Although, using the 50% AFA assumption, the predictive capacity of the BLM was already very promising, as reported in De Schamphelaere et al. (2002, chapter 3), it may be suggested that BLM-predictions could still be further improved by accounting for the variability of Cu binding properties of natural DOM. Therefore, the acute Cu-BLM for *D. magna* was used to estimate for each DOM (for both RO-isolates and natural waters), the optimal %AFA (% AFA_{opt}) that yields an exact match between observed and predicted 48-hour EC50s. In other words, *D. magna* was used as a biological speciation probe to estimate copper complexation capacity of different natural DOMs.

Tables 7-3 and 7-4 show the $\%AFA_{opt}$ that was calculated for each test. Table 3 also shows the mean % AFA_{opt} for each isolated DOM. The $\%AFA_{opt}$ varied between 31.8% and 108% for isolated DOMs and between 17.2% and 85.5% for natural waters. These calculations demonstrate that there is about factor 6 difference in the copper binding capacity of the DOMs tested. This is comparable to most other studies that have assessed variability in copper binding capacity of natural organic matter: i.e. factor 2 for 13 UK natural water samples (Table 2 in Dwane and Tipping, 1998), factor 2-3 for 14 UK natural water samples (Table 4 in Bryan and Tipping, 2002, without outlier value for the river Derwent), factor 3-10 for eight Norwegian isolated DOMs (Abbt-Braun and Frimmel, 1999; Takacs et al., 1999). Using *Oncorhynchus mykiss* as test species, Richards et al. (2001) observed a factor of 2 difference in both metal binding to gills and metal toxicity for 3 North American isolated DOM types. Based on these and the present study, we suggest that the variability of natural DOM with regard to metal binding is important both chemically, biologically and toxicologically.

7.3.3. Introducing DOM variability into the acute Cu-BLM

The variability of natural DOM points to the importance of assessing the potential improvement of metal bioavailability models as the BLM by accounting for this variability. However, not to limit the wide applications of a potentially improved BLM, the metal binding capacity of natural DOM (e.g. expressed as %AFA) should preferably be derived from a relative simple measure, i.e. one that is easily measurable on a routine basis.

Table 7-3 Physico-chemical characteristics[1] of the test media for tests with isolated natural dissolved organic matter (DOM) and corresponding 48-hour EC50s of copper for *Daphnia magna*. Optimal % active fulvic acid ($\%AFA_{opt}$) was calculated for each individual test as explained in text. Absorbance coefficients (ε_{350}) and specific absorption coefficients (SAC) for each DOM were determined as explained in text.

DOM-source	pH [2]	DOC (mg L^{-1})	IC (mM)	Na (mM)	SO_4 (mM)	Cl (mM)	48h-EC50 [3] (µg L^{-1})	$\%AFA_{opt}$	$\%AFA_{opt}$ (Mean ± SE)	ε_{350} (mg L^{-1} cm^{-1})	SAC (cm^2/g)
	6.90	3.04	0.243	2.12	0.500	4.08	81.8 (74.6-91.3)	50.2			
Big Moose	6.92	4.53	0.211	2.17	0.500	4.12	128 (108-153)	54.5	52.3 ± 2.1	0.0119	27.4
	6.91	8.96	0.245	2.34	0.500	4.24	< 295[4]	< 66.0			
	7.01	1.95	0.233	2.14	0.502	4.02	129 (122-138)	122			
Newport	7.00	5.11	0.201	2.36	0.505	4.07	261 (226-304)	96.8	108 ± 7	0.0213	49.1
	6.99	11.7	0.230	2.82	0.510	4.17	638 (593-687)	104.4			
	6.98	1.97	0.261	2.29	0.502	4.01	86.6 (79.4-94.3)	78.1			
Suwannee	6.97	5.35	0.187	2.78	0.505	4.03	192 (164-226)	67.2	67.2 ± 6.3	0.0126	29.0
	6.99	10.8	0.207	3.56	0.510	4.06	332 (282-390)	56.4			
	6.94	1.95	0.201	3.84	0.501	6.23	53.8 (50.2-57.6)	42.0			
Bihain	6.92	8.54	0.194	10.1	0.503	13.5	311 (280-345)	58.6	50.6 ± 4.8	0.0104	24.0
	6.99	15.4	0.221	16.3	0.505	21.1	542 (498-589)	51.2			
	7.08	2.08	0.263	2.50	0.500	4.62	50.6 (43.8-58.4)	36.0			
Ossenkolk	7.03	9.22	0.239	4.22	0.500	6.46	275 (248-306)	52.3	50.4 ± 7.4	0.0126	29.0
	6.98	16.9	0.217	6.06	0.501	8.44	607 (586-639)	62.9			
	7.06	2.58	0.253	2.65	0.571	4.64	60.6 (56.3-65.2)	36.5			
Ankeveen	7.07	13.7	0.258	5.44	0.879	7.04	212 (187-240))	25.3	31.8 ± 3.4	0.0063	14.5
	7.10	17.8	0.273	6.47	0.992	7.93	372 (353-392)	33.7			

DOC = dissolved organic carbon; IC = inorganic carbon

[1] Only variable physico-chemical characteristics are reported. In all test media Ca, Mg and K concentrations were equal: Ca = 2 mM, Mg = 0.5 mM, K = 0.078mM

[2] pH values refer to the pH at the start of the test, pH at the end of the test was always within 0.1 pH units from initial pH

[3] values between brackets indicate 95% confidence limits

[4] all organisms were immobilized at 295 µg Cu L^{-1}

Table 7-4 Physico-chemical characteristics of the test media for tests with natural waters and 48-hour EC50s of copper for *Daphnia magna*. Optimal % active fulvic acid (%AFA_{opt}) was calculated for each individual test as explained in text. Absorbance coefficients (ε_{350}) and specific absorption coefficients (SAC) for each natural water were determined as explained in text.

Sampling date (dd/mm/yy)	Site ID + Country	pH [1]	DOC (mg L^{-1})	IC (mM)	Ca (mM)	Mg (mM)	Na (mM)	K (mM)	SO_4 (mM)	Cl (mM)	EC50 [2] (µg L^{-1})	ε_{350} (mg L^{-1} cm^{-1})	SAC (cm^2/g)	Optimal %AFA
05/09/01	Ankeveen A - NL	7.75	36.6	0.940	0.831	0.235	0.426	0.220	0.311	0.680	1090 (1030-1150)	0.00750	17.3	50.6
09/11/01	Ankeveen A - NL	7.75	27.4	0.912	0.938	0.251	0.583	0.0256	0.738	0.601	932 (891-974)	0.0118	27.1	53.6
14/06/01	Bihain – B	6.06	9.63	0.0335	0.137	0.0494	0.257	0.0512	0.0344	0.223	97.9 (91.0-105.3)	0.00959	22.1	50.9
31/08/01	Bihain –B	5.98	4.29	0.0323	0.0574	0.0535	0.100	0.0512	0.0177	0.147	64.0 (58.9-70.0)	0.0129	29.7	85.5
10/09/01	Le Voyon - FR	7.35	7.73	0.845	0.621	0.281	0.257	0.0512	0.271	0.203	236 (218-255)	0.00733	16.9	58.4
29/08/01	Marais St. Boutien - FR	7.91	3.64	1.34	3.24	0.259	0.418	0.0512	0.236	0.914	70.8 (67.1-74.6)	0.00595	13.7	17.2
22/05/01	Markermeer - NL	8.30	8.24	2.69	1.66	0.702	3.34	0.242	1.12	3.58	257 (231-286)	0.00574	13.2	37.8

DOC = dissolved organic carbon; IC = inorganic carbon

[1] pH values refer to the pH at the start of the test, pH at the end of the test was always within 0.2 pH units from initial pH

[2] values between brackets indicate 95% confidence limits

[4] all organisms were immobilized at 295 µg Cu L^{-1}

Richards et al. (2001) suggested that the UV absorbance of organic matter may be a good measure of biologically relevant differences in metal-binding strength and/or capacity of natural organic matter. Their evidence, however, was based on only three isolated DOM samples. In the present study, this hypothesis was tested using 6 isolated DOMs (tested in reconstituted water) and 7 natural water samples.

In Table 7-3 and Table 7-4 the specific absorbance at 350 nm (ε_{350}) and the SAC at 350 nm are given. For the isolated DOMs ε_{350} were between 0.0063 and 0.0213 L mg^{-1} cm^{-1} (SAC between 14.5 and 49.1 cm^2 g^{-1}), for the natural water samples ε_{350} varied between 0.00574 and 0.0129 L mg^{-1} cm^{-1} (SAC between 13.2 and 29.7 cm^2 g^{-1}). Similar ranges were observed for 3 isolated North American DOMs (SAC between 11 and 38 cm^2 g^{-1}; Richards et al., 2001). Hence, according to Richards et al. (2001) it may be suggested that our samples probably also range from mainly autochtonous, less aromatic, low-metal binding (low SAC) to mainly autochtonous, more aromatic, high metal-binding (high SAC).

Linear regression analysis ($\%AFA_{opt}$ = slope · ε_{350} + intercept) revealed that the intercepts for both the tests with isolated DOM samples and those with natural waters were not significant ($\alpha = 0.05$). Hence, regression analyses were repeated with intercept = 0 (Figure 7-1, Table 7-5):

$$\%AFA_{opt} = \text{slope} \cdot \varepsilon_{350} \quad (4)$$

The intercept being zero offers the advantage that no measurement of DOC concentrations of a test water is necessarry, but that only a measure of A_{350} at a certain path length (d) is required to determine which DOC_{input} for the BLM-software has to be used for accurate copper toxicity predictions. Indeed, combining Eqn. (1), (3) and (4) yields:

$$DOC_{input} = (\text{slope} \cdot A_{350}) / (100 \cdot d) \quad (5)$$

This may be important in the cost-effective implementation of the Cu-BLM for regulatory purposes, since it offers the opportunity to account for both the effect of DOC concentration and DOM quality by a relatively simple measurement such as UV-absorption.

Table 7-5 Regression analysis of the equation: $\%AFA_{opt}$ = slope . ε_{350} (Eq. 4). Analyses were performed using $\%AFA_{opt}$ (optimal % active fulvic acid) and ε_{350} values (UV absorbance at 350 nm) reported in Tables 7-3 (isolated dissolved organic matter, DOM) and 7-4 (natural waters). $\%AFA_{opt}$ was calculated as explained in text from acute toxicity data with *Daphnia magna*. The "lumped" regression equation was calculated based on data from isolated DOMs (mean $\%AFA_{opt}$ was used for each DOM, Table 7-3) and natural waters, together. The regression equation for the green alga *Pseudokirchneriella subcapitata* was calculated based on data of De Schamphelaere et al. (2003, chapter 6).

	N	slope ± SE	r^2	*p*
D. magna (isolated DOMs)	17	4850 ± 188	0.8459	<0.001
D. magna (natural water)	7	5731 ± 510	0.6110	<0.001
D. magna (isolated DOMs + natural water; "lumped")	13	5267 ± 252	0.8349	<0.001
P. subcapitata (isolated DOMs from Europe)	3	5725 ± 432	0.6923	0.005

SE = Standard Error

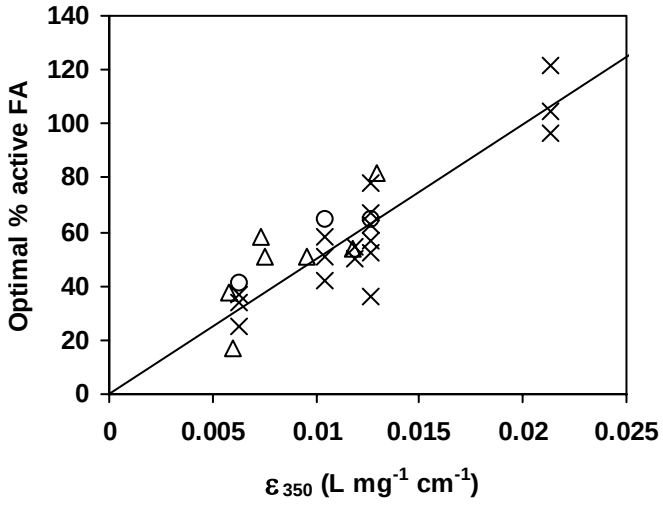

Figure 7-1 Relation between the optimal % active fulvic acid and the UV-absorption coefficient (ε_{350}) for acute *Daphnia magna* tests with isolated dissolved organic matter (X) and natural waters (Δ) and for chronic toxicity tests with *Pseudokirchneriella subcapitata* (De Schamphelaere and Janssen, 2003, chapter 6) (○); the regression line is shown for *D. magna* (lumped regression, see Table 7-5).

Slopes of the regression lines for tests with isolated DOM and natural water were statistically compared according to Green and Margerison (1978) and were not significantly different ($\alpha = 0.05$). This indicates that the RO-isolation method does not alter the relation between ε_{350} and the (biologically relevant) copper binding capacity of aquatic DOM. Hence; results of metal toxicity studies that use RO-isolates of aquatic DOM can safely be used to draw conclusions for natural waters testing. Since the two regressions were not significantly different, it was decided to calculate a "lumped" regression line, i.e. combining both data from isolated DOMs as from natural waters. This equation is also shown in Table 7-5 and will be used further on to investigate the effect of accounting for DOM variability on the predictive capacity of the BLM.

7.3.4. Effect of incorporating DOM variability on predictive capacity of the BLM

Two series of predictions of 48-hour EC50s were conducted with different DOM assumptions: (1) using 50% AFA for the EC50-predictions for all tests (both isolated DOMs and natural waters; i.e. all DOMs are considered to have the same properties, Cf. De Schamphelaere et al., 2002, chapter 3), (2) using the % AFA estimated with the lumped regression equation (Table 7-5) for the EC50-predictions for all tests (the ε_{350} values reported in Tables 7-3 and 7-4 were used as input for this regression equation).

The predictive capacity of the BLM for one single toxicity test is best described in terms of the factor difference between observed and predicted 48-hour EC50: the lower the factor difference, the better the prediction. For a set of tests, parameters indicative of the predictive capacity include the maximum and the 90th percentile of this factor difference. For all tests (N=24, 17 tests with isolated DOMs and 7 with natural waters, see Tables 7-3 and 7-4) the maximum difference was a factor of 2.18 for the 50% AFA assumption, whereas it was only 1.66 when the lumped regression equation was used for estimating the %AFA. The 90th percentile of the difference was a factor of 2.01 for the 50% AFA assumption and 1.28 when the lumped regression equation was used for estimating the %AFA. This indicates that when the relation between ε_{350} and the % AFA is taken into account, BLM predictions are significantly better than when the 50% AFA assumption is used. The predictive capacity of the BLM using the "lumped" regression equation to estimate the % AFA for all tests is shown in Figure 2.

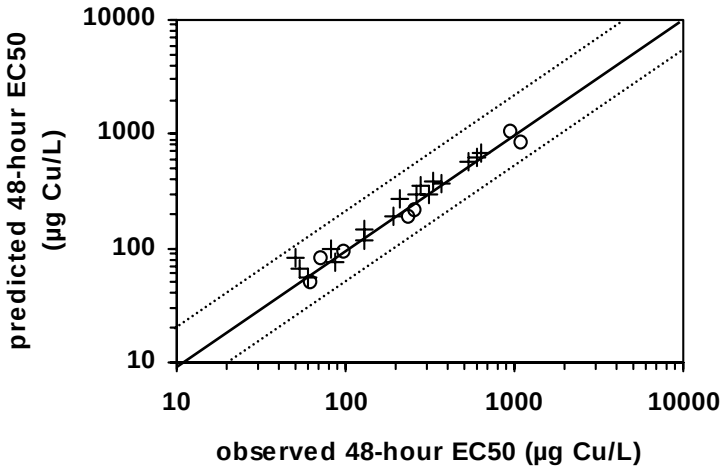

Figure 7-2 Predictive capacity of the acute Cu-BLM for *Daphnia magna* as shown by the relation between observed and predicted 48-hour EC50s; prior to the BLM predictions for tests with isolated dissolved organic matter (+) and with natural waters (o) the optimal %AFA was first calculated with the lumped regression equation mentioned in Table 75, using UV-absorption coefficients (ε_{350}) form Table 7-3 and Table 7-4. The full line represents perfect agreement with observed and predicted EC50s; the dashed line represents a factor of 2 error between observed and predicted EC50s.

7.3.5. Applicability to other organisms?

For DOM variability to be included in risk assessments of copper, it is necessarry that a simple measure, such as UV-absorption, be predictive of the copper complexation properties of this DOM. Furthermore such approach should not only be able to improve predicitions of copper speciation but also of effect concentrations of copper (e.g. EC50, NOEC). The latter should not only be applicable to an acute toxicity endpoint of one species, but prefarably to chronic toxicity endpoints for a number of different species from different trophic levels. The latter is discussed in this section.

A previous study (De Schamphelaere et al., 2003a, chapter 6), using the same three European DOMs revealed a similar pattern of the effect of DOM source on chronic copper toxicity to the alga *Pseudokirchneriella subcapitata*. As in the present study, Ankeveen DOM was observed to reduce copper toxicity less than Bihain and Ossenkolk DOM. In the same

study, $\%AFA_{opt}$ was also determined based on ion selective electrode measurements of Cu^{2+} activity in the toxicity tests with the green alga Cu^{2+}-measurements (more detail is provided in De Schamphelaere et al., 2003); they were 50.6% (±4.8%; standard error), 50.4% (±7.4%) and 31.8% (±7.4%) for Bihain, Ossenkolk and Ankeveen DOM, respectively. For each DOM, the $\%AFA_{opt}$ in a previous study (De Schamphelaere et al., 2003, chapter 6) were not significantly different from the $\%AFA_{opt}$ derived in the present study (t-tests, $\alpha = 0.05$). Additionally, the slope of the linear regression equation ($\%AFA_{opt}$ vs. ε_{350}, Table7- 5) for the algae-tests was not significantly different from that obtained in the present study.

