EFFECTS OF CADMIUM ON CALCIUM HOMEOSTASIS AND PHYSIOLOGICAL CONDITIONS OF THE FRESHWATER MUSSEL *ANODONTA ANATINA*

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

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Bayreuth, July 2008

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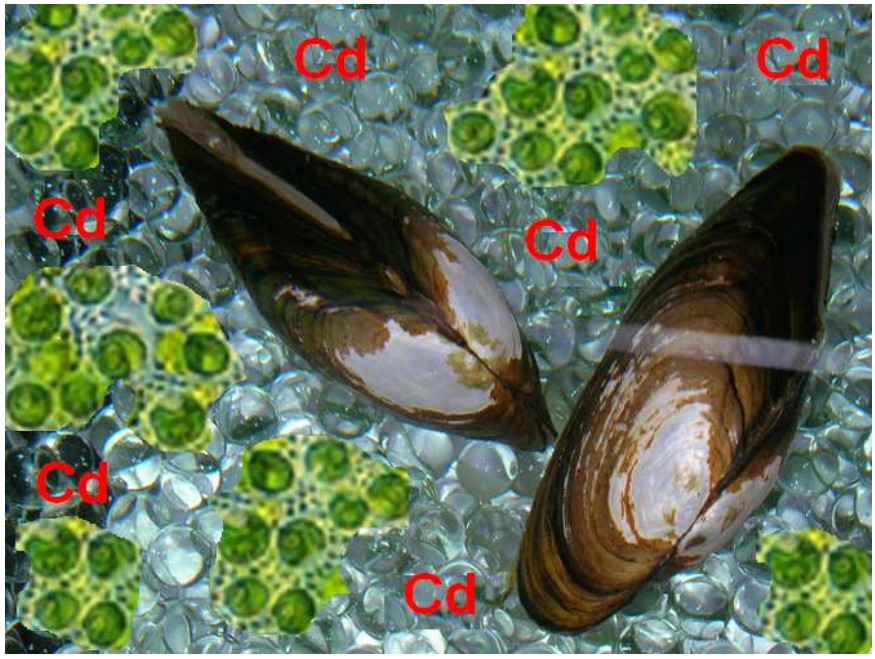

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Vollständiger Abdruck der von der Fakultät für Biologie, Chemie und Geowissenschaften der Universität Bayreuth genehmigten Dissertation zur Erlangung des akademischen Grades eines Doktors der Naturwissenschaften (Dr. rer. nat.).

Promotionsgesuch eingereicht am:	16. Juli 2008
Wissenschaftliches Kolloquium am:	17. November 2008

Prüfungsausschuss:

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Die vorliegende Arbeit wurde in der Zeit von April 2004 bis Juli 2008 an der Universität Bayreuth am Lehrstuhl für Umweltchemie und Ökotoxikologie, unter Betreuung von Prof. Dr. Hartmut Frank angefertigt.

Verfügbar als PDF unter/ available as PDF at: http:/opus.ub.uni-bayreuth.de

To my husband Nguyen Quoc Dinh

and my daughter Nguyen Ngoc Minh Hanh

SUMMARY

As the result of the worldwide decline of freshwater mussels, some significant wetland and riverine habitats are threatened. It has been shown that cadmium (Cd), among other heavy metals, is present at surprisingly high levels in freshwater pearl mussels from mountain brooks in central Europe. This metal is likely to be one of the factors involved in the decline because of its high toxicity, bioaccumulation potential and transfer through food chains. In natural ecosystems, aquatic animals are usually exposed to Cd from both the dissolved- and the particulate-phase, and at lower levels than those used in standard toxicity testing. The aim of this study was to investigate the effects of environment-like Cd levels on its bioaccumulation, tissue distribution and on the redistribution of background Cd (Cd present in animals prior to the experiment) among body compartments of the freshwater mussel *Anodonta anatina* by exposure to both algae- and water-borne ^{111}Cd (about 0.2 µg/L) for 35 days followed by 120 days of depuration. Furthermore, the effects of Cd on calcium (Ca) homeostasis, condition index, energy reserves, carbonic anhydrase activity and their correlations were examined and evaluated. Before mussels were exposed to Cd, a Cd-exposure experiment with the green algae *Parachlorella kessleri* was carried out for producing ^{111}Cd-loaded algae suitable for exposed mussels as food of the same quality as non-contaminated algae.

P. kessleri were grown at four Cd concentrations (0, 0.5, 2, 8 and 32 µg/L) for 5 days, starting from day 2 after inoculation. At 8 µg Cd/L and higher, Cd showed clear effects on algal growth, cell morphology, size and algal physiological state. The minimum Cd concentration at which the algae were significantly different from the control group was about 3 µg/L. At a Cd concentration of 2 µg/L, algae exhibited a comparable physiological state to the control which was used to grow ^{111}Cd-carrying food for the test mussels.

In the mussel exposure experiment, different types of samples were taken. Hemolymph (HML), extrapallial fluid (EPF), gills, mantle, digestive gland, kidney and other tissues were used to determine total Cd, the ^{114}Cd/^{111}Cd isotope ratio for studying Cd accumulation, distribution, elimination and redistribution of background Cd, and its effects on other element profiles (Ca, Mg and Zn). Effects of Cd on energy

reserves (glycogen, glucose and proteins) and on carbonic anhydrase were investigated in the body fluids (HML and EPF), gills, mantle and digestive gland.

During the exposure phase, newly incorporated Cd increased in all body compartments, the highest levels occurring in the kidney. Elimination of newly incorporated and background Cd was slow and mainly from the digestive gland; the increase of newly incorporated Cd in the mantle and of newly incorporated and background Cd in the kidney during exposure and the first half of depuration indicated the mobilization of the Cd pools from other body compartments and deposition in this organ.

Ca concentrations in the body fluids increased during Cd exposure; at the same time, they were lowered in most organs, remaining unchanged only in the gills. Simultaneously, depletion of glycogen in the mantle and digestive gland accompanied by a rise in glucose and a decrease in protein levels in the HML and EPF were observed. This entailed a sharp decrease in condition index and relative dry weight (dw). Significant correlations of Cd with glycogen (mantle, digestive gland) and of Ca with glucose (body fluids) and glycogen (mantle, digestive gland) were found. Concerning enzymatic effects, significantly lower levels of carbonic anhydrase activities were observed in all tested tissues, especially in the gills and digestive gland; strong fluctuations were observed in the HML and EPF with a significant increase right after the rise of glucose in these compartments. The effects of Cd exposure on carbonic anhydrase activities in *A. anatina* were confirmed by significant negative correlations of both total and cytoplasmic carbonic anhydrase activities with newly incorporated Cd.

In conclusion, *A. anatina* readily accumulates Cd at low exposure levels. This results in unfavorable effects on Ca profiles, energy reserves and carbonic anhydrase activities in tissue-specific and time-dependent manners. The effects are long lasting and entail interactions which finally influence Ca metabolism, e.g. adverse effects on energy reserves and enzyme activities. Among the organs, the digestive gland appears to be actively involved in the uptake of Cd from the outside environment and in distributing it to other organs via the HML. This makes it more sensitive to Cd exposure than other organs, reflected by a strong depression of glycogen, carbonic anhydrase activity and Ca level. HML and EPF are important compartments playing a crucial role in uptake and distribution of Cd, revealed by its effects on all tested

parameters, i.e. Ca, glucose, proteins and carbonic anhydrase. The gills seem to be less sensitive due to their high amounts of calcareous concretions. The kidney acts as Cd sink for later excretion. Overall, these findings show that Cd at environment-like levels has distinct biochemical, toxicological and pathological effects which may constitute a critical component in the multitude of environmental factors leading to the observed general decline of freshwater mussel populations.

Keywords: *Anodonta anatina;* Cd bioaccumulation; Cd redistribution; Ca homeostasis; freshwater mussel; stable isotope ^{111}Cd; condition index; glycogen; glucose; protein; energy reserves; carbonic anhydrase; subcellular distribution of carbonic anhydrase; *Parachlorella kessleri;* Cd-loaded algae.

ZUSAMMENFASSUNG

Flussperlmuscheln sind europaweit von Aussterben bedroht. In Zentraleuropa sind sie überraschend hoch mit Cadmium (Cd) und anderen Schwermetallen belastet sind. Cd ist aufgrund seiner hohen Toxizität und seines Bioakkumulations-Potentials in Nahrungsketten mit hoher Wahrscheinlichkeit am Populations Rückgang beteiligt. Aquatische Organismen sind diesem Metall in der gelösten und in der festen Phase ausgesetzt, normalerweise in Konzentrationen, die weit niedriger sind als die bei üblichen Expositionsexperimenten. Das Ziel der vorliegenden Studie war es, die Wirkung von Cd bei Konzentrationen, wie sie in der Umwelt auftreten, auf die Süßwassermuschel *Anodonta anatina* zu untersuchen. Dies betraf besonders die Bioakkumulation, Gewebeverteilung und Diposition des im Tier vorhanden Cd und des neu aufgenommenen. Dazu wurden die Muscheln 35 Tage über Algen und Wasser mit ^{111}Cd (ca. 0,2 µg/L) exponiert, gefolgt von 120 Tagen Depuration. Die Auswirkungen der Cd-Exposition auf die Calcium-(Ca)-Homöostase, Gewebe-Trockenmasse in Bezug auf das Schalenlange (Konditions-Index), Energiereserven, Carboanhydrase-(CA)-Aktivität wurden untersucht.

Zuerst wurden die Bedingungen zur Kultivierung der Grünalge *Parachlorella kessleri* etabliert, um ^{111}Cd-beladene Algen zu züchten, die als Futter für die Muscheln ebenso geeignet sind wie unbelastete Algen. Dazu wurde *P. kessleri* 2 Tage nach Inokulation über 5 Tage mit 4 verschiedenen Cd-Konzentrationen (0; 0,5; 2; 8 und 32 µg/L) kultiviert. Ab einer Konzentration von 8 µg/L zeigten sich starke Wirkungen auf Wachstum, Zellmorphologie, Größe und Physiologie. Die niedrigste Cd-Konzentration, bei der sich exponierte Algen von Kontrollen unterschieden, betrug 3 µg/L. Bei 2 µg/L kultivierte Algen entsprechen bezüglich des Gehaltes photosythentischer Pigmente und Morphologie unbelasteten Algen und sind als Futter für die Muschel Expositionsexperimente geeignet.

Im Verlauf dieser Experimente wurden Proben von Hämolymphe (HML), extrapalliale Flüssigkeit (EPF), Kiemen, Mantel, Verdauungsdrüse, Niere und restliche Gewebe genommen, in denen das ^{114}Cd/^{111}Cd-Isotopenverhältnis, Gesamt-Cd, Cd-Akkumulation, Verteilung und Umverteilung des bereits zu Beginn im Körper vorhandenen Cd, sowie seine Effekte auf andere Elementprofile (Ca, Mg, Zn) bestimmt wurden. Die Wirkungen auf Energiereserven (Glykogen, Glukose, Proteine)

und die Aktivitäten von CA in HML, EPF, Kiemen, Mantel und Verdauungsdrüse wurden ebenfalls untersucht.

Während der Exposition stiegen die Konzentrationen an neu akkumuliertem Cd in allen Körper-Kompartimenten, mit höchsten Werten in der Niere. Die Elimination von neu aufgenommenem und vorhandenem Cd war langsam und verlief zum Teil über die Verdauungsdrüse, zum Teil über die Niere. Die starke Zunahme von Cd im Mantel und in der Niere während der Depuration zeigten die Mobilisierung von Cd-Reservoirs aus anderen Körper-Kompartimenten.

Die Ca-Konzentrationen nahmen während der Cd-Exposition in den Körperflüssigkeiten zu, ab im Gewebe; in den Kiemen blieb sie fast unverändert. Damit ging eine Glykogen-Verarmung des Mantels und der Verdauungsdrüse einher, simultan stiegen die Glukose-Gehalte in HML und EPF, während Protein-Gehalte sowie relatives Trockengewicht fielen. Korrelationen von Cd zu Glykogen (Mantel, Verdauungsdrüse) und von Ca zu Glukose (Körperflüssigkeiten) und Glykogen (Mantel, Verdauungsdrüse) waren hoch signifikant. Besonders in den Kiemen und der Verdauungsdrüse wurden signifikant niedrige CA-Aktivitäten beobachtet. In den Körperflüssigkeiten HML und EPF traten starke Fluktuationen auf; besonders kam es direkt nach der Glukose-Zunahme zu einem signifikanten CA-Anstieg. Die Auswirkungen der Cd-Exposition spiegelten sich in seiner negativen Korrelation zur CA-Gesamt-Aktivität im Gewebe und im Cytoplasma wieder.

A. anatina akkumuliert bei niedrigen Expositions-Konzentrationen Cd stark. Dies hat biochemisch-physiologische Effekte zur Folge, besonders in Bezug auf Ca-Profile, Energiereserven und CA-Aktivitäten. Die Auswirkungen sind langanhaltend und ziehen Interaktionen nach sich, die letztendlich den Ca Metabolismus beeinflussen, z.B. gegensätzliche Effekte auf Energiereserven und Enzymaktivitäten. Die Verdauungsdrüse ist das wichtigste Organ für Cd-Aufnahme aus der Umgebung; von dort wird Cd über die HML in andere Organe verteilt. Dies macht die Verdauungsdrüse empfindlicher als andere Organe, wie starke Rückgange von Glykogen, CA-Aktivität und Ca-Konzentration zeigen. HML und EPF sind Kompartimente, die bei Cd-Aufnahme und Verteilung eine wichtige Rolle spielen. Dies zeigt sich in den Auswirkungen auf Ca, Glucose und Protein sowie auf die CA-Aktivität. Die Kiemen sind am unempfindlichsten, wahrscheinlich wegen ihres hohen Anteils an Calciumcarbonat-Ablagerungen. Die Niere ist eine Cd-Senke.

Insgesamt zeigen die Ergebnisse, dass Cd in Konzentrationen, wie sie in der Umwelt auftreten, toxische Wirkungen hat. Es ist wahrscheinlich, dass die Cd-Belastung von Süßwassermuscheln einen wesentlichen Anteil am beobachteten Rückgang der Populationen beteiligt sind.

Schlüsselwörter: *Anodonta anatina*, Cd-Bioakkumulation, Cd-Umverteilung, Ca-Homöostase, Süßwassermuschel, stabiles Isotop ^{111}Cd, Konditions-Index, Glykogen, Glukose, Protein, Energiereserve, Carboanhydrase, subzelluläre Distribution, *Parachlorella kessleri*, Cd-belastete Algen.

ACKNOWLEDGEMENTS

It would not have worked without the never ending encouragement, support and unconditional love from my family including my parents and my parents-in-law, and especially the heart of my life, Dinh. I thank him for all his love and support, and sharing with me the difficult times during this long journey. A big and sweet thank goes to my daughter, Hanh, who gave me many sweet moments, fun and diversions which distracted me from work for a while.

I wish to express my sincere appreciation and gratitude to my supervisor, Prof. Dr. Hartmut Frank for his support and encouragement during my research at the Chair of Environmental Chemistry and Ecotoxicology, University of Bayreuth. I also appreciate the independence that I had in my studies.

Especially I would like to thank Dr. Silke Gerstmann who has spent time for helping me in many aspects of work and life. She also provided me with valuable instructions, hints and skepticism about my work.

I also want to thank Prof. Jorge Machado (Lab. Applied Physiology, University of Porto) for valuable discussions, critical readings and comments for the manuscripts.

I am very grateful to Dr. Gunter Ilgen and his team for assisting in metal analyses.

Furthermore, I am greatly indebted to Katharina and Robert Klupp who are always with me whenever I need help; they always make me happier with their deep sentiments.

I wish to express my appreciation to Susanne and Ortwin for their friendship, their kindness and for providing me uncountable help whenever needed, their understanding and encouragement during my stay in Germany.

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

I would like to express my gratitude to all of my colleagues at the Chair of Environmental Chemistry and Ecotoxicology, University of Bayreuth. I thank Dr. Radomir Cabala for valuable discussions, Elke Bär and Agnes Bednorz for their

technical support and kind-heartedness, Benjamin Schmidt for providing lots of technical assistance, Irmgard Lauterbach for her help in the administrative procedures during my study, Monika Weckert, Guillermo Restrepo and Nubia Quizros for their cordiality and support.

I also would like to express my sincere acknowledgements to all the people who assisted and supported me during these years, and to those I may have involuntarily forgotten to mention here.

Thanks to you all...

TABLE OF CONTENTS

LIST OF MANUSCRIPTS AND AUTHOR'S CONTRIBUTION

This dissertation is presented in cumulative form. It comprises four individual manuscripts which were all accepted for publication and are currently in press. Author's contribution to each manuscript is given below.

