COPPER EXPOSURE OF FRESHWATER MUSSELS

(*Anodonta anatina*):

SOME PHYSIOLOGICAL EFFECTS

Dissertation submitted to the
FACULTY OF BIOLOGY, CHEMISTRY, AND GEOSCIENCES
UNIVERSITY OF BAYREUTH, GERMANY

to obtain the academic degree of

DR. RER. NAT.

presented by

ANDHIKA PUSPITO NUGROHO

M. Sc.

born August 8, 1976

in Semarang, Indonesia

Bayreuth, November 2011

COPPER EXPOSURE OF FRESHWATER MUSSELS (*Anodonta anatina*): SOME PHYSIOLOGICAL EFFECTS

by

Andhika Puspito Nugroho

Environmental Chemistry and Ecotoxicology

University of Bayreuth

Germany

Supervisor:

Prof. Dr. Hartmut Frank

This doctoral thesis was prepared at the Department of Environmental Chemistry and Ecotoxicology, University of Bayreuth, from November 2010 until November 2011, supervised by Prof. Dr. Hartmut Frank.

This is a full reprint of the dissertation submitted to attain the academic degree of Doctor of Natural Sciences (Dr. rer. nat.) and approved by the Faculty of Biology, Chemistry and Geosciences of the University of Bayreuth.

Acting Dean:	Prof. Dr. Beate Lohnert
Date of submission:	November 9, 2011
Date of defense (disputation):	June 19, 2012

Doctoral Committee

Prof. Dr. Hartmut Frank	1st reviewer
Prof. Dr. Klaus H. Hoffmann	2nd reviewer
Prof. Dr. Ch. E.W. Steinberg	3rd reviewer
Prof. Dr. Gerhard Gebauer	Chairman
Prof. Dr. Stefan Peiffer	
Prof. Dr. Britta Planer-Friedrich	

ACKNOWLEDGEMENTS

Many people contributed to this dissertation in innumerable ways, and I am grateful to all of them. First and foremost I am heartily thankful to my supervisor, Prof. Dr. Hartmut Frank, for his time, advice, support, encouragement, and patience during my research at the Chair of Environmental Chemistry and Ecotoxicology, University of Bayreuth.

I would like to thank Dr. Silke Gerstmann for all the guidance, instruction, and helpful discussion about my work.

The support of Prof. Dr. Britta Planer-Friedrich is greatly appreciated by allowing me to use her laboratory facilities for the mussel exposure experiments and the preparation of the metal analyses.

I also want to thank Prof. Dr. Klaus H. Hoffmann and Dr. Martina Meyering-Vos for all their help in the laboratory and giving me access to other facilities during sample analyses and writing my dissertation.

I am grateful to Dr. Gunter Ilgen and Barbara Scheitler for assisting in metal analyses.

My thanks also go to all of my Indonesian friends for their nice friendship given to me during these years.

I wish to express my appreciation to all of my colleagues at the Chair of Environmental Chemistry and Ecotoxicology, University of Bayreuth. I thank Dr. Huong Ngo Thi Thuy for helpful discussions; Fabian Iltzsche and Stefan Will for providing lots of technical assistance, Irmgard Lauterbach for all her help in the administrative affairs during my study; Michael Fischer, Mario Kiesewetter, Andreas Bantle, and Michael Heyers for all their support with laboratory work.

I am pleased to thank to all of my colleagues at the Chair of Animal Ecology I, University of Bayreuth. I thank Ahmad Alamer, Dr. Judith Lorenz, Hassan El-Damanhouri, Marion Preiß, Dorothea Wiesner, Carmela Herrmann, Ursula Wilczek, and Intisar Taha for their kindness and help.

Finally, I would like to thank my parents, wife, parents-in-law, brothers, and sisters-in-law for supporting and encouraging me always.

TABLE OF CONTENTS

LIST OF PUBLICATIONS AND AUTHOR'S CONTRIBUTIONS

This dissertation is presented in cumulative form. It comprises four individual manuscripts, from which all was published. The author's contributions to each manuscript are given below.

1. Nugroho, A.P., and H. Frank, 2011. Producing Cu-loaded algae for feeding experiments: effects of copper on *Parachlorella kessleri*. Toxicological and Environmental Chemistry, published (Publication I)

   Own contribution: idea (60%), method development (90%), data analysis and calculations (100%), writing (100%) and editing the paper (60%)

2. Nugroho, A.P., and H. Frank, 2011. Uptake, distribution, and bioaccumulation of copper in the freshwater mussel *Anodonta anatina*. Toxicological and Environmental Chemistry, published (Publication II)

   Own contribution: idea (70%), method development (90%), data analysis and calculations (100%), writing (100%) and editing the paper (60%)

3. Nugroho, A.P., and H. Frank, 2012. Effects of copper exposure on calcium, carbohydrate, and protein levels in the freshwater mussel *Anodonta anatina*. Toxicological and Environmental Chemistry, published (Publication III)

   Own contribution: idea (70%), method development (100%), data analysis and calculations (100%), writing (100%) and editing the paper (60%)

4. Nugroho, A.P., and H. Frank, 2012. Effects of copper on metallothionein, glutathione, lipid peroxidation, and antioxidative enzymes in the freshwater mussel *Anodonta anatina*. Toxicological and Environmental Chemistry, published (Publication IV)

   Own contribution: idea (80%), method development (100%), data analysis and calculations (100%), writing (100%) and editing the paper (60%)

LIST OF ABBREVIATIONS

APW	Artificial pond water
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
CAT	Catalase
DNA	Deoxyribonucleic Acid
DTNB	5,5'-Dithio-bis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
dw	Dry weight
EDTA	Ethylenediaminetetraacetic Acid
EF	Enrichment Factor
EPF	Extrapallial fluid
GHL	Gonads, Heart, Labial Palps
GPX	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Glutathione
HML	Hemolymph
IC	Inhibition Concentration
LOEC	Lowest-observed-effect concentration
MDA	Malondialdehyde
MT	Metallothionein
NOEC	No-observed-effect concentration
OD	Optical density
PBS	Phosphate-buffered Saline
PMSF	Phenylmethylsulphonyl Fluoride
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
TF	Transfer Factor
ww	Wet weight

SUMMARY

Copper (Cu), a transition metal, has the tendency to increase in its concentration in freshwater ecosystems over natural levels, due to industrial and other anthropogenic sources. In water, copper can exist in dissolved form or associated with suspended food particles. Freshwater mussels living at the interface of the free-flowing water and the sediment phase can take up copper directly from the water or by consumption of lower trophic level organisms laden with copper. For mussels, copper is essential at low concentration as cofactor of metalloenzymes involved in growth regulation and development, but it may be toxic at higher levels by disturbing calcium (Ca) homeostasis. The duck mussel *Anodonta anatina* is a freshwater species found in abundance in limnic and lotic European ecosystems and is used as test organism in ecotoxicological studies. The potential involvement of Cu in the general decline of many European freshwater mussel species is the major motivation for this work.

This research aims to study the relevance of Cu exposure pathways on its uptake, distribution, bioaccumulation, and elimination in the freshwater mussel *A. anatina* and its various potential physiological impacts. The work is started with raising Cu-loaded algae using the stable isotope ^{63}Cu as marker for feeding of mussels without affecting the nutritional value of the algal food. In these latter experiments, mussels are exposed to ^{63}Cu via water or via food to investigate the relative importance of Cu uptake to its distribution and accumulation among the mussel's organs. Its consequences on calcium homeostasis, soluble carbohydrate and protein levels in various tissues, metallothionein induction, glutathione levels, activities of antioxidative enzymes and glutathione reductase, and on lipid peroxidation are examined.

In the algal experiment, *Parachlorella kessleri* is grown at six ^{63}Cu concentrations (0, 5.9, 11.7, 23.5, 47, and 94 µmol L^{-1}) for 4 days, starting from day 3. When exposed to Cu at a level of up to 6 µmol L^{-1}, *P. kessleri* is largely unchanged in its nutritional values; so this concentration is used to grow ^{63}Cu-carrying food for mussel experiment. Concentrations above 6 µmol L^{-1} decrease significantly in the algal growth and alter the other physiological parameters.

Three groups of 21 mussels each are used, one as control and two for exposure, receiving copper as the stable isotope ^{63}Cu via the water at 0.3 µmol

L^{-1} or via the food (1.5 mg L^{-1} freeze-dried Cu-loaded algae, equivalent to 0.06 µmol L^{-1} Cu) for 24 days, followed by 12 days of depuration. For analysis, three mussels each are taken randomly from every group at days 0, 6, 12, 18, 24, 30, and 36. The mussels are anaesthetized and hemolymph and extrapallial fluid are sampled before the mussels are dissected into gills, mantle, kidney, digestive gland, foot, adductors, intestines, and the remainder (gonads, heart, and labial palps).

During copper exposure, the levels of exogenous copper (^{63}Cu) and total Cu increase in all body compartments. Uptake via the water leads to higher Cu levels than via the food, but in relative terms food uptake is more efficient taking the five-fold lower nominal concentration of copper into consideration. Upon exposure via the water, the metal is compartmentalized mainly in the mantle, the gills, and the digestive gland, upon exposure via the food the major recipients are the digestive gland and the intestines. Upon depuration for two weeks, copper is quickly but not completely eliminated.

Simultaneously with increasing Cu levels, Ca levels are increased in all body compartments, accompanied by decreases in soluble carbohydrates and proteins in the gills, mantle, digestive gland, and kidney. At the same time, Cu exposure results in increases in malondialdehyde levels, decreases in glutathione levels, strong increases in metallothionein levels, and changes in the activities of the antioxidative enzymes superoxide dismutase, catalase, and glutathione peroxidise, and of glutathione reductase in the gills, mantle, digestive gland, and kidney. During depuration, most parameters tend to normalize but do not return to control values.

In conclusion, the overall pictures suggest that the considerable physiological stress elicited by low-level copper exposure may contribute to the factors involved in the decline of many European freshwater mussels.

Keywords: Copper, *Parachlorella kessleri*, *Anodonta anatina*, Bioaccumulation, Elimination, Ca homeostasis, Carbohydrates, Proteins, Metallothionein, Glutathione, Superoxide Dismutase, Catalase, Glutathione Peroxidase, Glutathione Reductase, Lipid Peroxidation (Malondialdehyde)

ZUSAMMENFASSUNG

Kupfer (Cu), ein Übergangmetall, hat die Tendenz, in seinen Konzentrationen in Süßwasser-Ökosystemen gegenüber natürlichen Werten anzusteigen, bedingt durch seine zahlreichen industriellen und elektro-, bau- und agrar-technischen Anwendungen. In Wasser kann Kupfer gelöst in freier ionischer Form und in verschiedenen komplexartigen Verbindungen vorliegen, und es kann gebunden an suspendiertem Sediment auftreten. Süßwassermuscheln, die an der Schnittstelle von frei fließendem Wasser und Sediment-Phase leben, können es direkt aus dem Wasser oder durch Verzehr von Organismen niedrigerer trophischer Ebenen, die mit Kupfer überladen sind, zu sich nehmen. Für Muscheln ist Kupfer in geringer Konzentration als Cofaktor von Metalloenzymen zur Regulation des Wachstums und der Entwicklung unerlässlich, aber auf hohem Niveau ist Kupfer giftig. Die Teichmuschel *Anodonta anatina* ist eine Süßwasser-Arten, die sich in vielen limnischen und fliessenden Europäischen Ökosystemen befindet. Die mögliche Beteiligung von Cu am allgemeinen Niedergang vieler europäischer Süßwassermuscheln ist die wesentliche Motivation für diese Arbeit.

Die hier beschriebene Forschungsarbeit zielt darauf ab, einige Aspekte der Bedeutung der Cu-Exposition für Aufnahme, Verteilung, Bioakkumulation und Elimination in der Süßwassermuschel *A. anatina* und seine potentiellen patho-physiologischen Auswirkungen zu untersuchen. Die Arbeit begint mit der Aufzucht ^{63}Cu-beladener Algen als Futter für die Muscheln, ohne dass der Nährwert der Algen beeinträchtigt wird. In den folgenden Experimenten werden Muscheln dem stabilen Isotop ^{63}Cu durch Wasser oder durch die Algen als Nahrung ausgesetzt, um die relative Bedeutung der Cu-Aufnahmewege für dessen Verteilung und Anreicherung in den Organen der Muschel zu untersuchen. Die Auswirkungen auf die Calcium-Homöostase, auf lösliche Kohlenhydrate und Protein-Konzentrationen in den verschiedenen Geweben, auf die Metallothionein-Induktion und die Höhe der Glutathion-Konzentrationen, auf die Aktivitäten der antioxidativen Enzyme und der Glutathion-Reduktase, und auf die Lipidperoxidation werden überprüft.

In einem Vor-Experiment wird *Parachlorella kessleri* bei sechs Cu-Konzentrationen (0; 5,9; 11,7; 23,5; 47 und 94 µmol L^{-1}) für 4 Tage kultiviert. Bei

einer Cu Konzentration von bis 6 µmol L^{-1} ist des Wachstum von *P. kessleri* weitgehend unverändert und als ^{63}Cu-tragende Nahrung für das Muschel-Experiment geeignet. Exposition bei Cu-Konzentrationen über 6 µmol L^{-1} hat offensichtliche Auswirkungen auf das Wachstum und den physiologischen Zustand der Algen.

Drei Gruppen von je 21 Muscheln werden in diesem Muschel-Expositions-Experiment verwendet. Eine Gruppe dient als Kontrolle, und bei den beiden anderen wird das stabile Isotop ^{63}Cu mit dem Wasser bei 0,3 µmol L^{-1} oder mit der Nahrung (1,5 mg L^{-1} gefriergetrocknete, Cu-beladene Algen, das entspricht ca 0,06 µmol L^{-1} Cu) für 24 Tage gegeben, gefolgt von 12 Tagen Ausscheidungsphase. Für die Analysen werden nach dem Zufallsprinzip aus jeder Gruppe an den Tagen 0, 6, 12, 18, 24, 30, und 36 drei Muscheln entnommen. Die Muscheln werden betäubt und Hämolymphe und Extrapallial-Flüssigkeit werden isoliert, bevor die Muscheln in Kiemen, Mantel-, Nieren-, Verdauungsdrüse-, Fuß-, Adduktoren, Darm und den Rest (Gonaden, Herz und labialen Palpen) seziert werden.

Während der Kupfer-Exposition steigt die Menge des exogenen (^{63}Cu) und des gesamten Kupfers in allen Kompartimenten. Die Aufnahme aus dem Wasser führt zu einer höheren Cu Konzentrationen als aus der Nahrung, aber dennoch ist der letztere Weg in relativer Betrachtung effizienter, wenn man die fünf-fach niedrigere nominale Konzentration von Kupfer bedenkt. Das Metall wird bei Exposition der Muscheln mit dem Wasser vor allem in den Mantel, die Kiemen und die Verdauungsdrüse verteilt, bei Aufnahme über die Nahrung vor allem in die Verdauungsdrüse und den Darm. In der Ausscheidungsphase von zwei Wochen wird Kupfer schnell aber nicht vollständig eliminiert.

Mit zunehmenden Cu Konzentrationen steigen die Ca Konzentrationen in allen Kompartimenten, begleitet von Rückgängen der löslichen Kohlenhydrate und Proteine in den Kiemen, im Mantel, in der Verdauungsdrüse und in der Niere. Gleichzeitig führt die Cu Exposition zum Anstieg von Malondialdehyd, zur Abnahme von Glutathion, starkem Anstieg der Metallothionein-Konzentrationen, und Veränderungen in den Aktivitäten der antioxidativen Enzyme Superoxiddismutase, Katalase, und Glutathion-Peroxidase, und der Glutathion-Reduktase in den Kiemen, im Mantel, in der Verdauungsdrüse und der Niere.

Während der Ausscheidungsphase beginnen sich die meisten Parameter zu normalisieren, aber nicht vollständig zurück zu Normal-Werten.

Der durch erhöhte Kupfer-Exposition verursachte erhebliche physiologische Stress könnte also einer der Faktoren in der ökotoxikologischen Kausalkette sein, die zu den kontinuierlich abnehmenden Populationen einer ganzen Reihe von Europäischen Süßwassermuscheln führen.

Schlüsselwörter: Kupfer, *Parachlorella kessleri, Anodonta anatina*, Bioakkumulation, Ca-Homöostase, Kohlenhydrate, Proteine, Metallothionein, Glutathion, Superoxiddismutase, Katalase, Glutathionperoxidase, Glutathionreduktase, Lipidperoxidation (Malondialdehyd)

1. General Introduction

1.1. Copper in aquatic ecosystems and its transfer in food chains

Copper (Cu) is a transition metal having the atomic number 29 and belongs – along with silver and gold – to Group IB of the Periodic Table of the elements. The metal occurs either in metallic form or in oxidized form in many minerals such as cuprite (Cu_2O), malachite ($Cu_2CO_3 \bullet Cu(OH)_2$), azurite ($2CuCO_3 \bullet Cu(OH)_2$), chalcopyrite ($CuFeS_2$), chalcocite ($Cu_2S$), and bornite ($Cu_3FeS_4$). Natural copper consists of an isotopic mixture of 69% ^{63}Cu and 31% ^{65}Cu (Momčilović 2004).

The metal occurs naturally in all aquatic ecosystems. Concentrations of copper in non-contaminated freshwater ecosystems range from 0.02 to 0.3 µmol L^{-1} (1 – 20 µg L^{-1}) (Momčilović 2004). In water, copper generally exists in two oxidation states, i.e. Cu^+ and Cu^{2+}, mostly complexed to organic (e.g. fulvic and humic acids) and inorganic ligands (e.g. hydroxide, carbonate, chloride, arsenite, and sulfide), with only 5% in the free ionic form. Copper can also be associated with suspended particles, and taken up and concentrated by algal cells (Abbe and Sanders 1990; Arunakumara and Xuecheng 2008; Cuppett et al. 2006; Moore and Ramamoorthy 1984; Pinto et al. 2003).

The availability of copper at low concentrations is essential for aquatic life. Mussels require copper as part of the oxygen-binding site in hemocyanin (Birge and Black 1979), as cofactor of cytochrome-c oxidase, tyrosinase, dopamine β-hydroxylase, alcohol dehydrogenase, prolyl and lysyl oxidase, and in other enzymes involved in growth regulation and development (Amiard-Triquet et al. 2006; Company et al. 2008; Debelius et al. 2009; Viarengo et al. 2002).

Trace metal uptake by mussels occurs via the gills, the mantle, and the digestive tract. Organs serving as the sites for intake exhibit high potentials for bioaccumulation. Hemocytes (blood cells) play an important role in transporting metals among the mussels' organs. In balance with trace metal accumulation, mussels may eliminate them from their bodies via renal and intestinal excretion and by diapedesis. These overall mechanisms are species-, organ-, and metal-specific (Deb and Fukushima 1999; Marigómez et al. 2002).

Mussels take copper up from water, from copper-containing food items, or from both. Microalgae as primary producers at the basis of aquatic food chains

accumulate copper from water, transferring it to grazing species at the next trophic level. This contributes to biomagnification along aquatic food chains. In addition, grazing species can take copper up from the water (Connell and Sanders 1999; Edding and Tala 1996; Pinto et al. 2003). Bioconcentration occurs via uptake and retention of metals from water, across gill membranes or other external body surfaces (Kaoud and El-Dahshan 2010). The concentration of the metals in the soft tissue or particular organs can be considered as a relative measure of ambient concentrations due to the ability of mussels to accumulate copper (Kumari and Nair 1992).

Since the middle of the last century, the continuously increasing technological and industrial use of copper has led to globally increased mining and translocation of copper from the earth's crust to the surface and the corresponding trend of rising concentrations in all compartments of the anthroposphere over natural levels, especially in freshwater ecosystems of industrialized and industrializing countries. Metallurgic activities, its use in machinery such as electrically propelled locomotives, in the building sector as roofing material, for water pipes and kitchenware, for overland high-voltage power lines, and in directly dissipative ways of using it as fungicide, algicide, and molluskicide, the disposal of copper-containing waste waters, and its release and deposition of atmospheric particulate matter from coal combustion (Mohammed and Markert 2006; Momčilović 2004), all this has led to a continuously increasing Cu-burden of the biosphere. This condition entails copper transfer through food chains and its bioconcentration in mussels' bodies. Copper accumulation in cells over the physiological requirements leads to toxic effects, depending upon its bioaccumulation beyond the optimum level within the respective organism (Nott 1998).

1.2. Effects of copper on calcium homeostasis and cellular defense mechanisms of freshwater mussels

Calcium (Ca) is an essential macronutrient for mussels. It has an almost universal importance for nerve conduction, mussel contraction, as second messenger for regulation of carbohydrate metabolism such as controlling the activation of glycogenesis, regulation of mitochondrial electron transport, the metabolism of carbohydrate intermediates of the tricarboxylic acid cycle, and of

almost all aspects of cellular metabolisms and growth (Albert et al. 1994; Sick et al. 1979). Moreover, calcium is required by young and adult mussels for shell formation (calcification) (Sick et al. 1979). Mussel cells require calcium (Ca) in specific limits of cytosolic concentrations (≤ 10^{-7} M). Calcium homeostasis is maintained by extrusion and compartmentalization systems (Viarengo et al. 1993). In addition, mussels require carbohydrates as main energy source for their metabolic processes (Honkoop et al. 1999) and for shell formation (Marie et al. 2007; Marin and Luquet 2004). Proteins are also required by mussels for catalyzing biochemical reactions, transport and storage of molecules in and out or within cells, and have structural and mechanical functions (Albert et al. 1994).

Exposure of mussels to copper at 0.35 µmol L^{-1} leads to an interference with the systems responsible for maintaining Ca homeostasis in gills, digestive gland, and kidney (Santini et al. 2011). This is followed by uncontrolled, increased cytosolic Ca concentrations activating various Ca-dependent catabolic processes such as phospholipid hydrolysis, protein degradation, and DNA fragmentation, ultimately leading to cell death (Viarengo et al. 2002; Viarengo 1994). In addition, high copper levels can entail decreased carbohydrate levels in gills and mantle (Satyaparameshwar et al. 2006).

Mussels have developed detoxification mechanisms to cope with copper challenge. In the cytosol, glutathione (GSH), a tripeptide which contains sulfhydryl (SH) groups with strong affinity for copper cations and found in high concentrations (0.2 – 10 mM) (Monostori et al. 2009), can provide a first line of defense against increased cytosolic levels of free copper by binding the metal to its SH-groups. Increased copper also induces synthesis of metallothioneins (MT), specific SH-rich proteins having the capacity to bind copper (Conners and Ringwood 2000; Viarengo et al. 2002).Increased cytosolic copper can induce oxidative stress because copper may be involved in the formation of reactive oxygen species (ROS) by catalyzing the generation of $^{\bullet}OH$ from H_2O_2 and $O_2^{\bullet-}$ through a Haber-Weiss cycle (Lackner 1998; Pinto et al. 2003). During aerobic respiration, oxygen is reduced to water through four steps of electron transfer resulting in oxygen intermediates which are highly reactive and toxic ROS, i.e. the superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical

($^{\bullet}OH$) (Lackner 1998). In healthy aerobic cells, there is a balance between ROS production, molecular oxidation, and antioxidant consumption.

Mussel cells have a wide range of antioxidative enzymes, neutralizing ROS and keeping their concentrations at very low levels. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) represent a group of enzymes having antioxidative roles (Isani et al. 2003; Pinto et al. 2003; Viarengo et al. 2002). SOD catalyzes the disproportionation of $O_2^{\bullet-}$ to O_2 and H_2O_2, whereas CAT and GPX catalyze the production of H_2O from the degradation of H_2O_2 and ROOH, respectively. SOD is the cell's first line of defense against ROS because it controls $O_2^{\bullet-}$ which can be a precursor to several other highly reactive species (Pinto et al. 2003). If these mechanisms are challenged beyond their protective capacities and ROS production rates are higher than the rates of its inactivation by antioxidant defense systems, oxidative stress conditions arise. In such case, free ROS can react quickly and indiscriminately with biomolecules such as lipids, proteins, and nucleic acids, resulting in lipid peroxidation, formation of protein carbonyl groups, and DNA strand breaks. Determination of lipid peroxidation allows to assess oxidative stress levels in cells (Company et al. 2008; Lackner 1998).

1.3. Freshwater mussels and their status

Freshwater mussels are invertebrate animals which have two shells (bivalve shell) as mirror images of each other, connected by a hinge-like ligament. Adult mussels have a variety of sizes, colours, and shapes, depending on the species. They are sedentary and inhabit the bottom of freshwater ecosystems such as creeks, rivers, streams, ponds, and lakes. They have a muscular foot which helps them anchor against strong currents and allows limited movements. Mussels are ecologically important in aquatic ecosystems comprising a significant proportion of the total standing crop in freshwater benthic communities, cycling calcium in lakes, removing suspended detritus and cleaning the water, mixing surficial sediments through bioturbation, and serving as food for aquatic mammals (Box et al. 2006; Naimo 1995; Nedeau and Victoria 2003).

The family of Unionidae is the most endangered of all aquatic animal species. Alterations of mussel habitats potentially influence the survival of the

mussels because several stages of the mussels' life histories such as sperm release by adult males into the water column, uptake of sperms by siphoning females, fertilization of ovae, release of viable larvae (glochidia) from females, and attachment of glochidia to suitable host fish by encystations for transformation to free-living juvenile mussels are critical stages which ultimately can all contribute to a decreased mussel reproduction and population development. Laboratory experiments have shown that the early life stages of freshwater mussels are sensitive to many chemicals including copper and ammonia (Cope et al. 2008). Due to the importance of mussels in aquatic ecosystems, protective and conservative actions are required for maintaining healthy mussel populations and for recovering endangered ones by investigation of freshwater mussel biology, the preservation of water and riparian resources, and the control and/or elimination of threats to these animals (Watters et al. 2009).

1.4. Ecotoxicological studies with Anodonta anatina

Ecotoxicological investigations concerning the effects of contaminants are devoted to reveal at which dose or concentrations they become toxic. Investigations on contaminant uptake and elimination rates, distribution among mussel tissues and organs, and the relationship between contaminant accumulation and biological responses at each level of organization are required.

Anodonta anatina is a freshwater mussel species of the family Unionidae which is widely distributed in Europe in a variety of freshwater ecosystems and is used for biological monitoring surveys (Mäkelä et al. 1995; Mäkelä and Oikari 1990). The species has been used for uptake and body distribution studies of contaminants such as chlorinated phenolics (Mäkela and Oikari 1990), for accumulation and monitoring studies of 2,4,6-trichlorophenol (Englund and Heino 1996), pentachorophenol (Mäkela and Oikari 1995), other chlorinated phenolics (Mäkelä et al. 1991), ^{45}Ca accumulation (Pynnönen 1991), and uptake and cadmium accumulation and depuration (Holwerda et al. 1988). Other ecotoxicological studies on contaminant impacts at biochemical and physiological levels of *A. anatina* have been reported, such as the effects of copper on Ca-ATPase and carbonic anhydrase (Santini et al. 2011), effects of

cadmium on calcium metabolism (Ngo et al. 2011), and effects of crude oil on cytogenetic damage (Baršienė et al. 2006).