Contrarily, in chronic toxicity tests with *D. magna* (De Schamphelaere and Janssen, 2003b, chapter 4) the same three European DOMs (Bihain, Ossenkolk and Ankeveen) all exhibited a similar protective effect on copper toxicity. As a possible explanation it was suggested that copper complexes with Bihain and Ossenkolk DOM (but not with Ankeveen DOM) might have been bioavailable to some extent and that in this chronic exposure scenario the effect of the higher complexation capacity of Bihain and Ankeveen DOM was annihilated. Thus it seems that, at least for the three studied isolated European DOMs, the relation between UV-absorbance and copper complexing properties is not applicable with regard to chronic copper toxicity to *D. magna*. However, since this observation is based on only three DOMs, it would be advisable to determine if this also applies to other isolated DOMs and to natural surface water samples. However, the fact that toxicity predictions with the chronic Cu-BLM were relatively accurate for natural surface water samples, even without taking into account possible bioavailability of Cu-DOM complexes, indicates that the latter may be of minor importance.

Results of other studies, which have reported on the effects of DOM quality on metal toxicity or uptake, are more difficult to compare directly and quantitatively with our results. First, qualitatively, Richards et al. (2001) observed an increased protective effect of DOM on metal accumulation and toxicity in rainbow trout with increasing UV-absorbance, which coincides with the results of our study. An accurate quantitative comparison with the present study is, however, not possible because Richards et al. (2001) studied the effect of DOM quality on metal accumulation and metal toxicity in rainbow trout for a mixture of 6 metals including copper, not for copper only. Second, Pempkowiak et al. (1999) have shown for eight different freshwater sources of organic matter that the reduction of copper uptake by blue mussels (*Mytillus trossulus*) in the presence of organic matter was related to a higher

carbon content of the organic matter used. They speculated that increased carbon content is indicative of a higher degree of aromaticity, resulting in the ability of the organic matter to from more chelates with metals, for example via carboxylic groups attached to aromatic rings (1999). Since a higher aromaticity of an organic matter is generally reflected by a higher UV-absorbance, these findings also seem to corroborate with the results of our study.

7.4. Conclusion

The present study and other studies indicate that UV-absorbance may be a good measure of biologically relevant differences in copper complexing properties of DOM. In this study, using *Daphnia magna* as a model organism, it has been shown that the predictive capacity of the acute *D. magna* Cu-BLM is significantly improved by incorporating the factor DOM variability. Predictions errors were reduced from a factor of 2 (without DOM variability) to a factor of 1.3 (with DOM varaibility). Future research should focus on exploring in more detail the possibility of incorporating the factor DOM-variability into BLMs for other species, other metals and other exposure times/scenarios.

Chapter 8

Summary:

general conclusions and future research perspectives

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Summary: general conclusions and future research perspectives

This doctoral thesis is situated in the field of aquatic toxicology, a sub-discipline of ecotoxicology, which aims at evaluating hazards and risks of natural and anthropogenic substances to aquatic ecosystems. More specifically, the subject of this thesis is the ecotoxicology of metals in freshwater ecosystems and the use of ecotoxicological test data for risk assessment and water quality criteria setting procedures.

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. The focus of this study is on the toxicity of the essential element copper, i.e. on copper concentrations at which the homeostatic regulation capacity of organisms is exceeded. The potential toxicity of copper, together with its wide-spread use in numerous applications, indicates the necessity for the accurate assessment of the risks of copper for the environment.

To date, water quality criteria (WQC) and the risk assessment of metals are still predominantly based on total or dissolved concentrations. However, total or even dissolved concentrations of metals are no good predictors of potential harm to ecosystems. Indeed, several physico-chemical water characteristics such as dissolved organic carbon (DOC), pH and hardness can modify toxicity with several orders of magnitude. The latter is summarized with the term “bioavailability”.

The main idea behind the term “bioavailability” is that the toxic effect of a metal does not only depend on the total (or dissolved) concentration of that metal in the surrounding environment, but rather that it depends on a complex interaction between physico-chemical and biological factors. In other words, the same total metal concentration does not result in the same degree of toxic effect under all conditions.

This indicates that, if bioavailability is not taken into account, water quality criteria based on total or dissolved concentrations may be under-protective for one type of water and over-protective for another. In the context of sustainable development, neither over-protection nor under-protection is desirable as the former will result in increased societal costs involved with emission reduction and environmental sanitation measures, whereas the latter may result in harm to aquatic life and biodiversity.

Despite the body of evidence that has been generated during the past decades about the effects of physico-chemical water characteristics on metal toxicity, few regulatory systems have taken this into account. This is mainly a result of a lack of quantitative tools to account for bioavailability. In this context, the recently developed biotic ligand model (BLM) has gained increased interest from both the academic, industrial and regulatory community as this (conceptual) model is able to predict acute metal toxicity by integrating the most important effects of water chemistry.

Although the BLM, developed to predict acute metal toxicity, is currently considered for implementation in the WQC for copper in the U.S.A., it is not suitable for incorporation in the European Union (EU) regulatory frameworks, as these require chronic toxicity data. In the EU, the environmental management of copper is mainly addressed through a risk assessment approach. In the risk assessment a predicted environmental concentration (PEC) and a predicted no-effect concentration (PNEC) are derived and compared to asses the risk. The focus of this study is on the estimation of the PNEC for copper.

If bioavailability is to be incorporated into the EU risk assessment of copper in a quantitative manner, models are needed that can predict chronic copper toxicity to different organisms. Therefore, the main goal of this study was to develop bioavailability models that can predict chronic copper toxicity to the crustacean *Daphnia magna* and the green alga *Pseudokirchneriella subcapitata* as a function of the physico-chemical water characteristics.

It is envisaged that the development and validation of these models will result in the improvement of currently applied risk assessment and water quality setting procedures.

At the start of this doctoral study the BLM concept had only been evaluated for acute metal toxicity to fish species and only preliminary attempts were available to illustrate the possible extrapolation of the acute BLM to invertebrate species. However, no proof was available for the similarity of the BLM across species. Moreover, with small invertebrates it was methodologically impossible to develop a BLM based on measured gill concentrations of copper (which is the basis for the fish BLM). Therefore, before investigating the potential application of the BLM concept for chronic copper toxicity to invertebrates, a new mathematical methodology was developed to establish an acute Cu-BLM for *D. magna* based on toxicity data (chapter 2).

Experiments were carried out in which the individual effects of Ca, Mg, Na and pH on acute copper toxicity to *D. magna* were investigated. It was demonstrated that Ca and Na had a quantitatively similar protective effect as in fish. However, it was also shown that Mg protected against toxicity to the same extent as Ca, something that had not been observed for fish. Overall, 48-hour EC50s were between 3.7 and 21 µg Cu L^{-1}, indicating that the maximal variation of toxicity due to protective effects of Ca, Mg and Na was about factor 5 to 6.

Expressed as dissolved copper 48-hour EC50s increased about factor 10 between pH 6 and 8 (from 5.6 to 52 µg Cu L^{-1}). Expressed as Cu^{2+}-activity, the 48-hour EC50s were about factor 2 higher at pH 6 than at pH 8. It was demonstrated that this was due to $CuOH^{+}$ being bioavailable to some extent. In a further refinement of the BLM (chapter 3) it was demonstrated that for a broader pH range (i.e. 5.7 to 8.4) the $EC50_{Cu2+}$ exhibited even larger variations, i.e. between 3 and 69 nM (~factor 20), and this was attributed to the co-toxicity of $CuCO_3$. It was also demonstrated that $CuOH^{+}$ and $CuCO_3$ were 5 and 10 times less toxic than Cu^{2+}, respectively (chapter 3). Although it was suggested that the toxicity of these complexes could also be the result of the gill-microenvironment having a different physico-chemistry as the bulk solution, it was necessary to include their bioavailability into the BLM-framework by allowing these species to bind to the biotic ligand. This proved to be of utmost importance to attain accurate toxicity predictions at pH levels above 8.

This research indicated that the BLM-framework can easily be adapted for use with species. However, given the fact that some differences across species may exist and that this may become important for toxicity predictions under some specific conditions, care should be taken in extrapolating the BLM from one species to another based on limited datasets. The experimental design and the mathematical methodology presented provide a relatively rapid approach to investigate species-specific differences of BLM-constants (i.e. differences in effects of water chemistry on copper toxicity to different species) and hence to develop BLMs for different species and metals. It would also be worthwhile to investigate the possible importance of these species-specific differences, not only across standard laboratory species but also across field-collected species originating from different ecosystems. This would improve the ecological relevance of BLM-predicted metal toxicity.

The tests described above, which were used to derive the competition constants, were performed in laboratory test media with background DOC concentration (~ 0.3 mg L^{-1}) and the 48-hour EC50s ranged between 3.7 and 52 µg Cu L^{-1}. In a subsequent research phase (chapter 3), the acute Cu-BLM was validated with 25 reconstituted waters containing different concentrations of artificial organic carbon and with 19 spiked natural surface waters. This was aimed at assessing the predictive capacity of the developed BLM in the presence of dissolved organic carbon. In these tests a clear protective effect of DOC was observed. For DOC concentrations ranging from 1.4 to 23 mg DOC L^{-1}, the 48-hour EC50 increased from 41 to 1200 µg Cu L^{-1}. This, together with the fact that DOC explained about 80% of the observed variability of the 48-hour EC50s, indicates that if bioavailability corrections of WQC would have to be done using only one parameter, the most relevant one would definitely be a DOC-based correction and not the commonly applied hardness-based correction (see chapter 1).

Despite this large variation in 48-hour EC50s (factor 30 for the DOC-containing waters; factor 300 overall), the developed BLM could predict them all within a factor 2 from the observed values. This indicates that the BLM is a powerful tool to reduce uncertainty related to bioavailability. Within the BLM, copper complexing with DOC is computed via the WHAM V model and based on literature data, DOC in natural water was assumed to consist of 50% active fulvic acid and 50% organic matter inert for ion-binding. When no *a priori* information is available on copper complexing properties of natural organic matter, this assumption was demonstrated to be successful and is thus recommended. This assumption has

been made throughout this study whenever toxicity predictions needed to be carried out for natural surface waters.

Despite the demonstrated ability of the BLM to accurately predict acute copper toxicity to *D. magna*, the use of this model in the EU regulatory framework is limited as the EU approach requires chronic toxicity data to be used for risk assessment purposes. In the next research phase, it was attempted to investigate if chronic copper toxicity to *D. magna* could also be modelled with the BLM-concept (chapter 4).

The effects of pH (5.3 to 8.7), water hardness (25 to 500 mg $CaCO_3$ L^{-1}) and dissolved organic carbon (DOC) concentration (1.6 to18.4 mg L^{-1}) and source on the chronic (reproductive) toxicity of copper to *D. magna* were investigated using a multi-factorial, central composite test design. Natural dissolved organic matter (DOM) was collected at three sites in Belgium and the Netherlands using reverse osmosis. For a total number of 35 toxicity tests performed, 21-day NOECs ranged from 29.4 to 228 µg Cu L^{-1} and 21-day EC50s from 41.5 to 316 µg Cu L^{-1} (factor 8).

Statistical analysis revealed that DOC concentration and pH had a significant effect on chronic copper toxicity but hardness did not. In general, an increase in pH or DOC resulted in a linear increase of 21-day NOEC and EC50 values. All DOMs (originating from three different sources) reduced copper toxicity to the same extent. Statistical analysis revealed that DOC concentration is the most important factor determining chronic copper bioavailability in *D. magna* assays, explaining about 60% of the observed variability. pH only explained about 15% of the observed variability, whereas the effect of hardness was not significant. Again this indicates that the hardness-based correction of WQC may not be appropriate for copper, but that a DOC-based correction would be more relevant.

Next to empirical regression models, the obtained toxicity data were also used to investigate the possibility of BLM modelling of chronic copper toxicity to *D. magna*. BLM constants were derived and compared with those obtained for the acute BLM: 1) The effect of Ca and Mg was not important for chronic toxicity, 2) the competitive effect of Na was similar and 3) proton competition and the bioavailability of $CuOH^+$ and $CuCO_3$ was more important in chronic exposures.

The fact that Ca and Mg did not affect chronic toxicity (as opposed to acute toxicity), whereas Na did indeed exhibit similar protective effects on chronic copper toxicity as on acute toxicity, suggests an increased importance of the physiology of the test organism in chronic exposures, i.e. the disturbance of the Na-balance of the organism.

We also observed an increased proton competition and the increased bioavailability of $CuOH^+$ and $CuCO_3$ in chronic exposures. Although a number of hypotheses can be put forward to explain this observation, it should be underlined that the developed BLM cannot be interpreted as a model that exactly describes the mechanisms underlying the observed relations. Instead, the derivation of the BLM is aimed at integrating all mechanisms in such a way to maximize the correlation between observed and predicted toxicity. In other words, the increased $CuOH^+$ and $CuCO_3$ bioavailability and proton competition observed in the chronic tests might be the result of a number of processes, some of which are described below.

The organisms may acclimate to sub-lethal copper concentrations during the chronic exposure which may alter the BLM-constants. The reduced reproduction and increased mortality may also be induced by a number of different mechanisms, next to the disturbance of the sodium balance. Examples include, but are probably not limited to, redox cycling of accumulated copper resulting in decreased survival time and increased maintenance costs and feeding inhibition, both resulting in decreased reproductive output. The investigation of the exact chronic toxicity mechanisms that determine the observed bioavailability relations, would certainly result in an improved bioavailability model and would help in the regulatory acceptance of the BLM.

Next to these mechanistic considerations, some evidence was also found for some (limited) toxicity of complexes of copper with isolated dissolved organic matter. As the latter was only a minor effect, which did not seem to affect the accuracy of toxicity predictions in spiked natural surface waters, this factor was not included in the chronic Cu-BLM.

Both the empirical regression model and the BLM exhibited a similar predictive capacity with regard to the 21-day EC50s and NOECs in natural water samples: most of these effect cocnentrations were predicted within a factor 2 of the observed values.

For one specific surface water, 21-day NOECs and EC50s were over-estimated with a factor > 5 (i.e. toxicity was under-estimated). Since this is not really desirable if the chronic Cu-BLM is to be implemented into the risk assessment procedure, a science-based should be provided. The specific surface water, a Swedish lake, exhibited some water characteristics which could indeed have resulted into the under-estimation of toxicity. It had a low pH (5.5) and was characterized by a high iron and aluminium content. It is generally known that iron and aluminium can effectively compete with copper for strong binding sites on organic matter, especially at low pH levels. This may result in more-than-expected free copper ion in solution and hence a higher copper toxicity. Since these competition effects are more likely to become important at low copper concentrations (i.e. concentrations around which WQC are expected), this issue certainly deserves further investigation. In this context it may be necessary to introduce the more advanced WHAM-6 speciation model into the BLM, as this model is specifically adapted to predict metal speciation at low-metal concentrations. Until then, it is discouraged to use the developed chronic Cu-BLM for waters with a combination of low pH and high Fe and/or Al concentrations.

Another important issue and a current matter of intensive debate with regard to chronic copper toxicity mechanisms is the potential toxicity of dietary copper, i.e. copper associated with food particles (e.g. algae) that are present in standard chronic toxicity tests. The chronic Cu-BLM was developed to predict chronic toxicity of waterborne copper and it was hypothesized that, if dietary copper would contribute to chronic copper toxicity, the developed model would only be valid under conditions in which dietary copper toxicity is not significant.

For that reason, in chapter 5, experiments were carried out to assess the possible importance of dietary copper on the predictive capacity of the developed chronic Cu-BLM. The alga *P. subcapitata* was cultured for three days at 6 different copper concentrations (35 to 200 µg Cu L^{-1}) and a control in a test medium containing 10 mg DOC L^{-1}, and having pH = 7 and a hardness of 250 mg $CaCO_3$ L^{-1}. Equilibrium copper burdens of the algae were reached within 2 days and were between about $6 \cdot 10^{-16}$ and $200 \cdot 10^{-16}$ g Cu $cell^{-1}$ and remained more or less constant throughout the 21-day storage period.

Subsequently, *D. magna* was exposed to copper in three types of standard 21-day chronic exposures 1) waterborne exposure (copper in solution and control food-algae, 2)

dietary exposure (control solution and copper-laden food algae) and 3) waterborne + dietary exposure (copper in solution and copper-laden food-algae). It was recognized that in the waterborne exposure (in which control algae were used as the food source and which is representative for standard chronic toxicity tests with daphnids), a possible dietary exposure might occur. Indeed, the food-algae can take up dissolved copper from the exposure solution before being ingested by the feeding daphnids. It was also recognized that in the dietary exposure, the food-algae could release part of the accumulated copper before being ingested. Therefore, a preliminary kinetic uptake and elimination model was developed for algae exposed to copper. As such, based on the knowledge of the applied feeding regime and measured algal ingestion rates of the daphnids, average (and ranges of) copper burdens of the algae and copper ingestion rates by the daphnids could be calculated.

These calculations demonstrated that algal copper burdens and copper ingestion rates of the daphnids in the so-called waterborne exposure could reach similar or even higher levels than in some dietary or waterborne + dietary exposures. Second, the ranges of algal copper burdens and copper ingestion rates could be relatively large: variations of about factor 3 to 4 for waterborne exposure, a factor 2 for dietary exposure, and less than factor 1.5 for the waterborne + dietary exposure were observed.

Without this type of analysis, the correct interpretation of the test results (i.e. reproduction, growth and copper accumulation by the daphnids) would have been very difficult. Exposure to dietary copper resulted in an increased copper body burden of the daphnids but also in an increased growth and reproductive output, indicating a stimulatory rather than an inhibitory effect of dietary copper.