1. Ngo, H.T.T., Gerstmann, S., Frank, H., 2008. Toxicity of cadmium to the green alga *Parachlorella kessleri*: Producing Cd-loaded algae for feeding experiments. Toxicol. Environ. Chem., in press (Appendix I)

Own contribution: idea (70 %), method development (90 %), data analysis and calculations (100 %), writing (100 %) and editing the paper (50 %)

2. Ngo, H.T.T., Gerstmann, S., Frank, H., 2008. Sub-chronic effects of environment-like cadmium levels on the bivalve *Anodonta anatina* (Linnaeus 1758): I. Bioaccumulation, distribution and effects on calcium metabolism. Submitted to Toxicol. Environ. Chem., in press (Appendix II)

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

3. Ngo, H.T.T., Gerstmann, S., Frank, H., 2008. Sub-chronic effects of environment-like cadmium levels on the bivalve *Anodonta anatina* (Linnaeus 1758): II. Effects on energy reserves in relation to calcium metabolism. Submitted to Toxicol. Environ. Chem., in press (Appendix III)

Own contribution: idea (80 %), method development (100 %), data analysis and calculations (100 %), writing (100 %) and editing the paper (70 %)

4. Ngo, H.T.T., Gerstmann, S., Frank, H., 2008. Sub-chronic effects of environment-like cadmium levels on the bivalve *Anodonta anatina* (Linnaeus 1758): III. Effects on carbonic anhydrase activity in relation to calcium metabolism. Submitted to Toxicol. Environ. Chem., in press (Appendix IV)

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

LIST OF ABBREVIATIONS

APW	Artificial pond water
AR	Accumulation rate
ATP	Adenosine triphosphate
BAF	Bioaccumulation factor
Ca	Calcium
Cd	Cadmium
DR	Depuration rate
dw	Dry weight
ECE	Economic Commission for Europe
EDTA	Ethylene Diamine tetra-Acetic Acid
EGTA	Ethylene Glycol tetra-Acetic Acid
EPF	Extrapallial fluid
Eqn	Equation
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
HML	Hemolymph
IC	Inhibiting concentration
IUCN	International Union for Conservation of Nature
KCl	Potassium chloride
KOH	Potassium hydroxide
LOEC	Lowest-observed-effect concentration
MSD	Minimum significant difference
MT	Metallothionein
NOEC	No-observed-effect concentration
OD	Optical density
OME	Outer mantle epithelium
r_s	Spearman coefficient
SD	Standard deviation
ww	Wet weight

1. General introduction

1.1. Freshwater mussels and their status

Freshwater mussels are bivalves, belonging to the phylum Mollusca, which have two shells hinged together. They live on the bottom of rivers, brooks, streams, lakes and ponds. They can have smooth shells or shells with bumps or knobs depending on the type of bottom of their habitat. Most of them live 20 – 30 years, some up to 200 years. Freshwater mussels feed on microscopic animals and plants by sucking the water through siphons to filter out food particles. As filtering organism, they clean the water and remove algae, particulates, toxicants and potential disease agents. This in turn enables sunlight to penetrate deeper; therefore, growth of submerged aquatic vegetation is favoured and better nursery areas for benthic fish are created (Phelps, 1994). Mussels also serve as food for a wide variety of animals of the higher trophic levels and help to stabilize and modulate the flow of sediment and substrate along the bottom of rivers and streams (Hauer and Lamberti, 2006). Over the last decades, the number of mussels has been declining so severely that many are now considered as endangered species or as threatened with extinction, e.g. the freshwater pearl mussel *Margaritifera margaritifera* which is now on the IUCN Red List (Groombridge and Mace, 1993). The extinction of freshwater mussels has negative effects on the aquatic ecosystem which they inhabit. Some factors leading to this deterioration are related largely or in part to anthropogenic disruptions of habitat (channelization, siltation and water pollution) and life-cycle restrictions (loss of host fish) (Bogan, 1993). There have been a number of efforts to conserve and restore habitats, propagate juveniles in captivity, and provide refuges for threatened and endangered species. Nevertheless, the fact remains that many species are still declining. This calls for further investigations to understand the involvement of persistent pollutants such as heavy metals in the multi-facetted sequence of events leading to toxic stress of such benthic populations. Understanding their roles in freshwater mussel decline can be helpful in contributing to management and early warning of population health disturbance, and development of successful intervention strategies for alleviation and prevention of further decline. Among others, the heavy metal cadmium (Cd) is potentially involved because of its high toxicity, bioaccumulation and transfer through

food chains; its presence at alarmingly high levels has been shown in the endangered species *M. margaritifera* (Frank and Gerstmann, 2007).

1.2. Cd in aquatic systems and its toxicity

Cd is persistent and known to be one of the most toxic heavy metals for a wide range of organisms, from microorganisms to terrestrial animals and human beings, in which it accumulates mainly in liver and especially in the kidney and skeleton (Willden and Hyne, 1974). At high dose, it gives rise to human health effects on the respiratory tract and kidney problems associated with osteoporosis which can be fatal. This element enters the aquatic environment from different anthropogenic sources such as non-ferrous smelting and refining, manufacturing of metals and chemicals, emissions of coal-fired power plants, its former presence in phosphate fertilizers, its use in various consumer products, and its release via waste waters and industrial effluents; natural processes such as forest fires, volcanic activities and weathering of rocks also play a role in the Cd cycle. Once released into water bodies through atmospheric deposition, direct discharges, run-off and natural processes, Cd is deposited in sediments from where it may be taken up by benthic organisms, bioaccumulated in their organs, and then transferred to animals of higher trophic levels via food chains. Acidification may result in increased mobilization of Cd from soils and sediments and lead to augmented levels in surface and ground waters (WHO, 1986). The toxicity of Cd to freshwater organisms varies considerably depending on the exposure level and duration, species, and life-stage, its toxicity being related to its free ionic concentration. Early life-stages are the most vulnerable; freshwater organisms are affected at lower Cd concentrations than marine organisms. The presence of Cd in sediments and in particulate matter makes it dangerous for benthic filtering organisms, particularly for freshwater bivalves which often have bioaccumulation factors (BAF) in the order of thousands.

1.3. Adverse effects of Cd exposure on physiological condition and Ca metabolism of freshwater mussels

Like other heavy metals, Cd has been known to affect the physiological condition of exposed molluscs specified by the depletion of energy reserves (Ansaldo et al., 2006; Leung and Furness, 2001), changes of intermediary metabolism in different organs (Reddy and Bhagyalakshmi, 1994), suppression of carbonic

anhydrase activity (Lionetto et al., 2006), and especially interference with calcium (Ca) metabolism (Faubel et al., 2008) by inducing renal tubular damage which results in excessive loss of Ca (Brzóska and Moniuszko-Jakoniuk, 1998). Besides the direct inhibition of Ca re-absorption from the primary urine, Cd also competes with Ca in its binding calmodulin, a Ca-specific protein, in the cell membrane (Flik et al., 1987) and may affect Ca-ATPase (Verbost et al., 1988). Freshwater mussels often live in a poor Ca environment and need to maintain a Ca concentration in the hemolymph (HML) of about 8 – 10 times higher than that of the outside environment (Van der Borght and Van Puymbroeck, 1966). Therefore, Ca uptake in these animals, besides that from food, mainly involves active transports which require high metabolic energy, i.e. the phosphate-bond energy in ATP (Schatzmann, 1973). Energy is also needed for the movement of Ca from a compartment with low chemical activity to the one with high activity. Thus, depletion of energy reserves is another factor indirectly disturbing Ca uptake and transport.

The high concentration of Ca in the HML helps to create a chemical gradient across the outer mantle epithelium (OME) favoring the movement of ionic Ca into extrapallial fluid (EPF) for shell formation (Coimbra et al., 1993). Ca is also stored transiently in calcareous deposits in the gills and mantle (Machado et al., 1988) for biomineralization (Moura et al., 1999), shell formation and larval development. Shell mineralization takes place in the EPF of the mantle cavity in which the precipitation of $CaCO_3$ is induced by organic components produced by the OME, and is also regulated through the indirect control of Ca solubility and Ca concentration at saturation condition in the EPF (Coimbra et al., 1993). These processes are known to be facilitated by carbonic anhydrase (Freeman, 1960; Medakovic, 2000; Wilbur and Saleuddin, 1983). Therefore, any disturbance of energy reserves, carbonic anhydrase activity, Ca uptake and transport would influence the calcification processes, hence lowers animal fitness and hampers the development of populations. The questions arise whether the disturbance of Ca metabolism in freshwater mussels by Cd occurs at environment-like concentrations and whether this constitutes a relevant aspect of the potential causes of population decline.

1.4. The freshwater mussel Anodonta anatina and ecotoxicological studies

Anodonta anatina (class *Bivalvia*, order *Unionoida*, family *Unionidae*) is a formerly abundant but now declining freshwater mussel. Looking for the potential causes of this mussel's decline, the results can be extrapolated to other freshwater mussels which are now threatened with extinction and are under strict conservation, i.e. *M. margaritifera.* While many ecotoxicological studies were conducted on marine bivalves, freshwater mussels have received less attention. There are a couple of studies on *A. anatina*, e.g. on the uptake and accumulation of chlorinated phenols (Mäkelä and Oikari, 1990; Mäkelä et al., 1991), of pentachlorophenol (Mäkelä and Oikari, 1995), testing of viability of glochidia exposed to selected metals (Hansten et al., 1996) and on Cd kinetics (Holwerda et al., 1988; Holwerda et al., 1989; Streit and Winter, 1993).

Toxicant burdens of bivalve tissues only show the estimation of environmental exposures of concerned animals, not the biological effects of the respective toxicant. Therefore, supplemental measures of effect, biomarkers and bioindicators, are used in efforts to estimate the biological impacts of toxicants on individuals and populations, respectively (Schlenk, 1999). Also, while acute toxicity testing with lethal endpoints can provide fast and strong responses, chronic and sub-chronic exposures at low toxicant levels warrant more sensitive and ecologically more relevant endpoints, such as changes in behaviour, metabolism or growth (Rand et al., 1995). Furthermore, experiments at population and community levels are needed because the ultimate goal of environmental protection is the ecological integrity (Clements, 1997), even though individual-level responses can sometimes be used to predict population-level effects (Maltby, 1999).

1.5. Objectives of the research

Although there are several ecotoxicological studies on *A. anatina* (see 1.4), the effects of Cd on Ca uptake and transport, on the general fitness of animals (energy reserves and condition index), carbonic anhydrase activity and eventually Ca metabolism have not been assessed, especially not at environmental Cd levels. In this study, the general aim was to investigate the effects of Cd at environment-like levels on compartmental Ca profiles and metabolism, and enzyme activity, and the general physiological condition of this mussel.

Freshwater mussels are filter feeders that rely on detritus, bacteria, and planktonic algae for nutrients (Cahoon and Owen, 1996; Gatenby et al., 1996; Nichols and Garling, 2000). Size, shape, and nutritional value of algal species are factors in determining their suitability as food (Gatenby et al., 1996). Therefore, before the experiment with mussels, green algae *Parachlorella kessleri* were tested with different Cd concentrations to determine a suitable Cd level to be used in preparing ^{111}Cd-loaded food which has a similar physiological state and nutritional value as non-exposed algae to avoid secondary effects, e.g. altered feeding behaviour (Appendix **I**). In mussel exposure experiment, first, the uptake, bioaccumulation, distribution of newly incorporated Cd, and the redistribution of background Cd were investigated (Appendix **II**). Secondly, effects of Cd on some biomarkers, i.e. carbonic anhydrase activity and energy reserves, and ultimately effects on Ca metabolism and general physiological conditions were examined (Appendices **II – IV**). These objectives are summarized in Fig. 1:

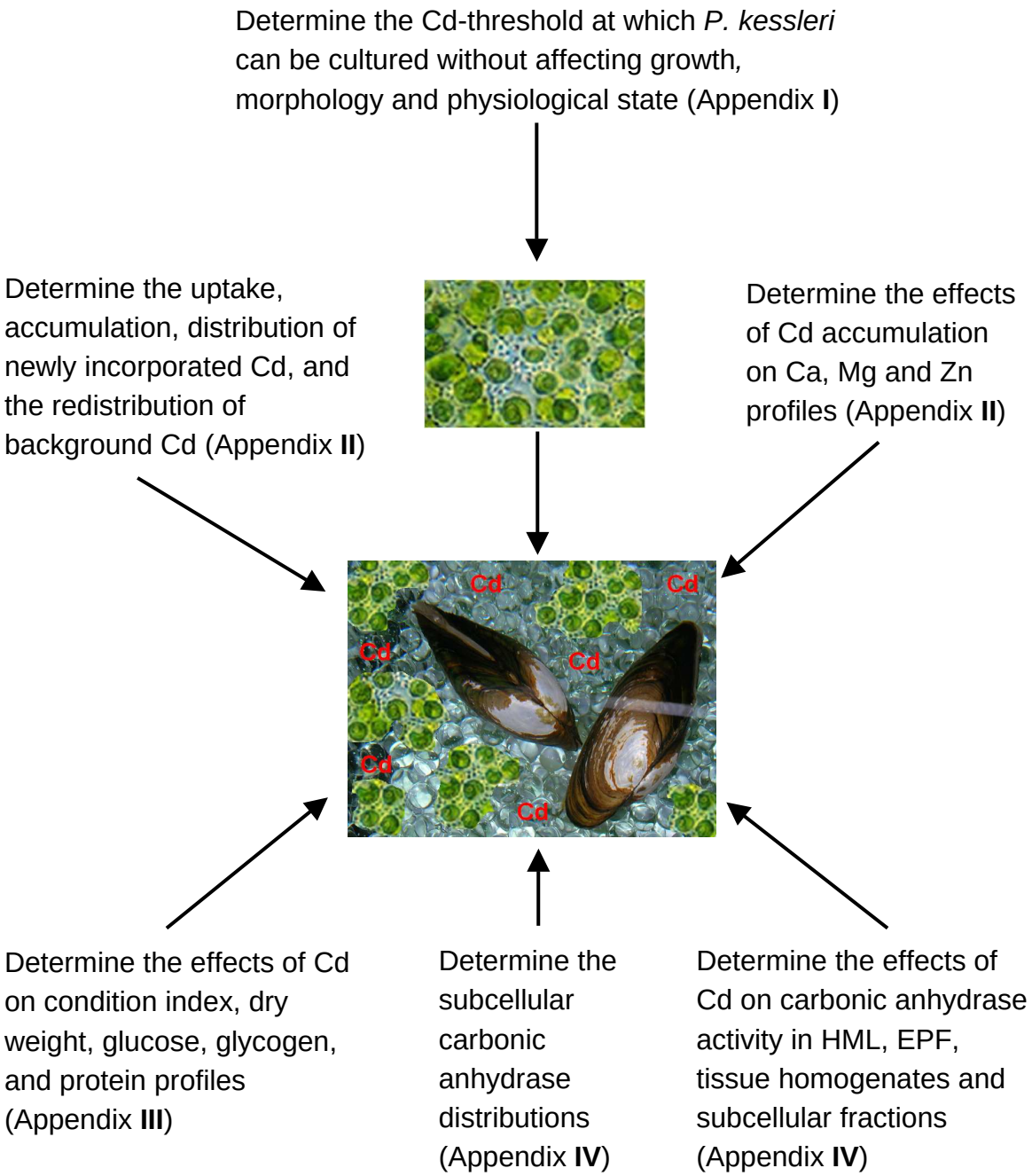

Figure 1. A schematic representation of the aims of the studies presented in several manuscripts (Appendices **I** – **IV**).

2. Materials and methods

2.1. Test compound and other chemicals

Isotopically enriched (96 %) ^{111}Cd metal (CK GAS product Ltd, Hampshire, UK) was used as a test compound for all experiments; all chemicals used for analytical purpose were of ultrapure grade (Sigma-Aldrich, München, Germany); other chemicals were of analytical grade (VWR, Darmstadt, Germany). Preparation of ^{111}Cd stock and working solution are described (Appendix **I** & **II)**.

2.2. Cd exposure experiment with green algae P. kessleri

Green algae *P. kessleri* (University of Göttingen, Germany) were exposed to different ^{111}Cd concentrations (0.5, 2, 8, and 32 µg/L) to find the most suitable concentration which can be used to produce ^{111}Cd-loaded algae suitable as food. Details of experimental designs and measurements are described in Appendix **I**. Briefly, a number of parameters were measured to investigate the growth rate, physiological state and nutritional value of algae, i.e. cell density, chlorophyll (a, b), pheophytin-a, optical density ratios of $OD664_b/OD665_a$, polysaccharides, proteins and their ratio as well as cell size and morphology.

2.3. Cd exposure experiment with freshwater mussel Anodonta anatina

2.3.1. Animals and design of experiment

A randomized experimental design with high external validity, which may be generalized causal interferences to other conditions and populations, was carried out. *A. anatina* of similar length and weight were used as test animals which were obtained from a pond (ZOO-Erlebnis Online Shop, Großefehn, Germany) with a Cd concentration in the water below the limit of quantification (0.03 µg/L), in suspended solids at 0.03 µg/L. ^{111}Cd was administered to the exposed group through both pathways as in nature: water- and particle-borne ^{111}Cd (0.2 µg ^{111}Cd/L plus 1 mg ^{111}Cd-loaded algae/L/day). A high level of similarity between the control and the exposed group was created to make sure that artifacts influencing the results were prevented, e.g. physicochemical condition and food quality. The design of the experiments was described in a series of manuscripts (Appendices **II** – **IV**). In

summary, three mussels were sacrificed for sampling of body fluids and tissue samples before the exposure experiments to serve as control for determination of the actual background levels; the latter were in good agreement with those of the control group. Mussels of similar size (weight: 44 ± 6 g, length: 80 ± 4 mm) and of similar age (6 – 8 year-old) were acclimatized in artificial pond water (APW) (Dietz et al., 1994b) for one month, then they were kept in APW for five weeks of exposure and four months of depuration. Several parameters and endpoints were determined in different body compartments during both phases (Table 1). Due to the low weight of the kidney, it could only be used for metal analyses, not for biochemical assays.