1.5. Objectives of the research

This present research project aims to study the importance of two different copper exposure pathways, i.e. via water or food, on uptake, distribution, accumulation, and elimination in the freshwater mussel *Anodonta anatina*, and their potential physiological impacts. In order to reveal the effects of copper via food, the mussel must be fed by copper-containing algae which have normal nutritional value to avoid secondary effects. Therefore, before the actual mussel experiments, microalgae *Parachlorella kessleri* are raised while being exposed to various copper concentrations, to find the limit at which the physiological state and nutritional value is comparable to non-exposed algae and to be used as ^{63}Cu-loaded food for the mussels (Publication I). In the following experiments, *A. anatina* are exposed to Cu via water or food. Distribution, bioaccumulation, and elimination of the trace metal among the organs of the exposed mussels are investigated (Publication II). The effects of elevated copper levels on the levels of calcium, soluble carbohydrates and proteins (Publication III), on metallothionein induction and glutathione levels, on the activities of antioxidative enzymes, and on lipid peroxidation (Publication IV) in various organs and tissues are examined.

1.6. Methodological requirements

Since mussels contain endogenous copper, the stable isotope ^{63}Cu is used as tracer to differentiate between endogenous and exogenous copper. According to Croteau et al. (2004), stable isotopes of metals can be used as markers to help investigating directional uptake pathways and their bioaccumulation and elimination from aqueous and dietary sources. Use of inductively-coupled plasma mass spectrometry (ICP-MS) as analytical tool allows to determine the isotopes at low concentrations as individual masses.

2. Materials and methods

2.1. Copper experiment with microalgae Parachlorella kessleri

P. kessleri (SAG Culture Collection, University of Goettingen, Germany) is exposed to Cu at various concentrations for 96 hours to find the most suitable Cu concentration which does not affect its nutritional values, being used to produce ^{63}Cu-loaded algae as food for mussel experiment. Details of copper experiments with the algae are described in Publication I.

2.2. Copper exposure experiment of mussels

The duck mussel *A. anatina* can take copper up from water or food, so three groups of 21 mussels each are used, one as control and the two other groups to be exposed to the stable isotope ^{63}Cu via water or food for 24 days followed by 12 days of depuration. Seven samplings of three mussels each every sixth days are taken to study the time-dependent Cu accumulation and elimination. Copper exposure for 24 days represents a long-term copper exposure (Company et al. 2008), while the depuration period of 12 days allows to investigate how fast the levels return to control values, reflecting also the half-life of copper. Details of copper exposure experiments with the mussel are described in Publication II.

During the experiments, total copper and isotopic copper (^{63}Cu and ^{65}Cu) are determined in the hemolymph (HML), the extrapallial fluid (EPF), gills, mantle, kidney, digestive gland, foot, adductors, intestines, and the collective remaining organs, i.e. gonads, heart, and labial palps (GHL) (Publication II). Effects of copper on calcium homeostasis, proteins, and carbohydrates are studied in all these body compartments. The relationships between copper and Ca, carbohydrate, and protein levels respectively, and between calcium and proteins are examined in the compartments (Publication III). Effects of copper on metallothionein, glutathione, lipid peroxidation, and antioxidative enzymes are also examined (Publication IV).

2.3. Analytical methods

2.3.1. Determinations of total Cu, isotopic Cu, and total calcium

Total Cu and isotopic Cu in lyophilized tissue fractions are determined by inductively-coupled plasma mass spectrometry (ICP-MS), total Ca by inductively-coupled plasma atomic emission spectroscopy (ICP-AES). Determinations and calculations of the concentrations of the elements are described in detail in Publications II and III.

2.3.2. Determinations of carbohydrates and proteins

Carbohydrates are determined by the phenol-sulfuric acid assay (Masuko et al. 2005), proteins by the dye-binding assay (Kruger 1994). Details of the determinations are described in Publication III.

2.3.3. Determination of metallothionein

Metallothionein (MT) concentrations in the gills, mantle, digestive gland, and kidney are determined using the spectrophotometric method described by Viarengo et al. (1997) and modified by Verlecar et al. (2008). Details of metallothionein determination are described in Publication IV.

2.3.4. Determinations of glutathione, antioxidative enzyme activities, and lipid peroxidation

Glutathione levels are determined according to Anderson (1985). The activities of the antioxidative enzymes catalase, glutathione peroxidase, and superoxide dismutase are assayed according to the methods of Rao et al. (1996), Paglia and Valentine (1967), and Beauchamp and Fridovich (1971), glutathione reductase activities are assayed following the method of Massey and William (1965). For lipid peroxidation assay, the method of Buege and Aust (1978) is employed. Details of the determinations are described in Publication IV.

2.4. Statistical analysis

Data of mussel experiments are transformed to log (X+1) units before statistical analysis for homogeneity of variance and normality. The variability of all parameters with exposure time and copper exposure pathways are tested in

each organ by two-way analysis of variance (ANOVA). Details of statistical analyses were described in Publications II and III, and Manuscript IV.

3. Results

3.1. Studies of copper effects on the green alga *Parachlorella kessleri*: Producing Cu-loaded algae for feeding experiments

Exposure of the algae to copper above 6 µmol L^{-1} leads to increased Cu levels in the algae, inhibition of algal growth, and significant alterations of biochemical-physiological parameters, strongest effects being observed at highest concentration (Publication I, Figure 1, 2, and 3, Table 1). Chlorophyll contents and growth rate are the most sensitive indicators. At 5.9 µmol L^{-1} Cu, the observed parameters do not differ significantly from control values.

3.2. Studies of different copper exposure pathways on the freshwater mussel *Anodonta anatina*

3.2.1. Studies of copper uptake, distribution, bioaccumulation, and elimination

Mobilization of the stable isotope ^{63}Cu among mussel organs reveals that Cu uptake from water occurs via the gills and mantle and from the food via the digestive gland (Publication II, Figure 4). Exogenous Cu (^{63}Cu) and total (exogenous and endogenous) Cu increase in all body compartments, highest levels being observed at day 24. Upon exposure via the water, high total Cu levels are found in the gills, mantle, digestive gland, kidney, and GHL while upon exposure via the food highest levels are found in the digestive gland and kidney (Publication II, Figure 1, 2, 3, and 4). During depuration, total and exogenous Cu decrease in all body compartments, except for total Cu in the mantle and intestines for which even further increases were observed within the first six days of depuration.

3.2.2. Studies of copper effects

3.2.2.1. Change in Ca levels

Ca levels in all body compartments increase in parallel to increased Cu concentrations, reaching highest levels at day 24 (Publication III, Figure 1 and 2). Upon depuration, Ca concentrations in the body fluids decline fast, returning

to control values within the first six days although Cu levels are still elevated. In the organs, Ca levels tend to normalize, although not fully back to controls.

3.2.2.2. Changes in carbohydrate and protein levels

Soluble carbohydrates and soluble proteins decline in all organs upon Cu exposure and in parallel to its concentrations, reaching lowest levels at day 24 (Publication III, Figure 3). When Cu administration is terminated, the levels in the studied organs start to increase, although not fully back to control within the 12 days.

3.2.2.3. Effects on metallothionein, glutathione, lipid peroxidation, and antioxidative enzymes

Exposure of *A. anatina* to copper induces increases in metallothionein (MT) in all organs, reaching highest levels at day 24 (Publication IV, Figure 1). For glutathione (GSH), the levels decrease at similar rates in all organs, reaching lowest levels at day 24. Simultaneously with the Cu elimination, MT levels decrease in all organs, for GSH being increased slowly. Thiobarbituric acid-reactive substances (TBARS) increase strongly upon Cu exposure via the water, reaching highest levels at day 24. During depuration, TBARS levels decrease slowly.

Activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione reductase (GR) are expressed in two ways, i.e. relative to protein contents and to tissue wet weight. In relation to protein contents, all enzyme activities increase reaching highest levels at day 24. Strongest effects were found in the digestive gland (Publication IV, Figure 2). In terms of tissue wet weight, activities of SOD, GPX, and GR decline in all organs, reaching lowest levels at day 24. For CAT, the activities remain unchanged for both pathways except for the kidney in which the activity is increased, reaching highest level at day 18. During depuration, most parameters tend to normalize but do not return to control values.

4. General discussion

Declines in chlorophyll contents of *P. kessleri* upon Cu exposure suggest three possibilities, i.e. inhibition of chlorophyll synthesis, increased lipid

peroxidation on chloroplast membranes, and degradation of chlorophyll-a confirmed by increased pheophytin-a (Sandmann and Böger 1980; Tripathi and Gaur 2006). This condition may affect photosynthesis rates, lowering glucose synthesis and ultimately resulting in inhibition of algal growth. Decreases in carbohydrates and proteins may be a result of increased hydroxyl radical formations induced by copper via the Haber-Weiss reaction. The radicals are highly reactive, oxidizing, and breaking apart biological macromolecules (Nikookar et al. 2005).

Use of the stable isotope ^{63}Cu can represent exogenous Cudistribution and the redistribution of endogenous Cu among body compartments. Redistribution of endogenous Cu causes pronounced alterations in total Cu in the organs, such as increases in the mantle and intestines upon exposure via the food (Publication II, Figure 2, 3, and 4).

Copper elimination can occur due to the difference in gradient Cu concentration between the mussel and APW (Publication II, Figure 2 and 3). High Cu level in the kidney indicates that this organ plays an important role in elimination. Declines of exogenous ^{63}Cu levels during depuration indicate that the isotope is in a relatively easily exchangeable form while the endogenous Cu is more tenaciously retained (Publication II, Figure 4).

Disturbance of Ca homeostasis upon Cu exposure (Publication III, Figure 1 and 2) can occur due to mobilization of $CaCO_3$ from the shell, most likely due to Cu-induced metabolic acidosis (Antunes et al. 2002; Faubel et al. 2008; Lopes-Lima et al. 2008). Inhibition of Ca extrusion and of intracellular compartmentalization systems may be another complication (Pattnaik et al. 2007; Viarengo et al. 2002; Viarengo et al. 1994). Decreased protein levels (Publication III, Figure 3) may be due to increased Ca levels, activating Ca-dependent catabolic processes such as protein degradation (Viarengo et al. 1994). A strong decrease of carbohydrate levels is attributed to cell hypoxia caused by copper, leading to increased activities of glycolytic enzymes involved in anaerobic ATP production (Satyaparameshwar et al. 2006; Martínez et al. 2006).

Increases of MT levels in all observed organs upon Cu exposure (Publication IV, Figure 1) confirm the role of MT in copper metabolisms. Strong decreases in GSH levels within the first 6 days of exposure indicate that GSH is

consumed for early cellular protection against copper and as precursor for MT-synthesis. In respect to activities of antioxidative enzymes and glutathione reductase, since the overall protein synthetic capacity is hampered (Publication III, Figure 3), the activities of the enzymes relative to tissue wet weight – and thus relative to copper – are depressed. This indicates that the mussels are under considerable oxidative stress. Increased TBARS levels as indicator of lipid peroxidation confirm the generation of reactive oxygen species upon copper exposure.

5. Conclusions, contributions, and perspectives

5.1. Conclusions

Anodonta anatina shows the ability to take up copper as water-dissolved ^{63}Cu or from ^{63}Cu-loaded algae. Copper is accumulated mainly in the mantle, gills, and digestive gland upon exposure via the water, from the food in the digestive gland. The digestive gland and the kidney are the main organs for accumulation and elimination.

At the same time, increased Cu concentrations in the mussels' organs result in pathophysiological consequences, such as alterations of Ca homeostasis and decreases of carbohydrates and proteins. Strong induction of MT and depletion of GSH confirm their biological roles in response to copper as a first line of defense against cell toxic effects. The attempt to activate a second line of defense by diverting protein synthetic capacity to antioxidative enzyme synthesis is futile, leading to their decline in relation to tissue weight; this indicates that the mussel *Anodonta anatina* is under considerable oxidative stress at such environmentally relevant Cu-concentrations. The overall pictures indicate that copper may be one of contributory factors in the presently observed decline of many European freshwater mussels.

5.2. Contributions

This study develops an ecotoxicological model for freshwater ecosystems, integrating biological, physical, and chemical factors. In order to study the effects of copper on mussels, the model establishes the interactions between mussels and dissolved copper, and mussels and Cu-contaminated food in representation of Cu transfer via food chains. During exposure, the levels of copper, calcium, carbohydrate, protein, metallothionein, glutathione, glutathione

reductase, and antioxidative enzymes are examined in the mussels' organs. The results can give information about the potential risks elicited by copper on aquatic ecosystems and may offer an early warning tool to evaluate the impact of this transition metal on environmental quality.

5.3. Perspectives

This study indicates that copper may be one of the factors involved in the decline of freshwater mussels including the pearl mussel *Margaritifera margaritifera*. Further parameters such as the effects of copper on calcium storage as concretions and DNA damage should be investigated, in order to improve the understanding of the underlying mechanisms. In aquatic ecosystems, mussel populations are exposed to a mixture of contaminants in water and sediments. Further laboratory experiments and field studies are necessary to investigate the synergistic and antagonistic effects of such contaminants on the biochemical, enzymological, endocrinological, and physiological conditions. Finally, the results of the laboratory experiments should be applied in selected watersheds to recover mussel populations and be used to decide proper policies related to environmental protections and biodiversity conservations.

References

Abbe, G.R., and J.G. Sanders. 1990. Pathways of silver uptake and accumulation by the American oyster (*Crassostrea virginica*) in the Chesapeake Bay. *Estuarine, Coastal and Shelf Science* 31:113-123.

Albert, B., D. Bray, J. Lewis, M. Raff, K. Robert, and J.D. Watson. 1994. *Molecular biology of the cell*. 3rd edition. New York: Garland Publishing.

Amiard-Triquet, C., B. Berthet, L. Joux, and H. Perrein-Ettajani. 2006. Significance of physicochemical forms of storage in microalgae in predicting copper transfer to filter-feeding oysters (*Crassostrea gigas*). *Environmental Toxicology* 21:1-7.

Anderson, M.E. 1985. Determination of glutathione and glutathione disulfide in biological samples. *Methods in Enzymology* 113:543-553.

Antunes, C., T. Magalhães-Cardoso, G. Moura, D. Gonçalves, and J. Machado. 2002. Effects of Al, Ni, Co, Zn, Cd, and Cu metals on the outer mantle epithelium of *Anodonta cygnea* (Unionidae). *Haliotis* 31:71-84.

Arunakumara, K.K.I.U., and Z. Xuecheng. 2008. Heavy metal bioaccumulation and toxicity with special reference to microalgae. *Journal of Ocean University of China* 7:60-64.

Baršienė, J., L. Andreikėnaitė, and A. Rybakovas. 2006. Cytogenetic damage in perch (*Perca fluviatilis* L.) and duck mussel (*Anodonta anatina* L.) exposed to crude oil. *Ekologija* 1:25-31.

Beauchamp, C., and I. Fridovich. 1971. Superoxide dismutase: improved assays and an assay applicable to polyacrylamide gels. *Analytical Biochemistry* 44:276-286.

Birge, W.J., and J.A. Black. 1979. Effects of copper on embryonic and juvenile stages of aquatic animals. In *Copper in the Environment. Part II. Health effects*, ed. J.O. Nriagu, 373-399. New York: John Wiley & Sons.

Box, J.B., J. Howard, D. Wolf, C. O'Brien, D. Nez, and D. Close. 2006. Freshwater mussels (Bivalvia: Unionoida) of the Umatilla and Middle Fork John Day Rivers in Eastern Oregon. *Northwest Science* 80:95-107.

Buege, J.A., and S.D. Aust. 1978. Microsomal lipid peroxidation. *Methods in Enzymology* 52:302-310.

Company, R., A. Serafim, R.P. Cosson, A. Fiala-Médioni, L. Camus, A. Colaço, R. Serrão-Santos, and M.J. Bebianno. 2008. Antioxidant biochemical responses to long-term copper exposure in *Bathymodiolus azoricus* from Menez-Gwen hydrothermal vent. *Science of the Total Environment* 389:407-417.

Connell, D.B., and J.G. Sanders. 1999. Variation in cadmium uptake by estuarine phytoplankton and transfer to the copepod *Eurytemora affinis*. *Marine Biology* 133:259-265.

Conners, D.E., and A.H. Ringwood. 2000. Effects of glutathione depletion on copper cytotoxicity in oysters (*Crassotrea virginica*). *Aquatic Toxicology* 50:341-349.

Cope, W.G., R.B. Bringolf, D.B. Buchwalter, T.J. Newton, C.G. Ingersoll, N. Wang, T. Augspurger, F.J. Dwyer, M.C. Barnhart, R.J. Neves, and E. Hammer. 2008. Differential exposure, duration, and sensitivity of unionoidean bivalve life stages to environmental contaminants. *Journal of the North American Benthological Society* 27:451-462.

Croteau, M.N., S.N. Luoma, B.R. Topping, and C.B. Lopez. 2004. Stable metal isotopes reveal copper accumulation and loss dynamic in the freshwater bivalve *Corbicula*. *Environmental Science and Technology* 38:5002-5009.

Cuppett, J.D., S.E. Duncan, and A.M. Dietrich. 2006. Evaluation of copper speciation and water quality factors that affect aqueous copper tasting response. *Chemical Senses* 31:689-697.

Deb, S.C., and T. Fukushima. 1999. Metals in aquatic ecosystems: Mechanisms of uptake, accumulation and release-ecotoxicological perspectives. *International Journal of Environmental Studies* 56:385-417.

Debelius, B., J.M. Forja, Á. DelValls, and L.M. Lubián. 2009. Toxicity and bioaccumulation of copper and lead in five marine microalgae. *Ecotoxicology and Environmental Safety* 72:1503 – 1513.

Edding, M., and F. Tala. 1996. Copper transfer and influence on a marine food chain. *Bulletin of Environmental Contamination and Toxicology* 57:617-624.

Englund, V.P.M., and M.P. Heino. 1996. The freshwater mussel (*Anodonta anatina*) in monitoring of 2,4,6-trichlorophenol: Behaviour and environmental variation considered. *Chemosphere* 32:391-403.

Faubel, D., M. Lopes-Lima, S. Freitas, L. Pereira, J. Andrade, A. Checa, H. Frank, T. Matsuda, and J. Machado. 2008. Effects of Cd^{2+} on the calcium metabolism and shell mineralization of bivalve *Anodonta cygnea*. *Marine and Freshwater Behaviour and Physiology* 41:93-108.

Holwerda, D.A., J. Hemelraad, P.R. Veenhof, and D.I. Zandee. 1988. Cadmium accumulation and depuration in *Anodonta anatina* exposed to cadmium chloride or cadmium-EDTA complex. *Bulletin of Environmental Contamination and Toxicology* 40:373-380.

Honkoop, P.J.C., J.V. der Meer, J.J. Beukema, and D. Kwast. 1999. Reproductive investment in the intertidal bivalve *Macoma balthica*. *Journal of Sea Research* 41:203–212.

Isani, G., M. Monari, G. Andreani, M. Fabbri, and E. Carpenè. 2003. Effect of copper exposure on the antioxidant enzymes in bivalve mollusc *Scapharca inaequivalis*. *Veterinary Research Communications* 27:691-693.

Kaoud, H.A., and A.R. El-Dahshan. 2010. Bioaccumulation and histopathological alterations of the heavy metals in *Oreochromis niloticus* fish. *Nature and Science* 8:147-156.

Kruger, N.J. 1994. The Bradford method for protein quantitation. In *Methods in Molecular Biology: Basic protein and peptide protocols vol. 32*, ed. J.M. Walker, 9-15. New Jersey: Humana Press, Inc.

Kumari, L.K., and V.R. Nair. 1992. Uptake and bioconcentration of copper and zinc by the molluscs *Saccostrea cucullata* (Born) and *Cerithium rubus*

(Desh) from the coastal waters of Bombay. *Indian Journal of Marine Sciences* 21:74-76.

Lackner, R. 1998. “Oxidative stress” in fish by environmental pollutants. In *Fish Ecotoxicology*, ed. T. Braunbeck, D.E. Hinton, and B. Streit, 203-224. Switzerland: Birkhäuser Verlag.

Lopes-Lima, M., R. Bleher, T. Forg, M. Hafner, and J. Machado. 2008. Studies on a PCMA-like protein in the outer mantle epithelium of *Anodonta cygnea*: Insights of calcium transcellular dynamics. *Journal of Comparative Physiology B* 178:17-25.

Mäkelä, T.P., and A.O.J. Oikari. 1995. Pentachlorophenol accumulation in the freshwater mussels *Anodonta anatina* and *Pseudanodonta complanata*, and some physiological consequences of laboratory maintenance. *Chemosphere* 31:3651-3662.

Mäkelä, T.P., T. Petänen, J. Kukkonen, and A.O.J. Oikari. 1991. Accumulation and depuration of chlorinated phenolics in the freshwater mussel (*Anodonta anatina* L.). *Ecotoxicology and Environmental Safety* 22:153-163.

Mäkelä, T.P., and A.O.J. Oikari. 1990. Uptake and body distribution of chlorinated phenolics in the freshwater mussel *Anodonta anatina* L. *Ecotoxicology and Environmental Safety* 20:354-360.

Marie, B., G. Luquet, J.P.D. Barros, N. Guichard, S. Morel, G. Alcaraz, L. Bollache, and F. Marin. 2007. The shell matrix of the freshwater mussel *Unio pictorum* (Paleoheterodonta, Unionoida): Involvement of acidic polysaccharides from glycoproteins in nacre mineralization. *FEBS Journal* 274:2933-2945.

Marigómez, I., M. Soto, M.P. Cajaraville, E. Angulo, and L. Giamberini. 2002. Cellular and subcellular distribution of metals in molluscs. *Microscopy Research and Technique* 56:358-392.

Marin, F., and Luquet, G. 2004. Molluscan shell proteins. *Comptes Rendus Palevol* 3:469-492.

Martínez, M.L., C. Landry, R. Boehm, S. Manning, A.O. Cheek, and B.B. Rees. 2006. Effects of long-term hypoxia on enzymes of carbohydrate metabolism in the Gulf killifish, *Fundulus grandis*. *The Journal of Experimental Biology* 209:3851-3861.

Massey, V., and C.H. Williams. 1965. On the reaction mechanism of yeast glutathione reductase. *The Journal of Biological Chemistry* 240:4470–4481.

Masuko, T., A. Minami, N. Iwasaki, T. Majima, S. Nishimura, and Y.C. Lee. 2005. Carbohydrate analysis by a phenol–sulfuric acid method in microplate format. *Analytical Biochemistry* 339:69-72.

Mohammed, M.H., and B. Markert. 2006. Toxicity of heavy metals on *Scenedesmus quadricauda* (Turp.) de Brébisson in batch cultures. *Environmental Science & Pollution Research* 13:98-104.

Momčilović, B. 2004. Copper. In *Elements and their compounds in the environment: Metals and their compounds (Vol. 2)*, ed. E. Merian, M. Anke, M. Ihnat, and M. Stoeppler, 731-750. Weinheim: Wiley-VCH Verlag GmbH&Co. KGaA.

Monostori, P., G. Wittmann, E. Karg, and S. Túri. 2009. Determination of glutathione and glutathione disulfide in biological samples: An in-depth review. *Journal of Chromatography B* 877:3331–3346.

Moore, J.W., and S. Ramamoorthy. 1984. *Heavy Metals in Natural Waters*. New York: Springer-Verlag New York, Inc.

Naimo, T.J. 1995. A review of the effects of heavy metals on freshwater mussels. *Ecotoxicology* 4:341-362.

Nedeau, E.J., and J. Victoria. 2003. A field guide to the freshwater mussels of Connecticut. Hartford: Connecticut Department of Environmental Protection, Bureau of Natural Resources – Wildlife Division.

Nikookar, K., A. Moradshahi, and L. Hosseini. 2005. Physiological responses of *Dunaliella salina* and *Dunaliella tertiolecta* to copper toxicity. *Biomolecular Engineering* 22:141-146.

Ngo, H.T.T., S. Gerstmann, and H. Frank. 2011. Subchronic effects of environment-like cadmium levels on the bivalve *Anodonta anatina* (Linnaeus 1758): I. Bioaccumulation, distribution and effects on calcium metabolism. *Toxicological and Environmental Chemistry* 93:1788-1801.

Nott, J.A. 1998. Metals and marine food chains. In *Metal metabolism in aquatic environments*, ed. W.J. Langston and M.J. Bebianno, 388. London: Chapman & Hall.

Paglia, D.E., and W.N. Valentine. 1967. Studies on quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *Journal of Laboratory and Clinical Medicine* 70: 158–169.

Pattnaik, S., G.B.N. Chainy, and J.K. Jena. 2007. Characterization of Ca^{2+}-ATPase activity in gill microsomes of freshwater mussel, *Lamellidens marginalis* (Lamarck) and heavy metal modulations. *Aquaculture* 270:443-450.

Pinto, E., T.C.S. Sigaud-Kutner, M.A.S. Leitão, O.K. Okamoto, D. Morse, and P. Colepicolo. 2003. Heavy metal-induced oxidative stress in algae. *Journal of Phycology* 39:1008-1018.

Pynnönen, K. 1991. Accumulation of ^{45}Ca in the freshwater unionids *Anodonta anatina* and *Unio tumidus*, as influenced by water hardness, protons, and aluminum. *Journal of Experimental Zoology* 260:18-27.

Rao, M.V., G. Paliyath, and D.P. Ormrod. 1996. Ultraviolet-B and ozone-induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. *Plant Physiology* 110:125-136.

Sandmann, G., and P. Böger. 1980. Copper deficiency and toxicity in *Scenedesmus. Zeitschrift für Pflanzenphysiologie* 98:53-59.

Santini, O., N. Chahbane, P. Vasseur, and H. Frank. 2011. Effects of low-level copper exposure on Ca^{2+}-ATPase and carbonic anhydrase in the freshwater bivalve *Anodonta anatina*. *Toxicological and Environmental Chemistry* 93:1826-1837.

Satyaparameshwar, K., T.R. Reddy, and N.V. Kumar. 2006. Study of carbohydrate metabolism in selected tissues of freshwater mussel, *Lamellidens marginalis* under copper sulphate toxicity. *Journal of Environmental Biology* 27:39-41.

Sick, L.V., C.C. Johnson, and C.A. Siegfried. 1979. Fluxes of dissolved and particulate calcium in selected tissues of *Crassostrea virginica. Marine Biology* 54:293-299.

Tripathi, B.N., and J.P. Gaur. 2006. Physiological behaviour of *Scenedesmus* sp. during exposure to elevated levels of Cu and Zn and after withdrawal of metal stress. *Protoplasma* 229:1–9.

Verlecar, X.N., K.B. Jena, and G.B.N. Chainy. 2008. Modulation of antioxidant defences in digestive gland of *Perna viridis* (L.), on mercury exposures. *Chemosphere* 71:1977-1985.

Viarengo, A., B. Burlando, and C. Bolognesi. 2002. Cellular responses to copper in aquatic organisms. In *Handbook of Copper Pharmacology and Toxicology*, ed. E.J. Massaro, 417-427. New Jersey: Humana Press, Inc.

Viarengo, A., E. Ponzano, F. Dondero, and R. Fabbri. 1997. A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic mollusks. *Marine Environmental Research* 44:69 – 84.

Viarengo, A. 1994. Heavy metal cytotoxicity in marine organisms: Effects on Ca^{2+} homeostasis and possible alteration of signal transduction pathways. *Advances in Comparative and Environmental Physiology* 20:85-110.

Viarengo, A., G. Mancinelli, M. Pertica, R. Fabbri, and M. Orunesu. 1993. Effects of heavy metals on the Ca^{2+}-ATPase activity present in gill cell plasma-membrane of mussels (*Mytilus galloprovincialis* Lam.). *Comparative Biochemistry and Physiology* 106C:655-660.