Regression analyses demonstrated that this stimulatory effect was not significantly correlated to the copper body burdens of the daphnids. Significant correlations were, however, observed with the average internal algal copper burden during the tests and with the mass specific copper ingestion rates of the daphnids. It is suggested that the latter is the result of a stimulation of digestive enzyme activity in the daphnids' gut invoked by the presence of copper in the gut, a process that has been demonstrated for benthic invertebrates.

Probably the most interesting conclusion was that the 21-day NOEC and the 21-day EC50 (about 95 and 110 µg Cu L^{-1}, respectively) obtained in the waterborne exposure were

similar to those in the waterborne + dietary exposure. This indicates that dietary copper does not alter the dissolved effect concentrations nor the predictive capacity of the chronic Cu-BLM.

It was demonstrated that mass specific ingestion rates of copper were between 1.9 and 124 µg Cu (g wet weight)$^{-1}$. The latter is higher than the mass specific copper ingestion rate for fish species that is demonstrated to result in a toxic effect, i.e. between 1 and 45 µg Cu (g wet weight)$^{-1}$ d^{-1}. Although, this could indicate a lower sensitivity of daphnids for dietary copper, the results of the present study were in agreement with the limited number of dietary copper toxicity studies with fish fed live diets. Indeed, these reports could not unambiguously demonstrate toxic effects of dietary copper up to 48 µg Cu (g wet weight)$^{-1}$ d^{-1}. Our study is the first to report on a clear stimulatory effect of dietary copper for freshwater organisms and this effect was significant at ingestion rates $\geq$ 20 µg Cu (g wet weight)$^{-1}$ d^{-1}.

However, given the limited and often apparently contradicting evidence on dietary metal exposure and toxicity available in literature, further research with regard to this issue should focus on the importance of factors such as food type, species-specific effects and water chemistry on dietary copper effects. With regard to the latter, it is noted that at higher pH levels algae can probably take up larger amounts of copper, resulting in a potentially higher dietary copper exposure.

Although the largest part of this doctoral thesis was dedicated to the development of BLMs for predicting acute and chronic copper toxicity to *D. magna*, another important goal of this study was the development of a chronic copper toxicity model for the green alga *P. subcapitata* (chapter 6). A similar experimental design as used for the development of the chronic Cu-BLM for *D. magna* (same test media) was applied.

For a total number of 35 toxicity tests performed, 72-hour E_bC10s (concentration resulting in 10% growth inhibition) ranged from 14 to 176 µg Cu L^{-1} (factor 12) and 72-hour E_bC50s from 26.9 to 507 µg Cu L^{-1} (factor 20). Statistical analysis demonstrated that DOC concentration, DOM source and pH had a significant effect on copper toxicity. Again, as for *D. magna*, hardness did not affect toxicity at the levels tested. In general, an increase in pH resulted in increased toxicity (on average explaining about 15% of the variability) whereas an increase of the DOC concentration resulted in decreased copper toxicity (on average

explaining about 60% of the variability). Thus, although the importance of DOC and pH for copper toxicity are similar for *D. magna* and *P. subcapitata*, it is extremely important to note that the pH-effect is completely opposite. Whereas toxicity is lower at higher pH levels for *D. magna*, toxicity is higher at higher pH levels for *P. subcapitata*. This has major implications for implementing bioavailability into the risk assessment of copper (see further).

When expressed as dissolved copper, significant differences of toxicity reduction capacity was noted across the three DOM sources tested (up to factor 2.5). However, when expressed as Cu^{2+} activity, effect levels were only significantly affected by pH (variation of over 4 orders of magnitude), indicating that Cu-DOM complexes were probably not bioavailable for the algae, as opposed to the observations in the chronic *D. magna* exposures. The differential effect of the different DOM sources was accounted for by calibrating the speciation model WHAM V to the measured Cu^{2+}-activities by adjusting the % active fulvic acid for each DOM source.

Linear relationships were observed between pH and the logarithm of the effect concentrations expressed as free copper ion activity: (1) Log (E_bC50_{Cu2+}) = - 1.431 pH + 2.050 (r^2 = 0.95), and (2) Log (E_bC10_{Cu2+}) = -1.140 pH - 0.812 (r^2 = 0.91). These relations also indirectly indicated that it was impossible and probably mechanistically incorrect to model this pH relationship according to the original BLM-concept (i.e. with proton competition at a single biotic-ligand site). It is suggested that the reason for the large pH-dependency of copper toxicity is the biochemical composition of the cell wall. Indeed, the algal cell wall carries a variety of functional groups, all of which become deprotonated at different pH levels and this may result in a continuous increase of copper binding with increasing pH. As cell wall binding may be the first step of a metal entering the cell and invoking a growth inhibition effect, the cell-wall may possibly be considered as the biotic ligand. Future research in this area should focus on investigating the relationship between water chemistry, copper bound to the cell wall, internal copper concentrations and toxic effects. To that end a detailed knowledge is needed on the pH-dependent copper binding characteristics of the cell-wall.

As an intermediate step, the following semi-empirical model was developed to predict copper toxicity. This model links the two above-mentioned equations to the WHAM V geochemical speciation model. Predicted 72-hour E_bC50 and E_bC10 values were within a

factor of 2 of the observed values for 97% of the reconstituted test waters and for 81% of the tested copper-spiked European surface waters. For the same Swedish water as used in the chronic *D. magna* Cu-BLM validation, toxicity was also under-estimated (about factor 4). Again, the competitive effect of Fe and Al on copper binding to DOM might explain this observation and this again indicates the importance of quantitatively taking into account this speciation effect in future toxicity modelling.

Given the importance of DOC for mitigating copper toxicity to *D. magna* and *P. subcapitata*, in chapter 7 the possible effect of the natural variability of DOM on the predictive capacity of the toxicity models was investigated. Prior to our study, most studies had focussed on the chemical variability of these natural DOM sources and not on the biologically and toxicologically relevant differences in metal binding properties. In the present study, acute toxicity tests with *D. magna* were carried out in artifical test water enriched with DOMs isolated from 6 locations in Europe and North America and in 7 natural European surface waters exhibiting a different physico-chemical composition and containing different concentrations of DOC. The acute Cu-BLM for *D. magna* was then used to estimate the copper complexing capacity of each DOM (expressed as % active fulvic acid). As opposed to the default assumption of 50% active fulvic acid (cf. above), a factor of 6 difference was observed between the lowest and the highest copper complexing capacity (i.e. between 17 and 109%), which is similar to differences found in other (mostly chemical) studies.

A significant linear relationship was observed between the UV-absorbance coefficient at 350 nm (ε_{350}) and the % active fulvic acid. Linking this relationship to the acute Cu-BLM resulted in a significant improvement of the predictive capacity of this BLM. Without accounting for DOM quality 90% of the predicted 48-hour EC50s were within a factor of 2 of the observed EC50s; taking DOM quality into account, 90% of the EC50s were predicted with an error of less than factor 1.3. The present study and other previously published studies seem to indicate that UV-absorbance may be a good measure of biologically and toxicologically relevant differences in the copper binding behaviour of DOM.

A similar relationship between UV absorbance and copper binding behaviour of DOM was observed for the alga *P. subcapitata*. It is proposed to use the same relationship for both species. As a consequence of the (limited) bioavailability of Cu-DOM complexes in chronic

exposures of *D. magna*, this relationship does not seem to be applicable here. However this bioavailability of Cu-DOM complexes was only observed for isolated DOM samples, whereas no such evidence was obtained for DOM in natural water samples. It is recommended that research is performed to investigate if the derived relation with UV-absorbance would be applicable to chronic exposures in natural surface water samples.

It is suggested that UV absorbance measurements (to account for toxicologically relevant copper complexing capacity of organic matter) may be a valid alternative for more expensive DOC-measurements and this could help in the routine application of the BLM.

In the above sections, a summary was given of the main results obtained in this doctoral study. However, one of the goals of this research was to develop tools that could contribute to the implementation of copper bioavailability into the EU risk assessment framework, and more specifically in the estimation of a bioavailability-corrected PNEC. The latter is discussed below.

First, it needs to be stressed again that the commonly applied hardness-based correction of WQC is probably not appropriate for copper, as it was demonstrated that it did not significantly affect chronic copper toxicity to either *D. magna* or *P. subcapitata*. Second, it was demonstrated that DOC explains about 60% of the variability of the chronic toxicity values and that pH explains about 15%. However, whereas an increased pH results in decreased toxicity (expressed as dissolved copper) for *D. magna*, an increased toxicity was observed for *P. subcapitata*. Both observations are in agreement with most reports on invertebrates and fish (decreased toxicity) and other algae (increased toxicity).

Within the risk assessment framework, the estimation of the PNEC is based on toxicity data for all these species groups and thus one can easily see that pH will not have a large effect on the copper sensitivity of ecosystems that consists of these species groups. Indeed, at low pH levels the invertebrates and fish will exhibit their largest sensitivity whereas at high pH levels the algae will exhibit their largest sensitivity. Most probably, the ecosystem sensitivity at high pH will be dominated by algal sensitivity whereas at low pH it will probably be dominated by invertebrate and fish sensitivity. Combined, this will probably result in only small effects of pH on ecosystem sensitivity to copper. Preferably should be confirmed by a thorough modelling analysis or by mesocosm-type toxicity assays. Our initial

analysis, however, indicates that the most important factor determining an ecosystems' sensitivity to copper is probably the concentration of dissolved organic carbon. This is not surprising, as Cu-DOC complexes are largely unavailable and usually make up the largest fraction of copper species in natural systems.

Although currently still a matter of debate, the following analysis could be carried out to derive bioavailability-corrected PNECs. The developed toxicity models (i.e. the chronic *D. magna* Cu-BLM and the toxicity model for *P. subcapitata*), together with a still-to-be developed chronic Cu-BLM for fish, would possibly allow to normalize the toxicity data used in the generic species sensitivity distribution (see Figure 1-6). Indeed, the generic SSD contains data obtained in test media with a large variation of physico-chemistry and thus bioavailability. One of the consequences of this is that the NOEC values for one species may vary up to 2 orders of magnitude. A preliminary analysis revealed that the uncertainty (i.e. the variation of NOEC values) was for most species largely due to differences in bioavailability. In the same study, the toxicity models developed in our study reduced this uncertainty to a large extent, which may illustrate the possible extrapolation of the developed models to other species. Hence, the models may be used to correct the toxicity data of all species of the generic SSD towards any given combination of physico-chemical characteristics (for a given surface water or for a given region). As such, a bioavailability-corrected SSD is obtained which may allow the calculation of a bioavailability-corrected PNEC, which may then be compared to a monitored or modelled PEC to assess the bioavailability-corrected risk of copper.

It is clear that the approach discussed above is rather pragmatic and that some of the assumptions made may be criticized. Probably the most contentious assumption is the extrapolation of toxicity models from one species to another without the availability of a large toxicity dataset for the latter organism. However, the fact that a large part of bioavailability-related uncertainty can be explained with the developed models demonstrates the potential usefulness of these models. Indeed, any reduction of uncertainty in risk assessment is better for both the environment and the society as it brings the risk assessment closer to sustainable development.

Clearly, more research is needed to clarify the mechanistic, chemical, physiological, biological and ecological processes that contribute to the complexity of metal bioavailabity in

freshwater environments. The introduction of bioavailability concepts and models such as the biotic ligand model into the risk assessment of metals and the recognition of their potential use by the academic, industrial and regulatory community should not be regarded as the finish line, but rather as the start of an era of new and innovative metal bioavailability research…

References

Abbt-Braun G, Frimmel FH. 1999. Basic characterization of Norwegian NOM samples - similarities and differences. Science of the Total Environment 25:161-180.

Aldenberg T, Slob W. 1993. Confidence limits for hazardous concentrations based on logistically distributed NOEC toxicity data. Ecotoxicology and Environmental Safety 25:48-63.

Allen HE, Hall RH, Brisbin TD. 1980. Metal speciation, effects on aquatic toxicity. Environmental Science and Technology 14:441.

Allen HE, Hansen DJ. 1996. The importance of trace metal speciation to water quality criteria. Water Environment Research 68:42-54.

Allen Y, Calow P, Baird DJ. 1995. A mechanistic model of contaminant-induced feeding inhibition in *Daphnia magna*. Environmental Toxicology and Chemistry 14:1625-1630.

Allison JD, Brown DS, Novo-Gradac KJ. 1991. MINTEQA2/PRODEFA2, a Geochemical Assessment Model for Environmental Systems: version 3.0 user's Manual. US environmental Protection Agency, Environmental Research Laboratory, Office of Research and Development.

Andrew RW, Biesinger KE, Glass GE. 1977. Effects of Inorganic Complexing on the Toxicity of Copper to *Daphnia magna*. Water Research 11:309-315.

ANZECC/ARMCANZ. 2000. Australian and New Zealand Guidelines for Fresh and Marine Water Quality. Environment Australia, Canberra, Australia.

ASTM. 1993a. Standard guide for conducting static 96 hour toxicity tests with microalgae. E1218-90. In: Annual Book of ASTM Standards, Vol. 11.04., American Society for Testing and Materials Philadelphia, PA, USA, pp 929-940.

ASTM. 1993b. Standard guide for conducting renewal life-cycle toxicity tests with *Daphnia magna*. E1993-87. In: Annual Book of ASTM Standards, Vol. 11.04. American Society for Testing and Materials, Philadelphia, PA, USA, pp 866-882.

Barata C, Baird DJ, Markich SJ. 1998. Influence of Genetic and Environmental Factors on the Tolerance of Daphnia magna Straus to Essential and Non-essential Metals. Aquatic Toxicology 42:115-137.

Barnes RD. 1987. Invertebrate Zoology. Fifth Edition. Saunders College Publishing, Philadelphia, PA, USA.

Belanger SE, Cherry DS. 1990. Interacting effects of pH acclimation, pH and heavy metals on acute and chronic toxicity to *Ceriodaphnia dubia* (Cladocera). Journal of Crustacean Biology 10:225-235.

Benedetti MF, Milne CF, Kinniburgh DG, Van Riemsdijk WH, Koopal LK. 1995. Metal ion binding to humic substance: application non-ideal competitive adsorption model. Environmental Science and Technology 29:446-457.

Bergman HL, Dorward-King EJ. 1997. Reassessment of metals criteria for aquatic life protection. SETAC Press, Pensacola, FL, USA.

Bianchini A, Wood CM. 2002. Physiological effects of chronic silver exposure in *Daphnia magna*. Comparative Biochemistry and Physiology C 133:137-146.

Blaylock BG, Frank ML, McCarthy JF. 1985. Comparative toxicity of copper and acridine to fish, *Daphnia* and algae. Environmental Toxicology and Chemistry 4: 63-71.

Blust R, Fontaine A, Decleir W. 1991. Effect of hydrogen ions and inorganic complexing on the uptake of copper by the brine Shrimp Artemia franciscana. Marine Ecology Progress Series 76:273-282.

Borgmann UW, Ralph KM. 1983. Complexation and toxicity of copper and the free metal bioassay technique. Water Research 17:1697-1703.

Bossuyt BT, Janssen CR. 2001. Acclimation of the freshwater crustacean *Daphnia magna* to copper: changes in tolerance and energy allocation. Mededelingen van de Faculteit Landbouwwetenschappen van de Univeristeit Gent 66:191-195.

Bossuyt BTA, Janssen CR. 2003a. Acclimation of *Daphnia magna* to copper background concentrations. In preperation

Bossuyt BTA, Janssen CR. 2003b. Influence of multi-generation acclimation to copper on tolerance, energy reserves and homeostasis of *Daphnia magna*. In preparation.

Bossuyt BTA, Janssen CR. 2003c. Influence of long-term sub lethal copper exposure on tolerance and physiology of *Pseudokirchneriella subcapitata* (Korshikov) Hindak. In preparation.

Boudou A, Ribeyre F. 1989. Aquatic Toxicology: Fundamental Concepts and Methodologies. CRC Press, Boca Raton, Florida, USA.

Box GEP, Draper NR. 1987. Empirical model-building and response surfaces. Wiley, New York, NY, USA.

Box GEP, Hunter WG, Hunter SJ. 1978. Statistics for experimenters: an introduction to design, data analysis, and model building. Wiley, New York, NY, USA.

Brix KV, DeForest DK. 1999. Critical review of the use of bioaccumulation potential for hazard classification of metals and metal compounds. Parametrix, Inc., Kirkland, Washington, USA.

Brown PL, Markich SJ. 2000. Evaluation of the free ion activity model of metal-organism interaction: extension of the conceptual model. Aquatic Toxicology 51:195-200

Brunori M, Girdina B, Bannister JV. 1979. Respiratory proteins. In: Hill HAO (Ed.) Inorganic biochemistry: a specialist periodical report. The Chemical Society, London, vol. 1:159-209.

Bryan GW, Gibbs PE. 1983. Heavy metals in the Fal estuary, Cornwall: a study of long-term contamination by mining waste and its effects on estuarine organisms. Marine Biology Association UK Ocassional Publication 2:1-112.

Bryan SE, Tipping E, Hamilton-Taylor J. 2002. Comparison of measured and modelled copper binding by natural organic matter in feshwaters. Comparative Biochemistry and Physiology C 133:37-50.

Bury NR, Wood CM. 1999. Mechanisms of branchial apical silver uptake by rainbow trout is via the proton-coupled Na^+ channel. American Journal of Physiology. R1385-R1391.

Cabaniss SE, Shuman MS. 1988. Copper binding by dissolved organic matter: II. Variation in type and source of organic matter. Geochimica Cosmochimica Acta 52:185-193.

Campbell PGC, Twiss MR, Wilkinson KJ. 1997a. Accumulation of natural organic matter on the surfaces of living cells: implications for the interaction of toxic solutes with aquatic biota. Canadian Journal of Fisheries and Aquatic Sciences 54:2543-2554.