During the experimental course, the stable isotope ratio of $^{114}Cd/^{111}Cd$, the concentration of total Cd, total Ca, Mg and Zn were monitored in HML, EPF, gills, mantle, digestive gland, kidney and other tissues. Background and newly incorporated Cd were calculated according to Rodríguez-Cea et al. (2006), based on total Cd concentration and the stable isotope ratio of $^{114}Cd/^{111}Cd$ (Appendix **II**).

The effects of Cd on condition index and energy reserves were studied in some body compartments, i.e. HML, EPF, gills, mantle and digestive gland. The relationships between Cd levels and energy reserves and between energy reserves and Ca levels were also examined in these body compartments (Appendix **III**).

Studies of tissue and subcellular distributions of the key enzyme in the calcification process, carbonic anhydrase, effects of Cd on its activity in the HML, EPF, gills, mantle and digestive gland, and correlations of its activity with Cd and with Ca levels are reported in Appendix **IV**.

Table 1

Design of mussel exposure experiment (Appendices **II** – **IV**)

	Appendix **II**	Appendix **III**	Appendix **IV**
Test type	Semi-static with substrate and continuous aeration		
Temperature	17 ± 1℃		
Illumination (intensity)	12 h dark : 12 h light (13 – 19 µmol/m^2/s)		
Test chamber size	50 x 30 x 30 cm		
APW volume	32 L		
Water exchange	Every second day		
Size of mussels	Weight: 44 ± 6 g, length: 80 ± 4 mm		
Exposure phase:	35 days		
- Total no. of mussels	18 (control group) + 18 (exposed group)		
- Sample replicates	3 mussels/sampling point		
- Test endpoints	Cd conc. and its distribution, AR, BAF_{35d}, Ca conc.	CI, body dw (%), glycogen and protein conc.	CA activity, CA distribution
Depuration phase:	120 days		
- Total no. of mussels	9 (control group) + 9 (exposed group)		
- Sample replicates	3 mussels/sampling point		
- Test endpoints	Cd conc. and distribution, DR, Ca conc.	CI, body dw (%), glycogen and protein conc.	CA activity, CA distribution
Whole experiment:	155 days		
- Test endpoints	Correlation of Ca *vs.* Cd conc.	Correlation of glycogen *vs.* Cd and *vs.* Ca conc., protein *vs.* Cd and *vs.* Ca conc.	Correlation of CA *vs.* Cd and *vs.* Ca conc.

Notes: BAF_{35d} = bioaccumulation factor after 35 days exposure, conc. = concentration of elements or substances measured at different time points during both phases, CA = carbonic anhydrase, AR = accumulation rate of Cd during exposure, DR = depuration rate of Cd during depuration, CI = condition index of animals, and body dw (%) = percentage of the dry weight of the soft body relative to its wet weight (ww).

2.3.2. Biological sampling and analytical methods

As the species is declining, the minimum acceptable replicates (3 x) and sampling points were chosen. To compensate for the low number of animals to obtain a more complete picture of mussels' condition and the effects of Cd exposure, HML and EPF were taken more often (Appendix **II**, Table 1) using a nonlethal method (Gustafson et al., 2005). At given time intervals, three mussels from each group were taken for sampling of the body fluids (HML and EPF) and tissues of the gills, mantle, kidney, digestive gland and other tissues (foot, heart, reproductive gland and adductors) (Appendix **II**, Table 1). The gills, mantle and digestive gland were divided into three aliquots. The aliquot for element determination was freeze-dried; the two aliquots for glycogen and for protein and carbonic anhydrase analyses were frozen at -80℃ for further processing. Kidney and other tissues were lyophilized as a whole for element analyses.

2.3.2.1. Determination of elements

Sample preparation and analytical methods were described in Appendix **II**. Briefly, freeze-dried tissue samples were digested in a mixture of conc HNO_3 and HCl (4/1) at 40℃ for 1 h, then at 120℃ for 3 h, diluted with bidistilled water to 10 mL and filtered through 0.45 µm cellulose syringe filters. Body fluid samples were acidified with 0.5 mL HNO_3 and diluted with bidistilled water to 10 mL. Total Cd, $^{114}Cd/^{111}Cd$ ratio and Zn were determined by inductively coupled plasma mass spectrometry (ICP-MS). Ca and Mg were determined by ICP atomic emission spectroscopy (ICP-AES). The validity of the methods was checked periodically by analyzing the certified biological reference material from mussel tissue *Mytilus edulis* (EFM® - CE278, Geel, Belgium), all results being within the certification range (96 – 111 %). Procedural blanks (HNO_3/HCl, 4/1) of all analyzed elements were below detection limits.

2.3.2.2. Glycogen determination

Glycogen in the digestive gland, mantle and gills, and glucose in the HML and EPF (Appendix **III**) were determined by a modified phenol-sulphuric acid method (Dubois et al., 1956; Naimo et al., 1998) using glycogen Type VII as standard (Sigma-Aldrich, Munich, Germany). Briefly, the sample was digested in three volumes (v/v or v/w) of 30 % aqueous KOH in 2-mL polypropylene cryovials (Simport Plastic, Quebec, Canada) in a shaking water bath at 100℃ for 20 min. A mixture of digestate and

absolute ethanol (v/v, 1/1) in a scintillation vial was shaken in a water bath at 100℃ for another 15 min. The sample was then diluted with bidistilled water, vortexed and kept at room temperature for at least 5 min before being measured in triplicate using a microtiter plate reader (340-ATTC, Easy software, SLT Labinstruments, Crailsheim, Germany) at 492 nm.

2.3.2.3. Protein and carbonic anhydrase determination

Sample preparation and analysis were described in detail in Appendix **III** and **IV**. In a few words, the tissue samples were homogenized in three volumes (v/w) ice-cold isotonic extraction buffer (buffer A), containing 10 mM HEPES, pH 7.5 with 250 mM sucrose, 1 mM EGTA, 25 mM KCl and protease inhibitors, using a Labsonic U tip sonicator (B. Braun Biotech International, Melsungen, Germany). All procedures were carried out at 0 – 4℃. The crude homogenates were fractionated following Henry et al. (2003) and Depierre and Dallner (1976) into cytosol (S1), nuclei and cell debris (P1), then into cytosol (S2), mitochondria and lysosomes (P2), cytoplasm (S3), microsomes (P3). Body fluid samples were sonicated and centrifuged at 10,000 x g for 5 min to remove particulate matter. Thereafter, protein and carbonic anhydrase activity were assayed in body fluids, tissue homogenates and subcellular fractions.

Total proteins were analyzed in appropriately diluted samples (Appendix **III**) by the Bradford method (Bradford, 1976) using a microtiter plate reader (340-ATTC, Easy software, SLT Labinstruments, Crailsheim, Germany).

The pH method (Vitale et al., 1999) was employed to estimate the carbonic anhydrase activity (Appendix **IV**). The enzymatic assay was carried out in triplicate in a reaction medium (10 mM Tris-phosphate pH 7.4, 225 mM mannitol, 75 mM sucrose) into which a known volume (0.1 mL) of tissue-homogenate, subcellular fraction, body fluids or buffer A (blank) and 1 mL ice-cold CO_2-saturated bidistilled water was added and stirred. The catalyzed ($b_{\text{catalysed}}$) and non-catalyzed reaction rates ($b_{\text{non-catalysed}}$) were the slopes of linear regression of pH *vs.* time from samples and blanks, respectively. Specific carbonic anhydrase activities were calculated as units per mg protein (U/mg protein) (Vitale et al., 1999).

2.4. Data and statistical analyses

During the whole experiment, dry weight of the gills decreased slowly due to low glycogen contents and high proportions of Ca concretions, to about 55 % (Pynnönen et al., 1987), while total body weights of both control and exposed animals decreased significantly at the end of exposure and during depuration. Corrections for weight loss between these periods were needed before calculating Cd and Ca concentrations (Appendix **II)** to accurately determine Cd accumulation and its effects on Ca levels in mussel tissues. If not, the "concentration" parameter would be misleading with respect to Cd kinetics during exposure and depuration. Weight loss correction was calculated in relation to the respective gill weight according to Hemelraad and Herwig (1988). Concentrations of all elements were calculated on wet weight (ww) basis. In Appendix **III**, proteins and glycogen were calculated based on actual wet weight (before correcting for weight loss) therefore Cd and Ca concentrations before correcting for weight loss were used for testing correlation. For Appendix **IV**, carbonic anhydrase activities were calculated per mg protein hence Cd and Ca concentrations were converted to protein basis before correlation with enzyme activities.

In this study BAFs, ARs and DRs were calculated based on the newly incorporated Cd concentration of the respective tissue, not based on total Cd concentration as conventionally, to avoid misestimating their values due to redistribution of background Cd among organs.

Data are presented as means ± standard deviation (SD). Two-way analysis of variance was used to determine whether differences in each variable between groups and sampling times were significant. If significant differences were found, the paired t-test and post-hoc test (Newman-Keuls procedure) were used to detect significant differences between groups and between sampling times as independent variables (GraphPad Software, San Diego, CA).

Correlations between variables, i.e. tissue-Cd *vs.* tissue-Ca, tissue-Cd *vs.* tissue-glycogen or tissue-Ca *vs.* tissue-glycogen were tested with the linear regression test or nonparametric Spearman's rank correlation test ($p < 0.05$). Statistical significances were assigned at $p < 0.05$, $p < 0.01$ and $p < 0.001$.

3. Results

3.1. Studies of Cd-effects in green algae P. kessleri: Food quality for exposure experiment

Green algae *P. kessleri* exposed to different Cd concentrations showed adverse effects at very low level, from about 3 µg/L (MSD values, Appendix **I**, Table 3). At Cd concentrations of 2 µg/L, algae showed growth rates, food quality and physiological state comparable to control, reflected in all tested parameters, i.e. pheophytin-a and optical density ratios of $OD664_b/OD665_a$, protein/polysaccharide ratio, and cell morphology (Appendix **I**, Table 1, Fig. 1 and 2). Therefore, to produce ^{111}Cd-loaded algae suitable as food in mussel exposure experiment, the concentration of 2 µg Cd/L was used to ensure that the feeding behaviour of exposed mussels were not different from that of control animals. The food contained a concentration of 5.9 µg ^{111}Cd/g dw. This was confirmed by the results of a preliminary experiment in which mussels fed with the Cd-loaded algae showed a comparable clearance rate to animals fed with control algae.

3.2. Effects of environment-like Cd levels on freshwater mussels A. anatina

3.2.1. Studies of uptake, bioaccumulation, distribution and redistribution of Cd

During exposure, newly incorporated Cd increased with time in all body compartments (Appendix **II**, Fig. 1), especially in the body fluids where it increased immediately after 1 h to reach highest values (HML 1.1, EPF 1.0 µg/L) at the end of exposure (Appendix **II**, Table 2). Total Cd increased only in the body fluids, kidney, digestive gland and gills, not in the mantle and other tissues. Kidney contained a high initial concentration of Cd (about 2 mg/kg), 3- to 5-fold higher than that in the other organs; newly incorporated Cd increased most strongly with a high BAF (1300) at the end of exposure. Both newly incorporated and background Cd increased until day 60 of depuration, reaching almost double the initial value. The digestive gland showed a similar trend, also with a high BAF (1350) but total Cd increased more slowly than in the kidney. Other organs showed quite slow increases in newly incorporated and total Cd, except for the gills where total Cd increased relatively fast, being about 50 % of the initial level at the end of exposure (Appendix **II**, Fig. 1).

During depuration, newly incorporated Cd increased further in the kidney and mantle. Total Cd also increased strongly in the kidney during the first half of depuration and started to decrease only towards the end. Excretion of newly incorporated Cd was fastest for the digestive gland followed by the gills, mantle and other tissues (Appendix **II**, Table 3).

During the whole experiment, background Cd (i.e. total minus newly incorporated Cd) was mobilized in the exposed animals and redistributed among organs by incoming Cd (Appendix **II**, Table 4). After the onset of Cd exposure, the digestive gland and other tissues lost some background Cd (negative values of mobilized Cd) to the gills and especially to the kidney, as shown by the increasingly positive values of mobilized Cd (from +0.3 to +3.5 at day 60 of depuration) in the kidney. The balance of mobilized Cd indicates that it was excreted from the end of exposure onwards. Overall elimination of both background and newly incorporated Cd from the mussels' body was slow (together from 170 down to 150 µg/kg soft body weight within 4 months) and occurred only during the second half of depuration (Appendix **II**, Table 3).

3.2.2. Studies of Cd effects

3.2.2.1. Changes in Ca homeostasis and other element profiles

A fast and significant increase in Ca was observed both in HML and EPF (HML 25 %, EPF 20 % at 5 h) followed by gradual decreases during depuration to reach control levels at day 5 (Appendix **II**, Table 5). Concurrently, Ca concentrations in most mussel tissues (Appendix **II**, Fig. 1) decreased with time, being about 30 – 40 % lower in the mantle, digestive gland and other tissues ($p < 0.05$) than in the control at day 5 of exposure and during last two months of depuration; in the kidney it was lowest when the newly incorporated and total Cd concentrations were highest at day 60 of depuration ($p < 0.05$).

Mg and Zn were also disturbed by Cd exposure (Appendix **II**, Fig. 2). Mg tended to decrease in the digestive gland and mantle upon Cd exposure but less than Ca. Zn decreased significantly in the kidney from the end of exposure till the end of depuration. This effect was also observed in all other organs but not as strong as in the kidney.

3.2.2.2. Changes in dry weight and condition index

Control mussels lost about 1.5 % of their dry weight, while exposed mussels lost about 3 % towards the end of the whole experiment (Appendix **III**, Table 1). Concurrent with the dry weight loss, the condition index of the exposed group was also lower at the end of exposure, becoming more marked ($p < 0.05$) towards the end of depuration (1.7 *vs.* 0.9), but not in control group ($p > 0.05$).

3.2.2.3. Changes in energy reserves

During depuration, glycogen decreased in the mantle and digestive gland of exposed animals (Appendix **III**, Fig. 1), becoming only about 20 % of the initial values at the end. As a result, an increase of glucose in both HML and EPF were observed in exposed animals, reaching 147 % of the initial value at the end of exposure, while it remained nearly unchanged in the control (Appendix **III**, Table 2).

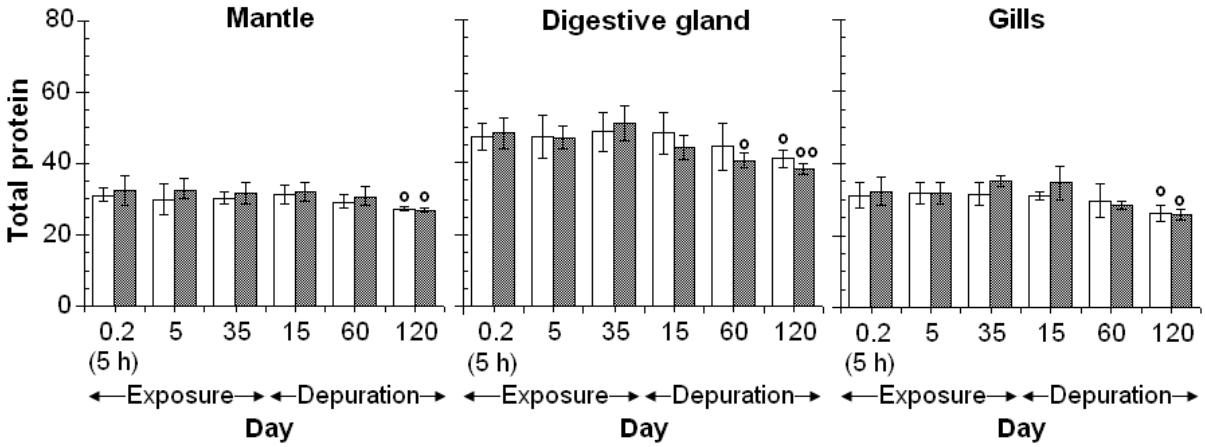

Figure 2. Protein concentrations (g/kg ww) in the mantle, digestive gland and gills of control (open bars) and exposed (shaded bars) animals (mean ± SD, n = 3). Statistically significant differences in comparison to the same group in the beginning of experiment (^{o} $p < 0.05$; ^{oo} $p < 0.01$) are shown.