Watters, G.T., M.A. Hoggarth, and D.H. Stansbery. 2009. The freshwater mussels of Ohio. Ohio: The Ohio State University Press.

PUBLICATIONS

Toxicological & Environmental Chemistry
Vol. 93, No. 3, March 2011, 537–548

Taylor & Francis
Taylor & Francis Group

Producing Cu-loaded algae for feeding experiments: effects of copper on *Parachlorella kessleri*

Andhika Puspito Nugroho[ab] and Hartmut Frank[a]*

[a]*Department of Environmental Chemistry and Ecotoxicology, University of Bayreuth, 95440 Bayreuth, Germany;* [b]*Faculty of Biology, Laboratory of Ecology, Gadjah Mada University, Yogyakarta 55281, Indonesia*

(*Received 18 September 2010; final version received 2 November 2010*)

Microalgae require several essential metals for optimum growth, which at elevated concentrations may interfere with biochemical and physiological processes, one of them being copper (Cu). The aim of this study is to raise Cu-loaded *Parachlorella kessleri* as feed for mussels. In order to spike the algae with Cu without lowering their nutritional quality, it is important to know the highest Cu-concentration at which the main parameters remain unaffected, especially in respect to proteins and polysaccharides. The dependence of growth rate, biomass, chlorophyll-a and -b, pheophytin-a, protein, and polysaccharide contents on Cu concentrations are determined. The tests show that *P. kessleri* is largely unchanged in its nutritional value when exposed to Cu at levels of up to 6 μmol L^{-1}. Above 10 μmol L^{-1}, toxic effects become obvious, with chlorophyll contents and growth rate being the most sensitive indicators.

Keywords: copper; *Parachlorella kessleri*; growth rate; biomass; chlorophyll-a; chlorophyll-b; pheophytin-a; proteins; polysaccharides

Introduction

Microalgae are fundamental constituents of food chains in almost all aquatic ecosystems, serving as food for organisms of the next trophic levels and as source of oxygen for respiration. For optimum growth, algae require a number of essential metals, some of which may be toxic above certain levels. A typical example is Cu; usually, it is present in natural fresh waters at concentrations ranging from 0.02 to 2 nmol L^{-1} (0.001–0.1 μg L^{-1}) and in ocean waters from 0.5 to 10 nmol L^{-1} (0.03–0.6 μg L^{-1}). To cope with its low availability, algae have mechanisms for active uptake and accumulation (Debelius et al. 2009; Lim et al. 2006; Wright and Welbourn 2002), based upon the strong complexation with functional thiol groups of the proteins involved in the active uptake of Cu (Levy et al. 2008; Nalimova et al. 2005; Stauber and Florence 1987; Yan and Pan 2002).

Copper is required as a cofactor of enzymes participating in oxygen metabolism and in redox reactions, e.g., plastocyanin, polyphenol oxidase, superoxide dismutase, ascorbate oxidase, cytochrome oxidase, lysyl oxidase, and diamine oxidase (Nalimova et al. 2005; Yilmaz, Işik, and Sayin 2005). Mollusks and other invertebrates also require Cu as a component of hemocyanine; the animals receive it from the water as well as from Cu-containing food (Amiard-Triquet et al. 2006; Company et al. 2008).

*Corresponding author. Email: hartmut.frank@uni-bayreuth.de

ISSN 0277–2248 print/ISSN 1029–0486 online

DOI: 10.1080/02772248.2010.537859
http://www.informaworld.com

Due to its use as fungicide, in the building sector as roofing material, for overland high-voltage power lines, and its presence in municipal and industrial waste waters, Cu concentrations in rivers, lakes, and estuaries have increased severalfold over natural levels (Mohammed and Markert 2006; Yilmaz, Işik, and Sayin 2005). High concentrations have been reported in rivers of Japan (1.1–3.5 μmol L^{-1} (0.07–0.22 mg L^{-1}), Pawlik-Skowrońska and Skowroński 2001), China (Yangtse river 0.2–0.8 mmol L^{-1} (15–50 mg L^{-1}), Xu et al. 2000), or Brazil (Jurujuba Sound 0.08–3.3 mmol L^{-1} (5–210 mg L^{-1}), Neto, Smith, and Mc Allister 2000). At such levels, Cu can have toxic effects to producers and consumers on the various stages of the aquatic food chain. For algae, toxicity thresholds (NOEC, no observed effect concentration) have been reported (Levy, Stauber, and Jolley 2007) to be in the range of 0.003–0.14 μmol L^{-1} (0.2–9 μg L^{-1}), lowest observed effect concentrations (LOECs) in the range of 0.004–0.63 μmol L^{-1} (0.3–40 μg L^{-1}), and the 72 h IC_{50} between 0.009 and 8.3 μmol L^{-1} (0.6–530 μg L^{-1}), depending on the strain.

The primary toxic effects of Cu on algal cells are altering the rate of deoxyribonucleic acid (DNA) synthesis, interfering with protein and carbohydrate metabolisms, mitochondrial electron transport, and adenosine triphosphate (ATP) production and respiration, disrupting cell division, and interfering with the uptake of Ca and Mg (Arunakumara and Xuecheng 2008; Debelius et al. 2009; Markina and Aizdaicher 2006; Pawlik-Skowrońska and Skowroński 2001; Stauber and Florence 1987; Tripathi and Gaur 2006). In *Chlorella pyrenoidosa*, Cu affects growth rates, photosynthesis, and content of chlorophyll-a starting at concentrations of 4 μmol L^{-1} (0.25 mg L^{-1}), 1.6 μmol L^{-1} (0.1 mg L^{-1}), and 1.6 μmol L^{-1} (0.1 mg L^{-1}), respectively (Wong and Chang 1991). Yan and Pan (2002) reported the growth of *Scenedesmus obliquus*, *C. pyrenoidosa*, and *Closterium lunula* being inhibited at concentrations of 0.8, 1.0, and 3.0 μmol L^{-1} (50, 70, and 200 μg L^{-1}), respectively. In this context, it should be mentioned that under laboratory culture conditions, the onset of Cu toxicity depends also on initial cell density, composition of the medium, and physical conditions (Debelius et al. 2009).

The algal species *Parachlorella kessleri* used in this study is a common food source for herbivore consumers in freshwater ecosystems. They are easy to culture in the laboratory, often used in toxicity bioassays for predicting environmental impacts of pollutants, and known to have a remarkable ability to accumulate metals (Debelius et al. 2009; Kaduková and Virčíková 2005; Mallick 2003). In order to raise Cu-loaded algae for feeding experiments with mussels, it is important to assess the toxicological and pathophysiological threshold of Cu which does not affect significantly the nutritional value of Cu-loaded algae compared to control algae, especially in respect to protein and carbohydrate contents. NOECs of Cu, its effects on growth rate and biomass, on chlorophyll-a and -b, in the formation of pheophytin-a, and on the protein and polysaccharide contents are determined.

Materials and methods

Glassware and reagents

All glassware is rinsed twice with half-concentrated HNO_3 (65%; Sigma–Aldrich, Munich, Germany), deionized and bidistilled water, and sterilized in an autoclave (Certoclav CV-EL 18 O, Certoclav Sterilizer GmbH, Traun, Austria) at 120°C for 15 min. Lyophilized glycogen standard (Type VII, *Mytilus edulis*), Coomassie blue solution, bovine serum albumin (BSA), and all other chemicals (Sigma–Aldrich) are of analytical grade. For exposure experiments, a Cu solution is prepared by dissolving

0.1 g $CuCl_2 \cdot 2H_2O$ in bidistilled water in a 100 mL volumetric flask yielding a concentration of 5.9 μmol L^{-1}.

Test organism, culture conditions, and toxicity testing

Parachlorella kessleri is obtained from the Culture Collection of Algae (SAG) of the University of Goettingen, Germany. An algal stock culture is grown axenically in a sterilized K-medium (Kuhl and Lorenzen 1964), modified by containing the macronutrients KNO_3, $NaH_2PO_4 \cdot H_2O$, $Na_2HPO_4 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$ at 1.5 times, and $CaCl_2$ at 2.5 times increased concentrations, under omission of Cu^{2+} and ethylenediaminetetraacetic acid (EDTA), and with the pH value lowered to 6.5. The algae are kept suspended by gentle shaking, maintained at 22 ± 2°C, and illuminated continuously with fluorescent tubes at a photon intensity of 48–51 μmol m^{-2} s^{-1} in 2 L Erlenmeyer flasks connected to a Drechsel gas wash bottle to distribute air and CO_2; the latter is filled with potassium carbonate buffer (2 mol L^{-1} $KHCO_3/K_2CO_3$, 35/65 v/v).

For toxicity testing, aliquots of the stock culture are added to 1.8 L modified K-medium in 2 L Erlenmeyer flasks to establish an initial cell density of $1–2 \times 10^5$ cells mL^{-1}. The cultures are maintained as described above. At day 3 after inoculation, Cu is added using the stock solution to establish the following exposure concentrations: 0 (control), 5.9, 11.7, 23.5, 47, and 94 μmol L^{-1}. The algae are grown at these levels for 96 h, i.e., from day 3 to day 7. Growth is monitored daily and, in the end of the experiment (day 7), biomass, chlorophyll-a and -b, pheophytin-a, protein, and polysaccharide contents are determined, as well as Cu accumulation. For all analyses, three aliquots are taken.

Determination of growth and biomass

Growth is followed by measuring optical density. Three 3 mL aliquots are taken from each culture using Pasteur pipettes connected with silicon tubing to 1000 μL micropipettors (Carl Roth, Karlsruhe, Germany) and transferred to polystyrene cuvettes with 1 cm light path. The optical density is read at a wavelength of 686 nm (OD_{686}) (UVIKON 930 Spectrophotometer, Kontron Instruments, Munich, Germany). The specific growth rate is calculated according to Mei et al. (2006), taking the rate at the highest Cu concentration as 100% inhibition.

At the end of the experiment, the whole algal culture is centrifuged in six 300 mL centrifugation bottles at 10,000 rpm at 4°C for 10 min (Beckman Avanti J25, rotor JA-16.50). The supernatants are discarded and the algal pellets are washed by resuspension/centrifugation, once with fresh culture medium and once with phosphate-buffered saline (PBS). The pellets are combined and the algae are resuspended in 47 mL bidistilled water, transferred to a 50 mL polypropylene centrifugation tube of known weight, and centrifuged again (Beckman Avanti J25, rotor JA-16.50); the supernatant is discarded, and the fresh weight of the biomass is calculated by subtracting the weight of the empty tube from the weight of the tube containing the algae. The pellet is frozen at −80°C, freeze-dried at −40°C for 72 h, and weighed again to yield the dry weight.

Copper determination

For Cu determination, the lyophilized algae are homogenized by acid digestion as follows: three algal samples of 10 mg each are placed in 55 mL borosilicate glass tubes, and to each

tube, 5 mL of a mixture (4 : 1) of suprapure concentrated HNO_3 and suprapure concentrated HCl is added. The tubes are kept in an oven at 40°C for 1 h, followed by 95°C for 3 h. The digested samples are diluted with bidistilled water to 10 mL and filtered through a 0.45-μm cellulose syringe filter (Carl Roth). Copper is determined by inductively coupled plasma mass spectrometry (Agilent 7500ce, Cetac ASX-510, Agilent Technologies, Waldbronn, Germany) and expressed per kilogram dry weight. Copper concentration per kilogram wet weight is calculated by multiplying the determined concentration per dry weight with the ratio of algal wet weight *versus* dry weight.

Determination of chlorophyll-a, chlorophyll-b, and pheophytin-a contents

Chlorophyll-a and -b and pheophytin-a contents are determined according to the APHA method (APHA 1992). Lyophilized algae, 3 mg each, are suspended in 12 mL ice-cold acetone in 50 mL polypropylene centrifugation tubes and sonicated in an ice bath at 20 kHz, acoustic power 50 W (Labsonic U tip Sonicator, B. Braun Biotech International, Melsungen, Germany) for 160 s in eight 20 s periods, allowing equal time for cooling on ice. The homogenates are kept for 2 h at 4°C in the dark and centrifuged at 2500 rpm at 4°C for 15 min. The supernatants, 3 mL each, are transferred to 1 cm polystyrene cuvettes, and the optical densities at 750 and 664 nm (OD_{750b} and OD_{664b}) are read (UVIKON 930 Spectrophotometer, Kontron Instruments); OD_{664b} value should lie between 0.1 and 1.0. Subsequently, 0.1 mL of 0.1 mol L^{-1} HCl is added under gentle agitation, and 90 s later, the optical densities are read again at 750 nm and, this time, at 665 nm (OD_{750a} and OD_{665a}). The OD_{664b}/OD_{665a} ratio is calculated, and then chlorophyll-a and pheophytin-a are determined (APHA 1992):

$$\text{Chlorophyll-a}(\text{mg L}^{-1}) = 26.7[(OD_{664b} - OD_{750b}) - (OD_{665a} - (OD_{750a})]$$

$$\text{Pheophytin-a}(\text{mg L}^{-1}) = 26.7[1.7(OD_{665a} - (OD_{750a}) - (OD_{664b} - OD_{750b})]$$

For determination of chlorophyll-b, 3 mL of the supernatant is transferred to a 1 cm polystyrene cuvette and the optical densities at 750, 664, 647, and 630 nm are read. Chlorophyll-b is calculated according by the trichromatic method (APHA 1992):

$$\text{Chlorophyll-b}(\text{mg L}^{-1}) = 21.03(OD_{647} - OD_{750}) - 5.43(OD_{664} - OD_{750}) - 2.66(OD_{630} - OD_{750}).$$

Protein and polysaccharide contents

Lyophilized algae, 5 mg each, are placed in 2 mL Eppendorf tubes, 1 mL of PBS is added to each tube, and the samples are sonicated for 160 s in eight 20 s periods in an ice bath at 20 kHz, acoustic power 50 W, allowing equal time for cooling on ice to avoid protein denaturation. The homogenates are centrifuged at 4°C for 20 min at 15,000 rpm (Beckman Avanti J25, rotor JA-16.50).

The supernatants are used for determination of protein content by the dye-binding assay (Kruger 1994). Aliquots of 10 μL are filled into 1 mL disposable polystyrene cuvettes and 90 μL of bidistilled water and 1 mL of Coomassie blue solution are added. After gentle but thorough mixing, the samples are kept at room temperature for 15 min before

absorbances are read at 595 nm. Protein concentrations are determined from a calibration curve obtained with BSA.

Carbohydrate is determined by the phenol–sulfuric acid assay (Masuko et al. 2005). Aliquots of the supernatants, 50 μL each, are placed in 2 mL Eppendorf tubes, and 200 μL of bidistilled water and 750 μL of concentrated sulfuric acid are rapidly added to achieve complete mixing. Immediately afterward, 150 μL of a solution of 5% phenol in water is added. After incubation for 5 min at 90°C in a static water bath, the tubes are cooled to room temperature for 5 min in another water bath and wiped dry for spectrophotometric measurement at 490 nm. The concentrations of polysaccharides are determined using a calibration curve obtained with glycogen standard type VII (Sigma–Aldrich).

Data analysis

The data of all parameters are statistically analyzed by one-way analysis of variance (ANOVA), followed by the Duncan multiple comparison tests if significant differences are found. Data are transformed to log units before statistical analysis for homogeneity of variance and normality. Linear regression analysis is performed for evaluating the relationships between Cu concentration and physiological and biochemical parameters, followed by Pearson correlation analysis for testing the strength of linear relationships.

Toxicity is expressed as NOEC, estimated using the Dunnett's multiple comparison test after analysis by one-way ANOVA, while IC_{10} and IC_{50} values are determined using the inhibition concentration (ICp) approach (Version 2.0, Norberg-King 1993). Visual MINTEQ software is used to calculate Cu speciation in relation to pH of algal medium (Version 3.0, beta version; Gustaffsson 2010).

Results

Growth of *P. kessleri* is moderate and statistically, non-significantly inhibited (5% relative to control, $p > 0.05$) by Cu given between day 3 and day 7 at a concentration of 5.9 μmol L^{-1}. Copper exposures at 11.7, 23.5, 47, and 94 μmol L^{-1} cause significant decreases by 27, 34, 87, and 100% ($p < 0.05$). Inhibition plotted on the probit scale (Figure 1) shows a linear relationship to Cu concentration with a strong, positive correlation ($r = 0.96$; $p < 0.05$). It should be kept in mind that at the pH of the incubation medium of 6.5, the relative percentage of free Cu^{2+} is about 89–91% of the nominal concentration; decrease of pH to 6.3 at the end of exposure may increase the free Cu^{2+} to about 95% (Gustaffsson 2010).

Copper exposure at 5.9 μmol L^{-1} results in decrease in chlorophyll-a and -b contents and in OD_{664b}/OD_{665a} ratio at day 7 (Table 1) though statistically insignificant ($p > 0.05$). Reductions in chlorophyll-a and -b (42% and 32%) are found to be significant at 11.7 μmol L^{-1} Cu, for the OD_{664b}/OD_{665a} ratio at 23.5 μmol L^{-1} Cu. At the highest Cu concentration (94 μmol L^{-1}), chlorophyll-a and -b contents are strongly lowered (97% and 95% relative to control), the OD_{664b}/OD_{665a} ratio moderately. Regression and correlation analysis show strong, highly negative correlations between Cu exposure concentration and chlorophyll-a ($r = -0.908$; $p < 0.01$), chlorophyll-b ($r = -0.906$; $p < 0.01$), and OD_{664b}/OD_{665a} ratio ($r = -0.925$; $p < 0.01$). Pheophytin-a is increased ($r = 0.912$; $p < 0.01$) even at the lowest Cu concentration being significantly different from control (44%, $p < 0.05$). At the highest Cu concentration, pheophytin-a is increased by 800%. Biomass is reduced by

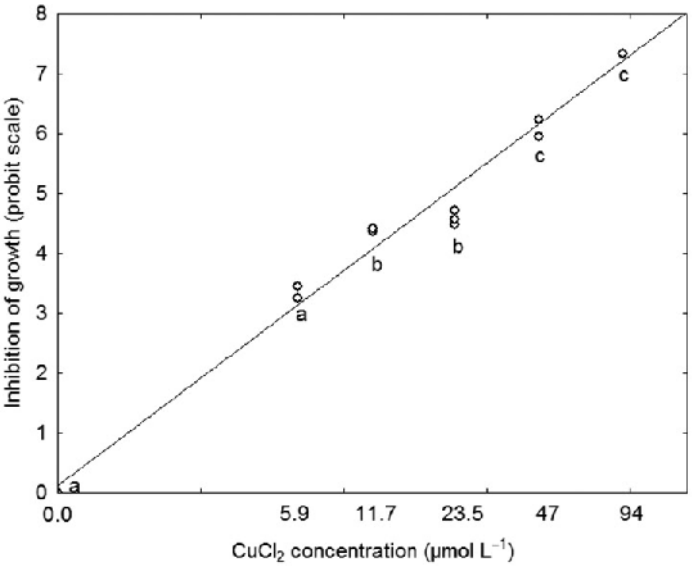

Figure 1. Probit plot of growth inhibition (between days 3 and 6) of *P. kessleri* at different $CuCl_2$ concentrations.
Note: Identical letters indicate that differences are not significant ($p > 0.05$).

Table 1. Effects of Cu exposure on the contents of chlorophyll-a, pheophytin-a, chlorophyll-b, on OD_{664b}/OD_{665a} ratio, and on biomass in *P. kessleri* on day 7, i.e., after 4 days of exposure.

Exposure $CuCl_2$ ($\mu mol\,L^{-1}$)	Effects				
	Chlorophyll-a ($mg\,g^{-1}$ dw)	Pheophytin-a ($mg\,g^{-1}$ dw)	Chlorophyll-b ($mg\,g^{-1}$ dw)	OD_{664b}/OD_{665a} ratio	Biomass ($g\,L^{-1}$)
0	$7.6^{a} \pm 1.31$	$0.09^{a} \pm 0.01$	$1.92^{a} \pm 0.38$	$1.67^{a} \pm 0.01$	$0.52^{a} \pm 0.10$
5.9	$6.7^{a} \pm 0.73$	$0.13^{b} \pm 0.01$	$1.71^{a} \pm 0.23$	$1.63^{a} \pm 0.02$	$0.44^{ab} \pm 0.04$
11.7	$4.4^{b} \pm 0.37$	$0.15^{b} \pm 0.02$	$1.30^{b} \pm 0.05$	$1.59^{a} \pm 0.01$	$0.41^{b} \pm 0.05$
23.5	$4.0^{b} \pm 0.46$	$0.25^{c} \pm 0.03$	$1.00^{c} \pm 0.04$	$1.48^{b} \pm 0.08$	$0.38^{b} \pm 0.02$
47	$0.3^{c} \pm 0.04$	$0.71^{d} \pm 0.05$	$0.14^{d} \pm 0.02$	$1.18^{c} \pm 0.01$	$0.30^{c} \pm 0.01$
94	$0.2^{c} \pm 0.04$	$0.73^{d} \pm 0.03$	$0.10^{d} \pm 0.02$	$1.13^{c} \pm 0.06$	$0.25^{c} \pm 0.01$

Note: Means $\pm$ standard deviations ($n = 3$). Identical letters indicate that the values are statistically not different ($p > 0.05$); dw = dry weight.

15% relative to control ($p > 0.05$) at $5.9\,\mu mol\,L^{-1}$ Cu; at $11.7\,\mu mol\,L^{-1}$ and above, reductions by 20% and more ($p < 0.05$) are found.

Exposure of algae to Cu results in strong accumulation of the metal (Figure 2) far above the natural level, the latter being about $0.01\,mmol\,kg^{-1}$ wet weight. On day 7 of the experiment, i.e., after 4 days of Cu exposure at $5.9\,\mu mol\,L^{-1}$, intracellular Cu reaches $2.5\,mmol\,kg^{-1}$ wet weight, the 410-fold of its concentration in the water. At the higher Cu exposure concentrations, similar accumulation factors are found, i.e., 280- to 510-fold.

The polysaccharide content is raised by 32% at $5.9\,\mu mol\,L^{-1}$ Cu, the level of protein is slightly and insignificantly elevated ($p > 0.05$) (Figure 3). Beyond a Cu exposure level of

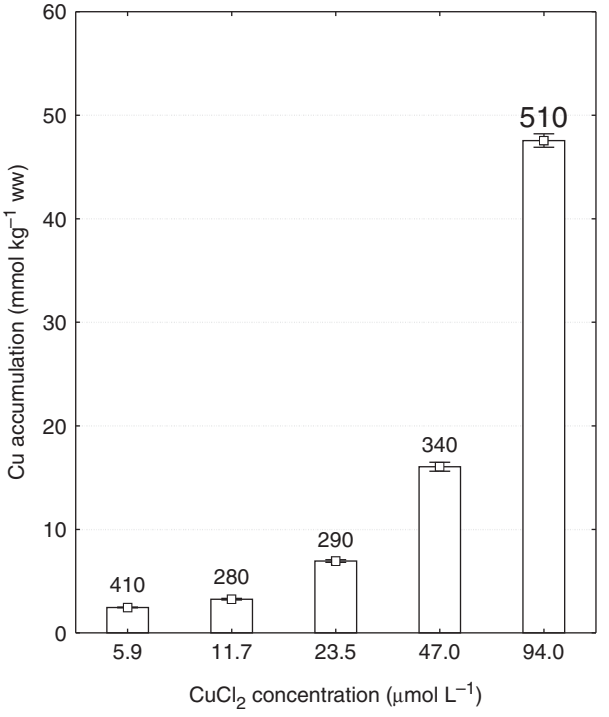

Figure 2. Copper accumulation by *P. kessleri* after 4 days of $CuCl_2$ exposure.
Note: Enrichment factors are given above each column.

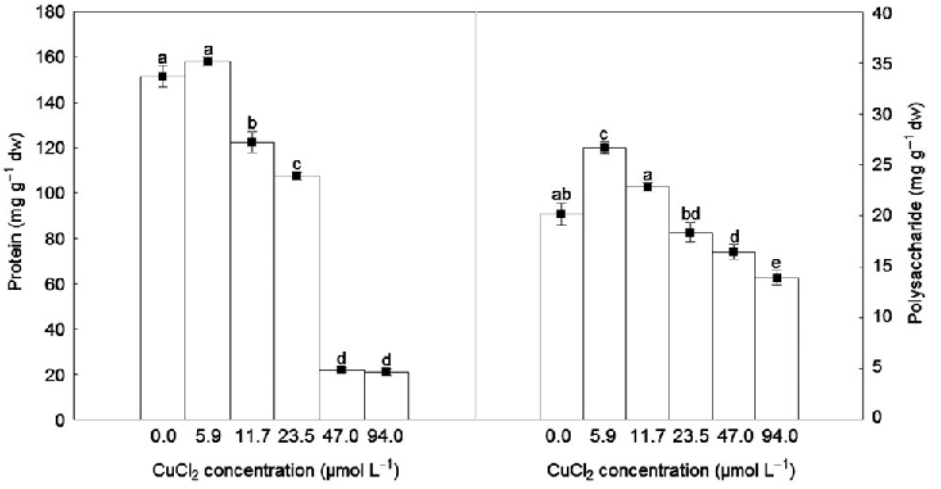

Figure 3. Concentration dependences of protein and polysaccharide contents in *P. kessleri* after 4 days of $CuCl_2$ exposure.
Note: Identical letters indicate that differences in these parameters are not significant ($p > 0.05$).

Table 2. IC_{10}, IC_{50}, and NOEC values ($\mu mol\,Cu\,L^{-1}$) after 4 days of $CuCl_2$ exposure based on chlorophyll-a, pheophytin-a, chlorophyll-b, OD_{686}, inhibition of growth, and biomass at day 7.

	Chlorophyll-a	Pheophytin-a	Chlorophyll-b	OD_{686}	Growth	Biomass
IC_{10}	12.1 ± 1.1	11.7 ± 0.6	9.4 ± 0.6	15.0 ± 0.4	9.0 ± 0.5	18 ± 7
IC_{50}	33.5 ± 0.5	32.1 ± 0.3	28.0 ± 3.0	78.5 ± 2.5	22.0 ± 3.0	85 ± 10
NOEC	5.9	5.9	5.9	5.9	5.9	11.7

Note: Data are expressed as mean ± standard deviation for IC_{10} and IC_{50}.

about $10\,\mu mol\,L^{-1}$, the levels of carbohydrates and proteins are declining, the latter to about 15% of control at the two highest Cu concentrations.

Toxicity of Cu is determined using various endpoints (Table 2). NOEC values based upon chlorophyll-a and -b and pheophytin-a contents, OD_{686} at day 7, and inhibition of growth are $5.9\,\mu mol\,Cu\,L^{-1}$; the NOEC for biomass is $11.7\,\mu mol\,Cu\,L^{-1}$. The toxicity markers are found to be increasingly sensitive in the following order: biomass and OD_{686} at day 7 < pheophytin-a < chlorophyll-a, chlorophyll-b, and growth rate. Polysaccharide and protein contents are obviously not useful as toxicity markers at low Cu-levels (Figure 3).