Campbell PGC, Lewis AG, Chapman PM, Crowder AA, Fletcher WK, Imber B, Luoma SN, stokes PM, Winfrey M. 1988. Biologically available metals in sediments (NRCC No. 27694), National Research Council Canada, Ottawa.

Campbell PGC. 1995. Interactions between trace metals and aquatic organisms: a critique of the free-ion activity model. In: Tessier A, Turner DR (eds.) Metal Speciation and Bioavailability in Aquatic Systems. John Wiley & Sons, Chichester, United Kingdom, pp 45-102.

Campbell PGC, Stokes PM. 1985. Acidification and toxicity of metals to aquatic biota. Canadian Journal of Fisheries and Aquatic Sciences 42:2034-2049.

Cass AEG, Hill HAO. 1980. Copper proteins and copper enzymes. In: Biological roles of copper. Ciba foundation symposium 79 (new series), Excerpt Medica, Amsterdam, The Netherlands, pp. 71-85.

Cavallaro N, McBride MB. 1984. Zinc and copper sorption and fixations by an acid soil clay: effect of selective dissolutions. Soil Science Society of America Journal 48:1050-1054.

Chen Z, Mayer LM, Weston DP, Bock MJ, Jumars PA. 2002. Inhibition of digestive enzyme activities by copper in the guts of various marine benthic invertebrates. Environmental Toxicology and Chemistry 25:32-36.

Cid A, Fidalgo P., Herrero C, Abalde J. 1996. Toxic action of copper on the membrane system of a marine diatom measured by flow cytometry. Cytometry 25: 32-36.

Cid A, Herrero C, Torres E, Abalde J. 1995. Copper toxicity on the marine microalgaa *Phaeodactylum tricornutum*: effects on photosynthesis and related parameters. Aquatic Toxicology 31:165-174.

Clearwater SJ, Farag AM, Meyer JS. 2002. Bioavailability and toxicity of dietary copper and zinc to fish. Comparative Biochemistry and Physiology C 132:269-313.

Clesceri LS;, Greenberg AE, Eaton AD. 1998. Standard Methods for the Examination of Water and Wastewater – 20th Edition; American Public Health Association, Washington, DC, USA.

Cowan CE, Jenne EA, Kinnison RRA. 1984. Methodology for determining the toxic chemical species of copper in toxicity experiments and natural waters. In: Hemphill DD (Ed.) Proceedings of the university of Missouris' 18th annual conference on trace substances in environmental health, University of Missouri, Columbia, Missouri, USA.

Crist RH, Martin JR, Guptill PW, Eslinger JM, Crist DR. 1990. Interactions of metals and protons with algae. Environmental Science and Technology 24:337-342.

Crist RH, Oberholser K, Schwartz D, Marzoff J, Ryder D, Crist DR. 1988. Interactions of metals and protons with algae. Environmental Science and Technology 22:755-760.

Curtis PJ, Schindler DW. 1997. Hydrological control of dissolved organic matter in low-order Precambrian Shield lakes. Biogeochemistry 36:125-138.

Cusimano RF, Brakke DF, Chapman GA. 1986. Effects of pH on the toxicities of cadmium, copper, and zinc to steelhead trout (*Salmo gairdneri*). Canadian Journal of Fisheries and Aquatic Sciences 43:1497-1503.

De Boeck G, Vlaeminck A, Balm PHM, Lock RAC, De Wachter B, Blust R. 2000. Morphological and metabolic changes in common carp *Cyprinus carpio* during short-term copper exposure: interaction between Cu^{2+} and plasma cortisol elevation. Environmental Toxicology and Chemistry 20:374-381.

De Coen WM, Janssen CR. 1997. The use of biomarkers in *Daphnia magna* toxicity testing. IV. Cellular Energy Allocation: a new methodology to assess the energy budget of toxicant-stressed Daphnia populations. Journal of Aquatic Ecosystems Stress and Recovery 6:43-55.

De Coen WM, Janssen CR. 1998. The use of biomarkers in *Daphnia magna* toxicity testing. III. Rapid toxicity testing of pure chemicals and sediment pore waters using ingestion and digestive enzyme activity. Chemosphere 37:2677-2694

De Schamphelaere KAC, Heijerick DG, Janssen CR. 2002. Refinement and field validation of a biotic ligand model predicting acute copper toxicity to *Daphnia magna*. Comparative Biochemistry and Physiology C 133:243-258.

De Schamphelaere KAC, Heijerick DG, Janssen CR. 2003b. The effect of pH buffering on the toxicity of copper and zinc to *Daphnia magna* and *Pseudokirchneriella subcapitata*. Ecotoxicology (accepted).

De Schamphelaere KAC, Janssen CR. 2002. A biotic ligand model predicting acute copper toxicity for *Daphnia magna*: the effects of calcium, magnesium, sodium, potassium and pH. Environmental Science and Technology 36:48-54.

De Schamphelaere KAC, Janssen CR. 2003a. Effects of dissolved organic matter concentration and source, pH and water hardness on chronic toxicity of copper to *Daphnia magna*. Environmental Toxicology and Chemistry (accepted).

De Schamphelaere KAC, Janssen CR. 2003b. Development and field validation of a biotic ligand model predicting chronic copper toxicity to *Daphnia magna*. Environmental Toxicology and Chemistry (Accepted).

De Schamphelaere KAC, Vasconcelos FM, Heijerick DG, Tack FMG, Delbeke K, Allen HE, Janssen CR 2003a. Development and field validation of a predictive copper toxicity model for the green alga *Pseudokirchneriella subcapitata*. Environmental Toxicology and Chemistry 22:2454-2465.

Di Toro DM, Allen HE, Bergman H, Meyer JS, Paquin PR, Santore CS. 2001. Biotic ligand model of the acute toxicity of metals. 1. Technical Basis. Environmental Toxicology and Chemistry 20:2383-2396.

Dunbar, L.E., 1996. Derivation of a site-specific dissolved copper criteria for selected freshwater streams in Connecticut. Technical report. Connecticut Department of Environmental Protection. Water Toxics Program, Falmouth, MA, USA.

Dwane GC, Tipping E. 1998. Testing a humic speciation model by titration of copper-amended natural waters. Environment international 24:609-616.

Elendt, B.P., Bias, W.R., 1990. Trace nutrient deficiency in daphnia magna cultured in standard medium for toxicity testing, effects of the optimization of culture conditions on life history parameters of D. magna. Water Research 24:1157-1167.

EPS. 1990. Biological test method: Acute lethality test using *Daphnia sp*. Report EPS 1/RM/11, Environmental Protection Series, Environment Canada.

Erickson RJ, Benoit DA, Mattson VR, Nelson HP Jr, Leonard EN. 1996. The effects of water chemistry on the toxicity of copper to fathead minnows. Environmental Toxicology and Chemistry 15:181-193.

European Commission. 1993a. Council regulation (EC) 793/93 of march 1993 on the evaluation and control of the environmental risk of existing substances. Off. J. Eur. Commun. L84.

European Commission. 1993b. Commission Directive 93/67/EEC of 20 july 1993, laying down the principles for the assessment of the risks to man and the environment of substances notified in accordance with Council Directive 67/548/EEC. Off. J. Eur. Commun. L227.

European Commission. 2003. Technical Guidance Document on Risk Assessment in support of Commission Directive 93/67/EEC on Risk Assessment for new notified substances, Commission Regulation (EC) No 1488/94 on Risk Assessment for existing substances and Directive 98/8/EC of the European Parliament and of the Council concerning the placing of biocidal products on the market.

Technical Guidance Document in support of Commission Directive 93/67/EEC on Risk Assessment for New Notified Substances and Commission Regulation (EC) No 1488/94 on Risk Assessment for Existing Substances, Part II. Office for Official Publications of the European Communities, Luxemburg.

Ferrando MD, Andreu E. 1993. Feeding behaviour as an index of copper stress in *Daphnia magna* and *Brachionus calyciflorus*. Comparative Biochemistry and Physiology C 106:327-331

Fish W, Morel FMM. 1983. Characterisation of organic copper-complexing agents released by *Daphnia magna*. Canadian Journal of Fisheries and Aquatic Sciences 40:1270-1277.

Fitch A. Stevenson FJ, Chen Y. 1986. Complexation of Cu(II) with a soil humic acid: response characteristics of the Cu(II) ion-selective electrode and ligand concentration effects. Organic Geochemistry 9:109-116.

Flickinger AL, Bruins RJF, Winner RW, Skilling JH. 1982. Filtration and phototactic behavior as indices of chronic copper stress in *Daphnia magna* Straus. Archives of Environmental Contamination and Toxicology 11:457-463

Flößner D. 2000. Die Haplopoda und Cladocera Mitteleuropas. Backhuys Publishers, The Netherlands.

Franklin NM, Stauber JL, Lim RP. 2001. Development of flow cytometry-based algal bioassays for assessing the toxicity of metals in natural waters. Environmental Toxicology and Chemistry 20:160-170.

Franklin NM, Stauber JL, Markich SJ, Lim RP. 2000. pH-dependent toxicity of copper and uranium to a tropical freshwater alga (*Chlorella* sp.). Aquatic Toxicology 48:275-289.

Franklin NM, Stauber JL, Apte SC, Lim RP. 2002. Effect of inital cell density on the bioavailability and toxicity of copper in microalgal bioassays. Environmental Toxicology and Chemistry 21:742-751

Fraunhofer Institute. 2001. Development of methods for the derivation of quality standards in the context of the water framework directive. Draft report of the study: Identification of quality standards for priority substances in the field of water policy. Department of Environmental Chemistry and Ecotoxicology, Fraunhofer Institute, Germany.

French P, Hunt DTE. 1987. The effects of inorganic complexing upon the toxicity of copper to aquatic organisms (principally fish). In: Hunt DTE (Ed.) Trace metal speciation and toxicity to aquatic organisms – a review, report TR 247, Water Research Centre, Marlow, Bucks, United Kingdom, pp.43-51.

Frimmel FH, Abbt-Braun G. 1999. Basic chracterization of reference NOM from central Europe – similarities and differences. Environment International 25:191-207.

Galvez F, Hogstrand C, Wood CM. 1998. Physiological responses of juvenile rainbow trout to chronic low level exposures of waterborne silver. Comparative Biochemistry and Physiology C 119:131-137.

Gamble DS, Schnitzer M. 1973. The Chemistry of Fulvic Acid and Its Reactions with Metal Ions. In Singer PC (ed.) Trace Metals and Metal Organic Interactions in Natural Waters. Ann Arbor Science, Ann Arbor, MI, USA.

Gensemer RW, Playle RC. 1999. The bioavailability and toxicity of aluminum in aquatic environments. Critical Reviews of Environmental Science and Technology 29:315-450.

Green JR, Margerison D. 1978. Statistical treatment of experimental data. Elsevier Scientific Publishing Company, Amsterdam, The Netherlands, pp. 221-235.

Grosell M, Nielsen C, Bianchini A. 2002a. Sodium turnover rate determines sensitivity to acute copper and silver exposure in freshwater animals. Comparative Biochemistry and Physiology C 133:287-303.

Grosell M, Brauner CJ, Kelly SP, McGeer JC, Bianchini A, Wood CM. 2002b. Physiological responses to acute silver exposure in the freshwater crayfish (*Cambarus diogenes diogenes*) - A model invertebrate? Environmental Toxicology and chemistry 21: 369-374.

Grosell M, Wood CM. 2002. Copper uptake across rainbow trout gills: mechanisms of apical entry. Journal of Experimental Biology. 205:1179-1188.

Hamilton MA, Russo RC, Thurston RV. 1977. Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. Environmental Science and Technology 11:714-719.

Happey-Wood CM. 1988. Ecology of freshwater planktonic green algae. In: Sandgren CD (ed.), Growth and Reproductive Strategies of Freshwater Phytoplankton. Cambridge University Press, Cambridge, USA, pp 175-226.

Heijerick DG, De Schamphelaere KAC, Janssen CR. 2002a. Biotic ligand model development predicting Zn toxicity to the alga *Pseudokirchneriella subcapitata*: possibilities and limitations. Comparative Biochemistry and Physiology C 133:207-218.

Heijerick DG, De Schamphelaere KAC, Janssen CR. 2002b. Predicting acute zinc toxicity for Daphnia magna as a function of key water chemistry characteristics: Development and validation of a biotic ligand model. Environmental Toxicology and Chemistry 21:1309-1315.

Heijerick DG, De Schamphelaere KAC, Janssen CR. 2003a. Application of biotic ligand models for predicting zinc toxicity in European surface waters. Project ZEH-WA-02. Prepared for International Lead Zinc Research Organization, Raleigh, NC, U.S.A.

Heijerick DG, Janssen CR, De Coen WM. 2003b. The Combined Effects of Hardness, pH, and Dissolved Organic Carbon on the Chronic Toxicity of Zn to *D. magna*: Development of a Surface Response Model. Archives of Environmental Contamination and Toxicology 44:210-217.

Hering JG, Morel FMM. 1988. Humic acid complexation of calcium and copper. Environmental Science and Technology 22:1234-1237.

Hille B, Woodhull AM, Shapiro BI. 1975. Negative surface charge near sodium channels of nerve – divalent ions, monovalent ions and pH. Philosphical Transactions of the Royal Society of London B 270:257-290.

Hogstrand C, Wood CM. 1998. Towards a better understanding of the bioavailability, physiology and toxicity of silver in fish: implications for water quality criteria. Environmental Toxicology and Chemistry 17:547-561.

Holm-Jensen I. 1948. Osmotic regulation in *Daphnia magna* under physiological conditions and in the presence of heavy metals. Det KGL. Danske Videnskabernes selvskab. Biologiske. Medelser. 20:4-64.

Hong S, Candelone JP, Patterson CC, Boutron CF. 1996. History of ancient copper smelting pollution during roman and medieval times recorded in Greenland ice. Science 272: 246.

Hook SE, Fisher NS. 2001a. Reproductive toxicity of metals in calanoid copepods. Marine Biology 138:1131-1140.

Hook SE, Fisher NS. 2001b. Sublethal effects of silver in zooplankton: importance of exposure pathways and implications for toxicity testing. Environmental Toxicology and Chemistry 20:568-574.

Hook SE, Fisher NS. 2002. Relating the reproductive toxicity of five ingested metals in calanoid copepods with sulfur affinity. Marine Environmental Research 53:161-174.

Hopkin SP. 1989. Ecophysiology of Metals in Terrestrial Invertebrates. Elsevier Applied Science Publishers, Barking, United Kingdom.

HydroQual. 1999. Biotic Ligand Model (BLM) version a008. HydroQual, Mahwah, NJ, USA.

HydroQual. 2002. BLM Windows Interface, version 1.0.0. HydroQual, Mahwah, NJ, USA.

IWP. 1999. Interdepartmental Working Party on Setting Integrated Environmental Quality Standards for Substances. 1999. Setting Integrated Environmental Quality Standards for Substances in The Netherlands – Environmental quality standards for soil, water & air. Interdepartmental Working Party on Setting Integrated Environmental Quality Standards for Substances, The Netherlands.

Irving H, Williams RJP. 1953. The stability of transition-metal complexes. III. Journal of the Chemical Society: 3192.

ISO. 1989. Water Quality – Fresh water algal growth inhibition test with *Scenedesmus subspicatus* and *Raphidocelis subcapitata*. ISO-8692. International Organisation for Standardisation, Geneva, Switzerland.

ISO. 1993. Water quality – Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea) – Acute toxicity test. In: Draft International Standard ISO/DIS 6341, International Organisation for Standardisation, Geneva, Switzerland, pp 1-11.

Janes N, Playle RC. 1995. Modeling Silver Binding to Fish Gills of Rainbow Trout (*Oncorynchus Mykiss*). Environmental Toxicology and Chemistry 14:1847-1858.

Janssen CR, De Schamphelaere K, Heijerick D, Muyssen B, Lock K, Bossuyt B, Vangheluwe M, Van Sprang P. 2000. Uncertainties in the environmental risk assessment of metals. Human and Ecological Risk Assessment 6:1003-1018.

Janssen CR, Heijerick DG. 2003. Algal toxicity testing for environmental risk assessments of metals: physiological and ecological considerations. Reviews of Environmental Contamination and Toxicology (in press).

Kamunde C, Grosell M, Higgs D, Wood CM. 2002. Copper metabolism in actively growing rainbow trout (*Oncorhynchus mykiss*): interactions between dietary and waterborne copper uptake. Journal of Experimental Biology 205:279-290.

Kandegedara A, Rorabacher DB. 1999. Noncomplexing tertiary amines as 'better' buffers covering the range of pH 3-11. Temperature dependence of their acid dissociation constants. Analytical Chemistry 71:3140-3144.

Keen C, Taubeneck M, Daston G, Rogers J, Gershwin M. 1993. Primary and secondary zinc deficiency as factors underlying abnormal CNS development. Annals of the New York Academy of Sciences 678:37-47.

Kikuchi S, Shiraishi K.1997. Ultrastructure and ion permeability of the two types of epithelial cell arranged alternately in the gill of the fresh water branchiopod *Caenestheriella gifuensis* (Crustacea). Zoomorphology 117:53-62.

Kikuchi S. 1983. The fine structure of the gill epithelium of a fresh-water flea, *Daphnia magna* (Crustacea: Phyllopoda) and changes associate with acclimation to various salinities. I. Normal fine structure. Cell and Tissue Research 22:253-268.

Kim SD, Ma H, Allen HE, Cha DK. 1999. Influence of dissolved organic matter on the toxicity of copper to *Ceriodaphnia dubia*: effect of complexation kinetics. Environmental Toxicology and Chemistry 18, 2433-2437.

Kicrhgessner M, Beyer MG, Steinhart H. 1976. Activation of pepsin (EC3.4.4.1.) by heavy metal ions including a contribution to mode of action of copper-sulfate in pig nutrition. British Journal of Nutrition 36:15-22.