Protein levels stayed almost unchanged in all tested tissues (Fig. 2) but decreased in the body fluids (Appendix **III**, Table 4) from the beginning of exposure. At the end of depuration, they decreased to 28 % in HML and 38 % in EPF in exposed animals while they were about 50 % each in the control group. Moreover, subcellular protein profiles showed some changes, especially in the microsomal fraction where they decreased in all examined tissues of exposed mussels during depuration (Appendix **III**, Fig. 3).

3.2.2.4. Changes in carbonic anhydrase activity

A decrease of carbonic anhydrase activity was obvious in EPF at 25 h and in HML at the end of exposure. Thereafter, carbonic anhydrase increased strongly being about 10-fold (HML) or 2-fold (EPF) higher than in control ($p < 0.05$), but afterwards it decreased again to reach control levels.

In all examined organs, Cd caused a decrease of carbonic anhydrase activity of 40 – 50 % at the end of exposure (Appendix **IV**, Fig. 1). Gills contained about 2 times more carbonic anhydrase than other organs and were also affected by Cd though to a lesser extent than the digestive gland. Mantle carbonic anhydrase was less affected. Concerning subcellular carbonic anhydrase, cytoplasmic and nuclear carbonic anhydrase activities (60 % and 16 % of the total tissue carbonic anhydrase activity, respectively - Appendix **IV**, Fig. 2) were most strongly affected by Cd exposure (Appendix **IV**, Fig. 3). This reflected by the depressions at the end of exposure which even continued during depuration, especially in the digestive gland. Microsomal carbonic anhydrase was least affected with a significantly lower value observed only in the digestive gland at day 60 of depuration.

3.2.3. Correlations between examined parameters

Spearman's rank correlation tests showed that newly incorporated Cd correlated to three out of four tested parameters (Ca, glycogen and carbonic anhydrase) while total Cd showed correlation only to Ca and glycogen in some compartments. Correlations to protein concentrations were not found in any tested organ. For Cd *vs.* Ca (Table 2), correlations were only observed in the kidney (newly incorporated Cd *vs.* Ca) and in the gills (total Cd *vs.* Ca). For Cd *vs.* energy reserves, the mantle and digestive gland (Appendix **III**, Fig. 2) showed negative linear correlations of glycogen *vs.* newly incorporated ($R^2 = 0.55$ and 0.68, respectively) and total ($R^2 = 0.49$ and 0.62, respectively) Cd concentrations (concentrations before correcting for weight loss, Appendix **V**, Table a), but not in the gills. Nevertheless, in the gills and digestive gland the increase in newly incorporated Cd was significantly correlated to the decrease in specific activity of total ($r_S = -0.61$ and -0.55, respectively) and cytoplasmic carbonic anhydrase ($r_S = -0.49$ and -0.59, respectively) (Appendix **IV**, Table 2) but not in the mantle ($p > 0.05$).

Table 2.

Correlation factors (Spearman r or r_s) of Cd *vs.* Ca levels in different tissues of *A. anatina* exposed to algae- and water-borne Cd for five weeks followed by four months depuration. Significant correlations are indicated (n = 21): * $p < 0.05$

	Newly incorporated Cd *vs.* Ca	Total Cd *vs.* Ca
Kidney	-0.47*	0.19
Gills	0.02	0.50*
Mantle	0.14	0.32
Digestive gland	0.09	0.31
Other tissues	-0.28	-0.01

Correlation tests between Ca *vs.* other endpoints were found for glycogen and carbonic anhydrase in some body compartments, but not for protein. Strongly positive correlations of Ca *vs.* glucose in the HML ($r_s = 0.73$) and EPF ($r_s = 0.66$), and significantly negative relationships of Ca *vs.* glycogen in the mantle ($r_s = -0.55$) and digestive gland ($r_s = -0.57$) were found, but not in the gills (Appendix **III**, Table 3). Significant correlations of Ca *vs.* carbonic anhydrase activities in the HML ($r_s = 0.36$) and mantle homogenates ($r_s = 0.45$) were observed.

4. General discussion

In the nature, mussels take up Cd and Ca from both sources, water and food (Fig. 3). It is proven by the present study that algal food is an important Cd source of exposed animals, especially when exposure experiments are carried out at ecologically relevant Cd levels. This is reflected by the order of the organs' ability to accumulate Cd from the outside environment: kidney = digestive gland >> gills ≥ mantle > other tissues. When *A. anatina* were exposed to water-borne Cd without food added, a different order was reported: kidney = gills >> digestive gland > mantle > muscle, reflecting that the main uptake was through the gills (Holwerda et al., 1988). Similar to the present findings, Cunningham (1979) concluded that trace elements enter the bivalve molluscs mainly through the digestive tract. Therefore, particulate Cd in the form of Cd-loaded food should be included in such exposure experiments.

By using ^{111}Cd, uptake of "new" Cd, its distribution, and the redistribution of background Cd between body compartments due to replacement by the newly incorporated Cd can be tracked at low exposure level of 0.2 µg/L, i.e. within the range (0.07 – 0.5 µg Cd/L) recommended by ECE for the maintenance of freshwater aquatic life, class II (40 – 50 mg $CaCO_3$/L) (UNECE, 1994). Using this tracer, a significant accumulation was found in the gills, digestive gland and mantle of *Mytilus galloprovincialis* exposed to a Cd level similar to that of the marine environment (1 µg/L) from the first day onwards (Labonne et al., 2002).

Due to the redistribution among organs of newly incorporated Cd during depuration and of background Cd during the whole course of the experiment, newly incorporated Cd continued to increase in the kidney, mantle and other tissues (Appendix **II**, Table 3); total Cd increased only in the kidney but decreased in the other organs. More specifically, increases of newly incorporated Cd were due to transfer from body fluids and the transport from other organs to the kidney, mantle and other tissues. The digestive gland and other tissues lost some background Cd early during Cd exposure to the gills and especially to the kidney, as shown by the increasingly positive values of mobilized Cd (Appendix **II**, Table 4).

The high BAF in the kidney, especially the continuous increase of both newly incorporated and background Cd until day 60 of depuration, indicates that this organ

is the most important one in seizing Cd from other body compartments. The digestive gland showed a similar trend as the kidney but this organ actively took up Cd from the environment while the kidney received Cd from the other organs. This is mirrored in the order of the ratio of newly incorporated Cd *vs.* background Cd at the end of exposure: digestive gland >> gills > mantle = other tissues >> kidney. On the other hand, the fastest release of newly incorporated Cd was observed also in the digestive gland which may be due to the active depuration and transport of Cd to other organs (Ballan-Dufrancais et al., 1985).

The Ca ion is of great importance not only in cellular functions and regulation mechanisms (Bronner, 1992) but also as a major component of the exoskeleton in molluscs, the shell. It is even more important for freshwater mussels which often live in relatively low-ionic environments and need to maintain Ca concentrations in the body fluids about 10 times higher than in the ambient water (Van der Borght and Van Puymbroeck, 1964; 1966) to ensure normal processes of shell formation and repair. It is well known that the processes of Ca uptake and transport in aquatic animals like freshwater mussels are often disturbed by Cd due to competition for the same binding sites on the cell membrane. In addition, Cd-induced acidosis results in mobilization of Ca from storage pools which is finally excreted due to kidney tubular damage by Cd (Fig. 3).

In the present study, the animals took up Cd directly from water and food via the gills, mantle and especially the digestive gland, and transferred it, via the body fluids, to other organs. The newly incorporated Cd made the background Cd being released from some organs, e.g. the digestive gland and other tissues, to move it to the kidney and partially to the gills (Appendix **II**, Table 4). This redistribution of background Cd caused pronounced alteration in total Cd in some tissues, e.g. decreases in the digestive gland and increases in the kidney. At the same time, the total Cd pools did not change in other organs, e.g. the mantle and other tissues. This suggests that the newly incorporated Cd is metabolically more active to exert effects on Ca profiles, glycogen metabolism and carbonic anhydrase activities. Anaerobic glycogen breakdown during Cd exposure and especially the inhibition of the carbonic anhydrase activity in the cytoplasm lead to metabolic acidosis due to the build-up of organic acids and CO_2, respectively (Crenshaw and Neff, 1969; Henry, 1996; Moura et al., 2000). This in turn entails the mobilization of Ca from the endoplasmic

reticulum and the mitochondrial storages, and the dissolution of Ca from calcareous concretions. These result in an increase of Ca in the extracellular compartments (HML and EPF) observed in this study. The damage of kidney tubule caused by high Cd levels (Appendix **II**, Fig. 1) may make it dysfunctional in re-absorption of Ca from the primary urine resulting in excessive losses (Brzóska and Moniuszko-Jakoniuk, 1998).

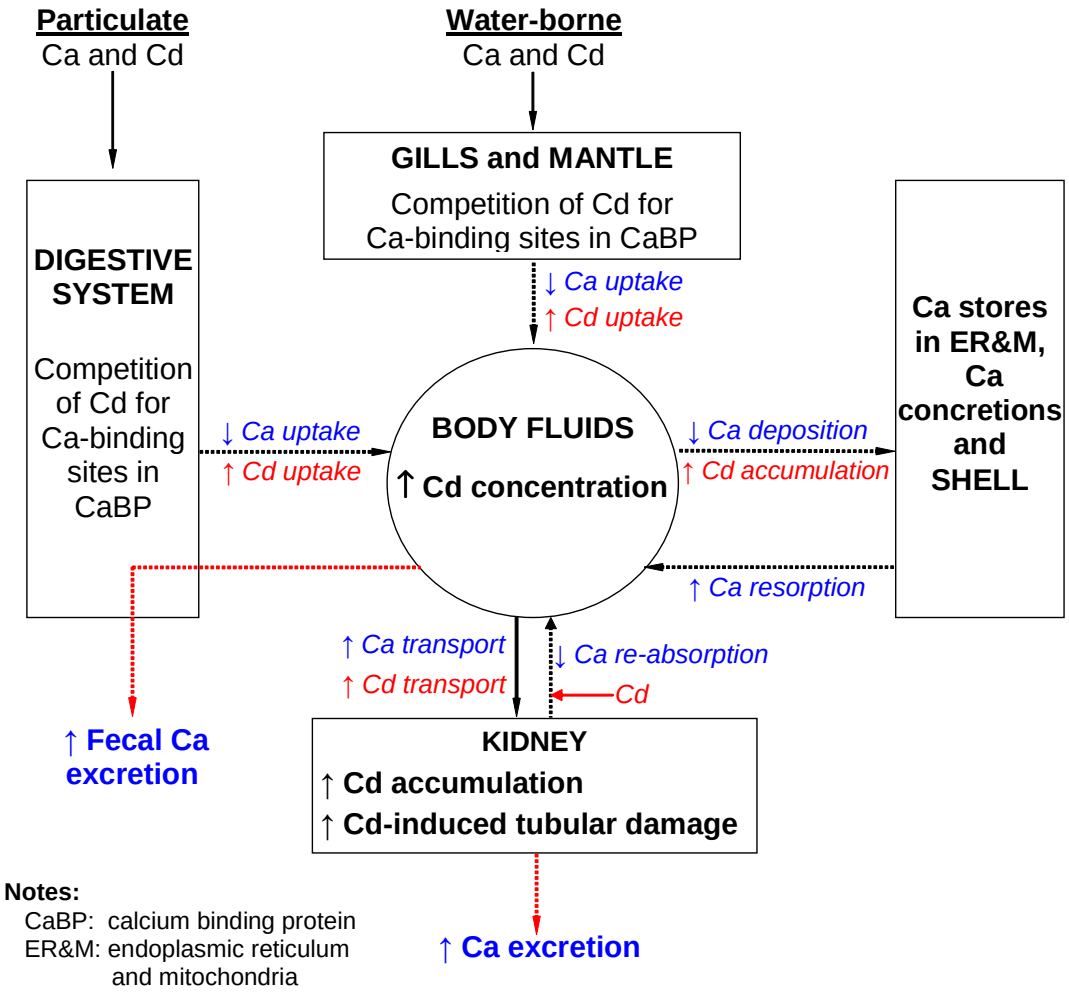

Figure 3. Influences of Cd on Ca uptake and homeostasis in aquatic bivalves (see text for detail).

Although the effects of Cd on Ca profiles were obvious in most tested tissues, correlations between these two parameters were only found for the kidney and the gills. This may be due to the fact that at this low Cd concentration, Cd accumulation in tissues exhibited its effect only at the beginning when its concentration started to

increase (Appendix **II**, Fig. 1). Thereafter, animals may adapt by inducing more metallothionein (MT) synthesis (Baudrimont et al., 2003; Marie et al., 2006) to capture Cd and impound it in non-toxic form. Positive correlation observed in the gills may be due to formation of new Ca concretions in this organ to sequester Cd.

Beside Ca, adverse effects of Cd on the two divalent cations Mg and Zn may cause additional problems for the mussels. Mg plays similar roles as Ca though its concentrations are lower. Zn plays an important role in many enzymes involved in the processes of calcification, e.g. carbonic anhydrase, was also disturbed by Cd exposure. This is similar to the findings in snails *Lymnaea stagnalis L.* exposed to both water-borne and particulate Cd (Présing et al., 1993) or in the freshwater mussel *Lammellidens marginalis* (Das and Jana, 1999). The reason for the reduction of Zn may be due to its displacement by Cd in some ligands like MTs or in Zn-containing enzymes like carbonic anhydrase (Présing et al., 1993; Pruski and Dixon, 2002).

Furthermore, deterioration of energy metabolism and physiological condition reflected by the lower condition index of exposed animals due to tissue-glycogen depletion (Appendix **III**, Fig. 1) may hamper Ca uptake and homeostasis which require high metabolic energy in the form of ATP (Schatzmann, 1973). The decrease of tissue glycogen is in accordance with the results for a closely related species, *A. cygnea,* exposed to water-borne Cd (Hemelraad et al., 1990). This glycogen reduction indicate the activity of glycogenolysis to fulfill the high energy demand of animals during stress (Pekkarinen and Suoranta, 1995). Tight correlations between the increases of newly incorporated and total Cd concentrations (Appendix **V**, Table a) in the digestive gland and the mantle to the depression of glycogen confirmed this. The absence of a correlation in the gills may be due to the high density (about 55 %) of Ca concretions (Pynnönen et al., 1987). Moreover, the unchanged glycogen levels during exposure and its decrease during depuration indicate the long-term effects of low Cd levels on energy storage of animals. Additionally, some alterations in subcellular protein patterns may affect other biological functions. These energy disturbances are related to Ca homeostasis, which was proven by significant correlations between these two parameters in several compartments, i.e. glucose *vs.* Ca (in the HML and EPF) and glycogen *vs.* Ca (in the mantle and digestive gland).

The toxicity of Cd on these animals was more obvious when carbonic anhydrase, an important enzyme present mainly in the cytoplasm, was found to be strongly depressed in the gills and digestive gland, the main organs for ion uptake, transport and regulation. The lower effect of Cd on the mantle carbonic anhydrase may be due to its high proportion of microsomal carbonic anhydrase (Appendix **IV**, Fig. 2) which is more stable than the other isozymes (Lionetto et al., 2000; Swenson et al., 2001). This indicates that carbonic anhydrase activities in different organs have different sensitivities toward Cd which is similar to findings with eel or estuarine crabs (Lionetto et al., 1998; Skaggs and Henry, 2002). The pronounced effects of Cd on carbonic anhydrase activities were confirmed by the significantly negative correlations of newly incorporated Cd to the total and cytoplasmic carbonic anhydrase activities in the gills and digestive gland, but not of total Cd *vs.* carbonic anhydrase in the gills. This indicates that the newly incorporated Cd is more efficiently suppressing carbonic anhydrase activity than its total pool which corresponds to the conclusion of Rainbow (2002) that there is no relationship between total heavy metal body burden and its toxicity. Due to its catalytic function in the reversible reaction of CO_2-hydration, carbonic anhydrase activity plays an important role in the ion exchange between Na^+/H^+ and Cl^-/HCO_3^- (Henry and Saintsing, 1983) in epithelial cells. As a consequence, inhibition of carbonic anhydrase caused by Cd may influence ion transports and osmoregulatory processes. The decrease of carbonic anhydrase in the mantle tissues also related to Ca homeostasis, showed by the significant positive correlation between carbonic anhydrase and Ca. A high levels of carbonic anhydrase (Appendix **IV**, Table 1) in body fluids just occurred after an increase in glucose (Appendix **III**, Table 2) which indicates the participation of carbonic anhydrase in glucose/glycogen re-synthesis and metabolism (Henry, 1996; Wang et al., 1998).

Overall, Cd at environment-like levels can exert profound effects, both directly and indirectly, on the transepithelial transport of ions of *A. anatina*, especially Ca. This transepithelial transport is crucial for freshwater mussels to take up essential elements from hypotonic environment and to re-absorb them from the primary urine. Disturbances in uptake, transport and/or re-absorption due to deterioration of interdependent processes, i.e. uptake competition, metabolic acidosis, kidney tubular damage, lack of energy reserves for active transports as well as limit of the key enzyme carbonic anhydrase facilitating for these processes, can eventually influence

the equilibrium and homeostasis of elements, especially of Ca, the major element of mussels' shell. This affects the overall physiological state of the mussels and causes the depletion of Ca storage pools reserved for a diversity of cell functions and for reproduction. As a result, a poor quality of offspring is entailed which consequently poses a threat for the development and survival of such populations.