Discussion

Decline in chlorophyll-a and -b contents of *P. kessleri* during exposure to Cu from about $10\,\mu mol\,L^{-1}$ upward suggests inhibited chlorophyll synthesis. With *Scenedesmus* sp., such inhibition is found at a Cu concentration as low as $2.5\,\mu mol\,L^{-1}$ (Tripathi and Gaur 2006). According to Sandmann and Böger (1980), decline in chlorophyll content of *Scenedesmus acutus* upon exposure to Cu exceeding $10\,\mu mol\,L^{-1}$ is correlated with increased lipid peroxidation resulting in chloroplast membrane damage. A similar behavior is observed for the floating macrophyte *Ceratophyllum demersum*, the chlorophyll contents of which being reduced by 20% upon Cu exposure at $2\,\mu mol\,L^{-1}$ for 24 h (Devi and Prasad 1998). In addition, Cu may replace the central Mg^{2+} ion in chlorophyll and inhibit the synthesis of δ-aminolevulinic acid and protochlorophyllide reductase, the latter two being important in chlorophyll biosynthesis (Perales-Vela et al. 2007).

Pheophytin-a is a sensitive indicator of toxicant effects on plants. It results from degradation of chlorophyll-a by replacement of Mg^{2+} for two protons (Küpper, Küpper, and Spiller 1998). Bačkor and Váczi (2002) reported that administration of Cu at $4\,mmol\,L^{-1}$ to lichen photobionts entails increased pheophytinization. According to APHA (1992), a small change in OD_{665}-value upon acidification reflects high pheophytin-a contents, also shown by the OD_{664b}/OD_{665a} ratio. When the latter is about 1.0, pheophytin content is high, while a value of about 1.7 is an indicator of excellent physiological condition.

Inhibition in growth of *P. kessleri* is conspicuous at $11.7\,\mu mol\,L^{-1}$ Cu and higher, similar to other algal species, i.e., $16\,\mu mol\,L^{-1}$ Cu for *Spirulina platensis* (Nalimova et al. 2005), $10\,\mu mol\,L^{-1}$ Cu for *Scenedesmus* sp. (Tripathi and Gaur 2006), $7.9\,\mu mol\,L^{-1}$ Cu for *Cylindrotheca fusiformis* (Pistocchi et al. 1997), but only $1.5\,\mu mol\,L^{-1}$ Cu for *C. pyrenoidosa* 251 (Wong and Chang 1991). Thus, *P. kessleri* is in respect to growth similarly sensitive to Cu as other algal species, although some are even more sensitive. Growth inhibition may be caused by interference with photosynthesis, directly by blocking

the photosynthetic electron transport in photosystem II or indirectly by inhibiting the biosynthesis of photosynthetic pigments. Inhibition of other anabolic enzymes by binding to SH groups affecting their catalytic action and/or by disrupting their structural integrity has been suggested (de Filippis and Pallaghy 1994; Juneau, Berdey, and Popovic 2002; Küpper et al. 2002), as well as interference with the homeostasis of the electrolyte metals calcium and magnesium (Arunakumara and Xuecheng 2008; Küpper, Küpper, and Spiller 1998; Pearlmutter and Lembi 1986). This may affect the rate of mitosis which finally causes poor algal growth. High Cu concentrations may also lead to oxidative stress and generation of free radicals which damage the cellular machinery (Arunakumara and Xuecheng 2008; Cid et al. 1995; Fernandes and Henriques 1991; Pawlik-Skowrońska and Skowroński 2001; Tripathi and Gaur 2006). In *Dunaliella salina* and *Dunaliella tertiolecta*, Cu exposure at 5 μmol L^{-1} for 24 h results in increased lipid peroxidation of 400% and 195% relative to control, indicating increased hydroxyl radical formation, presumably by Haber–Weiss reaction. The latter may also be the reason for the breakdown of polysaccharides and proteins (Nikookar, Moradshahi, and Hosseini 2005) in *P. kessleri* above 10 μmol L^{-1} Cu. Interestingly, proteins and polysaccharides are even elevated up to about 6–7 μmol L^{-1} Cu (Figure 3), indicating that *P. kessleri* is better suited for raising Cu-loaded feed than other algae, e.g., *Scenedesmus* sp. which is affected within 48 h at 2.5 μmol L^{-1} Cu by toxic action on the enzymes responsible for protein biosynthesis (Tripathi and Gaur 2006). In relation to Cu toxicity, the 96 h IC_{10} and IC_{50} values (Table 2) show differences in the sensitivity of the various endpoints. Chlorophyll loss, increase in pheophytin-a, and lowered growth rate are the most sensitive indicators. For *P. kessleri*, the NOEC for all endpoints, except biomass, is 5.9 μmol L^{-1} Cu (Table 2). This is much higher than for *D. tertiolecta* (0.13 μmol L^{-1} Cu), *Tetraselmis* sp. (0.11 μmol L^{-1} Cu), *Nitzschia closterium* (0.07 μmol L^{-1} Cu), *Phaeodactylum tricornutum* (0.02 μmol L^{-1} Cu), and *Emiliania huxleyi* (0.13 μmol L^{-1} Cu) (Levy, Stauber, and Jolley 2007).

Parachlorella kessleri has high accumulation capacity for Cu (Figure 2), at 5.9 μmol Cu L^{-1} 410 times increased over the concentrations in the medium. At concentrations between 11.7 and 47.0 μmol Cu L^{-1}, enrichment factors are slightly lower, but at the highest Cu concentration, enrichment is further increased. According to Mallick and Rai (2002) and Markina and Aizdaicher (2006), this non-linear dependence may be the consequence of Cu-exclusion as defense mechanisms, in combination with the induction of phytochelatin synthesis and metal sequestration into the nucleus and vacuole (de Filippis and Pallaghy 1994). For *Tetraselmis chuii*, Yilmaz, Işik, and Sayin (2005) have reported a 42-fold accumulation at Cu exposure at 4 μmol L^{-1} for 3 days.

The above experiments show that Cu-loaded micro algae of the species *P. kessleri* with acceptable nutritional value for feeding mussels, can be raised up to a Cu-concentration of about 6 μmol L^{-1}, and they can be recommended, especially as they can accumulate Cu efficiently (more than 400 times) without significantly affecting photosynthetic parameters (Figure 2), and even raising their protein and carbohydrate (Figure 3).

Conclusions

Parachlorella kessleri is a species suitable for the preparation of Cu-loaded algal feed if grown at moderate Cu concentrations of about 6 μmol L^{-1}, resulting in algae well loaded with Cu and equivalent to controls in respect to protein and polysaccharide contents. Above a Cu exposure level of 10 μmol L^{-1}, reductions in growth, biomass, chlorophyll-a

and -b, as well as proteins and polysaccharides are too severe to yield algae which are suitable as mussel feed.

Acknowledgments

We thank Dr Silke Gerstmann for facilitating helpful discussions. Financial support by Directorate General of Higher Education, Department of National Education of the Republic of Indonesia is highly appreciated.

References

Amiard-Triquet, C., B. Berthet, L. Joux, and H. Perrein-Ettajani. 2006. Significance of physico-chemical forms of storage in microalgae in predicting copper transfer to filter-feeding oysters (*Crassostrea gigas*). *Environmental Toxicology* 21: 1–7.

APHA. 1992. Chlorophyll. *Standard methods for the examination of water and wastewater*. 18th ed. New York: American Public Health Association.

Arunakumara, K.K.I.U., and Z. Xuecheng. 2008. Heavy metal bioaccumulation and toxicity with special reference to microalgae. *Journal of Ocean University of China* 7, no. 1: 60–4.

Bačkor, M., and P. Váczi. 2002. Copper tolerance in the lichen photobiont *Trebouxia erici* (Chlorophyta). *Environmental and Experimental Botany* 48: 11–20.

Cid, A., C. Herrero, E. Torres, and J. Abalde. 1995. Copper toxicity on the marine microalga *Phaeodactylum tricornutum*: Effects on photosynthesis and related parameters. *Aquatic Toxicology* 31: 165–74.

Company, R., A. Serafim, R.P. Cosson, A. Fiala-Médioni, L. Camus, A. Colaço, R. Serrão-Santos, and M.J. Bebianno. 2008. Antioxidant biochemical responses to long-term copper exposure in *Bathymodiolus azoricus* from Menez-Gwen hydrothermal vent. *Science of the Total Environment* 389: 407–17.

Debelius, B., J.M. Forja, Á. DelValls, and L.M. Lubián. 2009. Toxicity and bioaccumulation of copper and lead in five marine microalgae. *Ecotoxicology and Environmental Safety* 72: 1503–13.

de Filippis, L.F., and C.K. Pallaghy. 1994. Heavy metals: Sources and biological effects. In *Advances in Limnology 42: Algae and water pollution*, eds. L.C. Rai, J.P. Gaur, and C.J. Soeder, 59. Stuttgart: E. Schweizerbart'sche Verlagsbuchhandlung.

Devi, S.R., and M.N.V. Prasad. 1998. Copper toxicity in *Ceratophyllum demersum* L. (Coontail), a free floating macrophyte: Response of antioxidant enzymes and antioxidants. *Plant Science* 138: 157–65.

Fernandes, J.C., and F.S. Henriques. 1991. Biochemical, physiological, and structural effects of excess copper in plants. *The Botanical Review* 57: 246–73.

Gustaffsson, J.P. 2010. Visual MINTEQ version 3.0, beta version. KTH, Department of Land and Water Resources Engineering. Sweden. http://www.lwr.kth.se/English/OurSoftware/vminteq/index.html (accessed November 1, 2010).

Juneau, P., A.E. Berdey, and R. Popovic. 2002. PAM Fluorometry in the determination of the sensitivity of *Chorella vulgaris*, *Selenastrum capricornutum*, and *Chlamydomonas reinhardtii* to copper. *Archives of Environmental Contamination and Toxicology* 42: 155–64.

Kaduková, J., and E. Virčíková. 2005. Comparison of differences between copper bioaccumulation and biosorption. *Environmental International* 31: 227–32.

Kruger, N.J. 1994. The Bradford method for protein quantitation. In *Methods in molecular biology: Basic protein and peptide protocols*, Vol. 32, ed. J.M. Walker, 9–15. New Jersey: Humana Press.

Kuhl, A., and H. Lorenzen. 1964. Handling and culturing *Chlorella*. In *Methods in cell physiology*, ed. D.M. Prescott, 158. New York: Academic Press.

Küpper, H., F. Küpper, and M. Spiller. 1998. *In situ* detection of heavy metal substituted chlorophylls in water plants. *Photosynthesis Research* 58: 123–33.

Küpper, H., I. Šetlik, M. Spiller, F.C. Küpper, and O. Prášil. 2002. Heavy metal-induced inhibition of photosynthesis: Targets of *in vivo* heavy metal chlorophyll formation. *Journal of Phycology* 38: 429–41.

Levy, J.L., B.M. Angel, J.L. Strauber, W.L. Poon, S.L. Simpson, S.H. Cheng, and D.F. Jolley. 2008. Uptake and internalisation of copper by three marine algae: Comparison of copper-sensitive and copper-tolerant species. *Aquatic Toxicology* 89: 82–93.

Levy, J.L., J.L. Stauber, and D.F. Jolley. 2007. Sensitivity of marine microalgae to copper: The effect of biotic factors on copper adsorption and toxicity. *Science of the Total Environment* 387: 141–54.

Lim, C.Y., Y.H. Yoo, M. Sidharthan, C.W. Ma, I.C. Bang, and J.M. Kim. 2006. Effect of copper (I) oxide on growth and biochemical compositions of two marine microalgae. *Journal of Environmental Biology* 27: 461–6.

Mallick, N. 2003. Copper-induced oxidative stress in the chlorophycean microalga *Chlorella vulgaris*: Response of the antioxidant system. *Journal of Plant Physiology* 161: 591–7.

Mallick, N., and L.C. Rai. 2002. Physiological responses of non-vascular plants to heavy metals. In *Physiology and biochemistry of metal toxicity and tolerance in plants*, eds. M.N.V. Prasad and K. Strzalka, 111–47. Netherlands: Kluwer Academic Publishers.

Markina, Zh.V., and N.A. Aizdaicher. 2006. Content of photosynthetic pigments, growth, and cell size of microalga *Phaeodactylum tricornutum* in the copper-polluted environment. *Russian Journal of Plant Physiology* 53: 305–9.

Masuko, T., A. Minami, N. Iwasaki, T. Majima, S. Nishimura, and Y.C. Lee. 2005. Carbohydrate analysis by a phenol–sulfuric acid method in microplate format. *Analytical Biochemistry* 339: 69–72.

Mei, L., X. Xitao, X. Renhao, and L. Zhili. 2006. Effects of strontium-induced stress on marine algae *Platymonas subcordiformis* (Chlorophyta: Volvocales). *Chinese Journal of Oceanology Limnology* 24: 154–62.

Mohammed, M.H., and B. Markert. 2006. Toxicity of heavy metals on *Scenedesmus quadricauda* (Turp.) de Brébisson in batch cultures. *Environmental Science and Pollution Research* 13: 98–104.

Nalimova, A.A., V.V. Popova, L.N. Tsoglin, and N.A. Pronina. 2005. The effect of copper and zinc on *Spirulina platensis* growth and heavy metal accumulation in its cells. *Russian Journal of Plant Physiology* 52: 229–34.

Neto, J.A., B.J. Smith, and J.J. Mc Allister. 2000. Heavy metal concentrations in surface sediments in a near shore environment, Jurujuba Sound, Southeast Brazil. *Environmental Pollution* 109: 1–9.

Nikookar, K., A. Moradshahi, and L. Hosseini. 2005. Physiological responses of *Dunaliella salina* and *Dunaliella tertiolecta* to copper toxicity. *Biomolecular Engineering* 22: 141–6.

Norberg-King, T.J. 1993. A linear interpolation method for sublethal toxicity: The inhibition concentration (ICp) approach (Version 2.0). Duluth: National Effluent Toxicity Assessment Center Technical Report 03-93, Environmental Research Laboratory.

Pawlik-Skowrońska, B., and T. Skowroński. 2001. Freshwater algae. In *Metals in the environment: Analysis by diversity*, ed. M.N.V. Prasad, 70. New York: Marcel Dekker.

Pearlmutter, N.L., and C.A. Lembi. 1986. The effect of copper on the green alga *Phitophora oedogonia*. *Weed Science* 34: 842–49.

Perales-Vela, H.G., S. González-Moreno, C. Montes-Horcasitas, and R.O. Cañizares-Villanueva. 2007. Growth, photosynthetic and respiratory responses to sub-lethal copper concentrations in *Scenedesmus incrassatulus* (Chlorophyceae). *Chemosphere* 67: 2274–81.

Pistocchi, R., F. Guerrini, V. Balboni, and L. Boni. 1997. Copper toxicity and carbohydrate production in the microalgae *Cylindrotheca fusiformis* and *Gymnodinium* sp. *European Journal of Phycology* 32: 125–32.

Sandmann, G., and P. Böger. 1980. Copper deficiency and toxicity in *Scenedesmus*. *Zeitschrift für Pflanzenphysiologie* 98: 53–9.

Stauber, J.L., and T.M. Florence. 1987. Mechanism of toxicity of ionic copper and copper complexes to algae. *Marine Biology* 94: 511–19.

Tripathi, B.N., and J.P. Gaur. 2006. Physiological behaviour of *Scenedesmus* sp. during exposure to elevated levels of Cu and Zn and after withdrawal of metal stress. *Protoplasma* 229: 1–9.

Wong, P.K., and L. Chang. 1991. Effects of copper, chromium and nickel on growth, photosynthesis and chlorophyll a synthesis of *Chlorella pyrenoidosa* 251. *Environmental Pollution* 72: 127–39.

Wright, D.A., and P. Welbourn. 2002. *Environmental toxicology*. Cambridge: Cambridge University Press.

Xu, S., X. Jiang, X. Wang, Y. Tan, C. Sun, J. Feng, L. Wang, S. Martens, and B.M. Gawlik. 2000. Persistent pollutants in sediments of the Yangtse river. *Bulletin of Environmental Contamination and Toxicology* 64: 176–83.

Yan, H., and G. Pan. 2002. Toxicity and bioaccumulation of copper in three green microalgal species. *Chemosphere* 49: 471–76.

Yilmaz, A.B., O. Işik, and S. Sayin. 2005. Bioaccumulation and toxicity of different copper concentrations in *Tetraselmis chuii*. *Journal of Fisheries and Aquatic Sciences* 22: 297–301.

Toxicological & Environmental Chemistry
Vol. 93, No. 9, October 2011, 1838–1850

Taylor & Francis
Taylor & Francis Group

Uptake, distribution, and bioaccumulation of copper in the freshwater mussel *Anodonta anatina*

Andhika Puspito Nugroho[ab] and Hartmut Frank[a]*

[a]*Environmental Chemistry and Ecotoxicology, University of Bayreuth, D-95440 Bayreuth, Germany;* [b]*Laboratory of Ecology, Faculty of Biology, Gadjah Mada University, Yogyakarta 55281, Indonesia*

(*Received 10 February 2011; final version received 18 April 2011*)

Copper (Cu) is present in aquatic ecosystems in dissolved form, associated with suspended food particles, and as insoluble sulfide in the sediment. Due to its wide technical use and its presence in municipal and industrial waste waters, levels in rivers and sediments may be elevated. The aims of this study are to assess the relative importance of copper uptake by a typical freshwater mussel (*Anodonta anatina*), its distribution, accumulation among the mussel organs, and elimination. Using the stable isotope ^{63}Cu as tracer, the mussels are exposed via the water (0.3 μmol L^{-1} Cu) or via the food (1.5 mg L^{-1} Cu-loaded algae, equivalent to 0.06 μmol L^{-1} Cu) for 24 days. The levels of exogenous and total Cu increase in all body compartments. Relative increases are highest in the digestive gland, followed by mantle and gills. Upon depuration for 12 days, ^{63}Cu is quickly but not completely eliminated.

Keywords: copper; *Anodonta anatina*; bioaccumulation; elimination; water; freshwater bivalve

Introduction

Metals of technical importance are found in the environment at increasing concentrations resulting from mining and metallurgic activities, due to their wide technical use, or due to emissions from corrosion and from fossil fuel combustion. Therefore, many metals show the tendency to increase over natural levels and to accumulate in soil and aquatic compartments, especially in river sediments. Copper (Cu) is one of them; due to its use as fungicide, in the building sector as roofing material, for overland high-voltage power lines, and many other electrotechnical applications it may be present in municipal and industrial waste waters. Apparently, Cu is also spread in the environment as nonpoint source pollutant and can attain elevated levels in declining freshwater pearl mussels (Frank and Gerstmann 2007), sedentary animals living at the interface of free-flowing water and sediments of mountain streams. In water, copper can exist in dissolved form or associated with dissolved organic carbon and with suspended food particles (Vinot and Pihan 2005). In sediments it may be present as insoluble sulfide or dissolved in the interstitial water (Besser, Ingersoll, and Giesty 1996). Copper is essential to mussels up to 10 μmol kg^{-1} (0.6 mg kg^{-1}) body weight (Julshamn et al. 2001), as part of the oxygen-binding site in

*Corresponding author. Email: hartmut.frank@uni-bayreuth.de

ISSN 0277–2248 print/ISSN 1029–0486 online

http://dx.doi.org/10.1080/02772248.2011.582989
http://www.tandfonline.com

hemocyanin (Birge and Black 1979) and as cofactor of the prosthetic groups of enzymes such as of cytochrome-c oxidase, tyrosinase, dopamine β-hydroxylase, alcohol dehydrogenase, prolyl and lysyl oxidase, or others involved in growth regulation and development (Amiard-Triquet et al. 2006; Company et al. 2008). At higher concentrations, Cu is toxic to mussels, resulting in altered calcium (Ca) homeostasis of blood cells (Viarengo et al. 1994); the 96-hour LC_{50} for mollusks ranges between 6 and 30 $\mu mol\,L^{-1}$ (0.4–2 $mg\,L^{-1}$) (Crompton 1998).

Copper can be taken up by freshwater mussels with the water or the food. The route of uptake influences the distribution of the metal in the various organs, determines the dynamics of Cu-bioaccumulation and elimination, and has consequences on the pathophysiology of copper in the mussels (Croteau and Luoma 2005). In this work, duck mussels (*Anodonta anatina*) are used as model species to study the toxicological relevance of copper uptake via both pathways. The stable isotope ^{63}Cu is used as tracer to follow its distribution within the mussel and its elimination upon depuration.

Materials and methods

Algal food preparation

Algae (*Parachlorella kessleri*) are used as food for the mussels and, when grown at a ^{63}Cu-concentration of 5.9 $\mu mol\,L^{-1}$ (Nugroho and Frank 2010), for one experimental group as Cu-exposure source. Algae are grown in modified K-medium (Kuhl and Lorenzen 1964) for 7 days to produce normal or copper-loaded algae. Freeze-dried normal and copper-loaded algae contain 0.01 $mmol\,kg^{-1}$ Cu (0.6 $mg\,kg^{-1}$ Cu) and 40 $mmol\,kg^{-1}$ Cu (2.4 $mg\,kg^{-1}$ Cu) dry weight (dw).

Isotopic Cu stock solution preparation and labware

A ^{63}Cu stock solution (3.1 mmol L^{-1}, equivalent to 200 $mg\,L^{-1}$) is prepared by dissolving 25 mg isotopically enriched (99%) ^{63}Cu oxide (Euriso-top, Saarbrücken, Germany) in 1 mL suprapur HNO_3 (69%, Carl Roth, Karlsruhe, Germany) in a 100 mL glass beaker; 85 mL bidistilled water are added, and the pH of the solution is adjusted to 7.0 with aqueous ammonia (25%, VWR, Darmstadt, Germany). The solution is transferred to a 100 mL polypropylene (PP) volumetric flask which is filled to the mark with bidistilled water. Glassware and plastic equipments used for analytical purposes are rinsed twice with half-concentrated HNO_3 (65%; Sigma-Aldrich, Munich, Germany), and deionized and bidistilled water.

Organisms

About 70 duck mussels (*A. anatina*) (ZOO-Erlebnis Online Shop, Grossefehn, Germany) with shell lengths of 10–12 cm and weights of 100–200 g are brought to the laboratory in pond water. The mussels are brushed with dilute $KMnO_4$ solution (0.1 $mg\,L^{-1}$), rinsed with tap water, and placed in 38 L aerated tap water in 45 -L glass aquaria at dim light for 7 days. During this period they are not fed; every day, half of the water is exchanged. Then the mussels are marked, weighed, and the shell lengths are measured. They are fed with freeze-dried Cu-free algae, 1.0 $mg\,L^{-1}$ per day, and acclimatized for further 7 days to laboratory conditions at a temperature of $17 \pm 1^{\circ}C$ with a photoperiod of 12 h light per day, a photon flux of 13–19 $\mu mol\,m^{-2}\,s^{-1}$, in 38 L artificial pond water (APW) at pH 7.0 ± 0.3

(Ngo, Gerstmann, and Frank 2011) in 45-L glass aquaria covered with transparent polypropylene lids. The aquaria are equipped with inner bio-filters and stainless steel aeration tubes. Eight kilograms glass beads are used as substrate. Two-third of the water is exchanged every two days; a complete change is conducted on every sixth day.

Experimental design

Of these mussels, 63 are selected to match in size and divided into three groups consisting of 21 mussels each. They are placed in three 45 L aquaria containing 38 L artificial pond water (APW). Two-third of the water is exchanged every second day; a complete change is conducted on every sixth days. A control group (1) is kept in APW. Another group (2) is exposed to 0.3 μmol L^{-1} (20 μg L^{-1}) ^{63}Cu in the water using the ^{63}Cu stock solution; after each water change, the concentration is re-adjusted by adding appropriate volumes of the stock solution. A third group (3) receives daily 1.5 mg L^{-1} freeze-dried ^{63}Cu-loaded algae for 24 days, equivalent to a nominal copper concentration of 0.06 μmol L^{-1} (3.6 μg L^{-1}).

The mussels in the control and the exposure groups are fed with algae in amounts adjusted to their actual number. For 18 mussels, 1.5 mg L^{-1} of freeze-dried Cu-free (groups 1 and 2) or ^{63}Cu-loaded algae (group 3) are given per day. When the number of mussels is less than 18, 1.0 mg L^{-1} of freeze-dried Cu-free or ^{63}Cu-loaded algae are given daily (Ngo, Gerstmann, and Frank 2011) corresponding to a nominal concentration of 0.04 μmol L^{-1} (group 3). On day 24, the six mussels remaining in each group are transferred to APW-filled aquaria for 12 days of depuration, fed with 1.0 mg L^{-1} of freeze-dried Cu-free algae per day.

Actual Cu concentrations in the water including the suspended algae in each group are determined every second day. On the control group, Cu concentrations in the APW during experiment are below detection limit. For the experiment involving Cu exposure via the water, after exchange of water the concentration is adjusted to 0.32 ± 0.006 μmol L^{-1}, which falls to 0.03 ± 0.01 μmol L^{-1} within the next 2 days. By the food pathway (group 3), the nominal Cu concentration in the beginning and after each water exchange is 0.07 ± 0.01 μmol L^{-1}, falling to below detection limit within the next 2 days.

For sampling, three mussels of each group are taken for analysis at days 0, 6, 12, 18, and 24 (exposure), and at days 30 and 36 (depuration). The mussels are anaesthetized with an aqueous 2-phenoxyethanol solution (4 mL L^{-1}) for 30 min. Hemolymph (HML) and extrapallial fluid (EPF) are withdrawn using 5 mL syringes with 0.55×25 mm needles (B. Braun, Melsungen, Germany), transferred into 2-mL microtubes, and kept at −80°C. The mussels are dissected on ice into gills, mantle, kidney, digestive gland, foot, adductors, and intestines; the remainder is collected in a combined sample (GHL), i.e., gonads, heart, and labial palps. The tissues are washed twice with bidistilled water, dried using filter paper, placed in 15 mL polypropylene (PP) tubes of known weights, weighed to obtain the wet weights (ww), and lyophilized. After lyophilization, the tubes are weighed again for dry weights (dw). Tissue fractions and body fluids of the nine mussels taken at day 0 are used to calculate the respective percentages relative to the total weight of soft body (twsb).

Metal analyses

Each lyophilized tissue fraction of about 10–100 mg is placed in a 55 mL borosilicate glass tubes. 5 mL of a mixture (4 + 1) of suprapure concentrated HNO_3

(65%, Merck, Darmstadt, Germany) and suprapure concentrated HCl (30%, Merck, Darmstadt, Germany) are added to each tube. The tubes are kept in an oven at 40°C for 1 h and at 95°C for 3 h. The digested samples are diluted with bidistilled water to 10 mL and filtered through 0.45 µm cellulose syringe filters (Carl Roth, Karlsruhe, Germany). For the determination of Cu in HML and EPF, 0.4–1 mL of each are acidified with 0.5 mL suprapure concentrated HNO_3 in PP tubes, diluted to 10 mL with bidistilled water, and filtered through 0.45 µm cellulose syringe filters. Total Cu and its isotopes ^{63}Cu and ^{65}Cu are determined by inductively-coupled plasma mass spectrometry (ICP-MS, Agilent 7500ce, Cetac ASX-510, Agilent Technologies, Waldbronn, Germany). The detection limits for total Cu is 0.02 µmol L^{-1} for isotopic Cu 0.01 µmol L^{-1}.

Total copper in each tissue fraction is calculated in µmol kg^{-1} ww by multiplying the analytical data with the ratio of ww versus dw. The concentration of exogenous copper C_{63Cu} is calculated as $C_{63Cu} - 2.34 \times C_{65Cu}$, the concentration of endogenous copper as $3.33 \times C_{65Cu}$, considering the natural relative abundances of 69% ^{63}Cu and 31% ^{65}Cu. For body fluids, the Cu concentrations are given in µmol L^{-1}. Total and exogenous Cu-pools in the tissue fractions and body fluids are calculated in mmol kg^{-1} twsb by multiplying the concentration data with the weight fraction of the respective organ or body fluid.