Knauer K, Behra R, Sigg L. 1997. Adsorption and uptake of copper by the green alga *Scenedesmus subspicatus* (Chlorophyta). Journal of Phycology 33:596-601.

Kooijman SALM. 2000. Dynamic energy and mass budgets in biological systems, 2nd edition. Cambridge University Press, Cambridge, United Kingdom.

Korshikov OA. 1990. *Pseudokirchneriella subcapitata* Hindak, F-1990. Biologicke Prace 36:209.

Kramer KJM, Jak RG, Van Hattum B, Hooftman RN. 2001. Koper in de Nederlandse oppervlaktewateren - toxiciteit in relatie tot organisch materiaal. STOWA, Utrecht, The Netherlands.

Krogh A. 1938. The active absorption of ions in some freshwater animals. Z. Vergl. Physiol. 25:335-350.

Lampert w. 1987. Feeding and nutrition in *Daphnia*. In: Peters RH, De Bernardi R (Eds.) Daphnia. Memorie dell'Intituto Italiano di Idrobiologia 45:143-192.

Länderarbeitsgemeinschaft Wasser. 1997. Zielvorgaben zum Schutz oberirdischer Binnengewässer. Band II: Ableitung und Erprobung von Zielvorgaben zum Schutz oberirdischerBinnengewässer für die Schwermetalle Blei, Cadmium, Kupfer, Nickel, Quecksilber und Zink. Kulturbuchverlag Berlin GmbH, Berlin.

Landner L, Lindeström L. 1999. Copper in society and the environment. An account of the facts on fluxes, amounts and effects of copper in Sweden. Swedish Environmental Research Group. Centraltryckeriet AB, Boras, Sweden.

Laurén DJ, McDonald DG. 1985. Effects of Copper on Branchial Ionoregulation in the Rainbow Trout, *Salmo gairdneri* Richardson. Journal of Comparative Physiology B 155:635-644.

Laurén DJ, McDonald DG. 1987. Acclimation to copper by rainbow trout *Salmo gairdneri*: biochemistry. Canadian Journal of Fisheries and Aquatic Science 44:105-111.

Lepper P. 2002. Development of a methodological framework for the derivation of metal quality standards in the context of the EU Water Framework Directive. Presented at the "Bioavailability of metals in surface waters" Workshop, 7-8 february 2002, Gent, Belgium.

Lewis MA. 1993. Freshwater Primary Producers. In: Calow P (ed.), Handbook of Ecotoxicology. Blackwell Scientific Publications, Oxford, United Kingdom, pp 28-50.

Li J, Lock AC, Klaren PHM, et al. 1996. Kinetics of Cu^{2+} inhibition of Na^{+}/K^{+}-ATPase. Toxicology Letters. 87:31-38.

Li J, Quabius ES, Wendelaar Bonga SE, Flick G, Lock RAC. 1998. Effects of waterborne copper on branchial chloride cells and NA+/K+ ATPase activities in Mozambique Tilapia (*Oreochromis mozambicus*). Aquatic Toxicology 43:1-11.

Linder M. 1991. Nutrition and metabolism of the trace elements. In: Linder M (ed.). Nutritional Biochemistry and Metabolism with Clinical Applications. 2nd edition. Elsevier, New York, NY, pp 215-276.

Lock K, Janssen CR, De Coen WM. 2000. Multivariate test designs to asses the influence of zinc and cadmium bioavailability in soils to *Enchytraeus albidus*. Environmental Toxicology and Chemistry 19:2666-2671.

Lofts S, Tipping E. 2003. SCAMP – Surface Chemistry Assemblage Model for Particles. Centre for Ecology and Hydrology, CEH Windermere, Ferry House, Far Sawrey, Ambleside, Cumbria, UK. (http://www.ife.ac.uk/aquatic_processes/scamp.htm)

Lofts S, Tipping E. 2002. Windermere Humic Aqueous Model - Equilibrium chemical speciation for natural waters - Version 6.0. Centre for Ecology and Hydrology, CEH Windermere, Ferry House, Far Sawrey, Ambleside, Cumbria, UK. (http://www.ife.ac.uk/aquatic_processes/wham.htm)

Ma H, Allen HE, Yin Y. 2001. Characterization of isolated fractionations of dissolved organic matter from natural waters and a wastewater effluent. Water Research 35:985-996.

Ma H, Kim SD, Cha DK, Allen HE. 1999. Effects of kinetics of complexation by humic acid on toxicity of copper to *Ceriodaphnia dubia*. Environmental Toxicology and Chemistry 18:828-832.

MacCarthy P. 2001. The principles of humic substances. Soil Science 166:738-751.

Macfie SM, Tarmohamed Y, Welbourn PM. 1994. Effect of cadmium, cobalt, copper and nickel on growth of the green alga *Chlamydomonas reinhardtii*: the influence of cell wall and pH. Archives of Environmental Contamination and Toxicology 27:454-458.

MacRae RK, Smith DE, Swoboda-Colberg N, Meyer JS, Bergman HL. 1999. The Copper Binding Affinity of Rainbow Trout (*Oncorynchus Mykiss*) and Brook Trout (*Salvelinus fontinalis*) Gills: Implications for Assessing Bioavailable Metal. Environmental Toxicology and Chemistry 18:1180-1189.

Malcolm RL, MacCarthy P. 1986. Limitations in the use of commercial humic acids in water and soil research. Environmental Science and Technology 20:904-911.

Marinsky JA, Gupta S, Schindler P. 1982. The interaction of Cu(II) ion with humic-acid. Journal of Colloid and Interface Science 89:401-411.

Markich SJ, Jeffree RA. 1994. Absorption of divalent trace-metals as analogs of calcium by Australian freshwater vivalves – an explanation of how water hardness reduces metal toxicity. Aquatic Toxicology 29:257-290.

Martell AE, Smith RM, Motekaitis RJ. 1997. Critical stability constants of metal complexes database, version 4.0. NIST Standard Reference Database 46. National Institute of Standards and Technology, Gaithersburg, MD, USA.

Mason AZ, Jenkins KD. 1995. Metal Detoxification in Aquatic Organisms. In: Tessier A, Turner DR (eds.). Metal Speciation and Bioavailability in Aquatic Systems. John Wiley & Sons, New York, U.S.A., pp.479-608.

McKim JM, Christensen GM, Hunt EP. 1970. Changes in the blood of the brook trout (*Salvelinus fontinalis*) after short-term end long-term exposure to copper. J. Fish Res. Bd. Can. 27:1883-1889.

McKnight DM, Feder G, Thurman EM, Wrshaw RL, Westall JC. 1983. Complexation of copper by aquatic humic substances from different environments. Science of the Total Environment 28:65-76.

McLarnon-Riches CJ, Rolph CE, Greenway DLA, Robinson PK. 1998. Effects of environmental factors and metals on *Selenastrum capricornutum* lipids. Phytochemistry 49:1241-1247.

Meador JP, Sibley TH, Swartzman GL, Taub FB. 1998. Copper tolerance by the freshwater algal species *Oocystis pusilla* and its ability to alter free-ion copper. Aquatic Toxicology 44:69-82.

Meador, J.P., 1991. The interaction of pH, dissolved organic carbon and total copper in the determination of ionic copper and toxicity. Aquatic Toxicology 19, 13-32.

Meyer JS, Santore RC, Bobbit JP, Debrey LD, Boese CJ, Paquin PR, Allen HE, Bergman HL, Di Toro DM. 1999. Binding of Nickel and Copper to Fish Gills Predicts Toxicity when Water Hardness Varies, but Free Ion Activity Does Not. Environmental Science and Technology 33:913-916.

Meyer JS. 1999. A Mechanistic Explanation for the Ln(LC50) vs Ln(hardness) Adjustment Equation for Metals. Environmental Science and Technology 33:908-912.

Miller WE, Greene JC, Shiroyama T. 1978. *Selenastrum capricornutum* Printz Algal Assay Bottle Test: Experimental Design, Application, and Data Interpretation Protocol. EPA-600/9-78-018, U.S. Environmental Protection Agency, Corvallis, OR, USA.

Morel, F.M.M.,1983. Principles of Aquatic Chemistry. John Wiley and Sons, New York.

Mount DI, Norberg TJ. 1984. A seven-day life cycle Cladoceran toxicity test. Environmental Toxicology and Chemistry 3, 425-434.

Mount DR, Barth AK, Garrison TD, Barten KA, Hockett JR. 1994. Dietary and waterborne exposure of rainbow trout (*Oncorhynchus mykiss*) to copper, cadmium, lead and zinc using a live diet. Environmental Toxicology and Chemistry 13:2031-2041.

Munger C, Hare L, Craig A, Charest P. 1999. Influence of exposure time on the distribution of cadmium within the cladoceran *Cerodaphnia dubia*. Aquatic Toxicology 44:195-200.

Muyssen BTA, Janssen CR. 2001. Multi-generational zinc acclimation and tolerance in *Daphnia magna*: implications for water quality guidelines and ecological risk assessment. Environmental Toxicology and Chemistry 20:2053-2060.

Muyssen BTA, Janssen CR. 2002b. Zinc accumulation and regulation in *Daphnia magna* Straus: links with homeostasis and toxicity. Archives of Environmental Contamination and Toxicology 43:492-496.

Nalewajko C, Colman B, Olaveson M. 1997. Effects of pH on growth, photosynthesis, respiration and copper tolerance of three scenedesmus strains. Environmental and Experimental Botany 37:153-160.

Neter J, Wasserman W, Kutner MH. 1989. Applied linear regression models. Homewood, Irwin, IL, USA.

Newman MC, Jagoe CH. 1994. Ligands and the Bioavailibility of Metals in Aquatic Environments. In: Hamelink JL, Landrum PF, Bergman HL, Benson WH (eds.) Bioavailability: Physical, Chemical and Biological Interactions. Lewis Publishers, Boca Raton, U.S.A.

Nygaard G, Komárek J, Kristiansen J, Skulberg OM. 1986. Taxonomic designations of the bioassay alga NIVA-CHL-1 ("*Selenastrum capricornutum*") and some related strains. Opera Botanica 90:5-46.

O'Halloran T. 1993. Transition metals in control of gene expression. Science 261:715-725.

Ochiai EI. 1977. Bioinorganic chemistry: An introduction. (cited in Mason and Jenkins, 1995)

OECD. 1981. Test Guideline No. 101: UV-VIS Absorption Spectra (Spectrophotometric Method). Organization for Economic Cooperation and Development, Paris, France.

OECD. 1984a. Test Guideline No. 201: Alga growth inhibition test. Organization for Economic Cooperation and Development, Paris, France.

OECD. 1984b. Test Guideline No. 202: *Daphnia* sp. acute immobilisation test and reproduction test. Organization for Economical Cooperation and Development, Paris, France.

OECD. 1998. Test Guideline No. 211: *Daphnia magna* reproduction test. Organization for Economic Cooperation and Development, Paris, France.

Pagenkopf GK, Russo RC, Thurston RV. 1974. Effect of complexation on toxicity of copper to fishes. J. Fish. Res. Board Can. 31:462-465.

Pagenkopf GK. 1983. Gill surface interaction model for trace-metal toxicity to fishes: role of complexation, pH, and water hardness. Environmental Science and Technology 17:342-347.

Pagenkopf, G.K. Environmental Science and Technology **1983**, *17*, 342-347.

Paquin PR, Gorsuch JW, Apte S, Batley GE, Bowles KC, Campbell PGC, Delos CG, Di Toro DM, Dwyer RI, Galvez F, Gensemer RW, Goss GG, Hogstrand C, Janssen CR, McGeer JC, Naddy RB, Playle RC, Santore RC, Schneider U, Stubllefield WA, Wood CM, Wu KB. 2002a. The biotic ligand model: a historical overview. Comparative Biochemistry and Physiology C 133:3-36.

Paquin PR, Zoltay V, Winfield RP, Wu KB, Mathew R, Santore RC, Di Toro DM. 2002b. Extensions of the biotic ligand model of acute toxicity to a physiologically-based model of the survival time of rainbow trout (*Oncorhynchus mykiss*) exposed to silver. Comparative Biochemistry and Physiology *C* 133:305-343.

Paripatananont T, Lovel RT. 1995. Chelated zinc reduces the dietary zinc requirement of channel catfish, *Ictalurus punctatus*. Aquaculture 133:73-82.

Paripatananont T, Lovel RT. 1997. Comparative net absorption of chelated and inorganic trace minerals in channel catfish, *Ictalurus punctatus*. Journal of the World Aquaculture Society 28:62-67.

Pempkowiak J, Kozuch J, Grzegowska H, Gjessing ET. 1999. Biological vs. Chemical properties of natural organic matter isolated from selected Norwegian lakes. Science of the Total Environment 25:357-366.

Perrein-Ettajani H, Amiard JC, Haure J, Renaud C. 1999. Effects of metals (Ag, Cd, Cu) on the biochemical composition and compartmentalization of these metals in two microalgae *Skeletonema costatum* and *Tetraselmis suecica*. Canadian Journal of fisheries and Aquatic Sciences 56:1757-1765.

Perry SF. 1986. Carbon dioxide excretion in fishes. Canadian Journal of Zoology 64:565-572.

Peterson SM, Stauber JL. 1996. A new algal enzyme assay for the rapid assessment of aquatic toxicity. Bulletin of Environmental Contamination and Toxicology 56:750-757.

Playle RC, Dixon DG, Burnison K. 1993a. Copper and Cadmium Binding to Fish Gills: Modification by Dissolved Organic Carbon and Synthetic Ligands. Canadian Journal of Fisheries and Aquatic Sciences 50:2667-2677.

Playle RC, Dixon DG, Burnison K. 1993b. Copper and Cadmium Binding to Fish Gills: Estimates of Metal-Gill Stability Constants and Modeling of Metal Accumulation. Canadian Journal of Fisheries and Aquatic Sciences 50:2678-2687.

Playle RC, Gensemer RW, Dixon DG. 1992. Copper accumulation on gills of fathead minnows : influence of water hardness, complexation and pH of the gill micro-environment. Environmental Toxicology and Chemistry 11:381-391.

Playle RC. 1998. Modeling metal interactions at fish gills. Science ot the Total Environment 219:147-163.

Playle, R.C., Gensemer, R.W., Dixon, D.G., 1992. Copper accumulation on gills of fathead minnows: influence of water hardness, complexation and pH of the gill micro-environment. Environmental Toxicology and Chemistry 11, 381-391.

Potts WTW. 1994. Kinetics of sodium uptake in freshwater animals: a comparison of ion-exchange and proton-pump hypotheses. American Journal of Physiology 266:R315-R320.

Reuter JG, Morel FMM. 1981. The interaction between zinc deficiency and copper toxicity as it effects the silicic acid uptake mechanisms in *Thalassiosira pseudonana*. Limnology and Oceanography 26:67-73.

Richards JGPJ, Burnison, BK, Playle RC. 2001. Effects of natural organic matter source on reducing metal toxicity to rainbow trout (*Oncorhynchus mykiss)* and on metal binding to their gills. Environmental Toxicology and Chemistry 20:1159-1166.

Santore RC, Di Toro DM, Paquin PR, Allen HE, Meyer JS. 2001. Biotic ligand model of the acute toxicity of metals. 2. Application to acute copper toxicity in freshwater fish and *Daphnia*. Environmental Toxicology and Chemistry 20:2397-2402.

Santore RC, Mathew R, Paquin PR, Di Toro D, 2002. Application of the biotic ligand model to predicting zinc toxicity to rainbow trout, fathead minnow and *Daphnia magna*. Comparative Biochemistry and Physiology C 133:271-285.

Santore RC, Driscoll CT. 1995. The CHESS model for calculating chemical equilibria in soils and solutions, chemical equilibrium and reaction models. In: Loeppert R, Schwab AP, Goldberg S (Eds.) Chemical Equilibrium and Reaction Models, American Society of Agronomy, Madison, WI, pp. 357-375.

Schecher WD, McAvoy DC. 1992. MINEQL+: A software environment for chemical equilibrium modelling. Computers, Environ. Urban Syst. 16: 65-76.

Schubauer-Berigan MK, Dierkes JR, Monson PD, Ankley GT. 1993. pH-dependent toxicity of Cd, Cu, Ni, Pb and Zn to *Ceriodaphnia dubia*, *Pimephales promelas*, *Hyalella azteca*, *and Lumbriculus variegatus*. Environmental Toxicology and Chemistry 12:1261-1266.

Serkiz SM, Perdue EM. 1990. Isolation of dissolved organic matter from the Suwannee river using reverse osmosis. Water Research 24:911-916.

Shaw TL, Brown VM. 1974. The toxicity of some forms of copper to rainbow trout. Water Research 8:377-382.

Skou JC, Esmann M. 1992. The Na,K ATPase. Journal of Bioenergetics and Biomembranes 24:249-261.

Spry DJ, Hodson PV, Wood CM. 1988. Relative contributions of dietary and waterborne zinc in rainbow trout, *Salmo gairdeneri*. Canadian Journal of Fisheries and Aquatic Sciences 45:32-41.

Stagg RM, Shuttleworth TJ. 1982. The accumulation of copper in *Platichtys flesus* and its effect on plasma electrolyte concentrations. Journal of Fish Biology 20:491-500.

Stauber JL, Davies CM. 2000. Use and limitations of microbial bioassays for assessing copper bioavailability in the aquatic environment. Environmental Reviews 8:255-301.

Stauber JL, Florence TM. 1987. The mechanism of toxicity of ionic copper and copper complexes to algae. Marine Biology (Berlin) 94:511-519.

Stevenson, F.J., 1976. Stability-constants of Cu^{2+}, Pb^{2+}, and Cd^{2+} complexes with humic acids. Soil Science Society of America Journal 40:665- 672

Stumm W, Morgan JJ. 1996. Aquatic chemistry: chemical equilibria and rates in natural waters. 3rd Edition. John Wiley & Sons, New York, NY, USA.