5. Conclusions, contributions and perspectives

5.1. Conclusions

The results presented in this thesis demonstrate that *A. anatina* readily incorporated Cd at low exposure levels through both the water- and particulate-phase. Cd bioaccumulation during exposure and redistribution between organs during exposure and post-exposure caused alterations of Ca patterns in exposed-animals, with strong increases of Ca levels in body fluids and reductions of tissue-Ca. Among the organs, the kidney and digestive gland have the highest ability to concentrate Cd; the latter is more active in sequestering “new” Cd while the kidney is its “sink”. Moreover, the mass balance reveals that the newly incorporated Cd fraction constituted less than 20 % of the total Cd body burden although the former exerted profound derangements of Ca-homeostasis and physiological conditions. Also, it is noteworthy that the load of Cd accumulated over one month of exposure has not been fully eliminated during four months of depuration.

In addition, this low Cd level exerted strong effects on energy reserves and general fitness of animals, apparent through a striking decrease of glycogen in the digestive gland and mantle, and of proteins in the body fluids. At the same time, the glucose levels in the body fluids increased. Tight correlations of Ca *vs.* elevated glucose and lowered glycogen levels suggest that the carbohydrate metabolism is affected by Cd which in turn interferes with Ca metabolism.

Also, at the chosen Cd concentration the activity of carbonic anhydrase, an important enzyme for osmoregulation, acid-base regulation and calcification, was lowered in all examined compartments, especially in the HML, EPF, gills and digestive gland. These observations suggest indirect effects of Cd on Ca metabolism.

When looking at the general effects of Cd in various body compartments, the digestive gland appeared to be actively involved in the uptake of Cd from the environment and in its redistribution to other organs via HML. This coincided with the higher sensitivity of this organ to Cd exposure compared to others, mirrored by strong depressions of glycogen and carbonic anhydrase activity in this organ. HML and EPF were also important compartments due to their crucial role in the distribution of Cd; they showed Cd-effects in all tested parameters, i.e. increased Ca and glucose

levels, decreased protein concentrations and strong fluctuations of carbonic anhydrase activities. The mantle, an important organ for shell formation, showed moderate Cd-sensitivity; gills seemed to be the least sensitive organ in respect to Cd toxicity.

5.2. Contributions

Ecological risk assessments are commonly based upon toxicological laboratory tests. Thus, the obtained data are used to characterize risks associated with a given substance at relevant exposure levels and eventually to suggest procedures for risk management. The question is whether data obtained from standard tests, usually performed at high concentration, can be extrapolated to field conditions. Concentrations of Cd in the environment are well below the acute toxicity levels. In order to study the effects of Cd at environment-like level and its long-term effects on physiological conditions, in general, and on Ca metabolism, in particular, an attempt was made to create experimental conditions closer to natural ones, especially by supplying Cd-loaded algae as food. The study suggests that Cd-carrying food should always be included in exposure experiments to obtain a better insight into Cd-effects on freshwater mussels. The use of isotopically enriched ^{111}Cd allowed to elucidate the mechanisms of uptake, storage and redistribution of newly incorporated Cd and of background Cd which are normally not detected in toxicity tests using normal Cd.

At concentrations which are considered as safe for freshwater aquatic organisms, bioaccumulation of Cd can induce substantial effects on biological functions such as energy balance, carbonic anhydrase activity, Ca uptake and transport; all these effects, in some ways, influence the vitality of the organism. As a consequence, the overall fitness of animals is hampered and poor conditions of offspring are entailed. In the long-term, this can pose a threat to the populations and to the long-term survival of a species.

The methods developed in this study may be of practical use in the field for attempts to pinpoint the facets of causes leading to freshwater mussels' decline, in order to protect and conserve these animals.

5.3. Perspectives

In the present study, the steady-state concentration of Cd was not reached within the time frame of exposure. This suggests a need for longer-lasting exposure experiments in order to be able to calculate more valid steady-state BAFs and the actual half-life of Cd which is derived from these experiments in the range of several years.

Additional factors need to be included in such exposure experiments, e.g. other heavy metals and persistent organic pollutants, especially those which are known to be present at elevated levels in many benthic organisms, and which may have a multitude of synergistic and antagonistic effects on the biochemical, enzymological, endocrinological and physiological conditions.

Further parameters such as effects on the calcium-binding protein calmodulin or on the enzyme Ca^{2+}-ATPase should be investigated in order to improve the understanding of the underlying mechanisms.

Finally, practical conclusions should be drawn from these experiments, in particular with field studies to generate conditions in selected watersheds to lower the exposure of benthic organisms to pollutants predominantly absorbed to particulate organic matter in sediments of aquatic ecosystem.

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APPENDICES

Appendix I:

Toxicity of cadmium to the green alga *Parachlorella kessleri*: Producing Cd-loaded algae for feeding experiments

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(Received 07 February 2008; final version received 18 March 2008; accepted 18 April 2008)

Toxicological and Environmental Chemistry, in press

Abstract

The effects of Cd at environmental concentrations (0 – 32 µg/L) on the green alga *Parachlorella kessleri* were investigated. At about 3 µg Cd/L, toxic effects of Cd are becoming evident, much lower than reported previously. At 8 µg/L and higher, pronounced adverse effects on growth, cell morphology, size, and physiological state are seen. Therefore, levels not higher than 2 µg Cd/L should be employed to produce Cd-carrying algae for feeding experiments with organisms on the next trophic level, e.g. mussels, to avoid reduced food uptake. These findings also suggest that aquatic ecosystem conditions can be indirectly influenced via the impairment of the nutritional value of algae since they are the basic organisms of aquatic food chains.

Keywords: Parachlorella kessleri; Cadmium; algal growth; protein/polysaccharide ratio; pheophytin-a; algal physiological state.

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1. Introduction

Cadmium is a widely distributed metal which has considerable technological relevance (pigments, anti-corrosive, polymer-additive, batteries), and which may be present in coal and phosphate minerals. Due to its relative high volatility it is released with the flue-gases of coal-fired power plants by which it is widely distributed and diffusely deposited. In the aquatic environment it is mobile under oxic conditions but is retained in anoxic sediments. Although being non-essential it is taken up by algae, may substitute Mg as the central atom of chlorophyll (Küpper et al., 1998) and may cause ultrastructural changes (Nishikawa et al., 2003) of chloroplasts, an organelle containing numerous metal-sensitive enzymes (Rouch et al., 1995). If chlorophylls are converted to pheophytin, photosynthesis and growth will be influenced (Ilangovan et al., 1998; Lu et al., 2004; Mohammed and Markert, 2006). Cd is also known to alter lipid, protein and polysaccharide contents (Sathya and Balakrishnan, 1988) and the protein/polysaccharide ratio of algae (Thompson and Couture, 1991).

The green algae *Chlamydomonas reinhardtii*, *Scenedesmus quadricauda, Chlorella vulgaris, C. fusca, C. pyrenoidosa* or *C. ellipsoidea* have been used extensively in ecotoxicological studies, mostly at a few concentrations (e.g. 30 and 100 µg Cd/L; Thompson and Couture, 1993) or much higher than typical for the environment, i.e. 1000, 2000 and 5000 µg Cd/L (Ilangovan et al., 1998; Lam et al., 1999). So far no investigations have been conducted with Cd at concentrations as they occur in rivers and brooks of industrialized countries, especially not with *Parachlorella kessleri* (Krienitz et al. 2004), a unicellular freshwater green alga also known as *C. kessleri* (Fott and Nováková 1969) which can be grown on fully inorganic medium.

When ecotoxicological studies with aquatic animals are done with Cd, two uptake pathways need to be considered, i.e. via water and Cd-loaded algae as food. If their physiological state is affected they may have lowered nutritional value or may become fully unsuitable as food (Sathya and Balakrishnan, 1988). Preliminary feeding experiments have shown that freshwater mussels fed with algae grown at a Cd concentration of 32 µg/L filter the food three times slower than the control group. This will obviously influence the results of such experiments.

The aim of this study is to determine the Cd-threshold at which *P. kessleri* can be cultured without affecting growth, morphology and physiological state. The "health" status of such algae is examined microscopically and by determining cell density, chlorophyll-a and b, pheophytin-a and protein and polysaccharide contents.

2. Materials and methods

2.1. *Glassware and reagents*

All glassware was washed with half-conc HNO_3 and rinsed thoroughly with deionized water, finally with bidistilled water. All media and glassware were autoclaved before use. Lyophilized glycogen standard (Type VII, *Mytilus edulis*), Bradford solution, HEPES buffer, bovine serum albumin (BSA) (Sigma-Aldrich, Munich, Germany) for analytical purpose, and all other chemicals for culture medium and measurements were analytical grade. A $^{111}Cd^{2+}$ stock solution with a concentration of 100 mg/L was prepared by dissolving 0.01 g Cd metal 96 % ^{111}Cd enriched (CK GAS product Ltd, Hampshire, UK) in 0.4 mL conc HNO_3 (suprapur 65 %) in a 100-mL volumetric flask. Further 0.8 mL conc HNO_3 was added and bidistilled water was filled to the mark. A dilute solution of 1 mg $^{111}Cd^{2+}$/L was prepared from the stock solution for exposure experiments.

2.2. *Algal culture conditions*

P. kessleri (University of Göttingen, Germany) were cultured in K-medium according to Kuhl and Lorenzen (1964) modified to include one-tenth of the micronutrient concentrations, without Cu^{2+} and EDTA; the macronutrients were 1.5-fold elevated, $CaCl_2$ 2.5-fold elevated. The medium was adjusted to pH 7 with 10 % KOH solution. Axenic cultures were started with an inoculation density of 2 x 10^5 cells/mL and were illuminated continuously with fluorescent tubes at an intensity of 48 – 51 µmol/m^2/s; the temperature was 26 ± 2°C. Two days after in oculation, when algae were physiologically adapted and were at the beginning of the exponential growth phase, $^{111}Cd^{2+}$ was added to the medium using the 1-mg/L solution to establish exposure concentrations of 0.5, 2.0, 8.0, and 32.0 µg/L; the actual Cd^{2+} concentrations were determined 1 h and 2 days after administration and were found to be between 83 – 90 % of the nominal ones. All tests were conducted in three simultaneous replicates in 500-mL Erlenmeyer flasks with ground necks, closed with

silicone stoppers with two glass tubes for CO_2 supply and air exhaust. A dual-head membrane pump (VDE 0530 KNF, Neuberger, Germany) was connected to a pressure tank as equalizer; its outlet was connected to a manifold with five aeration lines, each joined with Teflon tubing to a Drechsel gas washing bottle filled with carbonate buffer 2 M $KHCO_3/K_2CO_3$ (35/65 v/v) to distribute air and CO_2 to three Erlenmeyer flasks per line. The cells were kept suspended by gentle shaking; pH was determined at the beginning and the end of the experiments (WTW GmbH and Co. KG, Model Multi 340i, Weilheim, Germany).

2.3. *Sampling and analyses*

Algal density was determined daily using an improved Neubauer Hemocytometer (Fisher Scientifics, Schwerte, Germany). After seven days of growth, i.e. five days of Cd exposure, the algae were harvested and cell density and biomass were determined. Specific growth rates and doubling times were calculated according to Reynolds (1984). The remaining algae were centrifuged at 10000 x g, 4°C for 10 min (Beckman Avanti J25, rotor JA-25.50), resuspended in fresh culture medium, again centrifuged twice, and washed once with PBS buffer (Phosphate Buffered Saline). Algal pellets were frozen (-80°C, 24 h), freeze-dried (48 h, -20°C), and kept in desiccators in the darkness for later use. Preliminary experiments showed that chlorophyll-a and b, pheophytin-a, protein and polysaccharide concentrations in fresh and freeze-dried samples did not differ ($p > 0.05$).

2.3.1. Chlorophylls and pheophytin-a determination

For determination of the concentrations of chlorophyll-a, chlorophyll-b and pheophytin-a (APHA, 1992; Jeffrey and Humphrey, 1975), lyophilized algae (about 10 mg) were suspended in 12 mL ice-cold acetone in 15 mL PP tubes and homogenized by sonication on ice at 20 kHz for 3 min (6 x 30 sec), acoustic power 50 W (Labsonic U tip sonicator, B. Braun Biotech International, Melsungen, Germany). Homogenates were kept for 24 h at 4°C before optical densities were measured (UVIKON 930 Spectrophotometer, Kontron Instruments, Munich, Germany) at 647, 664, and 750 nm ($OD647_b$, $OD664_b$ and $OD750_b$) and, after acidification with 30 µL 0.1 N HCl per mL extract solution, at 665 and 750 nm ($OD665_a$ and $OD750_a$).

2.3.2. Protein and polysaccharide analyses

About 10 mg of lyophilized algae were suspended in 1 mL 10 mM HEPES buffer, pH 7.5, in 1.5-mL Eppendorf tubes and were homogenized with a sonicator. Homogenates were centrifuged at 16,000 x g, 4℃ for 20 min (Beckman Avanti J25, rotor JA-25.50), supernatants were taken and stored at –80℃ till use. Aliquots of 50 µL supernatant were diluted with 450 µL 0.01 mol/L NaOH in 1.5-mL Eppendorf tubes; the mixtures were well blended and incubated at 60℃ for 30 min. Total protein content was determined (Bradford, 1976) using BSA as standard. Samples and standards were measured at 600 nm, reference filter 492 nm, using a microtiter plate reader (340-ATTC equipped with Easy software, SLT Labinstruments, Crailsheim, Germany).

Polysaccharide content was determined by a modified phenol-sulphuric acid method (Dubois et al. 1956; Naimo et al. 1998). Samples and standards were measured with a microtiter plate reader (340-ATTC) at a wavelength of 492 nm, reference filter 600 nm. Glycogen Standard type VII was used for calibration and for calculation of polysaccharide concentrations.

2.3.3. Cell size and morphology

At the end of the experiments, the cells were measured and visually examined with a microscope (1000-fold magnification, Leitz Laborlux, Leitz, Wetzlar, Germany) fitted with a digital camera (Leica DFC 320) and connected to a computer with appropriate software (Leica Application Suite).

2.4. *Data analyses*

Differences in variables were assessed by one-way ANOVA; if significant differences were found, the Tukey-Kramer multiple comparisons test was conducted (GraphPad InStat, San Diego, California, USA). Correlations between variables were tested with the Spearman's rank correlation test ($p < 0.05$). The no-observed-effect concentration (NOEC), the lowest-observed-effect concentration (LOEC), and the minimum significant difference (MSD) were estimated using Dunnett's test (USEPA, 2002). A linear interpolation method was used to estimate inhibition concentrations (IC_{10}) for algal growth and chlorophyll synthesis using software developed by Norberg-King (1993) (ICPIN, Version 2.0, USEPA, Duluth, MN, USA).

3. Results

The concentrations of chlorophylls, pheophytin-a and optical density ratios of $OD664_b/OD665_a$ determined in the control group and at the two lowest Cd concentrations (0.5, 2 µg/L) at the end of the experiments were closely similar (Table 1). In the 2-µg Cd/L-group, a trend towards lowered chlorophyll-b and increased pheophytin-a was apparent but not significant ($p > 0.05$), while in the 8 µg Cd/L group lowered pigment contents and $OD664_b/OD665_a$ ratio and elevated pheophytin-a levels were observed; in the 32 µg Cd/L group the effects were even stronger with 60 % decreases in chlorophyll-b and 109 % increase in pheophytin-a.

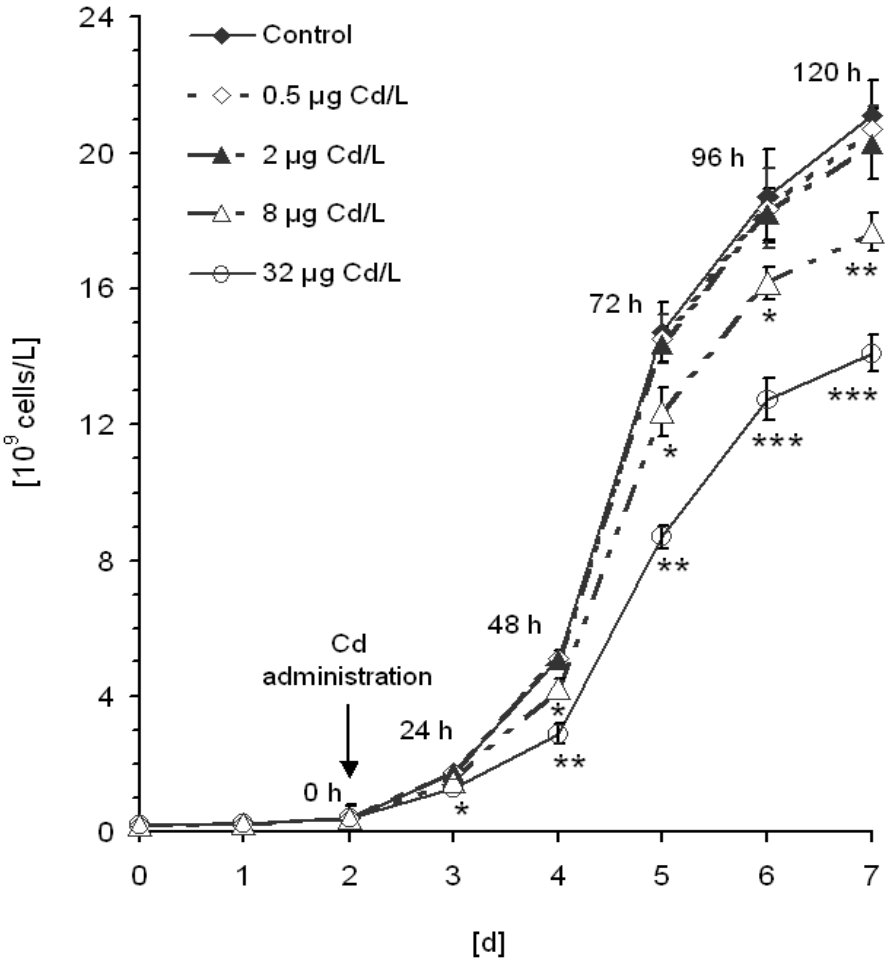

Fig. 1. Effects of Cd on the cell growth of *P. kessleri*. Error bars represent standard deviations (n = 3). Asterisks denote statistically significant differences from control values (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). The arrow indicates the time when Cd was added to the culture medium.