Statistical data analyses

Data are transformed to log units before statistical analysis for homogeneity of variance and normality. The data for total Cu are statistically evaluated by two-way analysis of variance (ANOVA) considering exposure time and Cu exposure pathways as independent variables; if significant differences are found, those between exposure times are tested by the Dunnett multiple comparison tests, between exposure pathways and controls using the Duncan multiple comparison tests. To assess the differences in exogenous Cu between exposure pathways, the independent *t*-test is performed.

Results

Exposure of *A. anatina* to Cu via the water results in rapid increases (Figure 1) of the concentrations of total Cu (solid lines) in the hemolymph (HML) and the extrapallial fluid (EPF) within the first 12 days, followed by slower increases until concentrations of 0.38 µmol L^{-1} are reached at day 24, about the 2.5-fold of control level. From the food, increases are more moderate, reaching about 0.25 µmol L^{-1}, the 1.7-fold of controls. In respect to exogenous Cu, the concentrations in both body fluids (dotted lines) increase similarly upon exposure via the water and the food within the first 6 days although the nominal concentration per liter water volume in food is considerably lower. Later on, exposure via the water entails faster uptake, especially in the HML between days 6–12 to reach 0.14 µmol L^{-1}, continuing until 0.17 µmol L^{-1} at day 24. Overall, increases during the first days are faster for the EPF than for the HML.

Within the 12 days of depuration, total Cu concentrations decline rapidly in EPF and in HML, in HML of animals having received the metal by the water pathway to about 50% over control; when having been exposed via the food, the Cu concentrations decline almost fully back to control values. For exogenous Cu, the concentrations in the HML and EPF of water- and food-exposed animals decline in similar relative rates. At the end of the depuration, the fraction of exogenous Cu, i.e., the excess of ^{63}Cu over the natural

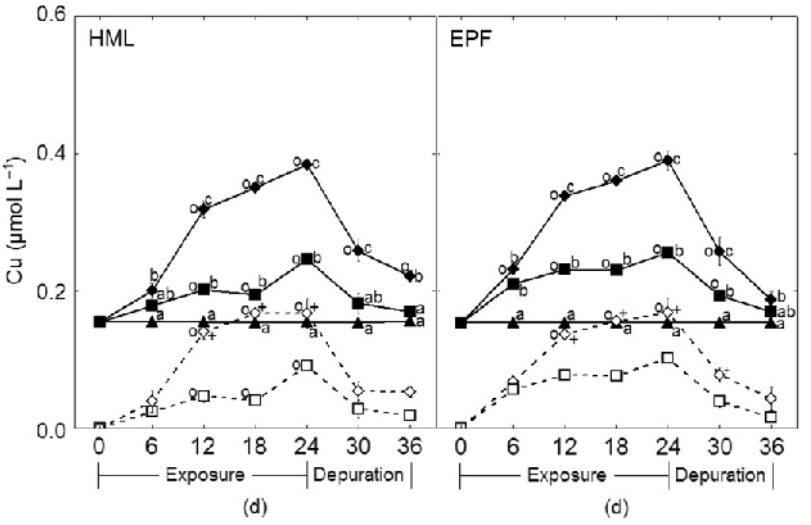

Figure 1. Concentrations of total (solid lines; ♦ = via water, ■ = via food; ▲ = control) and exogenous (dotted lines; ◊ = via water, □ = via food) Cu in hemolymph (HML) and extrapallial fluid (EPF) of *A. anatina* during exposure (days (d) 0–24) and depuration (days 24–36). Significant differences in comparison to control within each group are indicated by °. The same letters indicate that differences of Cu concentrations are not significant among groups at each time sampling (day) while the different letter indicate $p<0.05$. Significant differences between concentrations of exogenous Cu via food or water are indicated by $^{+}$.

abundance of this isotope, represents between 10% (food pathway) and up to 30% (water pathway) of total copper.

In the organ and tissue fractions (Figures 2 and 3), concentrations of endogenous Cu at day 0 are highest in the kidney and the digestive gland (63 and 58 µmol kg^{-1} ww). In the other organs, initial Cu levels are much lower, i.e., in mantle, intestines (both 15 µmol kg^{-1} ww), gills, and foot (both 12 µmol kg^{-1} ww). The mixed fraction of the gonads, heart, and labial palps (GHL) (20 µmol kg^{-1} ww) shows a fairly high initial copper level although nothing can be said about the distribution between the tissues contained in it. The adductors have the lowest concentration (7 µmol kg^{-1} ww), but this is still much higher than in HML and EPF (Figure 1, 0.17 µmol L^{-1}).

The development of the total copper concentrations is quite diverse for the various tissues/organs over time (Figures 2 and 3; solid lines) in relative and absolute terms. Upon uptake via the water, strongest relative increases are seen for the gills, the mantle, and the digestive gland, especially within the first 6 days. When ^{63}Cu is administered via the food, an almost equal increase of ^{63}Cu as via water is found for the digestive gland, although its nominal initial concentration is only a fifth of the concentration in the water in dissolved form. For other organs, uptake from food leads to moderate rise in the mantle, kidney, intestines, and GHL, almost none in the gills, adductors, and foot. In the digestive gland, highest concentrations, i.e., 120–140 µmol kg^{-1}, are reached within 24 days irrespective of exposure pathway. For other organs, exposure via water results in peak concentrations in the gills of 75 µmol kg^{-1} (6.5–fold relative to control), 70 µmol kg^{-1} in the mantle (4.2-fold), and 70 µmol kg^{-1} in the mixed fraction GHL (3.5–fold); moderate to low relative increases are seen in the foot, intestines, adductors, and kidney (2.8-, 1.7-, 1.6-, and 1.4-fold). Upon depuration, Cu concentrations fall immediately and strongly in most

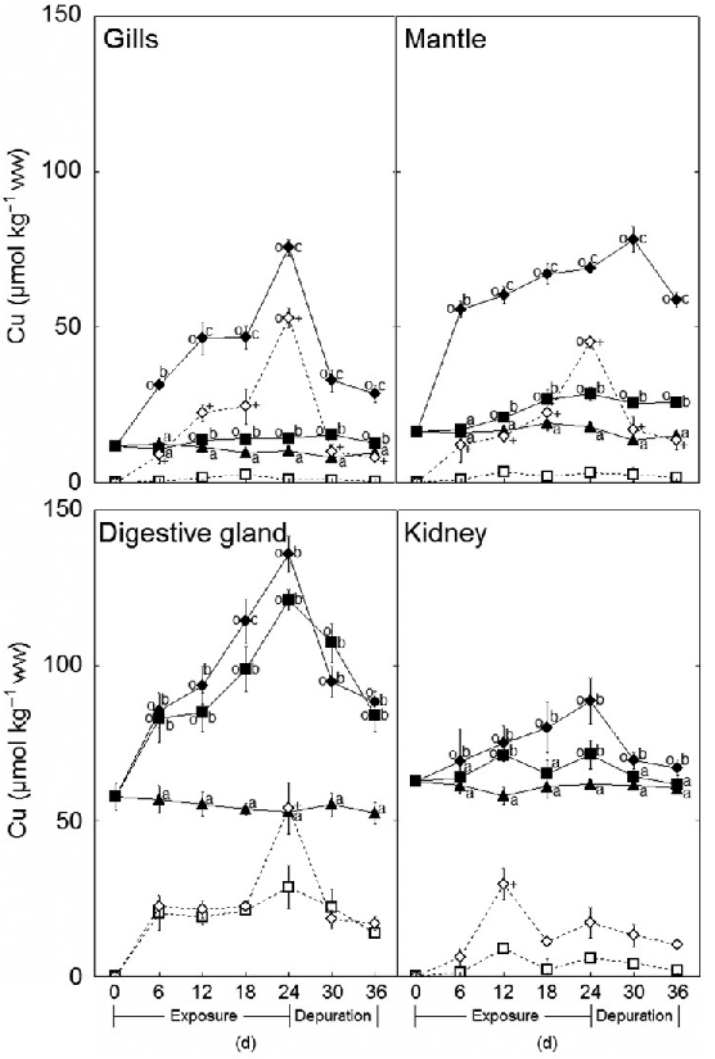

Figure 2. Concentrations of total (solid lines; ♦ = via water, ■ = via food; ▲ = control) and exogenous (dotted lines; ◊ = via water, □ = via food) Cu in the gills, mantle, digestive gland, and kidney of *A. anatina* during Cu exposure via water and food and during depuration. Significant differences in comparison to control within each group are indicated by °. The same letters indicate that differences of Cu concentrations are not significant among groups at each time sampling (day (d)) while the different letter indicate $p < 0.05$. Significant differences between concentrations of exogenous Cu via food or water are indicated by $^{+}$. Total and exogenous Cu are calculated by multiplication of the analytical data with the ratio of dry weight versus wet weight.

organs, except for the mantle and the intestines; for these even further increases are observed within the first 6 days of depuration.

In respect to exogenous ^{63}Cu (Figures 2 and 3; dotted lines), exposure to ^{63}Cu via water leads to rapid increases in the gills, mantle, digestive gland, and GHL within the first 6 days. In some organs, i.e., digestive gland, gills, and mantle, the increases continue until day 24 to reach a maxima of about 50 µmol kg^{-1} ww. Exogenous copper in the kidney,

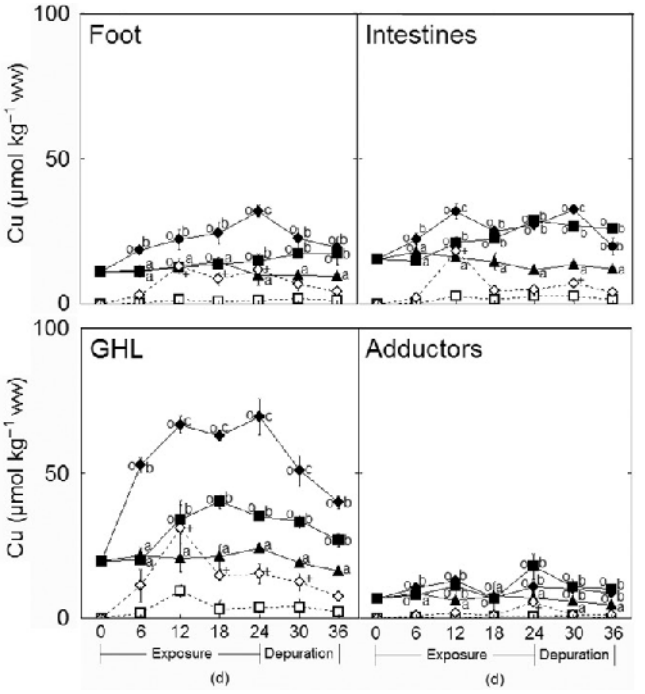

Figure 3. Concentrations of total (solid lines; ♦ = via water, ■ = via food; ▲ = control) and exogenous (dotted lines; ◊ = via water, □ = via food) Cu in the GHL (gonads, heart, and labial palps), intestines, foot, and adductors of *A. anatina* during Cu exposure via water and food and during depuration. Significant differences in comparison to control within each group are indicated by °. The same letters indicate that differences of Cu concentrations are not significant among groups at each time sampling (day (d)) while the different letter indicate $P<0.05$. Significant differences between concentrations of exogenous Cu via food or water are indicated by $^{+}$. Total and exogenous Cu are calculated by multiplication of the analytical data with the ratio of dry weight versus wet weight.

foot, intestines, and GHL shows maximum concentrations at day 12, followed by declines until the end of exposure. Via the food, exogenous ^{63}Cu initially increases in the digestive gland as fast as via the water, followed by slight further increase to reach a maximum of 20 µmol kg^{-1} ww on day 24. In the gills, mantle, adductors, and foot, after slight increases during the first 12 days of exposure, exogenous Cu remains relatively unchanged until the end of the experiment.

During depuration, in the gills, mantle, and digestive gland, the levels of exogenous copper drops within the first 6 days by 85, 70, and 60%. For animals having received the metal via food, similar patterns of decrease are observed for the kidney and GHL, only the levels being lower, i.e. about a third.

The isotope ratios of ^{63}Cu/^{65}Cu and its deviation from the natural ratio (2.33) are also monitored (Figure 4). Complementary to Figures 2 and 3, this allows to follow the movement of exogenous Cu within the body. When ^{63}Cu is administered via water, the relative abundance of ^{63}Cu in the body fluids HML and EPF rise up to 4.0 at day 12, then remain constant. Upon depuration, the ratio declines to about 3.0. In the kidney, GHL, foot, and intestines, peaks of ^{63}Cu are reached at day 12, while in the adductors, digestive gland, mantle, and gills, maximum isotope ratios are found at the end of exposure at day 24. During depuration, the relative abundance of ^{63}Cu in all organs declines, but not

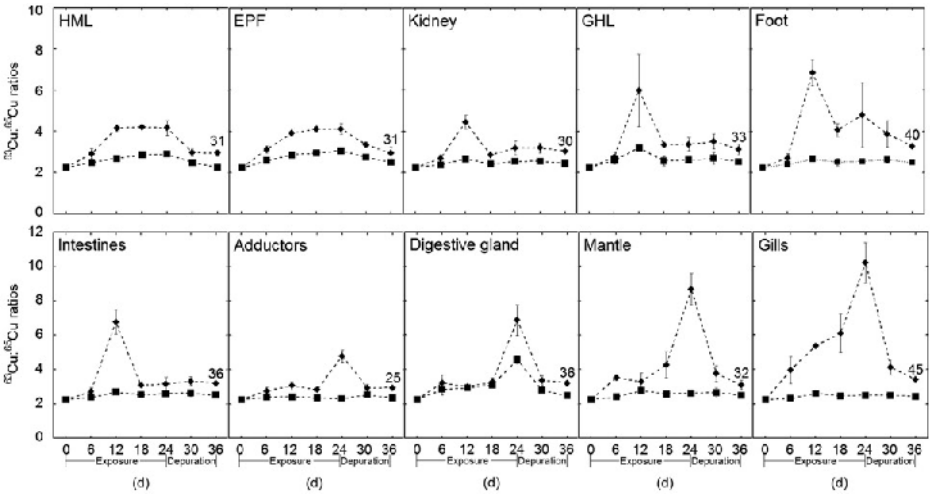

Figure 4. Isotope ratio of $^{63}Cu/^{65}Cu$ in organs and body fluids of *A. anatina* during exposure to ^{63}Cu via water (♦) or food (■), and following depuration (GHL = gonads, heart, and labial palps). The relative increases of ^{63}Cu in percent over the natural ratio upon exposure via water are given for day (d) 36.

totally back to the natural ratio remaining about 25–45% higher than before exposure. When ^{63}Cu is administered via food, the increase in the isotope ratio is pronounced for the digestive gland, while all the organs show only small increases.

Calculating the Cu-pools in the body compartments gives interesting insights (Figure 5). Although HML and EPF together constitute about 70% ($33 \pm 5\%$ and $37 \pm 4\%$) of the total soft body volume (Figure 5, A), both are insignificant as Cu-pools. The mantle, the gills, and the intestines are the largest solid organs; together they represent about 18% (6.4 ± 0.5, 5.9 ± 0.6, and $5.4 \pm 0.8\%$) twsb. Smaller body fractions are the adductors ($2.9 \pm 0.2\%$ twsb), the digestive gland ($2.8 \pm 0.3\%$ twsb), the foot ($2.2 \pm 0.4\%$ twsb), the mixed fraction GHL ($3.9 \pm 0.6\%$ twsb), and the kidney ($0.5 \pm 0.06\%$ twsb). In the beginning (Figure 5, B), the total pool of Cu (endogenous Cu) is about 6 μmol kg^{-1} twsb, the largest being in the digestive gland, followed by the mantle, gills, intestines, and GHL (Figure 5, B). Upon exposure via water (W), the total Cu-pools increases, continuing until day 24 to reach a maximum of 25 μmol kg^{-1} twsb, i.e., the four-fold of the initial pool size; uptake via the food (F) entails a total Cu-pool of only 9 μmol kg^{-1} twsb at day 24, i.e., slightly less than double the control, the largest pool being in the digestive gland. The exogenous ^{63}Cu-pool increases in parallel to total Cu-pool upon exposure via the water, reaching a maximum of about 12 μmol kg^{-1} twsb at day 24. For the food pathway, it increases only slightly, the maximum level being at about 1.5 μmol kg^{-1} twsb (Figure 5, C). During the 12 days of depuration, all the pools are rapidly emptied, particularly the gills. The mantle and the digestive gland retain the Cu-pools relatively long (as also reflected in Figures 2 and 3), in the latter most tenaciously. Similar patterns are found for exogenous Cu.

Discussion

The experiments show that ^{63}Cu is highly available to *A. anatina* (Figures 2 and 3, a and b), both in water-dissolved form or from ^{63}Cu-loaded algae. Calculation of Cu speciation

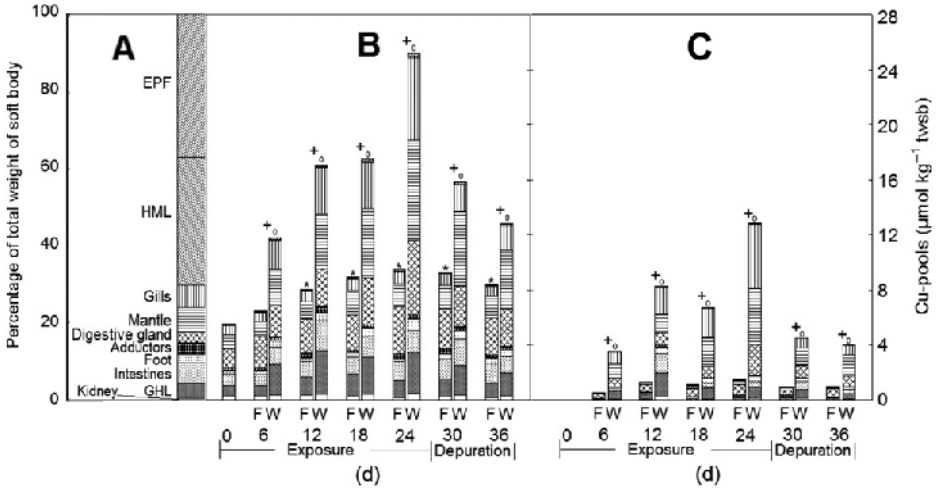

Figure 5. A: Percentages of total weight of soft body (twsb) (EPF = extrapallial fluid; HML = hemolymph; GHL = gonads, heart, and labial palps), and B: total and C: exogenous Cu-pools (right ordinate) in *A. anatina* during Cu exposure via food (F) or water (W) and during depuration. Significant differences in comparison to control (day (d) 0) within each group (water = °; food = *), and between Cu exposure via food and water are indicated by $^{+}$. The total Cu-pools in the body fractions are calculated by multiplication of the concentration data (Figure 2) with the respective percentages.

in artificial pond water (APW) at a pH of 7.0 and at 17°C shows that the metal is completely in the free Cu^{2+} ionic form, ready for uptake (Gustaffsson 2010). By the food pathway, the low nominal Cu concentration in the APW may be the main factor responsible for the low Cu accumulation in the mussel in absolute terms, but in relative terms it is obviously even more efficient.

During the 24 days of water-borne Cu exposure, exogenous ^{63}Cu levels in the organs increase differently, strongest in the gills to represent about 70% of total Cu (Figures 2 and 3, a and b). There is evidence of mobilization and re-distribution of endogenous Cu among the organs derived from the time pattern of the $^{63}Cu/^{65}Cu$ isotope ratio (Figure 4). The ratios are highest in the gills, mantle, and digestive gland (both pathways) at the sixth day of exposure, showing that the exogenous ^{63}Cu is initially taken up into these organs. The peaks of highest ratios at day 12 in the kidney, GHL, foot, and intestines indicate that these organs first receive fairly high amounts of exogenous Cu but – as the exposure continues – become recipients of endogenous copper mobilized from the other organs, presumably mobilized by exogenous ^{63}Cu. Later, exogenous and mobilized endogenous Cu is mainly stored in the digestive gland, gills, adductors, and mantle, the latter serving as transient recipient even beyond the exposure phase. The observation of copper being particularly strongly retained in the mantle is noteworthy as it is one of the most important organs for regulating the calcium household and for building the protective shell of the bivalve (Lopes-Lima et al. 2008).

In the body fluids, the isotope ratio remains relatively constant at about 4.0 during days 12–24, reflecting the roles of HML and EPF as transitory exchange and transport compartments, being small as pools (Figure 5). Upon depuration the isotope ratios tend to fall strongly, indicating that a large fraction of exogenous ^{63}Cu remains in a relatively easily exchangeable form while the endogenous Cu is more tenaciously retained.

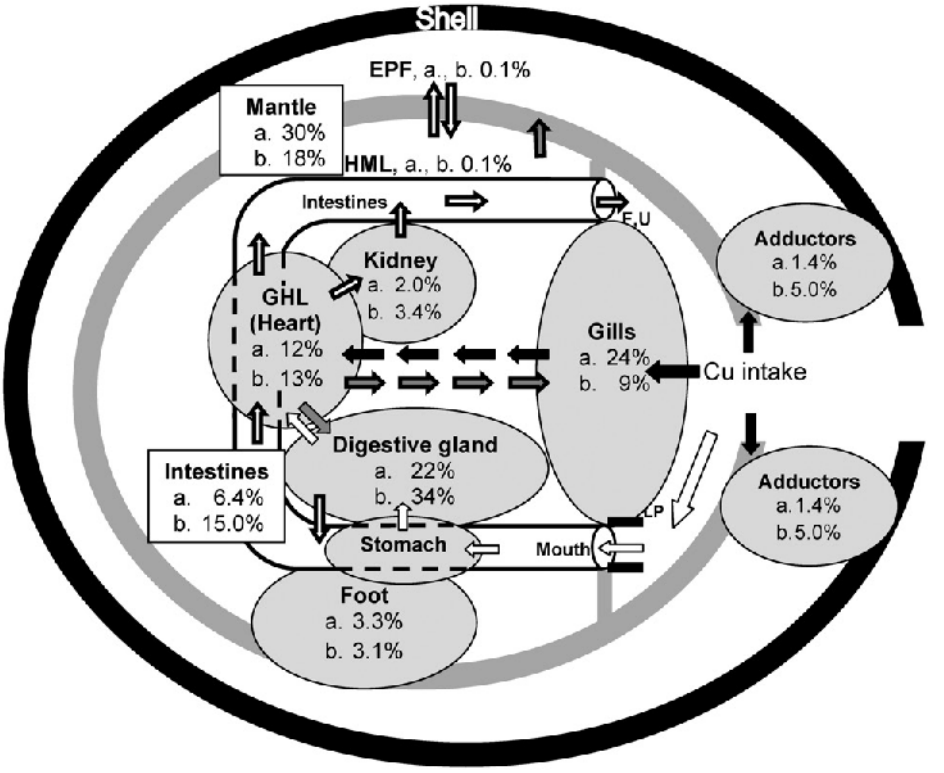

Figure 6. Scheme illustrating the distribution of the total Cu burden (Cu-pools) among the body fractions (EPF = extrapallial fluid; HML = hemolymph; GHL = gonads, heart, and labial palps; LP = labial palps; F = feces; U = urine) of *A. anatina* after exposure to Cu via water (a. black arrow (routes to the heart) and grey arrow (routes from the heart to the other organs) or food (b. white arrow (routes to the heart) and grey/heavy-lined (routes from the heart to the other organs)) (per kg of total weight of soft body). White/heavy-lined arrows show the routes of both Cu exposure pathways. The figure is adopted from Marigómez et al. (2002).

Nevertheless, at the end of the depuration, the relative abundance of ^{63}Cu taken up via the water pathway is between 25% and 45% higher than in the beginning in various body compartments, indicating that about a third of the functional Cu pool has been exchanged for exogenous ^{63}Cu.

Distribution of Cu in the mussel's body allows to assess the relative importance of the various copper pools (Figure 6). From the water it is mainly compartmentalized into the mantle (30%), the gills (24%), and the digestive gland (22%), altogether three quarters of the total Cu-pool. The former two organs have large surface areas and interact directly with the water coming into mantle cavity during filtration (Marigómez et al. 2002); the digestive gland is the major receiving organ for the hemolymph pathway. The mantle has a high secretor epithelium lined with acid mucopolysaccharides for digestion of trapped small particles (Machado 2011). By the food pathway, the digestive gland and the intestines are the major Cu-recipients. In addition, the former organ secretes high amount of digestive mucus to facilitate Cu storage (Machado 2011). High Cu levels in GHL suggest a role of the heart as ion recipient and its close anatomical relation to the intestines and the kidney (Gosling 2003; Machado 2011).

In relation to the organ pools of *A. anatina*, the size of the respective volumes is not directly related to Cu burden (Figure 5, A, B, and C). Binding to specific compounds and compartmentalization within the organs, and physiological and metabolic functions of the organs may play some roles (Otchere 2003). In any case, the organs which serve as the primary sites for uptake, i.e., gills, mantle, and digestive gland, tend to concentrate the copper.

During depuration, Cu is eliminated fairly fast from the body (Figures 2 and 3, a and b), due to the large differences in gradient Cu concentration between the mussel and APW. Han et al. (1993) reported that the initial rapid elimination can be caused by desorption of loosely bound, unassimilated copper, whereas slower elimination reflects the loss from pools (endogenous Cu) where copper is more tightly bound to tissue components. Rapid elimination is also observed in the gills and digestive gland of the marine clam *R. decussatus* within the first 10 days of depuration (Serafim and Bebianno 2009). In respect to Cu elimination from the digestive gland, the level in *A. anatina* drops to about 60% over control within 6 days (Figures 2 and 3, a). A similar pattern is observed in the marine mussels *Mytilus galloprovincialis* exposed to Cu at 0.63 μmol L^{-1} (40 μg L^{-1}) via water for 3 days (Viarengo et al. 1981). This confirms that the digestive gland is the main organ for metal elimination in bivalves (Marigómez et al. 2002). According to Marigómez et al. (2002), the release of metals from mussel body can occur via the digestive tract as a component of feces or via the kidney together with excretory concretions as a component of urine (Figure 6).

Copper accumulation in *A. anatina* during exposure via water or food represents two different processes, i.e., bioconcentration (water) and biomagnification (food). Calculation of the bioconcentration and biomagnification levels allows to assess the relative importance of exposure via water or food. Bioconcentration can be expressed as enrichment factor (EF), i.e., the ratio of the concentration of exogenous Cu kg^{-1} twsb (Figure 5, C) to the concentration in the water. Biomagnification is normally assessed as transfer factor (TF). The enrichment factor in the mussel at the end of the exposure (day 24) is about 43, by the food pathway a TF of 25 is reached (Cu concentration in the APW-added algal food is equivalent to 0.06 μmol L^{-1}, exogenous Cu-pools in the mussel = 1.5 μmol kg^{-1} twsb; Figure 5, C). Thus, exposure via water is more effective from this point of view. In respect to biomagnification, the TF is lower than the EF for algae which is about 400-fold (Nugroho and Frank 2010), indicating only weak biomagnification of copper along the food chain from the algae to the mussel. Overall, distribution and accumulation of copper in *A. anatina* are the results of exposure time, exposure pathways, and physiological functions of the respective organs. Food uptake is more efficient taking the five-fold lower nominal concentration of copper in these experiments into consideration.