Sun L, Perdue EM, McCarthy JF. 1995. Using reverse osmosis to obtain organic matter from surface and ground waters. Water Research 29:1471-1477.

Sunda WG, Engel DW, Thoutte RM. 1978. Effect of chemical speciation on toxicity of cadmium to grass shrimp, *Palaemonetes pugio*: importance of free Cd ion. Environmental Science and Technology 12:409-413.

Sunda WG, Huntsman SA. 1983. The response of a marine bacterium to cupric ion and its use to estimate cupric ion activity in seawater. *Journal of MarineResearch* 37:761-777.

Sunda WG, Lewis JAM. 1978. Effect of complexation by natural organic ligands on toxicity of copper to a unicellular alga, *Monochrysis lutheri*. Limnology and Oceanography 23:870-876.

Tao S, Xu S, Dawson R. 2000. Bioavailability of apparent fulvic acid complexed copper to fish gills. Bulletin of Environmental Contamination and Toxicology 64:221-227.

Takacs M, Alberts JJ, Egeberg PK. 1999. Characterization of Natural Organic Matter from Eight Norwegian Surface Waters: Proton and Copper Binding. Environment International 25:315-323.

Tao S, Xu S, Dawson R. 2000. Bioavailability of apparent fulvic acid complexed copper to fish gills. Bulletin of Environmental Contamination and Toxicology 64:221-227.

Taraldsen JE, Norberg-King TJ. 1990. New method for determining effluent toxicity using duckweed (*Lemna minor*). Environmental Toxicology and Chemistry 9:761-767.

Taylor G, Baird DJ, Soares AMVM. 1998. Surface binding of contaminants by algae: consequences for lethal toxicity and feeding to *Daphnia magna* Straus. Environmental Toxicology and Chemistry 17:412-419.

Taylor LN, McGeer, JC, Wood CM, McDonald DG. 2000. Physiological effects of chronic copper exposure to rainbow trout (*Oncorhynchus mykiss*) in hard and soft water: evaluation of chronic indicators. Environmental Toxicology and Chemistry 19:2298-2308.

Tessier A, Buffle J, Campbell PGC. 1994. Uptake of trace metals by aquatic organisms. In : Buffle J, de Vitre RR (Eds.) Chemical and biological regulation of aquatic systems, Lewis, Chelsea, MI, pp. 197-230.

Tipping E, Hurley MA, 1992. A unifying model of cation binding to humic substances. Geochimica Cosmochim Acta 56:3627-3641.

Tipping E. 1993. Modelling ion binding by humic acids. Colloids and Surfaces A: Physicochemical and Engineering Aspects 73:117-131.

Tipping E. 1994. WHAM - a chemical equilibrium model and computer code for waters, sediments, and soils incorporating a discrete site/electrostatic model of ion-binding by humic substances. Computers and Geosciences 20:973-1023.

Toussaint MW, Shedd TR, van der Schalie WH, Leather GR. 1995. A comparison of standard acute toxicity tests with rapid-screening toxicity tests. Environmental Toxicology and Chemistry 14:907-915.

Turner DR, Whitfield M, Dickson AG. 1981. The equilibrium speciation of dissolved components in freshwater and seawater at 25°C and 1 atm pressure. Geochimica Cosmochimica Acta 45:855-881.

US EPA. 1971. Algal assay procedure bottle test. National Eutrophication Research Program, Pacific Northwest, U.S. Environmental Protection Agency, Environmental Research Laboratory, Corvallis, OR, USA.

US EPA. 1985a. Ambient Water Criteria for Copper – 1984. Environmental Protection Agency, Office of Water, Criteria and Standards Division. Washington, DC, U.S.A. EPA-440-5-84-031.

US EPA. 1985b. Toxic substances control act test guidelines, final rules. Federal Register 50:797.1050, 797.1075, and 797.1060. U.S. Environmental Protection Agency, Washington, DC, USA.

US EPA. 1991. Methods for aquatic toxicity identification evaluation. Phase I Toxicity characterisation procedures (second edition). EPA/600/6-91/003. United States Environmental Protection Agency, Office of Water, Washington, DC.

US EPA. 1993. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, 4th edition. EPA/600/4-90/027F. U.S. Environmental Protection Agency, Cincinatti, OH, USA, pp 151-171.

US EPA. 1994. Interim guidance on determination and use of water-effect ratios for metals. US Environmental Protection Agency, Washington, DC. EPA-823-B-94-001.

US EPA. 1996. 1995 Updates: Water Quality Criteria Documents for the Protection of Aquatic Life in Ambient Water. Environmental Protection Agency, Office of Water, Washington, DC, U.S.A. EPA-820-B-96-001.

US EPA. 2001. Streamlined water-effect ratio procedure for discharges of copper. US Environmental Protection Agency, Office of Water, Washington, DC. EPA-822-R001-005.

Van Assche F, van Tilborg W, Waeterschoot H. 1997. Environmental risk assessment for essential elements, Case study: Zinc. In: Langley E, Mangas S (eds.). Zinc – Report of an International Meeting. National Environmental Health Forum Monographs, Metal Series No. 2, Adelaide, Australia, September 12-13 1996, pp 33-47.

Van Cutsem P, Gillet CA. 1981. Thermodynamic study of Cu(++)-Zn(++)ion exchange in the *Nitella flexillis* cell wall. Plant and Soil 62:367-375.

Van Ewijk PH, Hoekstra JA, 1993. Calculation of the EC50 and its confidence interval when subtoxic stimulus is present. Ecotoxicology and Environmental Safety 25:25-32.

Van Tilborg WJM. 2002. Natural background/ambient concentrations of metals and abiotic conditions of fresh waters in relation to risk assessment of metals. Report No. 0203. Van Tilborg Business Consultancy BV, Rozendaal, Netherlands.

Versteeg DJ, Belanger SE, Carr GJ. 1999. Understanding single-species and model ecosystem sensitivity: data-based comparison. Environmental Toxicology and Chemistry 18:1329-1346.

Vitale AM, Monserrat JM, Castilho P, Rodriguez EM. 1999. inhibitory effects of cadmium on carbonic anhydrase activity and ionic regulation of the estuarine crab *Chasmagnathus granulate* (Decapoda, Grasidae). Comparative Biochemistry and Physiology C 122:121-129.

Vlaamse Regering. 1995. Titel II van het VLAREM. Besluit van de Vlaamse regering van 1 juni 1995 houdende algemene en sectorale bepalingen inzake milieuhygiëne.

Wang W, Fisher NS. 1999. Assimilation efficiencies of chemical contaminants in aquatic invertebrates: a synthesis. Environmental Toxicology and Chemistry 18:2034-2045.

Wagner JG. 1979. Fundamentals of clinical pharmacokinetics. 2nd ed.. Drug intelligence Publications, Inc., Hamilton, IL, USA.

Welsh PG, Lipton J, Chapman GA, Podrabsky TL. 2000. Relative importance of calcium and magnesium in hardness-based modification of copper toxicity. Environmental Toxicology and Chemistry 19:1624-1631.

Wilson RW, Taylor EW. 1993. The physiological response of freshwater rainbow trout *Oncorhynchus mykiss* during acutely lethal copper exposure. Journal of Comparative Physiology B 163:38-47.

Winner RE. 1985. Bioaccumulation and toxicity of copper as affected by interactions between humic acid and water hardness. Water Research 19:449-455.

Wittmann GTW. 1979. Toxic metals. In: Forstner U, Witmann GTW (eds.) Metal Pollution in the Aquatic Environment. Springer-Verlag, Berlin Heidelberg, Germany, pp 3-68.

Wolterbeek HT, Viragh A, Sloof JE, Bolier G, van der Veer B, de Kok J. 1995. On the uptake and release of zinc (^{65}Zn) in the growing algae *Selenastrum capricornutum* Printz. Environmental Pollution 88: 85-90.

Wong SL, Nakomoto L., Wainwright JF. 1994. Identification of toxic metals in affected algal cells in assays of wastewaters. Journal of Applied Phycology 6:405-414.

Wood CM, Hogstrand C, Galvez F, Munger RS. 1996. The physiology of waterborne silver toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*). 2. The effects of silver thiosulfate. Aquatic Toxicology 35:111-125.

Wood CM. 1992. Flux measurements as indices of H+ and metal effects on feshwater fish. Aquatic Toxicology 22:229-264.

Xue HB, Stumm W, Sigg L. 1988. The binding of heavy metals to algal surfaces. Water Research 22: 917-926.

Xue HB, Sigg L. 1990. Binding of Cu(II) to Algae in a Metal Buffer. Water Research 9:1129-1136.

Zetino AM, Kirschner LB, Harvey M. 200. On the mechanism of sodium-proton exchange in crayfish. Comparative Biochemistry and Physiology A 128:863-872.

Zitko V, Carson WV, Carson WG. 1973. Prediction of incipient lethal levels of copper to juvenile Atlantic salmon in the presence of humic acid by cupric ion electrode. Bulletin of Environmental Contamination and Toxicology 10:265-271.

Zitko V, Carson WG. 1976. Mechanism of effects of water hardness on lethality of heavy metals to fish. Chemosphere 5:299-303.

Zuurdeeg BW. 1992. Natuurlijke achtergrondgehalten van zware metalen en enkele andere sporenelementen in Nederlands oppervlaktewater. Geochem Research Publications, Utrecht, The Netherlands.

Curriculum vitae

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Peer reviewed papers

De Schamphelaere KAC, Vasconcelos FM, Allen HE, Janssen CR. 2003. The effect of dissolved organic matter source on acute copper toxicity to *Daphnia magna*. Environmental Toxicology and Chemistry (accepted).

De Schamphelaere KAC, Janssen CR. 2003. Development and field validation of a biotic ligand model predicting chronic copper toxicity to *Daphnia magna*. Environmental Toxicology and Chemistry (accepted).

De Schamphelaere KAC, Janssen CR. 2003. Effects of dissolved organic matter concentration and source, pH and water hardness on chronic toxicity of copper to *Daphnia magna*. Environmental Toxicology and Chemistry (accepted).

De Schamphelaere KAC, Heijerick DG, Janssen CR. 2003. The effect of pH buffering on the toxicity of copper and zinc to *Daphnia magna* and *Pseudokirchneriella subcapitata*. Ecotoxicology (accepted).

De Schamphelaere KAC, Vasconcelos FM, Heijerick DG, Tack FMG, Delbeke K, Allen HE, CR Janssen. 2003. Development and field validation of a predictive copper toxicity model for the green alga *Pseudokirchneriella subcapitata*. Environmental Toxicology and Chemistry 22:2454-2465.

Verslycke T, Vangheluwe M, Heijerick D, De Schamphelaere K, Van Sprang P, Janssen C. 2003. The toxicity of metal mixtures to the estuarine mysid *Neomysis integer* under changing salinity. Aquatic Toxicology 64:307-315.

Janssen CR, Heijerick DG, De Schamphelaere KAC, Allen HE. 2003. Environmental risk assessment of metals: tools for incorporating bioavailability. Environment International 28: 793-800.

De Schamphelaere KAC, Heijerick DG, Janssen CR. 2002. Refinement and field validation of a biotic ligand model predicting acute copper toxicity to *Daphnia magna*. Comparative Biochemistry and Physiology C 133:243-258.

Heijerick DG, De Schamphelaere KAC, Janssen CR. 2002. Biotic ligand model development predicting Zn toxicity to the alga *Pseudokirchneriella subcapitata*: possibilities and limitations. Comparative Biochemistry and Physiology C 133:207-218.

Heijerick DG, De Schamphelaere KAC, Janssen CR. 2002. Predicting the acute zinc toxicity for *Daphnia magna* as a function of key water chemistry characteristics: development of a Biotic Ligand Model. Environmental Toxicology and Chemistry 21:1309-1315.

Lock K, De Schamphelaere KAC, Janssen CR. 2002. The effect of lindane on terrestrial invertebrates. Archives of Environmental Contamination and Toxicology 42:217-221.

De Schamphelaere KAC, Janssen CR. 2002. A biotic ligand model predicting acute copper toxicity for *Daphnia magna*: the effects of calcium, magnesium, sodium, potassium and pH. Environmental Science and Technology 36:48-54.

Janssen CR, De Schamphelaere K, Heijerick D, Muyssen B, Lock K, Bossuyt B, Vangheluwe M, Van Sprang P. 2000. Uncertainties in the environmental risk assessment of metals. Human and Ecological Risk Assessment 6:1003-1018.

Oral Presentations

Verdonck FAM, Deksissa T, De Laender F, De Schamphelaere KAC, Vandenberghe V, vincke S, Janssen C, Vanrolleghem PA. Uncertainty and variability in spatio-temporal probabilistic risk modelling. 13th annual meeting of SETAC Europe, April 27 - May 1, 2003, Hamburg, Germany.

De Schamphelaere KAC, Heijerick DG, Muyssen BTA, Bossuyt BTA, Janssen CR. How important is dietary exposure for predicting toxicity of essential metals to *Daphnia magna*? 23rd annual meeting of SETAC North America, November 16-20, 2002, Salt Lake City, UT, USA.

Janssen CR, De Schamphelaere KAC, Heijerick DG, Bossuyt B. Advances in the risk assessment of copper: reducing uncertainty through focussed scientific research. Copper Risk Assessment Workshop, June 13-14, 2002, Brussels, Belgium.

De Schamphelaere KAC, Heijerick D, Janssen CR. Modelling chronic copper toxicity to *Daphnia magna* and *Raphidocelis subcapitata* in natural waters: implications for risk assessment. 12th annual meeting of SETAC Europe, may 10-16, 2002, Vienna, Austria.

Janssen CR, De Schamphelaere KAC, Heijerick DG. Biotic ligand model of chronic toxicity: invertebrates and algae. SETAC Short Course, "Predicting the toxicity of metals to aquatic organisms". An introduction to the biotic ligand model", May 12, 2002, Vienna, Austria.

Janssen CR, De Schamphelaere KAC, Heijerick DG, Bossuyt B. The copper biotic ligand model: acute and chronic data. Workshop on "bioavailability of metals in surface waters: integrating science and regulations", February 7-8, 2002, Gent, Belgium.

Janssen CR, De Schamphelaere KAC, Heijerick DG. Biotic Ligand Models for predicting chronic copper toxicity to freshwater algae and crustaceans. 22nd annual meeting of SETAC America, November 11-15, 2001, Baltimore, Maryland, USA.

De Schamphelaere KAC, Heijerick D, Janssen CR. Combined effects of key water quality characteristics and different types of natural organic matter on the acute and chronic copper bioavailability and toxicity for *Daphnia magna* and *Selenastrum capricornutum*. Sixth international conference on the biogeochemistry of trace elements (ICOBTE), July 29 – August 2, 2001, Guelph, Ontario, Canada.

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), July 29 – August 2, 2001, Guelph, Ontario, Canada.

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

Heijerick DG, Bossuyt BT, De Schamphelaere KA, Janssen CR. Copper toxicity for *Daphnia magna*, *Raphidocelis subcapitata* and local crustaceans in six European surface waters. 11th annual meeting of SETAC Europe, May 6-10, 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. May 2000, San Juan, Puerto Rico.

Poster presentations

Unamuno VIR, De Schamphelaere KAC, Janssen CR, Vanderdeelen J, Tack FMG. Does reverse osmosis affect metal binding properties of natural dissolved organic matter? 7th ICOBTE Conference, June 15-19, 2003, Uppsala, Sweden.

De Schamphelaere KAC, Verdonck FAM, Heijerick DG, Van Sprang PA, Vanrolleghem PA, Janssen CR. Geography-referenced bioavailability modelling in risk assessment: a case study of copper in Swedish surface waters. 7th ICOBTE Conference, June 15-19, 2003, Uppsala, Sweden.

De Schamphelaere KAC, Janssen CR. Development and validation of a chronic Zn-BLM for the rainbow trout *Oncorhynchus mykiss*. 13th annual meeting of SETAC Europe, April 27 - May 1, 2003, Hamburg, Germany.

De Laender F, Verdonck FAM, De Schamphelaere KAC, Janssen CR, Vanrolleghem PA. Geography-referenced bioavailability modelling in risk assessment: a case study of copper in Swedish surface waters. 13th annual meeting of SETAC Europe, April 27 – May 1, 2003, Hamburg, Germany.

Heijerick DG, De Schamphelaere KAC, Janssen CR. Chronic biotic ligand models for algae and daphnids: species- and metal-specific considerations. 23rd annual meeting of SETAC North America, November 16-20, 2002, Salt Lake City, USA.

De Schamphelaere KAC Janssen CR. Combining single-species bioavailability models to predict the toxic effects of copper to multi-species systems. 23rd annual meeting of SETAC North America, November 16-20, Salt Lake City, USA.

De Schamphelaere KAC, Heijerick DG, Janssen CR. The integration of bioavailability into the risk assessment: The development of a bioavailability model predicting copper toxicity to the green alga *Pseudokirchneriella subcapitata*. Copper Risk Assessment Workshop, June 13-14, 2002, Brussels, Belgium.

De Schamphelaere KAC, Heijerick DG, Janssen CR. A copper bioavailability model for the green alga *Raphidocelis subcapitata*. 12th annual meeting of SETAC Europe, May 10-16, 2002, Vienna, Austria.

De Schamphelaere KA, Heijerick DG, Janssen CR. pH buffering using MOPS does not affect copper and zinc toxicity for *Daphnia magna* and *Selenastrum capricornutum*. 11th annual meeting of SETAC Europe, May 6-10, 2001, Madrid, Spain.

De Schamphelaere KA, Vasconcelos FM, Janssen CR, Allen HE. influence of water quality characteristics and natural organic matter on chronic copper bioavailability and toxicity for *Daphnia magna*: implications for biotic ligand modeling. 11th annual meeting of SETAC Europe, May 6-10, 2001, Madrid, Spain.