Table 1

Contents of photosynthetic pigments chlorophyll-a, chlorophyll-b, pheophytin-a [g/kg dw], $OD664_b/OD665_a$ ratio, biomass [g/L], protein and polysaccharide contents [g/kg dw] and the ratio of the latter two in *P. kessleri* after 5 days Cd-exposure.

Cd [µg/L]	Chlorophyll-a	Chlorophyll-b	Pheophytin-a	$OD664_b/OD665_a$ ratio	Biomass	Protein	Polysac-charide	Protein/poly-saccharide ratio
0	36.0 ± 0.9	13.0 ± 2.0	4.8 ± 0.3	1.62 ± 0.01	0.43 ± 0.02	224 ± 13	160 ± 11	1.38 ± 0.07
0.5	35.4 ± 1.7	12.3 ± 1.4	5.0 ± 0.7	1.61 ± 0.02	0.43 ± 0.02	225 ± 14	164 ± 9	1.37 ± 0.06
2.0	34.2 ± 1.4	10.5 ± 0.9	5.6 ± 0.8	1.60 ± 0.02	0.42 ± 0.02	218 ± 15	174 ± 12	1.26 ± 0.13
8 .0	29.7 ± 1.3**	7.6 ± 1.1***	7.2 ± 0.5**	1.56 ± 0.03**	0.36 ± 0.02**	192 ± 9*	193 ± 8**	1.00 ± 0.07**
32.0	23.3 ± 1.7***	5.4 ± 0.8***	9.9 ± 0.8***	1.48 ± 0.01***	0.30 ± 0.02***	162 ± 10***	212 ± 9***	0.76 ± 0.08***

Asterisks denote statistically significant differences from control values (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Data are presented as mean ± SD (n = 3).

The concentrations of proteins, polysaccharides and the ratios of the two in the 0.5 and 2 µg Cd/L-groups were not significantly different from control ($p > 0.05$). At 8 and 32 µg Cd/L, decreases in protein by 15 % and 28 %, and increases in polysaccharides by 20 % and 32 % were observed ($p < 0.05$), resulting in lower protein/polysaccharide ratios compared to control algae ($p < 0.01$; Table 2). The Spearman's rank correlation test ($p < 0.05$) showed a negative correlation between Cd exposure and protein concentrations ($n = 15$, $r = 0.90$) and a positive correlation between Cd and polysaccharide concentrations ($n = 15$, $r = 0.87$).

Table 2
Specific growth rates and doubling times of *P. kessleri* during the exponential phase (day 2 – 5)

Cd [µg/L]	Specific growth rate [d^{-1}]	Doubling time [h]
0	1.20 ± 0.01	13.8 ± 0.2
0.5	1.19 ± 0.02	13.9 ± 0.3
2.0	1.19 ± 0.02	14.0 ± 0.3
8 .0	1.15 ± 0.02*	14.5 ± 0.3*
32 .0	1.03 ± 0.01***	16.1 ± 0.2***

Asterisks denote statistically significant differences from control values (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Data are presented as mean ± SD ($n = 3$).

Growth rate and cell density (Fig.1, Table 2) did not differ significantly from control ($p > 0.05$) up to 2 µg Cd/L. At the two highest Cd concentrations, in the exponential phase doubling times were higher as growth rates were diminished ($p < 0.05$; Table 2). In the 8-µg/L group, a decrease in cell density and biomass was evident on the second day of exposure ($p < 0.05$), in the 32-µg/L group cell density was depressed by 25 % already on the first day of exposure. After 5 days, cell density and biomass were reduced by about 29 % and 33 %, respectively. The pH of the cultures at the end of the experiment was the same as in the beginning (pH 7.0 ± 0.1), except for the 32-µg/L group which showed a decrease to pH 6.6 ± 0.1.

The cells of the control and the two low-concentration groups were normally pigmented (Figs. 2A and 2B), but at 8 and 32 µg Cd/L cells were obviously bleached

(Figs. 2C and 2D). At such high Cd levels, algae aggregated and formed clusters of 4 to 6 cells; about half of them had altered shapes and surfaces (not round and not smooth; Fig. 2D) and were smaller (5.5 ± 0.9 µm versus 7.8 ± 1.3 µm of control cells).

The IC_{10} calculated from cell density and chlorophyll-a was 4.8 ± 1.2 and 4.2 ± 0.6 µg Cd/L, respectively; for chlorophyll-b and pheophytin-a it was 1.7 ± 0.9 and 1.5 ± 0.8 µg Cd/L, respectively (Table 3). The minimum significant difference (MSD), i.e. the lowest Cd concentration at which exposed algae are different from the control group, were determined as 2.4, 2.7 and 1.4 g/kg dw for chlorophyll-a, chlorophyll-b and pheophytin-a, respectively. This corresponds to IC_7, IC_{20} and IC_{15}, equivalent to 2.9 ± 0.7, 2.3 ± 1.1 and 2.5 ± 0.9 µg Cd/L (Table 3).

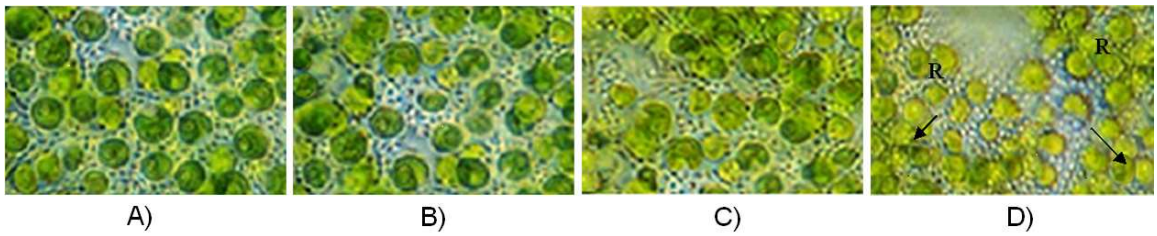

Fig. 2. *Parachlorella kessleri* control group (A), and grown for five days at Cd concentrations of 2 µg/L (B), 8 µg/L (C) and 32 µg/L (D). Arrows show clusters of algae, “R” indicates cells with rough surfaces.

Table 3

NOEC, LOEC, MSD and IC_{10} values [µg Cd/L] based on cell count, chlorophyll-a, chlorophyll-b and pheophytin-a contents after five days Cd-exposure. IC_{10}-values in parentheses are lower than the minimum significant difference (MSD)

	Cell counts	Chlorophyll-a	Chlorophyll-b	Pheophytin-a
NOEC	2.0	2.0	0.5	2.0
LOEC	8.0	8.0	2.0	8.0
IC_{10}	4.8 ± 1.2	4.2 ± 0.6	(1.7 ± 0.9)	(1.5 ± 0.8)
MSD	4.0 ± 1.2	2.9 ± 0.7	2.3 ± 1.1	2.5 ± 0.9

Data are presented as mean ± SD (n = 3).

4. Discussion

The presented data show clear toxicological effects on *P. kessleri* at surprisingly low Cd levels (2 – 3 µg/L) - lower than previously reported for other algal species. At the two highest Cd concentrations reduction of chlorophyll content and increase of pheophytin-a ($p < 0.05$) was observed. As a consequence, less mass and protein are synthesized leading to slower cell division and lower cell density. At the 0.5 µg/L Cd concentration, *P. kessleri* shows pigmentation, protein and polysaccharide content, doubling times and growth rate equal to control; at 2 µg/L, the onset of toxicity is noticed although statistically not significant. Much higher threshold (LOEC) values were reported for *Chlorella sp* (500 µg Cd/L) (Hatakeyama and Yasuno, 1981) and *C. vulgaris* (1000 µg Cd/L - Ilangovan et al*.,* 1998; 2000 µg Cd/L - Lam et al., 1999). The lowest Cd level reported previously to affect the growth of *C. vulgaris* is about twofold higher than in the present study, i.e. 5 µg Cd/L in the presence of humic acids (Pempkowiak and Kosakowska, 1998). *P. kessleri* has so far been considered as less sensitive towards heavy metals than other unicellular green algae (Rojickova-Padrtova and Marsalek, 1999; Lukavský et al., 2003), but our findings suggest that they are even more sensitive than others.

Ca- and Mn-concentrations in the medium used in this study are lower than previously used media, i.e. BBM, Bristol, and C-30 (Ilangovan et al., 1998; Lam et al., 1999; Pempkowiak and Kosakowska, 1998). Higher levels employed in former studies may entail diminished heavy metal toxicity (Kondo et al., 1974; Hart and Scaife, 1977); sometimes, EDTA or Fe-EDTA was used (Lam et al., 1999) whereby Cd uptake may have been slowed because of chelation or competition of Fe for transport sites. While in most studies Cd was added right at the beginning, in the present work exposure began two days after inoculation; at this stage, algae are adapted and enter the exponential growth phase with high metabolic activity, so Cd may be more efficiently taken up. At the highest concentration, the decrease in pH (6.6 ± 0.1) may further aggravate toxicity since the bioavailability of Cd is increased due to mobilization to the active free-ion state (Stumm and Morgan, 1981; Xian and Shokohifard, 1989). Last but not least, Cd tolerance may differ between algal strains (Kessler, 1986).

Sensitive indicators for a poor physiological state of algae are increased pheophytin-a concentrations, accompanied by lowered $OD664_b/OD665_a$ and

protein/polysaccharide ratios. These changes were highly correlated with Cd concentrations above 2 µg Cd/L after five days of exposure (Table 1). With *S. capricornutum*, protein/polysaccharide ratio was decreased at 30 µg Cd/L and higher after 6 h (Thompson and Couture, 1991), with *Nitzschia palea* at 20 µg Cd/L (Sathya and Balakrishnan, 1988). Increased numbers and volumes of starch grains (Nishikawa et al., 2003) have been suggested to be the main reasons.

With *P. kessleri a* trend of lowered chlorophyll-b and elevated pheophytin-a was observed in the 2 µg Cd/L group becoming significant at 8 µg/L, showing its relatively high sensitivity compared to *S. bijugatus* exposed to 10 and 30 µg Cd/L (Sathya and Balakrishnan, 1988). At high light intensity, conversion of chlorophyll-a to pheophytin-a by substitution of the Mg atom in the porphyrin ring by a free heavy metal cation might take place, especially in the PSII reaction center (Küpper et al., 1998). Disarrangement of chloroplasts, increase of vesicles in the cytoplasm and accumulation of starch grains under Cd stress (Walne, 1967) may finally be the cause for the observed altered cell surface.

Altered biochemical composition and morphology of algal cells reduces their food value (Pinto et al., 2003) which affects feeding behaviour of organisms on the next trophic level. Therefore, when intended as food for consumers on the next trophic stage, algae should be grown at Cd-concentrations in the medium not higher than 2 µg/L. This is also indicated by the calculated MSD's (Table 3). In most cases, the MSD was slightly lower than the IC_{10} except when standard deviations are high as for chlorophyll-b and pheophytin-a.

5. Conclusions

Cadmium exerts adverse effects on *P. kessleri* at concentrations lower than reported previously with other algal species. At exposure conditions close to environmental reality, *P. kessleri* exhibits high Cd-sensitivity. As morphology, pigment contents and protein/polysaccharide ratios are altered; algae with too high Cd-loading are unsuitable as food of animals on the next trophic levels (Sathya and Balakrishnan, 1988). Therefore, for exposure experiments with Cd-loaded algae as food, care must be taken that the phytoplankton is similar to non-exposed algae to avoid secondary effects such as altered feeding behaviour. When *P. kessleri* are

cultured for such purpose, a concentration of 2 µg Cd/L in the medium should not be exceeded.

Acknowledgements

We would like to thank Agnes Bednorz for technical assistance, and Dr. Radomir Cabala for valuable discussions.

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Appendix II:

Subchronic effects of environment-like cadmium levels on the bivalve *Anodonta anatina* (Linnaeus 1758):

I. Bioaccumulation, distribution and effects on calcium metabolism

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(Received 27 May 2008; final version received 24 July 2008; accepted 14 August 2008)

Toxicological and Environmental Chemistry, in press

Abstract

Cadmium bioaccumulation, tissue distribution, and its effects on Ca levels in the freshwater bivalve *Anodonta anatina* were examined. Mussels were exposed for 35 days to algae- and water-borne ^{111}Cd at an environment-like concentration and were allowed to depurate for posterior 120 days. Body fluids and tissue samples were taken during both phases to determine total Cd, the ^{114}Cd/^{111}Cd isotope ratio, the electrolytes Ca and Mg, and the essential trace metal Zn. Bioaccumulation and distribution of Cd and its effects on Ca levels were tissue-specific and time dependent. During exposure, newly incorporated ^{111}Cd increased in all body compartments, with highest levels in the kidney. During depuration, newly incorporated and background Cd both decreased slowly in the digestive gland and others while in the kidney it increased further, indicating mobilization from other body compartments and deposition in this organ. Following the uptake of Cd, Ca concentrations increased in body fluids within a few hours while it decreased in the digestive gland, mantle and other tissues; in the gills and kidney it remained largely unchanged. The derangement of Ca homeostasis, to some extent also of Mg and Zn, appears to be a major component of sub-chronic Cd toxicity in *A. anatina*.

Keywords: Anodonta anatina; Cd bioaccumulation; Cd redistribution; Ca homeostasis; freshwater mussel; stable isotope ^{111}Cd.

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1. Introduction

The present decline of numerous freshwater mussels, in spite of efforts to restore wetland and riverine habitats, calls for continued investigations to understand the involvement of persistent pollutants in the multi-facetted sequence of events leading to toxic stress of benthic populations. Those pollutants should be regarded with highest priority which are observed to reach relatively high levels in the respective ecosystem. Among others, the heavy metal cadmium (Cd) fulfills this condition as shown in a previous monitoring study on the declining freshwater pearl mussel *Margaritifera margaritifera* (Frank and Gerstmann, 2007). Cd is characterized by its ubiquitous environmental occurrence, its pronounced toxicity at low level, and its bioaccumulation potential and transfer through food chains.

Cd enters the aquatic environment with the fine particulate emissions of coal-fired power and metal smelting plants, by its presence in some phosphate fertilizers, its former use in batteries and various PVC products, and its release via waste waters and industrial effluents; natural processes also play a role, such as forest fires, volcanic emissions, weathering of soil, and release from bedrock. Once emitted, Cd is deposited and ends up in river sediments from where it may be taken up by benthic organisms and accumulated via food chains in animals of higher trophic levels. Cd has been reported to affect Ca metabolism in bivalve molluscs (Faubel et al., 2008) by renal tubular damage which may result in excessive loss of Ca (Brzóska and Moniuszko-Jakoniuk, 1998). It also competes for Ca binding sites in cell membranes and affects Ca-ATPase (Verbost et al., 1988); Ca uptake by active transport systems is a mechanism vitally important for all animals including freshwater mussels (Schatzmann, 1973). Once Ca has entered the body, it is stored in calcareous deposits in the gills and mantle (Machado et al., 1988) for biomineralization (Moura et al., 1999), shell formation and larval development.

Anodonta anatina is one of the declining freshwater mussels. Cd kinetics in this species have received some attention (Holwerda et al., 1988; Holwerda et al., 1989; Streit and Winter, 1993) but its effects on Ca metabolism have not been assessed, especially not at environmental Cd levels. In this study, *A. anatina* were sub-chronically exposed to environment-like Cd levels for 5 weeks via the water and the food (Cd-loaded algae), followed by 4 months of depuration. Bioaccumulation of newly absorbed Cd, its distribution and the redistribution of background Cd between

body compartments were determined by using ^{111}Cd as a tracer. Effects on Ca and Mg profiles as well as the essential metal zinc (Zn) in different body compartments were investigated.