These experiments will help understand the risks associated with copper exposure of freshwater mussels. Copper accumulation may promote the situation of metabolic acidosis leading to the dissolution of $CaCO_3$ deposits, inducing the increase of Ca concentration in the EPF (Antunes et al. 2002; Faubel et al. 2008; Lopes-Lima et al. 2008). Interference with Ca homeostasis by the inhibition of Ca-ATPase by Cu (Santini et al. 2011) may lead to physiological stress. These factors together with the involvement of copper in the formation of reactive oxygen species (Company et al. 2008) may be a contributory factor in the overall Europe-wide observed decline of freshwater bivalves.

Conclusions

Exposure of *A. anatina* to Cu via the water or via the food leads to enrichment of the transition metal in the mussel. Copper is mainly stored in the digestive gland, gills,

and mantle. The digestive gland and the kidney are the main organs for accumulation and elimination, although the latter represents only a small Cu-pool. Distribution and accumulation of copper are the results of exposure time, exposure pathways, and physiological functions of organ. Upon depuration, *A. anatina* eliminates Cu from the body quickly but not completely. Elevated exposure to the transition metal is suggested to be a main stress factor responsible for reduced viability of fresh water mussel populations.

Acknowledgments

We thank Dr Silke Gerstmann for helpful discussion. Financial support by Directorate General of Higher Education, Department of National Education of the Republic of Indonesia, is highly appreciated.

References

Amiard-Triquet, C., B. Berthet, L. Joux, and H. Perrein-Ettajani. 2006. Significance of physico-chemical forms of storage in microalgae in predicting copper transfer to filter-feeding oysters (*Crassostrea gigas*). *Environmental Toxicology* 21: 1–7.

Antunes, C., T. Magalhães-Cardoso, G. Moura, D. Gonçalves, and J. Machado. 2002. Effects of Al, Ni, Co, Zn, Cd, and Cu metals on the outer mantle epithelium of *Anodonta cygnea* (Unionidae). *Haliotis* 31: 71–84.

Besser, J.M., C.G. Ingersoll, and J.P. Giesty. 1996. Effects of spatial and temporal variation of acid-volatile sulfide on the bioavailability of copper and zinc in freshwater sediments. *Environmental Toxicology and Chemistry* 15: 286–93.

Birge, W.J., and J.A. Black. 1979. Effects of copper on embryonic and juvenile stages of aquatic animals. In *Copper in the environment. Part II. Health effects*, ed. J.O. Nriagu, 373–99. New York: John Wiley & Sons.

Company, R., A. Serafim, R.P. Cosson, A. Fiala-Médioni, L. Camus, A. Colaço, R. Serrão-Santos, and M.J. Bebianno. 2008. Antioxidant biochemical responses to long-term copper exposure in *Bathymodiolus azoricus* from Menez-Gwen hydrothermal vent. *Science of the Total Environment* 389: 407–17.

Crompton, T.R. 1998. *Toxicants in the aqueous ecosystem*. London: John Wiley & Sons Ltd.

Croteau, M.-N., and S.N. Luoma. 2005. Delineating copper accumulation pathways for the freshwater bivalve *Corbicula* using stable copper isotopes. *Environmental Toxicology and Chemistry* 24: 2871–8.

Faubel, D., M. Lopes-Lima, S. Freitas, L. Pereira, J. Andrade, A. Checa, H. Frank, T. Matsuda, and J. Machado. 2008. Effects of Cd^{2+} on the calcium metabolism and shell mineralization of bivalve *Anodonta cygnea*. *Marine and Freshwater Behaviour and Physiology* 41: 93–108.

Frank, H., and S. Gerstmann. 2007. Declining populations of freshwater pearl mussels (*Margaritifera margaritifera*) are burdened with heavy metals and DDT/DDE. *Ambio* 36: 571–4.

Gosling, E. 2003. Circulation, respiration, excretion and osmoregulation. In *Bivalve molluscs: Biology, ecology and culture*, ed. E. Gosling, 201–24. Oxford: Fishing News Books.

Gustaffsson, J.P. 2010. Visual MINTEQ version 3.0, beta version. KTH, Department of Land and Water Resources Engineering. Sweden. http://www.lwr.kth.se/English/OurSoftware/vminteq/download.html

Han, B.-C., W.-L. Jeng, Y.-N. Tsai, and M.-S. Jeng. 1993. Depuration of copper and zinc by green oysters and blue mussels of Taiwan. *Environmental Pollution* 82: 93–7.

Julshamn, K., E.K. Torpe, C. Børnes, L.J. Sæthre, and A. Maage. 2001. Cadmium, lead, copper and zinc in blue mussels (*Mytilus edulis*) sampled in the Hardangerfjord, Norway. *Journal of Environmental Monitoring* 3: 539–42.

Kuhl, A., and H. Lorenzen. 1964. Handling and culturing *Chlorella*. In *Methods in cell physiology*, ed. D.M. Prescott, 158. New York: Academic Press.

Lopes-Lima, M., R. Bleher, T. Forg, M. Hafner, and J. Machado. 2008. Studies on a PCMA-like protein in the outer mantle epithelium of *Anodonta cygnea*: Insights on calcium transcellular dynamics. *Journal of Comparative Physiology B* 178: 17–25.

Machado, J. 2011. Personal communication.

Marigómez, I., M. Soto, M.P. Cajaraville, E. Angulo, and L. Giamberini. 2002. Cellular and subcellular distribution of metals in molluscs. *Microscopy Research and Technique* 56: 358–92.

Ngo, H.T.T., S. Gerstmann, and H. Frank. 2011. Subchronic effects of environment-like cadmium levels on the bivalve *Anodonta anatina* (Linnaeus 1758): I. Bioaccumulation, distribution and effects on calcium metabolism. *Toxicological and Environmental Chemistry* (in press).

Nugroho, A.P., and H. Frank. 2010. Producing Cu-loaded algae for feeding experiments: Effects of copper on *Parachlorella kessleri*. *Toxicological and Environmental Chemistry* 93: 537–48.

Otchere, F.A. 2003. Heavy metals concentrations and burden in the bivalves (*Anadara (Senilia) senilis*, *Crassostrea tulipa* and *Perna perna*) from lagoons in Ghana: Model to describe mechanism of accumulation/excretion. *African Journal of Biotechnology* 2: 280–7.

Santini, O., N. Chahbane, P. Vasseur, and H. Frank. 2011. Effects of low-level copper exposure on Ca^{2+}-ATPase and carbonic anhydrase in the freshwater bivalve *Anodonta anatina*. *Toxicological and Environmental Chemistry* (in press).

Serafim, A., and M.J. Bebianno. 2009. Metallothionein role in the kinetic model of copper accumulation and elimination in the clam *Ruditapes decussatus*. *Environmental Research* 109: 390–9.

Viarengo, A., L. Canesi, M.N. Moore, and M. Orunesu. 1994. Effect of Hg^{2+} and Cu^{2+} on the cytosolic Ca^{2+} level in molluscan blood cells evaluated by confocal microscopy and spectrofluorimetry. *Marine Biology* 119: 557–64.

Viarengo, A., G. Zanicchi, M.N. Moore, and M. Orunesu. 1981. Accumulation and detoxification of copper by the mussel *Mytilus galloprovincialis* Lam.: A study of the subcellular distribution in the digestive gland cells. *Aquatic Toxicology* 1: 147–57.

Vinot, I., and J.C. Pihan. 2005. Circulation of copper in the biotic compartments of a freshwater dammed reservoir. *Environmental Pollution* 133: 169–82.

Toxicological & Environmental Chemistry
Vol. 94, No. 1, January 2012, 99–108

Taylor & Francis
Taylor & Francis Group

Effects of copper exposure on calcium, carbohydrate, and protein levels in the freshwater mussel *Anodonta anatina*†

Andhika Puspito Nugroho[ab] and Hartmut Frank[a]*

[a]*Environmental Chemistry and Ecotoxicology, University of Bayreuth, D-95440 Bayreuth, Germany;* [b]*Laboratory of Ecology, Faculty of Biology, Gadjah Mada University, Yogyakarta 55281, Indonesia*

(*Received 8 August 2011; final version received 6 October 2011*)

Exposure of the European freshwater mussel *Anodonta anatina* to Cu via water (0.3 μmol L^{-1} ^{63}Cu) or via food (^{63}Cu-loaded algae, equivalent to 0.06 μmol L^{-1}) for 24 days results in increased Ca concentrations in all body compartments, in time pattern and extent following the uptake of Cu. This is accompanied by decrease in protein and carbohydrate levels. During the subsequent 12 days of depuration, Cu is quickly eliminated and Ca, protein, and carbohydrate levels tend to normalize, although not fully back to controls.

Keywords: copper; *Anodonta anatina*; calcium homeostasis; carbohydrates; proteins

Introduction

Copper is one of the trace metals which occurs naturally in freshwater ecosystems at concentrations ranging from 0.02 to 2 nmol L^{-1} (0.001–0.1 μg L^{-1}) (Wright and Welbourn 2002). In freshwater ecosystems, elevated Cu levels can occur due to mining and metallurgic activities, use of copper as fungicide, disposal of copper-containing waste waters, deposition of atmospheric particulate matter from coal combustion, and many other sources. In rivers and lakes, copper can exist in dissolved form or associated with dissolved organic carbon and suspended food particles (Vinot and Pihan 2005), in sediments as insoluble sulfides or in solution in the interstitial water, depending upon the sediment oxygen status (Besser, Ingersoll, and Giesy 1996).

Duck mussels (*Anodonta anatina*) live at the interface of the free-flowing water and the sediment phase of many lentic and lotic freshwater ecosystems. As filter feeders, they are known to accumulate metals due their close contact to sediments and low rate of Cu-elimination (Streit and Winter 1993). Metal accumulation in mussels can reflect the pollution status over long time periods, making them useful for biomonitoring.

Copper is known to be essential for mussels at about 10 μmol kg^{-1} (0.6 mg kg^{-1}) body weight, being part of the active sites of some metalloenzymes and serving as oxygen binding principle in hemocyanin, the respiratory pigment in the hemolymph of mollusks (Birge and Black 1979; Demayo and Taylor 1981; Julshamn et al. 2001). When the mussels filter water, Cu present in dissolved form will enter the mantle cavity reaching all

*Corresponding author. Email: hartmut.frank@uni-bayreuth.de
†Originally intended for 93(9) Special Issue: 3rd Workshop on Pearl Mussels in Upper Franconia and Europe: Ecotoxicology of Freshwater Bivalves (Bayreuth, 10–11 December 2007). Under the auspices of "Oberfrankenstiftung".

ISSN 0277–2248 print/ISSN 1029–0486 online

http://dx.doi.org/10.1080/02772248.2011.630910
http://www.tandfonline.com

water-contacted body parts to be absorbed into the body via the gills and mantle. When Cu is associated with algal food and suspended particulate matter, it is taken up via the mouth into the digestive tract. Both pathways lead to Cu accumulation in various body compartments (Nugroho and Frank 2011b).

Freshwater mussels require calcium (Ca) for shell formation and for regulation of physiological processes, transporting Ca actively into their body (Lopes-Lima et al. 2008). Cells must maintain specific cytosolic Ca levels (Ca homeostasis) in relation to its almost universal importance for nerve conduction, muscle contraction, as second messenger for regulation of carbohydrate metabolism, and of almost all aspects of cellular metabolisms and growth (Albert et al. 1994). Carbohydrates are important nutrients required by the mussels as a main source of energy for their metabolic processes (Honkoop et al. 1999) and in shell formation (Marin and Luquet 2004; Marie et al. 2007). Proteins play important roles in biological processes such as catalyzing biochemical reactions, transport and storage of molecules in and out or within cells, and have structural and mechanical functions (Albert et al. 1994). Excess copper in cytosol can lead to biochemical and physiological alterations, such as interference with calcium (Ca) homeostasis and increase in carbohydrate and protein catabolisms (Viarengo et al. 1994; Viant et al. 2002). Santini et al. (2011) reported that toxic effects become obvious at a Cu-concentration of $0.35\,\mu mol\,L^{-1}$ in the water. These alterations are biological responses of fundamental importance for their health and population development. In this particular study, the concentrations of Ca, soluble carbohydrates, and soluble proteins upon Cu exposure via water or food in the organs and body fluids of *A. anatina* have been followed.

Materials and methods

Chemicals and labware

Concentrated HNO_3 (69%) and concentrated HCl (30%) are of suprapur grade (Merck, Darmstadt, Germany). Other chemicals, i.e., ethylenediamine tetraacetic acid (EDTA), dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF) (Carl Roth, Karlsruhe, Germany), Bradford solution, bovine serum albumin (BSA), and glycogen standard type VII (Sigma-Aldrich, Munich, Germany) have been used in the highest purity that is commercially available. Cleaning of labware and preparation of the Cu stock solution are described in a previous publication (Nugroho and Frank 2011b).

Animal and experimental design

About 70 duck mussels (*Anodonta anatina*) (ZOO-Erlebnis Online Shop, Grossefehn, Germany) with shell lengths of 10–12 cm and weights of 100–200 g were brought to the laboratory in pond water. Mussel handling, acclimatization, and experimental design are described in detail in a previous publication (Nugroho and Frank 2011b) as well as algal food preparation (Nugroho and Frank 2011a). Briefly, the mussels were divided into three groups consisting of 21 mussels each. The first group was kept in artificial pond water (APW), the second one was exposed to $0.3\,\mu mol\,L^{-1}$ ($20\,\mu g\,L^{-1}$) ^{63}Cu in the water, and the third group received daily $1.5\,mg\,L^{-1}$ freeze-dried ^{63}Cu-loaded algae ($40\,mmol\,kg^{-1}$ Cu dry weight) for 24 days, equivalent to a nominal copper concentration of $0.06\,\mu mol\,L^{-1}$ ($3.6\,\mu g\,L^{-1}$).

For sampling, three mussels of each group were taken for analysis at days 0, 6, 12, 18, and 24 (exposure), and at days 30 and 36 (depuration). Hemolymph (HML) and

extrapallial fluid (EPF) were sampled before the mussels were dissected on ice into gills, mantle, kidney, digestive gland, foot, adductors, and intestines; the remainder, i.e., gonads, heart, and labial palps, was collected in one sample (GHL). Aliquots of about 5–10 mg of each tissue fraction were placed in 2 mL microtubes of known weights for carbohydrate and protein determinations and frozen to −80°C for further analysis. The remaining samples were placed in 15 mL polypropylene (PP) tubes of known weights and are lyophilized.

Calcium determination

Each lyophilized tissue fraction, weight between 10 and 100 mg, was placed in a 55 mL borosilicate glass tube. Five mL of a mixture (4 + 1) of concentrated HNO_3 and concentrated HCl were added to each tube. The tubes were kept in an oven at 40°C for 1 h and at 95°C for 3 h. The digested samples were diluted with bi-distilled water to 10 mL and filtered through 0.45 µm cellulose syringe filters (Carl Roth, Karlsruhe, Germany).

For the determination of Ca in HML and EPF, 1 mL each was acidified with 0.5 mL concentrated HNO_3 in a 15 mL polypropylene (PP) tube, diluted to 10 mL with bidistilled water, and filtered through a 0.45 µm cellulose syringe filter. Ca was determined by inductively-coupled plasma atomic emission spectroscopy (ICP-AES) (Varian, Vista-Pro Radial) with a detection limit of 50 µg L^{-1} using a calibration curve obtained with a Ca standard solution (1000 mg L^{-1}; Merck, Darmstadt, Germany). Calcium concentrations in tissue fractions were calculated in mmol kg^{-1} wet weight (ww) by multiplying the analytical data with the ratio of dry tissue weight versus wet tissue weight. For body fluids, the analytical data are given in mmol L^{-1}.

Copper determination

Copper in lyophilized tissues was determined by inductively-coupled plasma mass spectrometry (ICP-MS). Details have been described previously (Nugroho and Frank 2011b).

Protein and carbohydrate determinations

The frozen tissue samples were thawed and each sample was immediately mixed with 1 mL 50 mmol L^{-1} phosphate buffer, pH 7.4, containing 1 mmol L^{-1} EDTA, 1 mmol L^{-1} DTT, 0.15 mol L^{-1} KCl, and 0.01% (w/v) PMSF. The tissues were homogenized in an ice bath with 12 strokes of a tip sonicator at 20 kHz, acoustic power 50 W (Labsonic U tip sonicator, B. Braun Biotech International, Melsungen, Germany). The homogenates were centrifuged at 4°C for 30 min at 10,000 × g (Heraeus Multifuge 1 L-R, Thermo Scientific, Osterode, Germany). For determination of soluble proteins by the dye-binding assay (Kruger 1994), aliquots of 10 µL were pipetted into the wells (240 µL) of a 96-well polystyrene microplate and 90 µL bi-distilled water and 100 µL Coomassie blue solution were added. The samples were kept at room temperature for 15 min. Absorbances of the samples were read at 595 nm with a microplate reader (Biotek Synergy HT, Bad Friedrichshall, Germany). The concentrations were determined using bovine serum albumin for calibration.

For determination of soluble carbohydrates by the phenol–sulfuric acid assay (Masuko et al. 2005), 10 µL of the supernatants were pipetted into the wells of a 96-well polystyrene

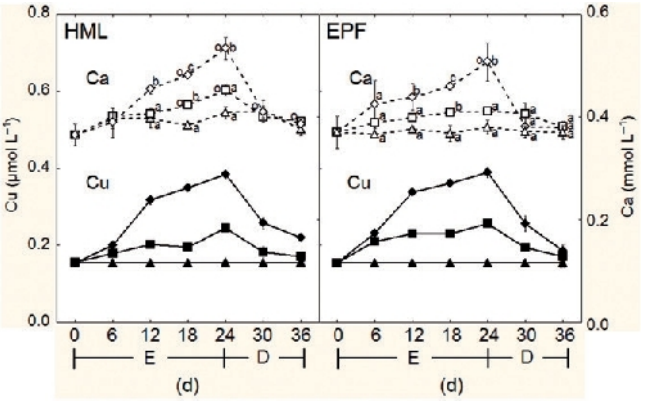

Figure 1. Concentrations of Ca (right ordinate, dotted lines; ◇ = upon exposure via water, □ = upon exposure via food, Δ = control) and Cu (left ordinate, solid lines; ◆ = via water, ■ = via food; ▲ = control) in hemolymph (HML) and extrapallial fluid (EPF) of *A. anatina* during Cu exposure (E) and depuration (D). Significant differences in comparison to control within each group are indicated by °. Similar letters indicate that differences of Ca concentrations are not significant among groups at each time sampling (day, d) while different letters indicate $p < 0.05$. Concentrations of copper are the same as in Nugroho and Frank (2011b).

microplate, and 40 µL bidistilled water and 150 µL concentrated sulfuric acid were added rapidly to achieve maximum mixing. Immediately afterwards, 30 µL of a solution of 5% phenol in bi-distilled water were added. After incubation for 5 min at 90°C in a static water bath, the microplate was cooled to room temperature for 5 min in a water bath and wiped dry for absorbance measurement at 490 nm. The concentrations were determined using a calibration curve obtained with glycogen standard type VII (Sigma-Aldrich, Munich, Germany).

Statistical data analyses

Data were transformed to log units before statistical analysis for homogeneity of variance and normality. The data for total Ca were statistically analyzed by two-way analysis of variance (ANOVA) considering exposure time or Cu exposure pathways as independent variables; if significant differences were found, those between exposure times were tested by the Dunnett multiple comparison test, and between exposure pathways and controls using the Duncan multiple comparison test. Linear regression analysis was performed for evaluating the relationships between Cu and Ca concentrations, carbohydrates, and proteins respectively, followed by Pearson correlation analysis for testing the strength of linear relationships.

Results

Exposure of *A. anatina* to water-dissolved Cu at a concentration of 0.3 µmol L^{-1} or to copper contained in the algal food at a nominal concentration of 0.06 µmol L^{-1} for 24 days induced time-dependent increases of Cu levels in all organs and body fluids, as shown previously (Nugroho and Frank 2011b). Concurrently, the calcium (Ca) concentrations were becoming elevated (Figures 1 and 2). In HML and EPF (Figure 1), at day 24 they reached maxima of about the 1.5-fold of control. Upon Cu-exposure via the food, Ca

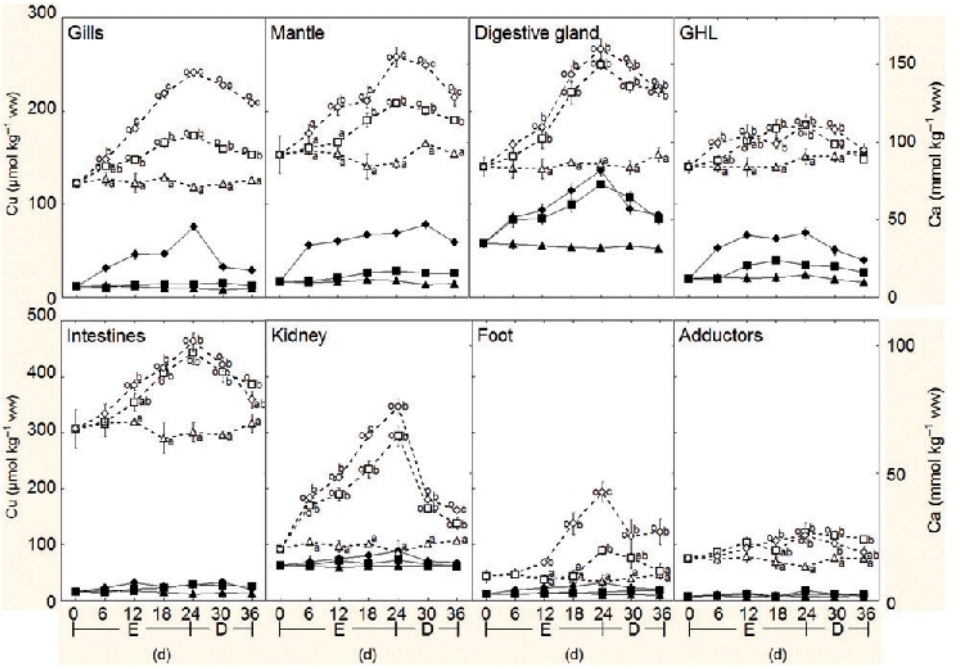

Figure 2. Concentrations of Ca (right ordinate, dotted lines; ◇ = upon exposure via water, □ = upon exposure via food, Δ = control) and Cu (left ordinate, solid lines; ◆ = via water, ■ = via food; ▲ = control) in the various organs of *A. anatina* during Cu exposure (E) and depuration (D) (GHL = gonads/heart/labial palps). Significant differences in comparison to control within each group are indicated by °. Similar letters indicate that differences of Ca concentrations are not significant among groups at each time sampling (day, d) while different letters indicate $p < 0.05$. Concentration of Ca is calculated by multiplication of the analytical data with the ratio of dry weight versus wet weight. Concentrations of copper are the same as in Nugroho and Frank (2011b).

increases were more moderate due to the lower Cu-intake, i.e., about the 1.2-fold of control at day 24. Ca levels in HML and EPF were correlated to total Cu concentration (r-food (HML) = 0.85, r-water (HML) = 0.90, r-food (EPF) = 0.61, r-water (EPF) = 0.73; $p < 0.05$). Upon depuration, Ca concentrations in the body fluids declined fast, returning to control values during the first six days although Cu was still elevated.

In the other organs and tissues (Figure 2), highest Ca concentrations at day 0 were found in the mantle (90 mmol kg^{-1} ww), the gills, the digestive gland, the mixed fraction containing gonads, heart, and labial palps (GHL) (all about 80 mmol kg^{-1} ww), and in the intestines (70 mmol kg^{-1} ww); much lower were the Ca-levels in the kidney, the adductors (both 20 mmol kg^{-1} ww), and the foot (10 mmol kg^{-1} ww). Upon Cu exposure via water or food, Ca levels increased in all organs except for the adductors. Highest Ca levels were found in the gills, mantle, and digestive gland upon exposure via the water, reaching about 140–160 mmol kg^{-1} ww (2-fold control) at day 24, highest relative increase being found in the kidney (4-fold, 80 mmol kg^{-1} ww). For GHL and the intestines, maximum Ca levels were at 110 (1.3-fold of control) and 100 (1.4-fold) mmol kg^{-1} ww, respectively. In the foot, Ca reached about the 4-fold (40 mmol kg^{-1} ww) of control at day 24 upon Cu-exposure via the water. When Cu-exposure took place via the food with its nominally lower Cu-levels per liter APW, Ca concentrations in the digestive gland, the intestines, the

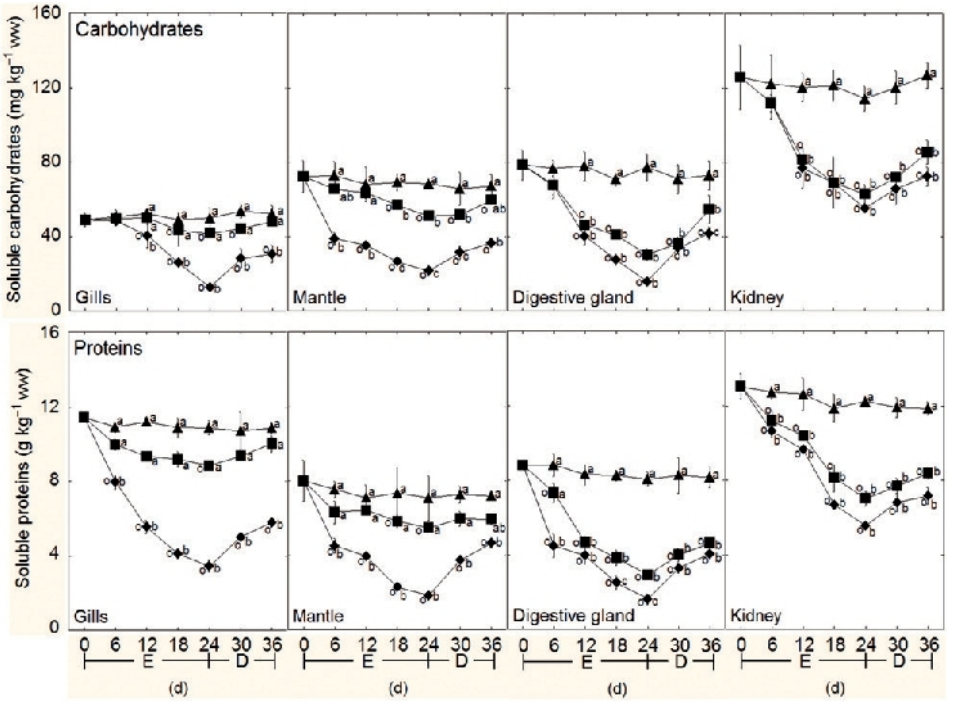

Figure 3. Contents of soluble carbohydrates and proteins (▲ = control; ■ = via food; ◆ = via water) in the gills, mantle, digestive gland, and kidney of *A. anatina* during Cu exposure (E) and depuration (D). Significant differences in comparison to control within each group are indicated by °. Similar letters indicate that differences of Ca concentrations are not significant among groups at each time sampling (day, d) while different letters indicate $p < 0.05$.

kidney, and the GHL increased almost equally as in the animals having received Cu via the water. In the adductors, Ca levels remained almost unchanged, as also found for Cu. In the gills, mantle, digestive gland, and kidney upon exposure via the water or the food, Ca concentrations were strongly and positively correlated to the Cu levels in the respective organs ($r > 0.7$; $p < 0.05$).

Upon depuration, Ca levels declined slowly, except for the kidney with fast elimination of excessive Ca. Upon the 12 days of depuration, Ca in the kidney, adductors, intestines, and GHL returned almost fully back to control values while in the gills, mantle, digestive gland, and foot the Ca levels declined to about 20–70% above control values.