Heijerick DG, De Schamphelaere KA, Janssen CR. Biotic ligand model predicting acute zinc toxicity to *Daphnia magna*: develoment and validation. 11th annual meeting of SETAC Europe, May 6-10, 2001, Madrid, Spain.

Bossuyt BT, Heijerick DG, De Schamphelaere KA, Janssen CR. Comparative sensitivity of laboratory-reared and field-collected organisms. 11th annual meeting of SETAC Europe, May 6-10, 2001, Madrid, Spain.

De Schamphelaere KAC, Janssen CR. A biotic ligand modeling approach to predict copper toxicity towards *Daphnia magna* as a function of Ca^{2+}, Mg^{2+}, Na^{+}, and H^{+} concentrations. Proceedings of the 21st Congress of the European Society for Comparative Physiology and Biochemistry. july 24-28, 2000, Liège, Belgium. Comparative Biochemistry and Physiology, 126A, Suppl. 1, S40

Conferences, workshops and symposia

"BIOSPEC Workshop", June 27, Wageningen, The Netherlands

7th ICOBTE Conference, June 15-19, Uppsala, Sweden.

"Hazard Identification Approach for Metals and Inorganic Metal Substances", May 2-9, 2003, Pensacola, FL, U.S.A. (invited student participant)

13th annual meeting of SETAC Europe, April 27 - May 1, 2003, Hamburg, Germany.

"EU Zinc Assessment Research Programme, Results Workshop", January 29-30, 2003, Bologna, Italy

"Meeting of the steering group on further testing of zinc and zinc compounds", January 20-21, 2003. Gent, Belgium

23rd annual meeting of SETAC North America, November 16-20, 2002, Salt Lake City, USA.

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

12th annual meeting of SETAC Europe, May 10-16, 2002, Vienna, Austria.

Workshop on "bioavailability of metals in surface waters: integrating science and regulations", February 7-8, 2002, Gent, Belgium.

Sixth International Conference on the Biogeochemistry of Trace Elements (ICOBTE), July 29 – August 2, 2001, Guelph, Ontario, Canada.

11th annual meeting of SETAC Europe, May 6-10, 2001, Madrid, Spain.

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

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

Foreign Research Visits

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Memberships of professional communities

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Awards

Science Award 2003. European Centre for Ecotoxicology and Toxicology of Chemicals. For the paper "De Schamphelaere KAC, Janssen CR. 2002. A biotic ligand model predicting acute copper toxicity for *Daphnia magna*: the effects of calcium, magnesium, sodium, potassium and pH. Environmental Science and Technology 36:48-54."

SETAC North America Travel Award 2002. 23rd annual meeting of SETAC North America, November 16-20, 2002, Salt Lake City, USA.

Poster Award, 2nd prize at the 7th ICOBTE Conference, June 15-19, 2003, Uppsala, Sweden for "De Schamphelaere KAC, Verdonck FAM, Heijerick DG, Van Sprang PA, Vanrolleghem PA, Janssen CR. Geography-referenced bioavailability modelling in risk assessment: a case study of copper in Swedish surface waters."

Samenvatting

Deze doctoraatsthesis is gesitueerd in het domein van de aquatische toxicologie, een subdiscipline van de ecotoxicologie. Het doel van aquatische ecotoxicologie is het evalueren van 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 en het gebruik van ecotoxicologische testgegevens voor procedures voor risicoschatting en het opstellen van waterkwaliteitscriteria.

In tegenstelling tot synthetische organische componenten 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 onderdeel geworden van metabolische processen die cruciaal zijn voor overleving, groei en reproductie van organismen. Elke soort kent voor elk essentieel element een optimaal concentratie-bereik waarbinnen ze optimaal kan functioneren. Wanneer de externe concentratie echter te hoog of te laag is zal de homeostatische regulatie falen en zal er toxiciteit of deficiëntie optreden, respectievelijk. Deze studie focusseert op de toxiciteit van het element koper. De potentiële toxiciteit van koper, samen met het wijdverspreide gebruik ervan in tal van applicaties, benadrukt de noodzaak voor een accurate inschatting van het risico ervan voor het milieu.

Nog steeds worden waterkwaliteitscriteria en risicoschatting van metalen voornamelijk gebaseerd op totale of opgeloste concentraties. Deze zijn echter geen goede voorspellers van hun potentiële schade voor ecosystemen. Verschillende fysico-chemische waterkarakteristieken zoals de concentratie van opgelost organisch koolstof (DOC), pH en hardheid kunnen de toxiciteit van metalen over verschillende grootteordes verschuiven. Dit effect wordt samengevat met de term "biobeschikbaarheid".

Het onderliggende idee van biobeschikbaarheid is dat het toxische effect van een metaal niet alleen afhangt van de totale (of opgeloste) concentratie van een metaal maar ook van een complexe interactie tussen fysico-chemische en biologische factoren. Met andere woorden, éénzelfde metaalconcentratie resulteert niet in hetzelfde effect onder alle condities.

Dit toont aan dat, als er geen rekening gehouden wordt met biobeschikbaarheid, waterkwaliteitscriteria gebaseerd op totale of opgeloste concentraties te conservatief kunnen zijn voor één type water en onvoldoende beschermend voor een ander. In de context van duurzame ontwikkeling zijn geen van beide wenselijk aangezien het eerste kan leiden tot een verhoogde economische kost ten gevolge van (mogelijke) emissiereductie- en saneringsmaatregelen en het tweede tot schade voor het aquatische milieu en een afname van biodiversiteit.

Ondanks de "body of evidence" omtrent de effecten van fysico-chemie op metaaltoxiciteit hebben weinig regelgevende systemen dit reeds in rekening gebracht. Dit is vnl. het gevolg van een gebrek aan modellen om biobeschikbaarheid te kwantificeren. In deze context wint het biotisch ligand model (BLM) steeds meer aan interesse vanwege academische, industriële en regelgevende instanties omdat dit model acute metaal toxiciteit kan voorspellen via het integreren van de belangrijkste effecten van fysico-chemie.

Hoewel in de Verenigde Staten momenteel overwogen wordt om het BLM voor acute metaaltoxiciteit te gebruiken voor het opstellen van waterkwaliteitscriteria voor koper, is het niet geschikt voor gebruik in de Europese Unie (EU) omdat hier chronische toxiceitsdata vereist zijn voor normstelling en risico-evaluatie. In de EU wordt koper beoordeeld via een risico-evaluatie. In deze benadering worden een "predicted environmental concentration" (PEC) en een "predicted no-effect concentration" (PNEC) afgeleid en met elkaar vergeleken om het risico te bepalen. Deze doctoraatsstudie is gericht op het afleiden van een correcte PNEC voor koper.

Als biobeschikbaarheid op een kwantitatieve manier in de EU dient geïntegreerd te worden, dan zijn er modellen nodig die chronische kopertoxiciteit kunnen voorspellen voor verschillende types organismen. Daarom was het belangrijkste doel van deze studie het ontwikkelen van biobeschikbaarheidsmodellen voor het voorspellen van chronische kopertoxiciteit voor de kreeftachtige *Daphnia magna* en de groen-alg *Pseudokirchneriella subcapitata* in functie van de fysico-chemische waterkarakteristieken. De ontwikkeling en validatie van deze modellen zou zeker bijdragen tot de verbetering van huidige procedures voor normstelling en risico-evaluatie.

Bij aanvang van deze studie was het BLM concept enkel geëvalueerd voor acute metaaltoxiciteit voor vissen en er waren slechts voorlopige pogingen ondernomen om het BLM te extrapoleren naar invertebraten. Er was echter geen bewijs dat het BLM toepasbaar was voor deze species, ook al omdat het met kleine invertebraten niet mogelijk was om het BLM-concept aan te tonen op basis van metingen van kieuwconcentraties van metalen (dé basis van het vis-BLM). Daarom werd het nodig geacht om, vóór de ontwikkeling van een chronisch BLM, een mathematische methodologie te ontwikkelen om een acuut Cu-BLM voor *D. magna* op te stellen uitsluitend op basis van toxiceitsdata (hoofdstuk 2).

Experimenten warden uitgevoerd om de individuele effecten van Ca, Mg, Na and pH op acute kopertoxiciteit voor *D. magna* te onderzoeken. Ca en Na hadden een kwantitatief gelijkaardig beschermend effect als bij vissen. Mg had bovendien een gelijkaardig beschermend effect als Ca, iets wat niet waargenomen was bij vissen. Globaal gezien varieerden de 48-uur EC50s tussen 3.7 and 21 µg Cu L^{-1}, wat aantoont dat de maximale variatie van de toxiciteit door de variatie van Ca, Mg and Na ongeveer factor 5 à 6 bedroeg.

Uitgedrukt als opgelost koper namen de 48-uur EC50s ongeveer met een factor 10 toe tussen pH 6 en 8 (van 5.6 tot 52 µg Cu L^{-1}). Uitgedrukt als Cu^{2+}-activiteit was de 48-uur EC50 ongeveer factor 2 hoger bij pH 6 dan bij pH 8. Er werd aangetoond dat dit vnl. te wijten was aan de biobeschikbaarheid van het $CuOH^+$ complex. Bij een verdere verfijning van het acute BLM werd aangetoond dat voor een groter pH bereik (nl. 5.7 to 8.4) de $EC50_{Cu2+}$ een grotere variatie vertoonde, nl. tussen 3 and 69 nM (~ factor 20). Dit werd toegeschreven aan de co-toxiciteit van het $CuCO_3$ complex (hoofdstuk 3). Er werd aangetoond dat $CuOH^+$ en $CuCO_3$ respectievelijk 5 en 10 keer minder toxisch waren dan Cu^{2+}. Hoewel gesuggereerd werd dat de toxiciteit van deze complexen veroorzaakt zou kunnen zijn door een verschil in fysico-chemie tussen het micromilieu van de kieuw en het blootstellingsmedium, was het toch noodzakelijk om hun biobeschikbaarheid in te bouwen in het BLM-concept via de binding van deze complexen op het biotisch ligand. Dit was uitermate noodzakelijk voor accurate toxiciteitsvoorspellingen voor pH > 8.

Dit onderzoek toont aan dat het BLM-concept kan aangepast worden voor andere species (en andere metalen). Het feit dat er verschillen kunnen bestaan tussen verschillende species en dat deze verschillen onder bepaalde omstandigheden belangrijk kunnen zijn, duidt echter aan dat de extrapolatie van het BLM naar andere species op basis van beperkte datasets

met de grootste voorzichtigheid moet gebeuren. Het in deze studie voorgestelde experimenteel opzet en de mathematische methodologie verschaffen een snelle manier om species-specieke verschillen in het BLM-concept aan te tonen en BLMs te ontwikkelen voor andere metalen en species. Het loont eveneens de moeite om het belang van deze species-specifieke verschillen te onderzoeken bij species afkomstig uit verschillende types ecosystemen. Dit zou de ecologische relevantie van het BLM zeker en vast ten goede komen.

De hierboven besproken testen, die gebruikt werden om het BLM te ontwikkelen, werden uitgevoerd in testmedia met uitsluitend achtergrond DOC (~ 0.3 mg L^{-1}). De 48-uur EC50s varieerden tussen 3.7 and 52 µg Cu L^{-1}. In de volgende onderzoeksfase (hoofdstuk 3) werd het acute Cu-BLM gevalideerd met 25 artificiële waters met verschillende concentraties van artificieel DOC en met 19 natuurlijke oppervlaktewaters. In deze testen werd een duidelijk beschermend effect van verhoogde DOC concentraties waargenomen: voor DOC concentraties tussen 1.4 en 23 mg DOC L^{-1} varieerde de 48-uur EC50 van 41 tot 1200 µg Cu L^{-1}. Bovendien verklaarde DOC ongeveer 80% van de waargenomen toxiciteitsvariatie. Dit alles toont aan dat, als waterkwaliteitscriteria zouden moeten gecorrigeerd worden voor biobeschikbaarheid op basis van één parameter, de meest relevante de DOC concentratie zou zijn en niet de hardheid, zoals momenteel veelvuldig toegepast door verschillende regelgevende instanties.

Ondanks de grote variatie van de 48-uur EC50s (factor 30 voor de DOC-bevattende waters, factor 300 globaal), kon het ontwikkelde BLM al deze waarden voorspellen met een fout van minder dan factor 2. Dit toont aan dat het acute BLM een krachtige tool is om onzekerheid, gerelateerd met biobeschikbaarheid, te reduceren. Omdat de EU echter chronische toxiciteistdata vereist voor risico-evaluatie, werd in een volgende fase onderzocht of ook chronische kopertoxiciteit voor *D. magna* kon gemodelleerd worden met het BLM-concept (hoofdstuk 4).

De effecten van pH (5.3 tot 8.7), waterhardheid (25 tot 500 mg $CaCO_3$ L^{-1}) en DOC concentratie (1.6 tot 18.4 mg L^{-1}) en herkomst van het DOC op de chronische (reproductieve) toxiciteit werden onderzocht met een multi-factorieel, centraal composiet design. Natuurlijk DOC werd gecollecteerd op drie plaatsen in België en Nederland d.m.v. omgekeerde osmose. Voor een totaal van 35 testen varieerden de 21-dagen NOECs van 29.4 tot 228 µg Cu L^{-1} en de 21-dagen EC50s van 41.5 tot 316 µg Cu L^{-1} (factor 8).

Statistische analyse toonde aan dat DOC concentratie en pH een significant effect hadden op de chronische toxiciteit van koper maar dat er geen effect was van hardheid. Meer specifiek resulteerde een toename van pH en DOC in een lineaire toename van de 21-dagen NOECs en EC50s. Alle DOCs reduceerden kopertoxiciteit in dezelfde mate. DOC concentratie was de belangrijkste factor en verklaarde ongeveer 60% van de variabiliteit, terwijl pH ongeveer 15% van de variabiliteit verklaarde. Dit toont opnieuw aan dat een hardheid-gebaseerde correctie van waterkwaliteitscriteria voor koper niet de meest geschikte is en dat een DOC-gebaseerde correctie waarschijnlijk meer relevant is.

Behalve empirische regressie-modellen werd ook de mogelijkheid van een chronisch Cu-BLM onderzocht. BLM konstanten werden afgeleid en vergeleken met die van het acute BLM: 1) het effect van Ca en Mg was niet belangrijk voor chronische toxiciteit, 2) het effect van Na was gelijkaardig en 3) competitie van H^+ en de biobeschikbaarheid van $CuOH^+$ en $CuCO_3$ was belangrijker in chronische blootstellingen.

De eerste twee observaties suggereren een toenemend belang van de fysiologie van het testorganisme in chronische blootstellingen, nl. via de verstoring van de Na-balans. Hoewel, voor de derde observatie verschillende hypotheses opgeworpen kunnen worden, moet het benadrukt worden dat het ontwikkelde chronische BLM niet mag beschouwd worden als een model dat alle mechanismen van chronische kopertoxiciteit exact weergeeft. Het ontwikkelde BLM is eerder een integratie van mechanismen die leidt tot een optimale correlatie tussen waargenomen en voorspelde toxiciteit. Een aantal van de mechanismen/processen die leiden tot verschillen in het acute en het chronische BLM zouden kunnen zijn: 1) de organismen kunnen acclimatiseren aan subletale koperconcentraties gedurende chronische blootstellingen, 2) de afname van de reproductie en de toename van de mortaliteit kunnen naast een verstoring van de Na-balans, ook het gevolg zijn van de cyclische productie van zuurstofradicalen door accumulatie van koper in de weefsels, 3) de organismen kunnen een inhibitie van de voedselopname vertonen en/of een toename van de energetische kosten voor onderhoud van het basismetabolisme. Het onderzoek naar de exacte mechanismen van chronische toxiciteit van koper zou zeker bijdragen tot een meer algemene aanvaarding van het BLM door regelgevende instanties.

Behalve deze mechanistische beschouwingen, werd er ook (beperkt) bewijs geleverd voor de mogelijke (beperkte) toxiciteit van Cu-DOC complexen in chronische blootstellingen met geïsoleerd DOC. Omdat dit echter slechts een kwantitatief onbelangrijk effect was, werd dit niet in rekening gebracht in het chronische BLM.

Zowel het empirische regressiemodel als het BLM vertoonden eenzelfde voorspellende capaciteit voor 21-dagen EC50s en NOECs in natuurlijke waters: de meeste effectconcentraties werden voorspeld binnen factor 2 van de waargenomen waarden.

Voor één oppervlaktewater werden de 21-dagen NOECs en EC50s overschat met een factor > 5 (m.a.w. toxiciteit werd onderschat). Aangezien dit niet echt wenselijk is als het chronische BLM moet geïmplementeerd worden in de risico-evaluatie, dringt een afdoende verklaring zich op. Dit oppervlaktewater, een Zweeds meer, vertoonde een aantal karakteristieken die inderdaad kunnen geresulteerd hebben in de onderschatting van toxiciteit: een lage pH en een hoog ijzer en aluminium gehalte. Ijzer en aluminium kunnen in competitie treden met koper voor bindingsplaatsen op het DOC, zeker bij lage pH en dit kan resulteren in hoger dan verwachte concentraties van het vrije koper-ion en dus in een hogere toxiciteit. Aangezien deze competitie effecten belangrijker worden bij lagere koperconcentraties, verdient dit onderwerp zeker verdere aandacht. In deze context kan het nodig zijn om het meer geavanceerde speciatie model WHAM-6 in het BLM te integreren (i.p.v. WHAM-5), aangezien dit model speciaal ontworpen werd om speciatie te voorspellen bij lagere metaalconcentraties. Tot dan wordt het gebruik van het ontwikkelde chronische koper-BLM afgeraden voor waters met een lage pH en hoge Fe en/of Al concentraties.