2. Materials and methods

2.1. Chemicals and labware

A ^{111}Cd stock solution (100 mg/L) was prepared by dissolving 0.01 g isotopically enriched (96 %) ^{111}Cd metal (CK GAS product Ltd, Hampshire, UK) in 0.4 mL suprapur HNO_3 in a 100-mL volumetric flask. Further 0.8 mL concentrated HNO_3 was added and the flask was filled to the mark with bidistilled water. All other chemicals were analytical grade (VWR, Darmstadt, Germany). Glassware and plastic equipments used for analytical purposes were soaked for 24 h in half-concentrated HNO_3, all others in a mixture of concentrated HNO_3 and bidistilled water (v/v, 1/9) and rinsed thoroughly with bidistilled water.

2.2. Food preparation

Cd-free and ^{111}Cd-loaded algae (*Parachlorella kessleri)* were grown for 7 days in a modified K-medium as previously described (Ngo et al., in press). Algal cells were harvested, centrifuged, freeze-dried, and kept in desiccators in the darkness for later use. Freeze-dried Cd-loaded algae contained 5.9 µg Cd/g dry weight.

2.3. Animal holding conditions

Anadonta anatina (ZOO-Erlebnis Online Shop, Großefehn, Germany), 6 – 8 year-old, were from a pond with a Cd concentration of less than 0.03 µg/L (limit of quantification) in the water and about 0.03 µg/L in the suspended solids (> 0.45 µm). The mussels were transported to the laboratory in a cool box, cleaned externally to remove epiphytes, and placed in 32 L aerated tap water in 45-L glass aquaria (50 x 30 x 30 cm) at dim light for seven days; half of the water was daily exchanged. After one week, the mussels were marked with numbers, weighed (44 ± 6 g), measured (80 ± 4 mm), and acclimatized for four weeks in 32 L artificial pond water (APW) (Dietz et al., 1994) in two 45-L glass aquaria covered with transparent polypropylene lids, equipped with an inner biofilter system, stainless steel aeration tubes and 8 kg

glass beads as substrate; water temperature was 17 ± 1°C, pH was 7.3 ± 0.3. Two thirds of the water was replaced every two days, a complete change was carried out every sixth day. Standard water parameters such as conductivity and dissolved oxygen were monitored every second day (Multi 340i, WTW, Weilheim, Germany); ammonia, nitrite and nitrate were checked once a week (Test Kits, Merck, Darmstadt, Germany). The light schedule was 12 h dark : 12 h light, with a photon intensity of 13 – 19 µmol/m^2/s. Mussels were fed with 1.5 mg/L/day freeze-dried Cd-free algae.

2.4. Design of experiment and sampling procedures

Before the start of the experiment, three mussels were taken for sampling of body fluids and tissue samples. The actual experiment involved the exposure of 18 mussels to water- and algae-borne ^{111}Cd for 35 days, followed by depuration for 120 days in Cd-free APW. The control group of 18 mussels was treated in the same way except that no Cd was added. On certain sampling points (Table 1), three mussels were randomly taken and sacrificed for sampling of hemolymph (HML), extrapallial fluid (EPF), and tissues.

The amount of food was adjusted to the number of mussels; to the exposed animals, ^{111}Cd was supplied via the water (0.2 µg ^{111}Cd/L) and the algal food. One mg Cd-loaded algae plus 0.5 mg Cd-free algae per litre were given daily to 18 or 15 mussels, 1.0 mg Cd-loaded algae per day to the 12 mussels remaining after removal of 3 each at the sampling times 0.2 d and 5 d (Table 1). Actual Cd concentrations were monitored every second day. After water exchange it was 0.22 ± 0.03 µg/L, with 94 % being dissolved in the filtered water (< 0.45 µm); within two days it fell to 0.16 ± 0.02 µg/L (about 0.01 µg/L in particulates). The nine mussels remaining in each group at the end of the exposure period were kept in Cd-free APW and were fed daily with control freeze-dried algae, 1 mg/L. After removal of another set of three mussels on day 15 of depuration (Table 1), 0.5 mg control freeze-dried algae per litre were given.

At the given times (Table 1), three mussels each of both groups were anaesthetized (White et al., 1996) in 2-phenoxyethanol solution (4 mL/L) for about 30 min, weighed, and HML (10 – 15 mL/mussel) and EPF (10 – 15 mL/mussel) were taken with a 5-mL syringe with a 0.55 x 25 mm needle (B. Braun, Melsungen, Germany). The mussels were dissected on ice into gills, mantle, kidney, digestive

gland and other tissues (foot, heart, reproductive gland and adductors) and weighed in petri dishes. The tissue samples were frozen at -80℃ and lyophilized. At other times (Table 1, case B), three animals each of both groups were removed temporarily for sampling of HML (0.3 – 1 mL/mussel) and EPF (0.3 – 1 mL/mussel) in a non-lethal manner (Gustafson et al., 2005a).

Table 1

Sampling schedule during respective experimental periods of Cd exposure and depuration with the number of animals per tank (n) decreasing after each sampling time A by three. A: Three randomly mussels were sacrificed for hemolymph, extrapallial fluid and tissues, B: Three mussels were temporarily taken out of the tank for non-lethal sampling of hemolymph and extrapallial fluid and returned to the tank

Day	**Exposure**	n	Day	**Depuration**	n
0.04 (1 h)	B	18	1	B	9
0.2 (5 h)	A	18	5	B	9
1	B	15	15	A	9
5	A	15	30	B	6
20	B	12	60	A	6
35	A	12	90	B	3
			120	A	3

2.5. Determination of elements

Tissue samples, between 10 – 100 mg dw, were digested in a mixture of conc. HNO_3 and conc. HCl (4/1) in 30-mL borosilicate glass tubes for 1 h at 40℃, followed by 3 h at 120℃. Digestates were diluted to 10 mL with bidistilled water and filtered through 0.45 µm cellulose syringe filters for metal analysis. HML and EPF, 0.2 – 1 mL each, were acidified with 0.5 mL conc HNO_3 in 15 mL PP tubes and diluted to 10 mL with bidistilled water.

Total Cd, $^{114}Cd/^{111}Cd$ ratios and Zn were determined by inductively-coupled plasma mass spectrometry (ICP-MS); detection limit for Cd was 0.03 µg/L, for Zn it

was 20 µg/L. Ca and Mg were determined by ICP atomic emission spectroscopy, detection limits being 0.05 mg/L each. The validity of the methods was checked periodically with certified reference material from mussel tissue *Mytilus edulis* (EFM® - CE278, Geel, Belgium), results being within the certification range (96 – 111 %). The procedural blanks (HNO_3/HCl, 4/1) for all analyzed elements were below detection limits. The concentrations of newly incorporated ^{111}Cd, background Cd (Cd the mussels had taken up during their previous life), and total Cd (newly accumulated + background + mobilized background Cd), and the amount of background Cd mobilized in exposed animals relative to control animals were calculated according to Rodríguez-Cea et al. (2006). Bioaccumulation factors (BAF) at the end of exposure were calculated as the ratio of the newly incorporated concentration of Cd in the respective tissue *versus* its concentration in the water. All data were calculated on wet weight basis.

2.6. Data analyses

Corrections for weight loss in organs during the experimental period were calculated in relation to the respective gill weight according to Hemelraad and Herwig (1988).

The data are presented as means ± SD (standard deviations). Two-way analysis of variance was used to determine whether differences in Cd and Ca concentrations between groups and sampling times were significant. If significant differences were found, the paired t-test and post-hoc test (Newman-Keuls test) were applied (GraphPad Software, San Diego, CA).

3. Results

In the body fluids (Table 2) of control mussels, total Cd was 2.2 µg/L in HML and 2.0 µg/L in EPF. In exposed animals, it started to increase within one day by almost 50 % (HML 4.1, EPF 3.8 µg/L) to become more than doubled after 35 days of exposure (HML 5.0, EPF 4.5 µg/L). During depuration, it decreased but even after 4 months (120 d) remained about 30 % higher than in the control group (HML 2.8 *vs.* 2.2; EPF 2.4 *vs.* 1.8 µg/L). Newly accumulated Cd also rose without delay to reach highest values of 1.1 µg/L (HML) and 1.0 µg/L (EPF) at the end of exposure; upon depuration, it decreased but did not come to nil within the following four months. In

the HML and EPF of the control group, the concentration of total Cd remained virtually constant throughout the experiment.

Table 2

Newly incorporated Cd and total Cd (µg/L) in the hemolymph (HML) and extrapallial fluid (EPF) in exposed animals, and total Cd in control animals during exposure and depuration (mean ± SD, n = 3)

Sampling time [d]	**HML**			**EPF**		
	Exposed		Control	Exposed		Control
	N. inc. Cd	Total Cd	Total Cd	N. inc. Cd	Total Cd	Total Cd
Exposure						
0.04 (1 h)	0.2 ± 0.1	2.3 ± 0.2	2.2 ± 0.2	0.1 ± 0.0	2.4 ± 0.1	2.0 ± 0.2
0.2 (5 h)	0.4 ± 0.1	2.3 ± 0.3	2.4 ± 0.3	0.4 ± 0.1	2.3 ± 0.2	2.2 ± 0.4
1	0.5 ± 0.1	4.1 ± 0.2	2.5 ± 0.2	0.5 ± 0.1	3.8 ± 0.2	1.9 ± 0.4
5	0.7 ± 0.1	4.0 ± 0.4	2.1 ± 0.2	0.6 ± 0.1	3.8 ± 0.3	1.8 ± 0.2
20	0.8 ± 0.1	4.8 ± 0.5	2.6 ± 0.4	0.7 ± 0.1	4.4 ± 0.2	1.7 ± 0.1
35	1.1 ± 0.1	5.0 ± 0.4	2.2 ± 0.3	1.0 ± 0.1	4.5 ± 0.2	1.9 ± 0.2
Depuration						
1 (24 h)	0.9 ± 0.1	5.1 ± 0.6	2.3 ± 0.1	0.8 ± 0.1	4.7 ± 0.6	1.9 ± 0.2
5	n.d.	4.5 ± 0.4	2.3 ± 0.4	n.d.	4.5 ± 0.3	1.9 ± 0.2
15	0.8 ± 0.1	4.3 ± 0.3	2.4 ± 0.4	n.d.	4.6 ± 0.4	2.4 ± 0.2
30	n.d.	3.3 ± 0.4	2.1 ± 0.2	n.d.	4.3 ± 0.5	2.3 ± 0.0
60	0.5 ± 0.1	3.1 ± 0.3	2.1 ± 0.2	0.6 ± 0.1	2.9 ± 0.3	2.1 ± 0.1
90	n.d.	3.0 ± 0.4	2.2 ± 0.3	n.d.	2.8 ± 0.2	n.d.
120	0.3 ± 0.1	2.8 ± 0.3	2.2 ± 0.3	0.4 ± 0.1	2.4 ± 0.2	1.8 ± 0.2

n.d.: not determinable due to small sample volumes; N. inc. Cd: Newly incorporated Cd.

In the tissues of control animals (Fig. 1), total Cd was highest in the kidney, i.e. 2.1 mg/kg; in the digestive gland it was 0.7 mg/kg, in the gills 0.5 mg/kg, in the mantle and other tissues about 0.4 mg/kg. Upon exposure, newly incorporated Cd increased in all tested organs ($p < 0.05$), most strongly in the kidney and in the digestive gland. In the kidney, total Cd increased even stronger, reaching almost double the initial levels after 60 days of depuration. In the gills, a significant increase of total Cd (50 %)

was also found although the accumulation of newly incorporated Cd was moderate. In the other organs, newly incorporated Cd and total Cd showed relatively slow increases. At the end of exposure, highest BAFs were found in the kidney and digestive gland (1300), followed by the gills (600), mantle (400) and other tissues (400). Upon depuration, newly incorporated Cd continued to increase in the kidney during the first two months, in the mantle during the first two weeks (Fig. 1). In the digestive gland, gills and other tissues it started to decrease immediately. Total Cd decreased in all organs except kidney.

The mass balance of background Cd and newly incorporated Cd (Table 3) per kg soft body weight was calculated, showing that at the end of exposure the fraction of newly incorporated Cd represents about 18 % of the total Cd burden of the body, being lower for the kidney (< 10 %) and higher for the digestive gland (≈ 30 %). Overall, the total body burden (sum of background and newly incorporated Cd) increased from 140 to 170 µg/kg during exposure, to return to 150 µg/kg after 4 months of depuration. This was also reflected by the amounts of background Cd mobilized (i.e. background Cd of a given organ of exposed animal minus total Cd in the respective organ of the control one) in exposed animals relative to control animals (Table 4). Although these data are statistically non-significant in some organs, they indicate clear tendencies, i.e. a continuous uptake of background Cd by the kidney (from +0.3 to +3.5 at day 60 of depuration), moderate uptake by the gills and mantle, and to a lesser extent by the HML and EPF. These loads stem mainly from the other tissues and to some extent also from the digestive gland, later on also from the mantle. The balance indicates that background Cd (negative = elimination) is starting to be excreted at the end of exposure, becoming more pronounced during depuration.

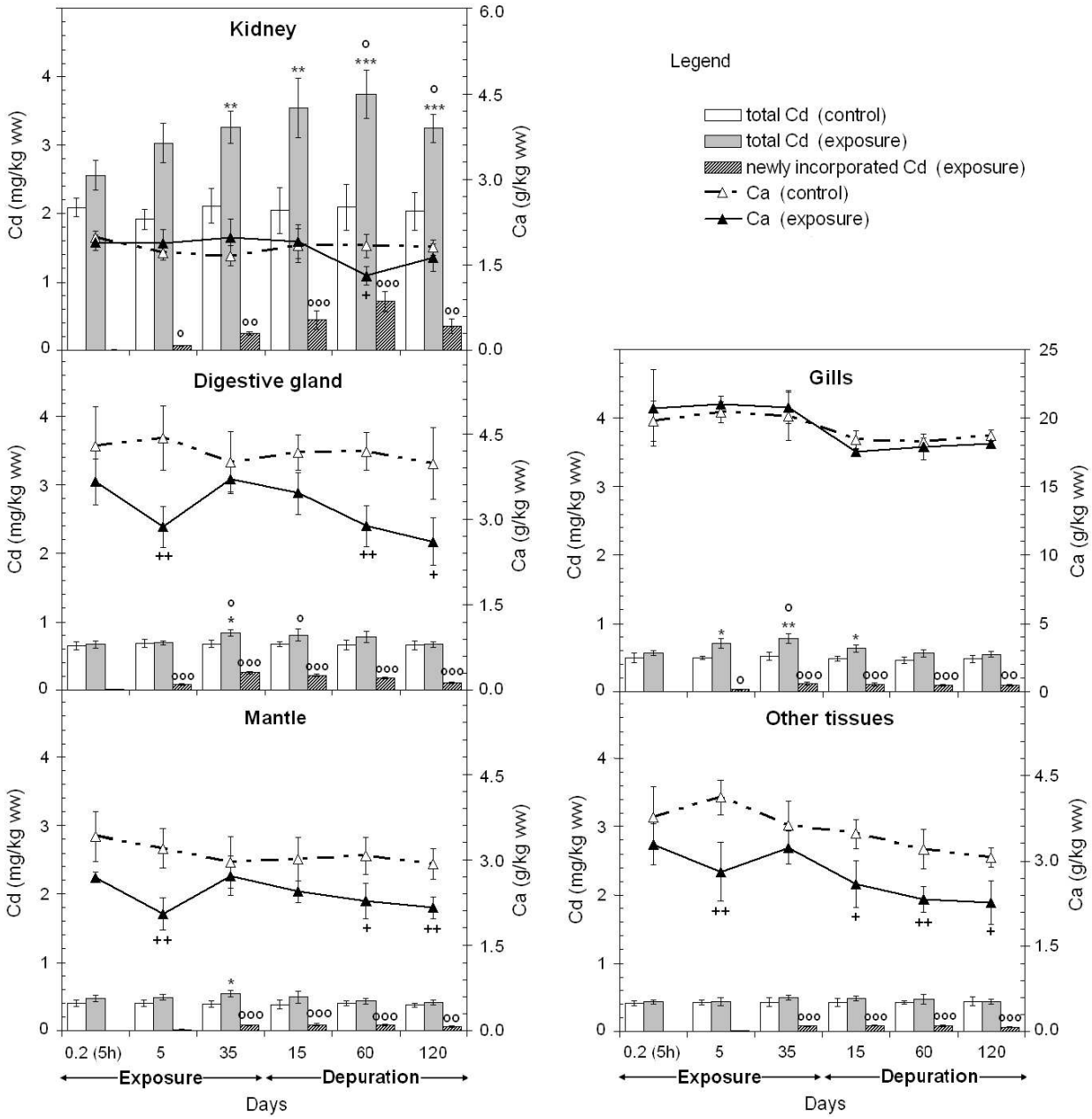

Figure 1. Concentrations of Cd (bars) and Ca (lines) in *A. anatina* during Cd exposure and depuration. Significant differences for Cd in comparison to control (*) and to the first 5 hours (^{o}) within a group, for Ca in comparison to the first five hours (+) (mean ± SD, n = 3, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and ^{o} $p < 0.05$, ^{oo} $p < 0.01$, ^{ooo} $p < 0.001$) are indicated.