Soluble carbohydrates and proteins in all organs were lowered upon Cu exposure and in parallel to its concentrations (Figure 3), strongest effects being observed when Cu was supplied via the water. Carbohydrate levels decreased drastically until the end of exposure with water-dissolved Cu, i.e. by 80% (gills) and 70% (kidney). Exposure to food-contained Cu had moderate effects except for the digestive gland and the kidney; these two organs showed only little differences between the two exposure pathways. For all other solid organs (not shown in Figure 3), soluble carbohydrates were decreased by 5–10%. Carbohydrates in HML and EPF at day 24 were lowered by only 5–10% upon Cu exposure via the food, the effects again being slightly stronger when Cu was taken up from the water (8–12%). Correlation analyses confirmed strong negative relationships between Cu and carbohydrate ($r > -0.6$; $p < 0.05$) in the gills, mantle (water pathway), digestive gland, and kidney (both pathways).

Soluble proteins in the gills, mantle, digestive gland, and kidney declined to between 20% and 45% of control levels at day 24. For the digestive gland and the kidney, the effects were almost equal for both exposure pathways while for the gills and the mantle great differences were found. Soluble protein levels in all other tissue compartments were decreased by not more than 5–10% (not shown in Figure 3). In the HML and EPF, levels of soluble proteins at day 24 were lowered by only 5–10% (HML: $550 \pm 40\,mg\,L^{-1}$; EPF: $390 \pm 40\,mg\,L^{-1}$; $n = 3$) for both pathways. Significant relationships of Cu and Ca with soluble protein levels ($r > -0.6$; $p < 0.05$) existed in the gills, mantle (water pathway), digestive gland, and kidney (both pathways). Upon depuration, soluble carbohydrate and protein levels in the studied organs started to increase, although not fully back to control within the 12 days.

Discussion

Exposure of *A. anatina* to copper via water or food causes increases of Ca levels in all body compartments, by the water pathway being stronger than via the food pathway. Nevertheless, when considering the nominally five-fold lower Cu concentration contributed by the algal suspension in the APW, the effects of the latter exposure pathway on the digestive gland and the kidney are surprisingly strong. Increase of Ca in all organs (Figure 2) suggests that Cu not only affects the enzymes regulating the internal Ca balance but also the overall Ca burden is strongly increased; such a flooding of the organism with excess Ca, mainly of the gills, mantle, and digestive gland, can only result from mobilization of $CaCO_3$ from the shell, most likely due to Cu-induced metabolic acidosis (Antunes et al. 2002; Faubel et al. 2008; Lopes-Lima et al. 2008). This is confirmed by the increase of Ca in the EPF between days 6 and 24. Interference of Cu with Ca homeostasis by affecting the mechanisms of Ca extrusion across cellular membranes may be another complication (Viarengo et al. 1994; Viarengo, Burlando, and Bolognesi 2002; Pattnaik, Chainy, and Jena 2007). Increase of Ca in the HML has also been reported by Viarengo (1994) for exposure of *Mytilus edulis* to Cu at 0.5–2 $\mu mol\,L^{-1}$.

A strong decrease of carbohydrate levels in *A. anatina* upon Cu exposure indicates that this is another sensitive toxicological endpoint associated with the disturbance of Ca homeostasis. A similar pattern is found in the gills and mantle of the freshwater mussel *Lamellindens marginalis* under copper stress at 2 $\mu mol\,L^{-1}$ (133 $\mu g\,L^{-1}$) for 3 days. This has been attributed to cell hypoxia (Satyaparameshwar, Reddy, and Kumar 2006) leading to increased activities of glycolytic enzymes involved in anaerobic ATP production (Martínez et al. 2006). Further decrease observed between days 12 and 24 (Figure 3) suggests that at lower carbohydrate levels gluconeogenesis is stimulated.

Strong correlation between increased Ca and low protein levels illustrate the key role of the electrolyte as intracellular signaling factor. According to Viarengo et al. (1994), elevated cytosolic Ca levels activated protein degradation. Decrease in protein levels was also found in the freshwater mussel *Anodonta woodiana* after exposure of Cu at 0.9 $\mu mol\,L^{-1}$ (0.06 $mg\,L^{-1}$) for 4 weeks (Kurnia, Purwanto, and Mahajoeno 2010). Similar to our findings, only minor effects on the soluble protein levels in HML and EPF were observed with the freshwater mussel *Anodonta cygnea* upon exposure to $CuSO_4$ at $10^{-6}\,mol\,L^{-1}$ for 1 month (Moura, Vilarinho, and Machado 2000).

Overall, Cu exposure at environmentally relevant levels leads to increased Ca levels in all body compartments of *A. anatina*, indicating that Cu interferes with Ca homeostasis. Dissolution of Ca from the shell upon Cu-induced metabolic acidosis (Antunes et al. 2002;

Lopes-Lima et al. 2008) and distribution of the electrolyte throughout the other body compartments are likely to contribute to its elevated levels. This can lead to the activation of Ca-dependent catabolic processes such as lipid hydrolysis, DNA fragmentation, and protein degradation, ultimately leading to cell death (Viarengo et al. 1994). Decreased carbohydrate and protein levels, being strongly inversely correlated to Ca levels, suggest that the mussels may not have sufficient energy and essential nutrients for normal reproduction, growth, and development. At the same time, carbohydrates and proteins are important components of the organic matrix which controls $CaCO_3$ polymorphism, size, and shape of the crystallites (Marin and Luquet 2004). These strong pathophysiological responses to environment-like levels of Cu may be one of the many factors involved in the presently observed decline of many European freshwater bivalves, including the freshwater pearl mussel *Margaritifera margaritifera* (Bauer 1986).

Conclusions

Copper exposure results in increases of Ca levels in all body compartments, accompanied by decreases in the levels of soluble proteins and carbohydrates. These effects may result in disturbance of mussel's reproduction, growth and development, and shell formation, leading to population decline.

Acknowledgments

We would like to thank Prof. Dr. Jorge P. Machado (Laboratory of Applied Physiology, University of Porto, Portugal) for helpful discussion. Financial support by the Directorate General of Higher Education, Ministry of National Education of the Republic of Indonesia, is highly appreciated.

References

Albert, B., D. Bray, J. Lewis, M. Raff, K. Robert, and J.D. Watson. 1994. *Molecular biology of the cell*. 3rd ed. New York: Garland Publishing.

Antunes, C., T. Magalhães-Cardoso, G. Moura, D. Gonçalves, and J. Machado. 2002. Effects of Al, Ni, Co, Zn, Cd, and Cu metals on the outer mantle epithelium of *Anodonta cygnea* (Unionidae). *Haliotis* 31: 71–84.

Bauer, G. 1986. The status of the freshwater pearl mussel *Margaritifera margaritifera* in the south of its European range. *Biological Conservation* 38: 1–9.

Besser, J.M., C.G. Ingersoll, and J.P. Giesy. 1996. Effects of spatial and temporal variation of acid-volatile sulfide on the bioavailability of copper and zinc in freshwater sediments. *Environmental Toxicology and Chemistry* 15: 286–93.

Birge, W.J., and J.A. Black. 1979. Effects of copper on embryonic and juvenile stages of aquatic animals. In *Copper in the environment. Part II. Health effects*, ed. J.O. Nriagu, 373–99. New York: John Wiley & Sons.

Demayo, A., and M.C. Taylor. 1981. *Guidelines for surface water quality. Vol. 1: Inorganic chemical substances – Copper*. Ottawa: Water Quality Branch, Inland Waters Directorate, Environment Canada.

Faubel, D., M. Lopes-Lima, S. Freitas, L. Pereira, J. Andrade, A. Checa, H. Frank, T. Matsuda, and J. Machado. 2008. Effects of Cd^{2+} on the calcium metabolism and shell mineralization of bivalve *Anodonta cygnea*. *Marine and Freshwater Behaviour and Physiology* 41: 93–108.

Honkoop, P.J.C., J.V. der Meer, J.J. Beukema, and D. Kwast. 1999. Reproductive investment in the intertidal bivalve *Macoma balthica*. *Journal of Sea Research* 41: 203–12.

Julshamn, K., E.K. Torpe, C. Børnes, L.J. Sæthre, and A. Maage. 2001. Cadmium, lead, copper and zinc in blue mussels (*Mytilus edulis*) sampled in the Hardangerfjord, Norway. *Journal of Environmental Monitoring* 3: 539–42.

Kruger, N.J. 1994. The Bradford method for protein quantitation. In *Methods in molecular biology: Basic protein and peptide protocols*, Vol. 32, ed. J.M. Walker, 9–15. New Jersey: Humana Press, Inc.

Kurnia, A.I., E. Purwanto, and E. Mahajoeno. 2010. Exposure copper heavy metal (Cu) on freshwater mussel (*Anodonta woodiana*) and its relation to Cu and protein content in the body shell. *Bioscience* 2: 48–53.

Lopes-Lima, M., R. Bleher, T. Forg, M. Hafner, and J. Machado. 2008. Studies on a PCMA-like protein in the outer mantle epithelium of *Anodonta cygnea*: Insights on calcium transcellular dynamics. *Journal of Comparative Physiology B* 178: 17–25.

Marie, B., G. Luquet, J.P.D. Barros, N. Guichard, S. Morel, G. Alcaraz, L. Bollache, and F. Marin. 2007. The shell matrix of the freshwater mussel *Unio pictorum* (Paleoheterodonta, Unionoida): Involvement of acidic polysaccharides from glycoproteins in nacre mineralization. *FEBS Journal* 274: 2933–45.

Marin, F., and G. Luquet. 2004. Molluscan shell proteins. *Comptes Rendus Palevol* 3: 469–92.

Martínez, M.L., C. Landry, R. Boehm, S. Manning, A.O. Cheek, and B.B. Rees. 2006. Effects of long-term hypoxia on enzymes of carbohydrate metabolism in the Gulf killifish, *Fundulus grandis*. *The Journal of Experimental Biology* 209: 3851–61.

Masuko, T., A. Minami, N. Iwasaki, T. Majima, S. Nishimura, and Y.C. Lee. 2005. Carbohydrate analysis by a phenol–sulfuric acid method in microplate format. *Analytical Biochemistry* 339: 69–72.

Moura, G., L. Vilarinho, and J. Machado. 2000. The action of Cd, Cu, Cr, Zn, and Pb on fluid composition of *Anodonta cygnea* (L.): Organic components. *Comparative Biochemistry and Physiology B* 127: 105–12.

Nugroho, A.P., and H. Frank. 2011a. Producing Cu-loaded algae for feeding experiments: Effects of copper on *Parachlorella kessleri*. *Toxicological and Environmental Chemistry* 93: 537–48.

Nugroho, A.P., and H. Frank. 2011b. Uptake, distribution, and bioaccumulation of copper in the freshwater mussel *Anodonta anatina*. *Toxicological and Environmental Chemistry* 93: 1838–50.

Pattnaik, S., G.B.N. Chainy, and J.K. Jena. 2007. Characterization of Ca^{2+}-ATPase activity in gill microsomes of fresheater mussel, *Lamellidens marginalis* (Lamarck) and heavy metal modulations. *Aquaculture* 270: 443–50.

Santini, O., N. Chahbane, P. Vasseur, and H. Frank. 2011. Effects of low-level copper exposure on Ca^{2+}-ATPase and carbonic anhydrase in the freshwater bivalve *Anodonta anatina*. *Toxicological and Environmental Chemistry* 93: 1826–37.

Satyaparameshwar, K., T.R. Reddy, and N.V. Kumar. 2006. Study of carbohydrate metabolism in selected tissues of freshwater mussel, *Lamellidens marginalis* under copper sulphate toxicity. *Journal of Environmental Biology* 27: 39–41.

Streit, B., and S. Winter. 1993. Cadmium uptake and compartmental time characteristics in the freshwater mussel *Anodonta anatina*. *Chemosphere* 26: 1479–90.

Viant, M.R., J.H. Walton, P.L. TenBrook, and R.S. Tjeerdema. 2002. Sublethal actions of copper in abalone (*Haliotis rufescens*) as characterized by *in vivo* ^{31}P NMR. *Aquatic Toxicology* 57: 139–51.

Viarengo, A. 1994. Heavy metal cytotoxicity in marine organisms: Effects on Ca^{2+} homeostasis and possible alteration of signal transduction pathways. *Advances in Comparative Environmental Physiology* 20: 85–110.

Viarengo, A., B. Burlando, and C. Bolognesi. 2002. Cellular responses to copper in aquatic organisms. In *Handbook of copper pharmacology and toxicology*, ed. E.J. Massaro, 417–27. New Jersey: Humana Press, Inc.

Viarengo, A., L. Canesi, M.N. Moore, and M. Orunesu. 1994. Effect of Hg^{2+} and Cu^{2+} on the cytosolic Ca^{2+} level in molluscan blood cells evaluated by confocal microscopy and spectrofluorimetry. *Marine Biology* 119: 557–64.

Vinot, I., and J.C. Pihan. 2005. Circulation of copper in the biotic compartments of a freshwater dammed reservoir. *Environmental Pollution* 133: 169–82.

Wright, D.A., and P. Welbourn. 2002. *Environmental toxicology*. Cambridge: Cambridge University Press.

Toxicological & Environmental Chemistry
Vol. 94, No. 5, May 2012, 918–929

Taylor & Francis
Taylor & Francis Group

Effects of copper on lipid peroxidation, glutathione, metallothionein, and antioxidative enzymes in the freshwater mussel *Anodonta anatina*

Andhika Puspito Nugroho[ab] and Hartmut Frank[a]*

[a]*Environmental Chemistry and Ecotoxicology, University of Bayreuth, D-95440 Bayreuth, Germany;* [b]*Laboratory of Ecology, Faculty of Biology, Gadjah Mada University, Yogyakarta 55281, Indonesia*

(*Received 20 August 2011; final version received 8 March 2012*)

Copper is an essential element to all animals. At elevated concentrations, it is toxic and can participate in the formation of reactive oxygen species, leading to cellular damage. In this study, the ecotoxicological relevance of copper was investigated with freshwater mussels, *Anodonta anatina*. When the mussels were exposed to copper at environmentally realistic concentrations, either via the water (0.3 μmol L^{-1} Cu) or fed with Cu-loaded algae (equivalent to 0.06 μmol L^{-1} Cu), the level of thiobarbituric acid-reactive substances rose and glutathione decreased. This was associated with the induction of metallothionein and, relative to total protein, of glutathione reductase and the antioxidative enzymes superoxide dismutase, catalase, and glutathione peroxidase. But, since the overall protein-synthetic capacity was hampered by the copper insult, the activities of the enzymes relative to tissue weight and copper concentrations were depressed. During depuration, most parameters started to normalize although not returning to control values within 12 days.

Keywords: copper; *Anodonta anatina*; thiobarbituric acid-reactive substances; glutathione; metallothionein; antioxidative enzymes

Introduction

Metals are brought to the earth's surface by mining for a multitude of agricultural, industrial, and technological applications. One of the technologically important metals is copper (Cu), used for electrical power installations and in the building sector, as animal feed additive or fungicide, as part of machineries, vehicles, electric appliances, and in many other consumer products. During its use, it is released by corrosion and/or abrasion, mobilized as particulate matter and dry or wet deposited, to some extent ending up in the sediments of freshwater ecosystems (Smolders et al. 2003). In non-contaminated freshwater ecosystems, its concentrations range from 0.02 to 0.3 μmol L^{-1} (1–20 μg L^{-1}) (Momčilović 2004). Close to mining activities, aquatic copper pollution can reach levels of up to 30 μmol L^{-1} (1.7 mg L^{-1}) (Smolders et al. 2003).

Mussels live at the interface of free-flowing waters and sediments and may be chronically exposed to copper for long time periods or intermittently at fluctuating levels, depending upon temporary hydrological conditions and extent of sediment oxygenation

*Corresponding author. Email: hartmut.frank@uni-bayreuth.de

ISSN 0277–2248 print/ISSN 1029–0486 online

http://dx.doi.org/10.1080/02772248.2012.675156
http://www.tandfonline.com

(Bhaduri et al. 2000; Poot, Gillissen, and Koelmans 2007). Copper is an essential element of their circulatory oxygen carrier hemocyanin (Momčilović 2004) and plays a role as cofactor of a number of enzymes such as cytochrome oxidase, superoxide dismutase (SOD), alcohol dehydrogenase, dopamine hydroxylase, tyrosinase, and lysyl oxidase (Serafim and Bebianno 2009). However, at excessive concentrations copper can participate in the formation of reactive oxygen species (ROS) through a Haber–Weiss cycle, producing hydroxyl radicals ($^{\cdot}OH$) from hydrogen peroxide (H_2O_2) and superoxide (O_2^{-}) (Bigot et al. 2011; Company et al. 2008). ROS may cause cellular damage by lipid peroxidation when the antioxidative defense systems of aquatic animals are overwhelmed, leading to inactivation of membrane enzymes, destruction of proteins (Remmer et al. 1989), and changes in the DNA structure (Company et al. 2008; Lackner 1998; Serafim and Bebianno 2009).

Mussels can cope with moderately elevated copper in various ways (Serafim and Bebianno 2009). In the cytosol, glutathione (GSH) and metallothionein (MT), the latter a family of cysteine-rich proteins (Ivanković et al. 2010), provide protection against increased concentrations through binding the copper ions to the thiol groups of their cysteine residues (Company et al 2008; Freedman, Ciriolo, and Peisach 1989). Other strategies against copper-induced oxidative toxicity is the induction of enzymes such as SOD, catalase (CAT), glutathione peroxidase (GPX), and glutathione reductase (GR) (Isani et al. 2003).

Copper has been observed in high concentrations in the tissue of freshwater pearl mussels *Margaritifera margaritifera* (Frank and Gerstmann 2007) and other European freshwater mussel species (Tallandini et al. 1986). Their populations are strongly affected Europe-wide and some are threatened with extinction (Cuttelod, Seddon, and Neubert 2011). Understanding the potential involvement of Cu in this phenomenon is the major motivation for this study.

In this work, *Anodonta anatina* is used as model species. In previous publications, it has been shown that *A. anatina* can accumulate copper from the water or by feeding on copper-containing algae (Nugroho and Frank 2011b). This article focuses on the effects of copper on MT and GSH and on antioxidative enzymes as response to oxidative stress, signaled by increased levels of thiobarbituric acid-reactive substances (TBARS).

Materials and methods

Chemicals

Isotopically enriched (99%) ^{63}Cu oxide (Euriso-top, Saarbrücken, Germany) was used. Concentrated HNO_3 (69%) and concentrated HCl (30%) were of suprapure grade (Merck, Darmstadt, Germany); other chemicals (Carl Roth, Karlsruhe, Germany; Sigma-Aldrich, Munich, Germany) were of analytical grade. Cleaning of labware and preparation of the Cu^{2+} stock solution are described in a previous publication (Nugroho and Frank 2011b).

Animals and experimental design

Seventy duck mussels (*A. anatina*) (ZOO-Erlebnis Online Shop, Grossefehn, Germany) with shell lengths of 10–12 cm and weights between 100 and 200 g were brought to the laboratory in pond water. Mussel handling, acclimatization, and experimental design

(Nugroho and Frank 2011b) as well as the preparation of normal and Cu-loaded algae have been described earlier (Nugroho and Frank 2011a). The mussels were divided into three groups consisting of 21 mussels each. The first group was kept in artificial pond water (APW); the second one was exposed to 0.3 μmol L^{-1} (20 μg L^{-1}) $^{63}Cu^{2+}$ in the water; the third group received daily 1.5 mg L^{-1} freeze-dried ^{63}Cu-loaded algae (40 mmol ^{63}Cu per kg dry weight) for 24 days, equivalent to a nominal concentration of 0.06 μmol (3.6 μg L^{-1}) ^{63}Cu per liter APW.

For sampling, three mussels of each group were taken for analysis at days 0, 6, 12, 18, and 24 (exposure), and at days 30 and 36 (depuration). The mussels' soft bodies were dissected on ice into gills, mantle, kidney, and digestive gland. Two aliquots of every tissue fraction, about 5–10 mg each, were placed in separate 2-mL microtubes of known weight. The first aliquot was used for the determination of MT and the second one for the determination of TBARS, GSH, enzyme activities, and proteins. All microtubes were kept in a freezer at −80°C until further analysis. The remainders of the tissues were placed in 15-mL polypropylene (PP) tubes of known weights and were lyophilized for copper determination.

Analytical methods

Sample preparation

Frozen tissue samples in microtubes were thawed and immediately mixed with 500 μL sucrose (0.5 mol L^{-1})/Tris-HCl (20 mmol L^{-1}; pH 8.6) buffer, to which leupeptine (6 μmol L^{-1}) and phenylmethanesulfonylfluoride (PMSF) (0.5 mmol L^{-1}) were added as anti proteolytic agents and β-mercaptoethanol (0.01%) as reducing agent. The mixtures were sonicated in an ice bath with 12 strokes of a sonicator (Labsonic U tip sonicator, B. Braun Biotech International, Melsungen, Germany) at 20 kHz, acoustic power 50 W. The homogenates were centrifuged at 4°C for 30 min at 10,000 × g (Heraeus Multifuge 1L-R, Thermo Scientific, Osterode, Germany). Supernatants were used for MT determination.

For the determination of TBARS, GSH, enzyme activities, and proteins, frozen tissue samples of 5–10 mg were thawed and immediately mixed with 500 μL phosphate buffer (50 mmol L^{-1}; pH 7.4) containing 150 mmol L^{-1} KCl, 1 mmol L^{-1} ethylenediaminetetra-acetic acid (EDTA), 1 mmol L^{-1} dithiothreitol (DTT), and 0.01% (w/v) PMSF. The samples were homogenized in an ice bath with 12 strokes of a sonicator at 20 kHz, acoustic power 50 W, and centrifuged at 4°C for 30 min at 10,000 × g. The supernatants were used for analysis.

Total copper

Total copper in lyophilized tissues and freeze-dried algal food, and – every second day – the actual copper concentrations in APW were determined by inductively-coupled plasma mass spectrometry. Details have been described previously (Nugroho and Frank 2011b).

Lipid peroxidation

Lipid peroxidation was determined following the method of Buege and Aust (1978) by measuring TBARS, expressed as malondialdehyde (MDA) equivalents. Absorbances of samples were read at 535 nm with a microplate reader (Biotek Synergy HT, Bad Friedrichshall, Germany). TBARS levels were estimated using a standard curve obtained

with 1,1,3,3-tetramethoxypropane (99%; VWR, Darmstadt, Germany) as stable precursor of MDA and expressed as μmol kg^{-1} tissue wet weight (tww).

Glutathione

GSH was determined according to Anderson (1985). Absorbances of samples were measured at 412 nm with a microplate reader. The GSH content was estimated using a standard curve obtained with reduced GSH and expressed as mmol kg^{-1} tww.

Metallothioneins

MT concentrations were determined by the spectrophotometric method of Viarengo et al. (1997) modified by Verlecar, Jena, and Chainy (2008). Absorbances of samples were read at 412 nm with a microplate reader. The MT content was determined using GSH (Carl Roth, Karlsruhe, Germany) as standard, assuming that 1 μmol GSH is equivalent to 0.055 μmol MT. Concentrations of MT were expressed as μmol kg^{-1} tww.

Enzyme activities

SOD activities were determined by the procedure of Beauchamp and Fridovich (1971), based on the inhibition of nitrotetrazolium blue reduction and measuring sample absorbances at 560 nm. CAT activities were assayed spectrophotometrically according to Rao, Paliyath, and Ormrod (1996) by monitoring the decrease in the absorbance of H_2O_2 at 240 nm. GPX activities were determined according to Paglia and Valentine (1967) and GR activities according to Massey and William (1965) in the presence of GSSG, in both cases following the rate of NADPH oxidation at 340 nm. Absorbances were measured with a microplate reader; enzyme activities were calculated in units per milligram protein and per gram tww.

Proteins

Proteins were determined by the dye-binding assay (Kruger 1994). Absorbances of the samples were read at 595 nm with a microplate reader. The concentrations were determined using BSA (≥96%; Sigma-Aldrich, Munich, Germany) for calibration.

Statistical data analyses

The variability of the observed parameters and of total Cu concentration in the different organs were tested by two-way analysis of variance (ANOVA) considering exposure time and copper exposure pathways as independent variables, followed by the Duncan multiple comparison tests ($p < 0.05$) if significant differences were found. Data were transformed to $\log(X+1)$ units before statistical analysis for the homogeneity of variance and normality. Linear regression analysis was performed for evaluating the relationship between Cu concentration and the observed parameters, followed by Pearson correlation analysis for testing the strength of linear relationship.

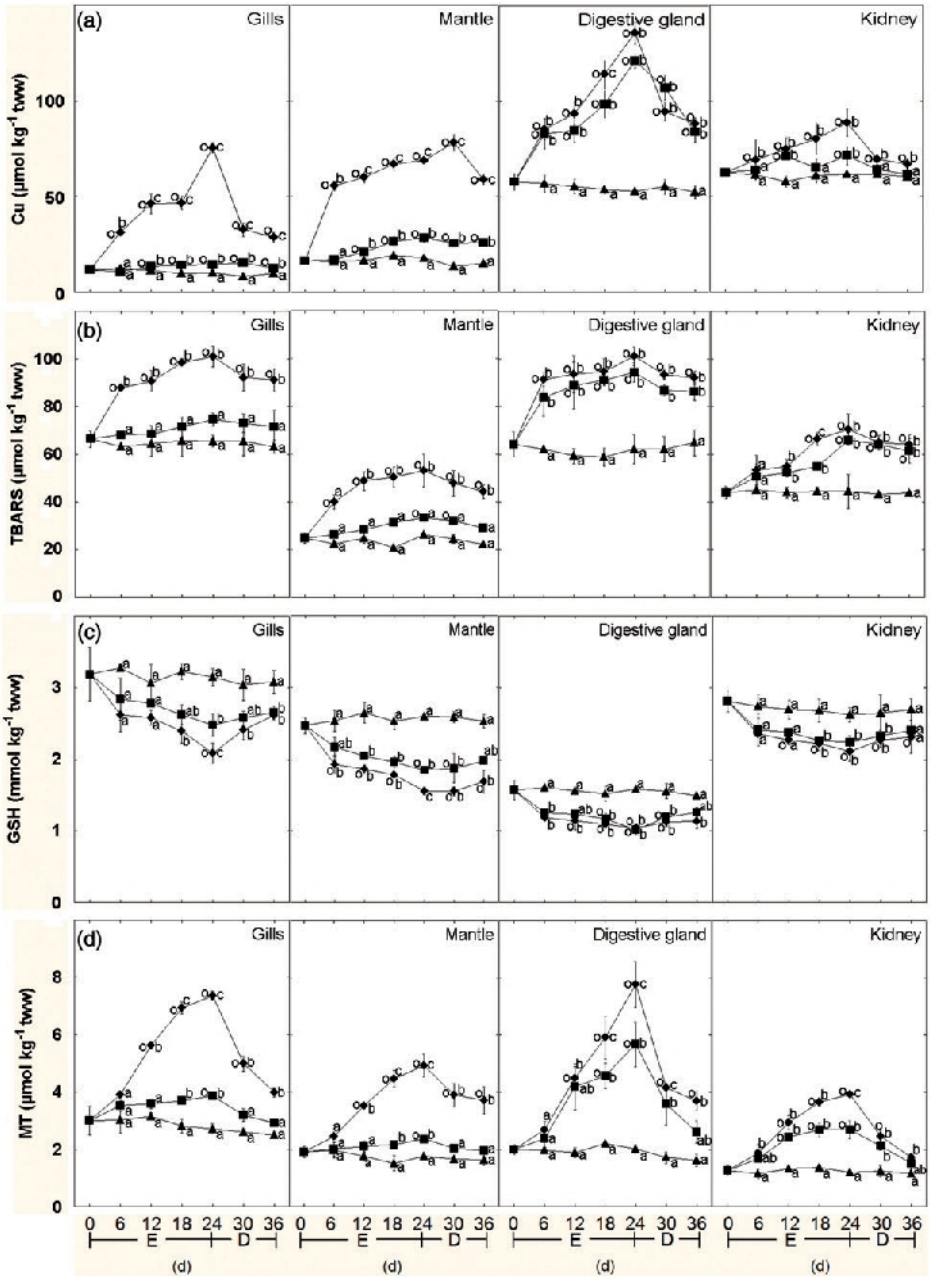

Figure 1. Concentrations of copper (a), TBARS (b), GSH (c), and MT (d) in the gills, mantle, digestive gland, and kidney of *A. anatina* during Cu exposure (E) and depuration (D) (◆ = exposure via water, ■ = exposure via food, ▲ = control). Significant differences in comparison to controls within each group are indicated by °. Same letters indicate that differences among groups are not significant at each time of sampling (day, d) while different letters indicate $p < 0.05$ ($n = 3$). Concentrations of copper were taken from a previous publication (Nugroho and Frank 2011b).