Een ander belangrijk onderwerp van debat m.b.t. chronische kopertoxiciteit is de potentiële toxiciteit van koper via het dieet van *D. magna* (koper geassocieerd met voedselpartikels zoals algen). Algen zijn steeds aanwezig in standaard chronische toxiciteitstesten en dus is er steeds de mogelijkheid van kopertoxiciteit via deze route. Het chronische Cu-BLM was ontwikkeld om toxiciteit van koper in de opgeloste fase te voorspellen en als dieet-koper zou bijdragen tot de toxiciteit, dan zou het ontwikkelde model enkel geldig zijn onder de specifieke condities van standaard toxiciteitstesten.

Daarom werden in hoofdstuk 5 experimenten uitgevoerd om het belang van dieet-koper voor de voorspellende capaciteit van het chronische Cu-BLM te onderzoeken. De alg

P. subcapitata werd gedurende 3 dagen blootgesteld aan 6 verschillende koperconcentraties (35 to 200 µg Cu L^{-1}) en een controle in een test medium met 10 mg DOC L^{-1}, pH = 6.8 en een hardheid van 250 mg $CaCO_3$ L^{-1}. Koperconcentraties in de algen varieerden van ongeveer $6 \cdot 10^{-16}$ tot $200 \cdot 10^{-16}$ g Cu cel^{-1}.

Vervolgens werd *D. magna* blootgesteld aan koper in 3 types van 21-dagen blootstellingen: 1) water-blootstelling (koper in de oplossing en controle algen als voedsel), 2) dieet-blootstelling (controle oplossing en koper-gecontamineerde algen als voedsel) and 3) water+dieet-blootstelling (koper in oplossing en koper-gecontamineerde algen als voedsel). Hierbij was het duidelijk dat in de water-blootstelling (representatief voor standard chronische testen), ook blootstelling via het dieet kon optreden aangezien de algen koper kunnen opnemen uit de oplossing vooraleer ze opgegeten worden. Anderzijds was het ook duidelijk dat in de dieet-blootstelling algen een deel van het koper kunnen verliezen vooraleer opgegeten te worden. Daarom werd een preliminair kinetisch opname- en eliminatiemodel ontwikkeld voor algen blootgesteld aan koper. Zodoende konden op basis van de kennis van het voedingsregime en gemeten voedselopnamesnelheden van *D. magna* gemiddelde koper concentraties in de algen en snelheden van koper-opname via het voedsel geschat worden.

Deze berekeningen toonden aan dat koperconcentraties in de algen en koper-opnamesnelheden via het voedsel in de zogenaamde water-blootstellingen gelijkaardige of zelfs hogere niveaus kon bereiken dan in de dieet- of de water+dieet-blootstellingen. Voorts varieerden de koperconcentraties in de algen en koper-opnamesnelheden via het voedsel tijdens de test ongeveer factor 3 tot 4 voor de water-blootstellingen, factor 2 voor de dieet-blootstelling en minder dan factor 1.5 voor de water+dieet-blootstellingen.

Zonder deze analyse was de correcte interpretatie van de resultaten (reproductie, groei, koperaccumulatie in *D. magna*) uiterst moeilijk. Nochtans was er een duidelijke trend dat blootstelling aan dieet-koper resulteerde in een toename van de interne koperconcentratie van *D. magna* en in een toename van de groei en de reproductie wat duidt op een veeleer stimulerend dan een toxisch effect van dieet-koper.

Regressie analyse toonde aan dat dit stimulerende effect niet gerelateerd was met de interne lichaamsconcentratie van koper in *D. magna*. Significante correlaties werden echter wel waargenomen met de gemiddelde interne koperconcentraties van de algen gedurende de

testen en met de massaspecifieke koperopnamesnelheden via het voedsel. Er werd gesuggereerd dat dit laatste mogelijk wijst op een stimulerend effect van koper op de activiteit van verteringsenzymen in het intestinaal kanaal van *D. magna*, zoals eerder aangetoond voor benthische invertebraten.

Waarschijnlijk de meest interessante conclusie van dit onderzoek was dat de 21-dagen NOEC en 21-dagen EC50 (ongeveer 95 en 110 µg Cu L^{-1}, respectievelijk) niet beïnvloed werden door blootstelling via het dieet. Dit duidt aan dat dieet-koper geen effect heeft op de voorspellende capaciteit van het ontwikkelde chronisch Cu-BLM.

Er werd aangetoond dat massaspecifieke koperopnamesnelheden via het voedsel tussen 1.9 en 124 µg Cu (g nat gewicht)$^{-1}$ lagen. Sommige waren dus hoger dan de snelheden bij vissoorten die resulteerden in een toxisch effect, nl. tussen 1 en 45 µg Cu (g nat gewicht)$^{-1}$ d^{-1}. Hoewel dit zou kunnen wijzen op een lagere gevoeligheid van *D. magna* voor dieet-koper, toch lijken de resultaten in overeenstemming met studies met vissen die gevoederd werden met levende diëten. In deze studies konden nl. ook geen eenduidige nadelige effecten van dieet-koper aangetoond worden tot een opnamesnelheid van 48 µg Cu (g nat gewicht)$^{-1}$ d^{-1}. Onze studie is echter de eerste die een duidelijk stimulerend effect van dieet-koper voor een zoetwater invertebraat beschrijft; dit effect was significant bij opnamesnelheden ≥ 20 µg Cu (g nat gewicht)$^{-1}$ d^{-1}.

Nochtans, door het beperkt aantal studies over blootstelling aan metalen via het dieet en doordat deze soms contradictorisch zijn, blijft verder onderzoek in dit domein uiterst belangrijk. Verder onderzoek zou zich moeten toespitsen op het belang van o.a. voedseltype, species-specifieke effecten en het belang van fysico-chemie op effecten van dieet-koper. M.b.t. deze laatste is het belangrijk op te merken dat algen bij hogere pH niveaus waarschijnlijk meer koper kunnen opnemen, wat potentieel kan resulteren in een groter belang van de blootstelling via het dieet.

Hoewel het grootste deel van dit eindwerk gericht was op het ontwikkelen van BLMs voor het voorspellen van acute en chronische kopertoxiciteit voor *D. magna* was een ander belangrijk doel om ook een toxiciteitsmodel te ontwikkelen voor de groen-alg *P. subcapitata* (hoofdstuk 6). Hiervoor werd een gelijkaardig experimenteel design aangewend als voor *D. magna* (zelfde test media, hoofdstuk 4).

Voor een totaal van 35 toxiciteitstesten varieerden de 72-uur E_bC10s tussen 14 en 176 µg Cu L^{-1} (factor 12) en 72-uur E_bC50s tussen 26.9 en 507 µg Cu L^{-1} (factor 20). Statistische analyse toonde aan dat DOC concentratie, DOC herkomst en pH een significant effect hadden op de kopertoxiciteit. Opnieuw, zoals voor *D. magna*, had hardheid geen significant effect. Een toename van de pH resulteerde in een toename van de toxiciteit (ongeveer 15% van de variabiliteit verklarend) terwijl een toename van de DOC concentratie resulteerde in een afname van de toxiciteit van koper (ongeveer 60% van de variabiliteit verklarend). Hoewel het relatieve belang van DOC en pH gelijkaardig was voor *D. magna* en *P. subcapitata*, is het toch belangrijk om vast te stellen dat *D. magna* gevoeliger is voor koper bij lage pH en dat *P. subcapitata* gevoeliger is bij hoge pH. Dit is uitermate belangrijk voor de implementatie van biobeschikbaarheid in de risico-evaluatie (zie verder).

Uitgedrukt als opgelost koper werden significante verschillen gevonden wat betreft de reductie van koper toxiciteit tussen de drie DOCs, afkomstig van verschillende locaties (tot factor 2.5). Uitgedrukt als Cu^{2+}-activiteit daarentegen werd toxiciteit enkel beïnvloed door pH (variatie van 4 grootteordes). Dit wijst erop dat Cu-DOC complexen waarschijnlijk niet biobeschikbaar zijn voor algen. Het verschillende effect van verschillende DOCs werd in rekening gebracht door het speciatie model WHAM 5 te calibreren door het % actief fulvozuur aan te passen (een maat voor de bindingcapaciteit).

Lineaire verbanden werden waargenomen tussen pH en het logaritme van de effect concentraties uitgedrukt als Cu^{2+}-activiteit: 1) log (E_bC50_{Cu2+}) = - 1.431 pH + 2.050 (r^2 = 0.95), en 2) log (E_bC10_{Cu2+}) = -1.140 pH - 0.812 (r^2 = 0.91). Deze relaties toonden indirect ook aan dat het mechanistisch niet correct was om deze pH-afhankelijkheid te modelleren met het originele BLM concept (nl. competitie van H^+ ter hoogte van één enkele biotisch ligand site). Er werd gesuggereerd dat de reden voor de grote pH-afhankelijkheid van kopertoxiciteit de samenstelling van de celwand zou kunnen zijn. De celwand van algen draagt inderdaad een grote verscheidenheid aan functionele groepen, die allemaal gedeprotoneerd worden bij verschillende pH niveaus. Dit kan resulteren in een continue toename van het aantal beschikbare bindingsplaatsen voor koper bij toenemende pH. Aangezien binding van metalen op de celwand de eerste stap kan zijn in de opname ervan en de daaropvolgende toxiciteit, kan de celwand mogelijk beschouwd worden als het biotisch ligand van algen. Toekomstig onderzoek in dit domein zou moeten focussen op de relatie tussen fysico-chemie, metaalbinding op de celwand en koperopname in de cel en toxische effecten. Daarvoor is in

de eerste plaats een gedetailleerde kennis nodig van de pH-afhankelijke binding van metalen op de celwand.

Als tussentijdse oplossing werd een semi-empirisch model ontwikkeld om chronische kopertoxiciteit te voorspellen. In het model werden de twee bovenvermelde vergelijkingen gekoppeld aan het speciatiemodel WHAM-5. Op die manier konden 72-uur E_bC50 en E_bC10 waarden binnen factor 2 van de waargenomen warden voorspeld worden voor 97% van de artificiële waters en voor 81% van de geteste natuurlijke Europese oppervlaktewaters. Voor hetzelfde Zweedse water als beschreven i.v.m. de validatie van chronische Cu-BLM voor *D. magna* werd toxiciteit opnieuw onderschat (~ factor 4). Opnieuw kan de competitie tussen Fe, Al en Cu voor bindingsplaatsen op het DOC deze waarneming mogelijk verklaren, wat opnieuw het belang van deze interactie in toekomstige toxiciteitsmodellen onderstreept.

Gezien het belang van DOC voor kopertoxiciteit voor *D. magna* en *P. subcapitata*, werd in hoofdstuk 7 aandacht besteed aan de effecten van natuurlijke variabiliteit van koperbindingseigenschappen van DOC op de voorspellende capaciteit van de ontwikkelde modellen. In deze studie werden acute toxiciteitstesten met *D. magna* uitgevoerd in artificiële test media aangerijkt met DOC, geïsoleerd uit oppervlaktewaters van 6 locaties in Europa en Noord Amerika en in 7 natuurlijke Europese oppervlaktewaters. Deze waters vertoonden een grote verscheidenheid qua fysico-chemie én DOC-concentraties. Het ontwikkelde acute Cu-BLM voor *D. magna* werd gebruikt om de koperbindingscapacaiteit van de verschillende DOCs te schatten (uitgedrukt als % actief fulvozuur). Er werd een factor 6 verschil gevonden tussen de laagste en de hoogste bindingscapaciteit, wat gelijkaardig is als het verschil gevonden in verschillende chemische studies.

Een significant verband werd gevonden tussen de UV-absorptiecoëfficient bij 350 nm en de koperbindingscapaciteit van het DOC. Het koppelen van deze relatie aan het acute Cu-BLM resulteerde in een significante verbetering van de voorspellende capaciteit. Zonder de variabiliteit in rekening te brengen werden 90% van de 48-uur EC50s voorspeld binnen factor 2 van de waargenomen waarden; mét het in rekening brengen ervan werd 90% voorspeld met een fout van minder dan factor 1.3. De huidige studie en andere, eerder gepubliceerde studies tonen duidelijk aan dat UV-absorbantie een goede maat kan zijn voor biologisch en toxicologisch relevante verschillen in koperbindingseigenschappen van DOC. Hoewel een

gelijkaardige relatie gevonden werd voor *P. subcapitata*, dient zulke relatie nog aangetoond te worden voor chronische blootstellingen van *D. magna*.

In de bovenstaande secties werd een samenvatting geschetst van de voornaamste resultaten van deze doctoraatsstudie. Eén van de belangrijkste doelen van deze studie was echter de implementatie van biobeschikbaarheid van koper in de EU risico-evaluatie van koper, meer bepaald voor de schatting van biobeschibaarheid-gecorrigeerde PNEC waarden.

Ten eerste dient het opnieuw benadrukt te worden dat de hardheid-gebaseerde correctie van waterkwaliteitscriteria niet geschikt is voor koper, aangezien de hardheid de chronische toxiciteit van koper niet beïnvloedt voor *D. magna* en *P. subcapitata*. Ten tweede, werd aangetoond dat DOC ongeveer 60% van de waargenomen variabiliteit in toxiciteit verklaart en pH ongeveer 15%. Hierbij werd reeds gewezen op de afnemende toxiciteit bij hogere pH voor *D. magna* en de hogere toxiciteit bij hogere pH voor *P. subcapitata*. Beide waarnemingen zijn in overeenstemming met de meeste studies met andere invertebraten en vissen (afnemende toxiciteit) en algen (toenemende toxiciteit).

Binnen het kader van risico-evaluatie is de PNEC berekening gebaseerd op toxiciteitsdata voor al deze groepen van species en daardoor kan er gemakkelijk afgeleid worden dat pH waarschijnlijk geen al te groot effect zal hebben op de chronische effecten van koper op ecosystemen die al deze species bevatten. Inderdaad, bij lage pH zullen invertebraten en vissen hun grootste gevoeligheid vertonen en bij hoge pH zullen de algen hun grootste gevoeligheid vertonen. Bijgevolg zal de ecosysteem-gevoeligheid bij hoge pH gedomineerd worden door de gevoeligheid van algen en bij lage pH door die van invertebraten en vissen. Gecombineerd leidt dit waarschijnlijk tot slechts kleine effecten van pH op ecosysteem gevoeligheid voor koper. Deze hypothese zou kunnen onderzocht worden d.m.v. intensieve modelleringsoefeningen en/of mesocosmos experimenten. Onze initiële analyse duidt echter aan dat de meest belangrijke factor voor de ecosysteem-gevoeligheid aan koper waarschijnlijk de DOC concentratie is. Dit is niet verassend aangezien Cu-DOC complexen grotendeels niet biobeschikbaar zijn en omdat deze normaal de grootste fractie van koper in natuurlijke oppervlaktewaters vormen.

Hoewel er momenteel sterk gedebatteerd wordt over de exacte methode om de ontwikkelde modellen toe te passen in het kader van de EU risico-evaluatie, kan de volgende

methode naar voor geschoven worden om biobeschikbaarheids-gecorrigeerde PNECs te bepalen. De ontwikkelde chronische modellen voor *D. magna* en *P. subcapitata*, tesamen met een nog te ontwikkelen chronisch Cu-BLM voor vissen, zou kunnen toelaten om de toxiciteitsdata van de generische SSD (zie Figuur 1-6) te normalizeren. De generische SSD bevat inderdaad toxiciteitsdata die verkregen zijn d.m.v. testen in media met een grote verscheidenheid aan fysico-chemische eigenschappen en dus grote verschillen in biobeschikbaarheid. Een gevolg is dat de NOEC waarden voor 1 species meer dan 2 grootte-ordes kunnen variëren. Een voorlopige analyse heeft al aangetoond dat deze variabiliteit voor de meeste species inderdaad grotendeels kan toegeschreven worden aan verschillen in biobeschikbaarheid. In dezelfde studie werd aangetoond dat de toxiciteitsmodellen ontwikkeld in dit eindwerk in staat waren om de onzekerheid in grote mate te reduceren, wat de mogelijkheid illustreert om deze modellen te extrapoleren naar andere species. Bijgevolg lijkt het mogelijk om de toxiciteitsdata van alle species van de generische SSD te normalizeren naar eender welke combinatie van fysico-chemische eigenschappen (b.v. voor een welbepaald water of een regio). Op die manier worden biobeschikbaarheid-gecorrigeerde SSDs bekomen die gebruikt kunnen worden voor de berekening van een biobeschikbaarheid-gecorrigeerde PNEC, die dan kan vergeleken worden met een PEC om het biobeschikbaarheid-gecorrigeerde risico van koper te bepalen.

Het is duidelijk dat deze benadering behoorlijk pragmatisch is en dat verschillende veronderstellingen die erbij gemaakt worden vatbaar zijn voor discussie. Waarschijnlijk de meest discutabele veronderstelling is het extrapoleren van toxiciteitsmodellen naar andere species op basis van beperkte datasets. Nochtans toont de reductie van de onzekerheid met deze modellen hun potentiële toepasbaarheid duidelijk aan. Inderdaad, elke onzekerheidsreductie is beter voor zowel milieu als maatschappij aangezien ze de risico-evaluatie dichter bij duurzame ontwikkeling brengt.

Het is duidelijk dat meer onderzoek nodig is om de mechanistische, biologische, chemische, fysiologische en ecologische processen te verklaren die aan de grondslag liggen van de complexiteit van de biobeschikbaarheid van metalen in zoewater ecosystemen. De introductie van biobeschikbaarheidsconcepten en –modellen zoals het biotisch ligand model in het kader van risico-evaluatie en normstelling en de erkenning van hun potentieel nut door zowel academische, industriële en regelgevende instanties mag niet gezien worden als een eindpunt maar eerder als de start van een nieuw tijdperk van innovatief onderzoek…