Results

Copper concentrations in all organs increased during exposure (Figure 1a, taken from the previous publication Nugroho and Frank 2011b). When Cu was administered via the water, the levels started to rise instantaneously, reaching a maximum of up to 6.5-fold of controls at day 24. From the food, Cu increased in the digestive gland to a similarly high level, but in the gills and mantle it was much lower; for the kidney uptake was moderate. Upon depuration, Cu was quickly but not completely eliminated, especially not from the mantle and the digestive gland.

Initial TBARS levels (Figure 1b) were highest in the gills and the digestive gland, i.e., about 60 $\mu mol\,kg^{-1}$ tww; in the mantle and the kidney they were between 25 and 40 $\mu mol\,kg^{-1}$ tww. Upon Cu exposure via the water, TBARS rose significantly within the first six days, except for the kidney. At day 24, highest levels in relative terms were reached in the mantle (about double of control), in absolute terms in the gills and digestive gland (100 $\mu mol\,kg^{-1}$ tww); in the kidney, TBARS were elevated by about 60%. Upon Cu exposure via the food, the effects on the digestive gland and the kidney were almost equally strong as for exposure via the water, while in the gills and the mantle the slightly increased levels were not significantly different from controls ($p > 0.05$). Correlation analyses between copper and TBARS confirmed strong relationships in all organs ($r > 0.6$; $p < 0.05$), except for the kidney. During depuration, the concentrations decreased slowly, the levels after 12 days of depuration being only slightly lower than in the beginning of depuration.

Initial levels of GSH (Figure 1c) were highest in the gills and the kidney, i.e., about 3.0 $mmol\,kg^{-1}$ tww, somewhat lower in the mantle, i.e., about 2.5 $mmol\,kg^{-1}$ tww, and lowest in the digestive gland, i.e., 1.6 $mmol\,kg^{-1}$ tww. Upon Cu uptake, GSH started to decrease within the first 6 days at similar relative rates in all organs, reaching lowest levels at day 24, especially when Cu-exposure took place via the water. In the mantle GSH decreased by about 40%, in the other organs between 20 (digestive gland and kidney) and 30% (gills) at day 24. Upon depuration, GSH-levels tended to increase but remained lower than in controls, even after 12 days.

Initial MT levels (Figure 1d) ranged from 1.5 (kidney) to 3.0 (gills) $\mu mol\,kg^{-1}$ tww. Upon Cu exposure via the water, MT increased in parallel to Cu, being significantly different from control on day 12 and later. Highest MT levels were reached on day 24, i.e., MT was increased by 300% from control in the digestive gland, 200% in the gills and mantle, and 100% in the kidney. Upon Cu-exposure via the food, MT increased in the digestive gland and the kidney almost to the same degree, while in the gills and the mantle MT levels were only slightly elevated. Correlation analyses between copper and MT confirmed their strong relation in all organs ($r > 0.6$; $p < 0.05$). Upon depuration and simultaneous with Cu elimination, MT decreased strongly in the gills, the digestive gland, and the kidney within the first 6 days; in the mantle, MT remained high, parallel to the slow elimination of Cu.

The antioxidative enzymes are presented in Figure 2 in two ways, i.e., relative to protein contents (open symbols, left ordinate) and to tissue wet weight (tww) (filled symbols, right ordinate). SOD (Figure 2a) had the highest initial activity in the kidney, i.e., about 9 units (U) per mg protein (120 U per g tww), for the other organs ranging from 3 to 5 U per mg protein (20–40 U per g tww). For CAT (Figure 2b), the initial levels were similar for all four organs at about 5 U per mg protein (65 U per g tww). GPX (Figure 2c) was initially highest in the kidney, i.e., about 0.04 U per mg protein (0.5 U per g tww), in

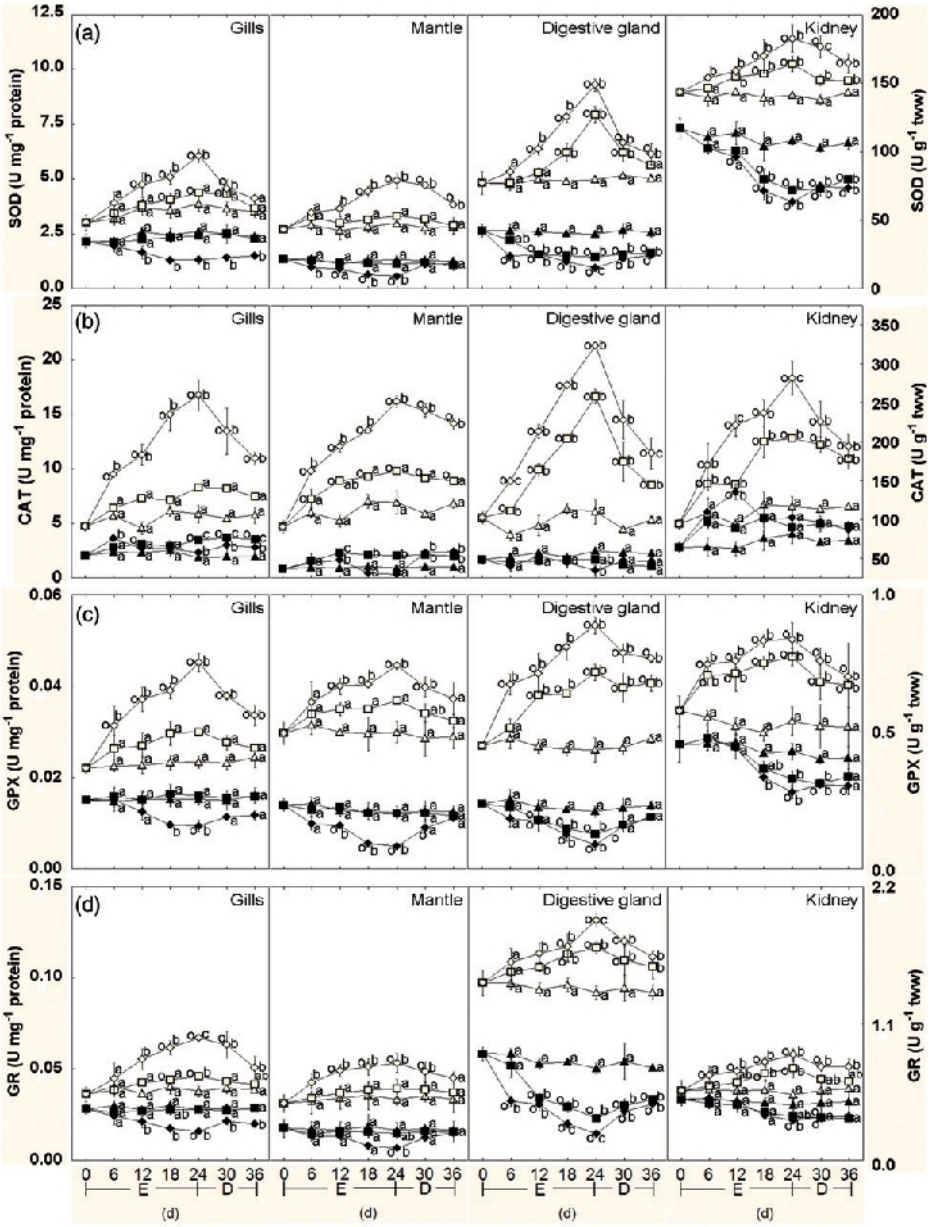

Figure 2. SOD (a), CAT (b), GPX (c), and GR (d) activities in the gills, mantle, digestive gland, and kidney of *A. anatina* during Cu exposure (E) and depuration (D) in units (U) per mg protein (left ordinate; ◇ = upon exposure via water, □ = upon exposure via food, △ = control) and in units per g tissue wet weight (tww) (right ordinate; ◆ = exposure via water, ■ = exposure via food, ▲ = control). Significant differences in comparison to controls within each group are indicated by °. Same letters indicate that differences among groups are not significant at each time of sampling (day, d) while different letters indicate $p < 0.05$ ($n = 3$).

the other organs at about 0.03 U per mg protein (0.25 U per g tww), taking the differences in protein contents among the organs into account (Nugroho and Frank 2012).

Upon Cu exposure via the water, the activities of the three enzymes relative to protein contents increased in all organs. CAT and GPX reached highest levels at day 24 in the digestive gland, the former being increased by 250% from control, the latter about double; SOD behaved similarly. Upon Cu-uptake from the food, the activities of all three enzymes in the digestive gland and the kidney increased almost equally as when exposed via the water, while in the gills and mantle only small effects were seen. Correlation analysis showed highly significant correlations ($r > 0.6$; $p < 0.05$) between the enzymes and copper in the gills and mantle (water pathway), and the digestive gland and kidney (water and food pathways). During depuration, the activities decreased, especially of SOD and CAT in the digestive gland, although not returning to control values until the end of depuration.

Relative to tissue wet weight, however, SOD and GPX activities decreased. This was strongest for SOD (Figure 2a) in the kidney, i.e., to about 50% of control at day 24, and for GPX in the mantle (40 % of control) and the digestive gland (50 % of control). Uptake of Cu from the food resulted in decreases in the digestive gland and kidney similar to uptake via the water, while in the gills and the mantle SOD and GPX remained at control levels. CAT activities were largely unchanged, except for the kidney in which it was doubled. During depuration, SOD and GPX activities increased in all organs, but not fully back to control values.

Initial GR levels (Figure 2d) relative to protein contents were highest in the digestive gland (0.1 U per mg protein), in the other organs being only a third (0.03 U per mg protein). Upon aqueous Cu exposure, increases were strong for the digestive gland and the gills; for the mantle and kidney they were moderate (~50%), but in all cases correlated to copper ($r > 0.6$; $p < 0.05$). Relative to wet weight, the GR activities declined upon Cu exposure via the water, strongest in the digestive gland to reach about 30% of control at day 24, in the other organs about 70%. Upon Cu exposure via the food, GR activities in the digestive gland were similarly depressed as via the water, in the gills and mantle they remained at control levels. During depuration, GR began to normalize although not fully back to control levels.

Discussion

As shown previously (Nugroho and Frank 2011b), uptake of Cu from the water (Figure 1a) led to a general and fast rise of its concentrations in all tissues and organs, while upon Cu-uptake with the food primarily the digestive gland and the intestines (not shown) were burdened.

In respect to lipid peroxidation, increased TBARS reflect the damage to biological membranes by ROS as a consequence of excess Cu (Company et al. 2008). The initial TBARS levels found in the digestive gland of *A. anatina* (7.0 nmol mg^{-1} protein) were about 7–10-fold higher than those reported by Sabatini et al. (2011) for the freshwater mussel *Diplodon chilensis* (0.7 nmol mg^{-1} protein) and by Bouskill et al. (2006) for the zebra mussel *Dreissena polymorpha* (1.0 nmol mg^{-1} protein). Parallel to the accumulation of copper, TBARS rose by 60–70% in all organs when the metal was taken up by the water pathway, while by the food pathway the effect was focused on the digestive gland; the latter has also been reported by Sabatini et al. (2011), although they used algae with a Cu-contamination level about 800 times higher than in our case and a different feeding schedule. Other researchers reported an increase in TBARS by about 100% in the whole

tissue (2.0 nmol mg^{-1} protein) of *D. polymorpha* upon exposure to water-dissolved Cu at 1.6 μmol L^{-1} (100 μg L^{-1}) for 7 days (Bouskill et al. 2006), i.e., at a Cu-concentration about 5-fold higher than in this study. For *A. anatina*, increases by about 50%, e.g., in the digestive gland, were observed within the same period of exposure.

Consumption of GSH can occur due to its role as a metal-complexing agent, and as an antioxidant and scavenger of reactive intermediates of lipid peroxidation (Canesi et al. 1999; Lackner 1998). The initial GSH-levels in the gills (300 nmol mg^{-1} protein) and the digestive gland (170 nmol mg^{-1} protein) of *A. anatina* were about 2–4-fold lower than in the same organs of the swollen river mussel *Unio tumidus* (500 and 600 nmol mg^{-1} protein) (Doyote et al. 1997). Copper exposure was associated with the depletion of GSH by about 20–40% in all organs of *A. anatina*, irrespective of exposure pathway. Although upon copper uptake via the food TBARS were predominantly increased in the digestive gland, GSH was lowered in all organs. This suggests a generalized, systemic mobilization and exchange of GSH between organs. For comparison, when *U. tumidus* was exposed to water-dissolved Cu (Doyotte et al. 1997) at about twice the concentration (0.5 μmol L^{-1}, 30 μg L^{-1}) used in our study, GSH in the gills and the digestive gland was lowered by about 15% within 3 days, comparable to the 20–25% we have observed with *A. anatina* after 6 days of exposure. The same authors have emphasized that the depletion was associated with decreased GR activities, similarly as we have observed with *A. anatina* (Figure 2d).

Binding of copper to MT is a detoxification process and meant to control its intracellular levels (Company et al. 2008; Serafim and Bebianno 2009). Initial MT levels in the gills and mantle of *A. anatina* (2–3 μmol kg^{-1} tww) were about 6–9-fold lower than that reported for the whole soft tissue of *D. polymorpha* (about 18 μmol kg^{-1} tww; Ivanković et al. 2010). In parallel to Cu uptake, MT levels were induced in all organs via both exposure pathways, showing dose–response relationships. The increase in molar concentrations of MT in the gills, mantle, and kidney of *A. anatina* (Figure 1d) were largely sufficient to complex the extra copper, considering a binding ratio of 12 (Adam et al. 2010; Eisler 1993); for the digestive gland, however, this ratio of extra Cu *versus* newly synthesized MT tended to be higher, especially upon Cu-exposure via the food pathway. In view of the fact that MT may additionally serve as an antioxidant, the induction of MT is rather limited and may constitute a considerable physiological challenge. Copper exposure induced an increase of MT by 10% in the whole soft tissue of *D. polymorpha* (20 μmol kg^{-1} ww; Ivanković et al. 2010) upon exposure to water-dissolved Cu at 0.5 μmol L^{-1} (30 μg L^{-1}) for 7 days, i.e., at a concentration about 1.5-fold higher than our study. Within the same period of exposure, the increases were slight lower than all organs of exposed *A. anatina* (20–30%). Bouskill et al. (2006) also reported a 10% increase of MT in the whole tissue (500 μg mg^{-1} protein) of *D. polymorpha* but exposed for 7 days to 1.6 μmol L^{-1} (100 μg L^{-1}) Cu in water, i.e., more than 5-fold higher than in this study. In field studies, MT in the gills of the giant floater *Pyganodon grandis* correlated positively with elevated Cu concentrations (Bonneris et al. 2005; Perceval et al. 2006). A similar study also showed a positive relationship between Cu and MT in the soft tissue of *D. polymorpha* found in the St Lawrence River, Canada (de Lafontaine et al. 2000).

Relative to protein contents, the major antioxidative enzymes were induced during copper exposure (Figure 2), SOD, and CAT in the digestive gland by 60% and 240%, respectively. Sabatini et al. (2011) reported the induction of SOD and CAT activities by only about 50% relative to protein in the digestive gland of *D. chilensis* after 4–5 weeks of exposure via food to much higher Cu concentration (equivalent to 50 μmol L^{-1}). However, relative to tissue wet weight – and thus relative to copper – the activities of SOD, GPX,

and GR were depressed, especially in the digestive gland (GPX by 30%, GR by 45 % after 6 days of exposure), since the overall protein-synthetic capacity was hampered (Nugroho and Frank 2012). Doyotte et al. (1997) also reported the decrease of GPX and GR activities in the digestive gland (by 15 and 30%) of *U. tumidus* exposed for 3 days to water-dissolved Cu at 0.5 μmol L^{-1} (30 μg L^{-1}), twice the concentration used in this study.

Overall, Cu-exposure of *A. anatina* obviously entailed considerable oxidative stress, depression of GSH, induction of MT, and perturbations in the activities of GR and the antioxidative enzymes. Exposure to Cu via the water affected all organs including the gills and mantle, by the food being mainly the digestive gland. Taking into account that in these experiments the nominal concentration of copper contained in the food was fairly low relative to the copper concentration in the water, exposure via the food and the impact on the digestive gland was particularly strong, the centrally important organ for digestion, catabolism, and uptake of nutrients and electrolytes such as Ca^{2+}. In conjunction with the effects on the kidney and the mantle lasting beyond the actual exposure, and together with the derangements of calcium homeostasis and of carbohydrate and protein metabolism (Nugroho and Frank 2012), the exposure to copper at moderately elevated environmentally realistic levels has profound pathobiochemical consequences.

Conclusions

Overall, mussels are under considerable oxidative stress when exposed to Cu-concentrations moderately elevated above natural conditions. Utilization of GSH and induction of MT constitute a first line of defense. The activation of a second line by induction of antioxidative enzymes is rather inefficient as protein-synthetic capacities are strongly affected. All this evidence suggests that copper at fairly low levels can affect the vitality of mussels at all life stages, making it likely to contribute to the population declines of European freshwater bivalves.

Acknowledgments

Financial support by Directorate General of Higher Education, Ministry of National Education of the Republic of Indonesia, is highly appreciated.

References

Adam, V., I. Fabrik, T. Eckschlager, M. Stiborova, L. Trnkova, and R. Kizek. 2010. Vertebrate metallothioneins as target molecules for analytical techniques. *Trends in Analytical Chemistry* 29: 409–18.

Anderson, M.E. 1985. Determination of glutathione and glutathione disulfide in biological samples. *Methods in Enzymology* 113: 543–53.

Beauchamp, C., and I. Fridovich. 1971. Superoxide dismutase: Improved assays and an assay applicable to polyacrylamide gels. *Analytical Biochemistry* 44: 276–86.

Bhaduri, B., J. Harbor, B. Engel, and M. Grove. 2000. Assessing watershed-scale, long-term hydrologic impacts of land-use change using a GIS-NPS model. *Environmental Management* 26: 643–58.

Bigot, A., L. Minguez, L. Giambérini, and F. Rodius. 2011. Early defense responses in the freshwater bivalve *Corbicula fluminea* exposed to copper and cadmium: Transcriptional and histochemical studies. *Environmental Toxicology* 26: 623–32.

Bonneris, E., O. Perceval, S. Masson, L. Hare, and P.G.C. Campbell. 2005. Sub-cellular partitioning of Cd, Cu and Zn in tissues of indigenous unionid bivalves living along a metal exposure gradient and links to metal-induced effects. *Environmental Pollution* 135: 195–208.

Bouskill, N.J., R.D. Handy, T.E. Ford, and T.S. Galloway. 2006. Differentiating copper and arsenic toxicity using biochemical biomarkers in *Asellus aquaticus* and *Dreissena polymorpha*. *Ecotoxicology and Environmental Safety* 65: 342–9.

Buege, J.A., and S.D. Aust. 1978. Microsomal lipid peroxidation. *Methods in Enzymology* 52: 302–10.

Canesi, L., A. Viarengo, C. Leonzio, M. Filippelli, and G. Gallo. 1999. Heavy metals and glutathione metabolism in mussel tissues. *Aquatic Toxicology* 46: 67–76.

Company, R., A. Serafim, R.P. Cosson, A. Fiala-Médioni, L. Camus, A. Colaço, R. Serrão-Santos, and M.J. Bebianno. 2008. Antioxidant biochemical responses to long-term copper exposure in *Bathymodiolus azoricus* from Menez-Gwen hydrothermal vent. *Science of the Total Environment* 389: 407–17.

Cuttelod, A., M. Seddon, and E. Neubert. 2011. *European red list of non-marine molluscs*. Luxembourg: Publications Office of the European Union.

de Lafontaine, Y., F. Gagné, C. Blaise, G. Costan, P. Gagnon, and H.M. Chan. 2000. Biomarkers in zebra mussels (*Dreissena polymorpha*) for the assessment and monitoring of water quality of the St Lawrence River (Canada). *Aquatic Toxicology* 50: 51–71.

Doyotte, A., C. Cossu, M. Jacquin, M. Babut, and P. Vasseur. 1997. Antioxidant enzymes, glutathione and lipid peroxidation as relevant biomarkers of experimental or field exposure in the gills and the digestive gland of the freshwater bivalve *Unio tumidus*. *Aquatic Toxicology* 39: 93–110.

Eisler, R. 1993. *Zinc hazards to fish, wildlife, and invertebrates: A synoptic r*eview. Laurel, MD: U.S. Department of the Interior, Fish, and Wildlife Service, Patuxent Wildlife Research Center.

Frank, H., and S. Gerstmann. 2007. Declining populations of freshwater pearl mussels (*Margaritifera margaritifera*) are burdened with heavy metals and DDT/DDE. *Ambio* 36: 571–4.

Freedman, J.H., M.R. Ciriolo, and J. Peisach. 1989. The role of glutathione in copper metabolism and toxicity. *The Journal of Biological Chemistry* 264: 5598–605.

Isani, G., M. Monari, G. Andreani, M. Fabbri, and E. Carpenè. 2003. Effect of copper exposure on the antioxidant enzymes in bivalve mollusk *Scapharca inaequivalvis*. *Veterinary Research Communications* 27: 691–3.

Ivanković, D., J. Pavičić, V. Beatović, R.S. Klobučar, and G.I.V. Klobučar. 2010. Inducibility of metallothionein biosynthesis in the whole soft tissue of zebra mussels *Dreissena polymorpha* exposed to cadmium, copper, and pentachlorophenol. *Environmental Toxicology* 25: 198–211.

Kruger, N.J. 1994. The Bradford method for protein quantitation. In *Methods in molecular biology: Basic protein and peptide protocols*, Vol. 32, ed. J.M. Walker, 9–15. New Jersey: Humana Press.

Lackner, R. 1998. "Oxidative stress" in fish by environmental pollutants. In *Fish ecotoxicology*, eds. T. Braunbeck, D.E. Hinton and B. Streit, 203–24. Switzerland: Birkhäuser Verlag.

Massey, V., and C.H. Williams. 1965. On the reaction mechanism of yeast glutathione reductase. *The Journal of Biological Chemistry* 240: 4470–81.

Momčilović, B. 2004. Copper. In *Elements and their compounds in the environment: Metals and their compounds*, Vol. 2, eds. E. Merian, M. Anke, M. Ihnat and M. Stoeppler, 731–50. Weinheim: Wiley-VCH Verlag.

Nugroho, A.P., and H. Frank. 2011a. Producing Cu-loaded algae for feeding experiments: Effects of copper on *Parachlorella kessleri*. *Toxicological and Environmental Chemistry* 93: 537–48.

Nugroho, A.P., and H. Frank. 2011b. Uptake, distribution, and bioaccumulation of copper in the freshwater mussel *Anodonta anatina*. *Toxicological and Environmental Chemistry* 93: 1838–50.

Nugroho, A.P., and H. Frank. 2012. Effects of copper exposure on calcium, carbohydrate, and protein levels in the freshwater mussel *Anodonta anatina*. *Toxicological and Environmental Chemistry* 94: 99–108.

Paglia, D.E., and W.N. Valentine. 1967. Studies on quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *Journal of Laboratory and Clinical Medicine* 70: 158–69.

Perceval, O., Y. Couillard, B. Pinel-Alloul, E. Bonneris, and P.G.C. Campbell. 2006. Long-term trends in accumulated metals (Cd, Cu and Zn) and metallothionein in bivalves from lakes within a smelter-impacted region. *Science of the Total Environmental* 369: 403–18.

Poot, A., F. Gillissen, and A.A. Koelmans. 2007. Effects of flow regime and flooding on heavy metal availability in sediment and soil of a dynamic river system. *Environmental Pollution* 148: 779–87.

Rao, M.V., G. Paliyath, and D.P. Ormrod. 1996. Ultraviolet-B and ozone-induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. *Plant Physiology* 110: 125–36.

Remmer, H., W. Kessler, H. Einsele, Th. Hintze, G. Diaz de Toranzo, A.M. Gharaibeh, and H. Frank. 1989. Ethanol promotes oxygen-radical attack on proteins but not on lipids. *Drug Metabolism Reviews* 20: 219–32.

Sabatini, S.E., I. Rocchetta, D.E. Nahabedian, C.M. Luquet, M.R. Eppis, L. Bianchi, M. del, and C. Ríos de Molina. 2011. Oxidative stress and histological alterations produced by dietary copper in the freshwater bivalve *Diplodon chilensis*. *Comparative Biochemistry and Physiology C* 154: 391–8.

Serafim, A., and M.J. Bebianno. 2009. Metallothionein role in the kinetic model of copper accumulation and elimination in the clam *Ruditapes decussatus*. *Environmental Research* 109: 390–9.

Smolders, A.J.P., R.A.C. Lock, G. Van der Velde, R.I.M. Hoyos, and J.G.M. Roelofs. 2003. Effects of mining activities on heavy metal concentrations in water, sediment, and macroinvertebrates in different reaches of the Pilcomayo River, South America. *Archives of Environmental Contamination and Toxicology* 44: 314–23.

Tallandini, L., A. Cassini, N. Favero, and V. Albergoni. 1986. Regulation and subcellular distribution of copper in the freshwater mussel *Anodonta cygnea* (L.) and *Unio elongatulus* (Pf.). *Comparative Biochemistry and Physiology C* 84: 43–9.

Verlecar, X.N., K.B. Jena, and G.B.N. Chainy. 2008. Modulation of antioxidant defences in digestive gland of *Perna viridis* (L.), on mercury exposures. *Chemosphere* 71: 1977–85.

Viarengo, A., E. Ponzano, F. Dondero, and R. Fabbri. 1997. A simple spectrophotometric method for metallothionein evaluation in marine organisms: An application to Mediterranean and Antarctic mollusks. *Marine Environmental Research* 44: 69–84.

DECLARATION

I hereby declare that this submission is my own account of my own research and that, to the best of my knowledge and belief, it contains neither material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of a university or any other institute of higher learning, except where due acknowledgment has been made in the text.

ERKLÄRUNG

Hiermit erkläre ich, dass ich die Arbeit selbstständig verfasst und keine anderen als die angegebenen Hilfsmittel verwendet habe.

Weiterhin erkläre ich, dass ich nicht anderweitig mit oder ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich einer Doktorprüfung zu unterziehen.

Bayreuth, den 9 November 2011 _____

Andhika Puspito Nugroho