Table 3

Mass balance of background (left column at each time point) and newly incorporated (right column) Cd (µg/kg soft body) in *A. anatina* during exposure and depuration. The values in parentheses at the organs are their average weight percentages of the total soft body weight. Relative standard deviations (omitted for clarity) are ± 15 %

	Exposure						**Depuration**					
	0.2 d (5 h)		5 d		35 d		15 d		60 d		120 d	
Kidney (0.3 %)	6.7	0.02	7.1	0.21	8.2	0.75	9.3	1.34	9.3	2.15	8.7	1.07
Digestive gland (2.0 %)	13.0	0.14	12.1	1.66	11.3	5.14	11.6	4.40	11.7	3.63	11.3	2.22
Gills (4.8 %)	24.1	0.14	25.0	1.63	26.6	5.89	25.5	5.71	22.8	4.59	22.0	4.39
Mantle (5.7 %)	23.0	0.28	23.8	0.99	24.4	4.65	23.2	5.08	19.9	4.62	20.1	3.37
Other tissues (17.2 %)	71.1	0.86	69.8	2.75	67.5	13.49	67.9	15.30	66.6	14.23	64.6	10.98
HML (35 %)	0.7	0.14	1.2	0.25	1.3	0.37	1.2	0.28	0.9	0.19	0.9	0.12
EPF (35 %)	0.7	0.14	1.1	0.21	1.2	0.34	-	-	0.8	0.20	0.7	0.12
Σ	**139**	**1.7**	**140**	**7.7**	**140**	**30.6**	**139**	**32.1**	**132**	**29.6**	**128**	**22.3**
Total	**141**		**148**		**171**		**171**		**162**		**150**	

Table 4

Background Cd[1] (µg/kg soft body weight) mobilized between organs in exposed mussels (relative to control) during exposure and depuration. Relative standard deviations (omitted for clarity) are ± 12 %

	Exposure			**Depuration**		
	0.2 d (5 h)	5 d	35 d	15 d	60 d	120 d
Kidney	+0.27	+1.65	+2.40	+3.15	+3.45	+2.55
Digestive gland	+0.00	-1.90	-2.50	-2.00	-1.70	-1.80
Gills	+0.12	+0.85	+2.80	+2.20	+0.70	-1.10
Mantle	+0.11	+0.85	+2.25	+1.40	-2.95	-1.10
Other tissues	-0.22	-2.25	-6.35	-5.85	-6.90	-11.10
HML	-0.18	+0.40	+0.60	+0.40	+0.18	+0.10
EPF	-0.11	+0.50	+0.55	-	+0.18	+0.10
Balance	**0.0**	**0.1**	**-0.3**	**-0.7**	**-7.0**	**-12.5**

[1] Background Cd concentration of a given organ of exposed animals minus Cd concentration in the respective organ of control animals.

Immediately and concurrent with Cd-uptake, Ca levels (Table 5) in the HML started to increase after 5 h, being about 25 % higher than in the control group and continuing to rise slowly during exposure. Upon depuration, it returned to control levels within 5 days. A similar increase was observed for the EPF, although somewhat retarded and slightly lower (20 %). Concurrently, in the mantle, digestive gland and other tissues Ca levels dropped immediately upon exposure (Fig. 1), being most pronounced (30 to 40 %) at day 5 ($p < 0.05$). In the kidney, Ca remained largely unchanged during Cd exposure; only when the concentrations of total and newly accumulated Cd-levels were highest at day 60 of depuration, it dropped significantly, returning to control level at day 120. In the gills, Ca concentration being about 6-fold higher than in other organs, was not significantly affected. In control mussels, Ca concentrations in the HML and EPF decreased slightly but non-significantly.

Table 5

Ca levels (mg/L) in hemolymph (HML) and extrapallial fluid (EPF) of *A. anatina* during Cd exposure and depuration (mean ± SD, n = 3)

	HML		**EPF**	
[d]	Exposed	Control	Exposed	Control
Exposure				
0.04 (1 h)	113 ± 24^{a}	126 ± 5^{a}	137 ± 8^{ab}	126 ± 14^{a}
0.2 (5 h)	158 ± 15*^{bc}	129 ± 12^{a}	157 ± 10^{b}	132 ± 22^{a}
1	168 ± 20^{c}	132 ± 33^{a}	158 ± 9*^{b}	129 ± 21^{a}
5	169 ± 21*^{c}	141 ± 11^{a}	166 ± 13*^{b}	143 ± 14^{a}
20	180 ± 28**^{c}	143 ± 13^{a}	168 ± 13**^{b}	144 ± 11^{a}
35	174 ± 20*^{c}	138 ± 14^{a}	162 ± 9*^{b}	142 ± 22^{a}
Depuration				
1 (24 h)	155 ± 10*^{bc}	131 ± 5^{a}	158 ± 14*^{b}	132 ± 12^{a}
5	130 ± 18^{ab}	130 ± 6^{a}	144 ± 10^{ab}	135 ± 12^{a}
15	147 ± 15^{b}	139 ± 8^{a}	150 ± 15^{ab}	144 ± 19^{a}
30	142 ± 8^{ab}	138 ± 13^{a}	148 ± 6^{ab}	138 ± 10^{a}
60	144 ± 15^{ab}	126 ± 4^{a}	139 ± 10^{ab}	130 ± 19^{a}
90	131 ± 13^{ab}	136 ± 21^{a}	126 ± 19^{a}	134 ± 18^{a}
120	130 ± 28^{ab}	131 ± 9^{a}	122 ± 10^{a}	128 ± 9^{a}

Asterisks denote statistically significant differences from control values (* $p < 0.05$; ** $p < 0.01$). Superscript letters (a-c) indicate that values are significantly different ($p < 0.05$; ANOVA followed by Student-Newman-Keuls test).

Cd exposure had also effects on other divalent cations (Fig. 2). In the body fluids, Mg increased slightly (not shown) up to day 5 of exposure (HML: 19 *vs.* 21; EPF: 17 *vs.* 20 mg/L at 5 h), then returned to control levels. In the gills, Mg was about two times higher than in the rest of the soft body. Mg tended to be lowered upon Cd exposure in the mantle and digestive gland, significantly at the end of exposure and during depuration ($p < 0.05$). Zn levels were clearly lower in the kidney at the end of exposure and remained low during depuration ($p < 0.05$); a similar but less pronounced reaction was seen for the digestive gland. In the mantle, Zn decreased

during exposure but recovered during depuration. For the gills with their 4- to 6-fold higher Zn-levels, an effect was discernible only towards the end (35 d) of exposure.

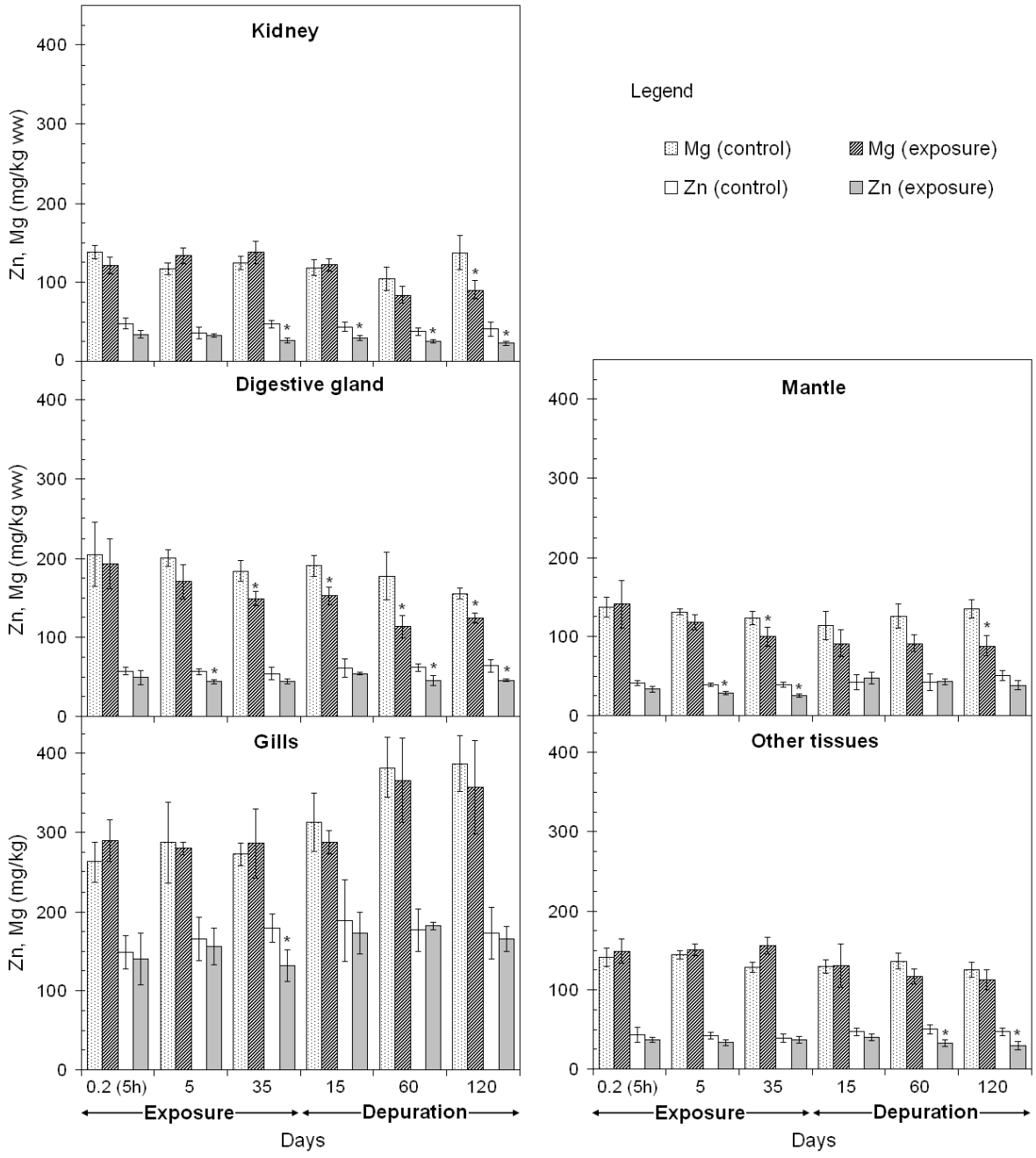

Figure 2. Tissue concentrations of Mg and Zn in *A. anatina* during Cd exposure and depuration. Significant differences in comparison to control (*) and to the first 5 hours (^{o}) within a group (mean ± SD, n = 3, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and ^{o} $p < 0.05$, ^{oo} $p < 0.01$) are indicated.

4. Discussion

One of the most obvious results is the time-dependent increase of newly accumulated Cd in all body compartments. By using ^{111}Cd as a tracer, the differentiation between newly incorporated Cd and its replacement and redistribution entailing the redistribution of background Cd can be followed at an environmental exposure level. In a previous study with *Mytilus galloprovincialis* exposed to Cd at a level similar to the marine environment (1 µg/L) and using the same tracer, Labonne et al. (2002) found significant uptake of Cd by the gills, digestive gland and mantle from the first day onwards.

In the present study, fast increases of newly accumulated Cd in the HML and EPF (Table 2) were observed during the first hours, as they are the primary compartments in the uptake and distribution route of Cd. Total Cd in these body fluids did not increase proportionally to the newly incorporated Cd, an indication that deposition of background Cd in the tissues took place due to the replacement of newly Cd. Thereafter, within one day of exposure, total Cd in HML and EPF increased strongly, probably due to mobilization of background Cd from the digestive gland and from other tissues on its way via the body fluids to the gills, mantle and kidney (Table 4). During the initial period of depuration, this redistribution was still continued but became attenuated towards the end.

Interestingly, the initial uptake of ^{111}Cd in the digestive gland in terms of concentration (Fig. 1) was similar to the kidney, but relative to its background Cd and in terms of weight, the digestive gland was more important (Table 3). Obviously, this organ serves as an intermediate store of relatively high capacity to be emptied during depuration. The kidney with its much smaller size and its role as excreting organ received Cd much longer, sequestering it from other parts of the body (Table 4) and leading to Cd concentrations being 4 – 6 times higher than in the other organs. The importance of the digestive gland in the turnover of Cd was mirrored by the highest ratio of newly incorporated Cd *versus* background Cd at the end of exposure, and the following sequence with respect to this ratio was found: digestive gland >> gills > mantle = other tissues >> kidney. Apparently, food plays an important role as it stays relatively long in the bivalve gut before being assimilated (Wang and Wong, 2003). Uptake of Cd in fed *Mytilus edulis* is three times higher than in starved animals

(Riisgard et al., 1987). The fast release of Cd from the digestive gland and the translocation to other organs in the beginning of depuration has also been reported by others (Ballan-Dufrancais et al., 1985).

Both background and newly incorporated Cd were not eliminated from the kidney up to the second half of the depuration period. In general, background Cd and the relative small fraction (20 % at the end of exposure) of newly incorporated Cd was started to be slowly excreted during the last two months of depuration. Nevertheless, even then it was so slow (Table 3) that a half-life of more than two years can be estimated. Strong retention of Cd was also observed for the bivalve *Isognomon* (Ringwood, 1989).

Apart from the digestive gland and other tissues (together 20 % of soft body weight) from which background Cd was mobilized early during exposure (Table 4), during the second half of depuration the mantle also released some of its Cd pool (Table 4) to be mainly transferred to the kidney and the gills. In the latter organ, Cd may be trapped in Ca concretions (Bonneris et al., 2005; Campbell et al., 2005) while in the kidney it may be seized by cytosolic metallothioneins (MTs), released via lysosomes and eventually excreted (George et al., 1983; Regoli et al., 1991).

When BAFs are calculated based on total Cd, in some organs (gills, mantle and kidney) they are overestimated, in others (digestive gland) underestimated, since redistribution of background Cd is not accounted for. BAFs calculated based on newly incorporated Cd were found to follow the following sequence: kidney = digestive gland >> gills ≥ mantle > other tissues. In a previous study with *A. anatina* being exposed only to water-borne Cd (16 µg/L) for 60 days, the sequence was as follows: kidney = gills >> digestive gland > mantle > muscle (Holwerda et al., 1988). Apparently, the way of Cd exposure determines its disposition in the body, illustrating the importance of Cd uptake via the food, in accordance with earlier findings (Cunningham, 1979).

Freshwater mussels, mostly living in water of low hardness, as all other animals they need Ca in order to maintain normal cellular functions (Bronner, 1992) and for building and repairing their shells. The present work showed that exposure to Cd at low concentration had profound effects on Ca homeostasis in the digestive gland, mantle and other tissues (Fig. 1). In the kidney an effect was seen when the Cd concentrations were highest: apparently, a certain level must be surpassed, perhaps

when the capacity to sequester and store Cd in nontoxic form is exceeded, e.g. bound to MTs. In the gills, control levels of Ca were highest, i.e. 18 – 20 g/kg, more than half of it being stored (55 %) in calcareous concretions (Pynnönen et al., 1987) which serve as reservoir during hypoxia and for reproduction (Silverman et al., 1983,1985); no alterations by Cd were seen in this organ.

Cd disturb Ca absorption directly by competition of Cd for Ca-binding sites in the epithelial cell membrane (Roesijadi and Unger, 1993). In addition, newly incorporated or mobilized Cd may lead to inhibition of the synthesis of calcium-binding protein due to its disturbance of vitamin D_3 metabolism (Brzóska and Moniuszko-Jakoniuk, 1998) and/or inhibition of plasma membrane Ca transport due to its high affinity to SH groups of Ca-ATPase of the endoplasmic reticulum (Verbost et al., 1988). All these effects may be involved in a faster cellular release and inhibited uptake of Ca from the body fluids. Since in the mantle, digestive gland and other tissues, Ca levels are about 40-fold higher than in the body fluids, any disturbance in the cellular uptake, sequestration and storage is mirrored by a strong increase in the body fluids as seen here (Table 5). Dissolution of calcareous deposits upon Cd-induced acidosis may further contribute to this process (Crenshaw and Neff, 1969; Moura et al., 2000; Antunes et al., 2002). Elevated Ca in the HML were also found in *Epilliptio complanata* living in metal-polluted regions (Gustafson et al., 2005b), or in *A. cygnea* exposed to water-borne Cd at higher concentrations (Hemelraad et al., 1990; Faubel et al., 2008). These disturbances of Ca homeostasis may be expected to affect normal cellular functions (Krampitz and Graser, 1988). Further, the Cd-induced renal tubular damage prevents tubular Ca re-absorption (Brzóska and Moniuszko-Jakoniuk, 1998) and leads to incipient Ca-deficiency.

The divalent cation Mg also tended to decrease upon Cd exposure, especially in the digestive gland and mantle. Mg plays a similar role in the mineralization process as Ca although its concentrations are lower. A decrease of Zn in the mantle and kidney upon Cd exposure detected here was also observed for the snail *Lymnaea stagnalis L.* exposed to both water-borne and particulate Cd (Présing et al., 1993), and for the freshwater mussel *L. marginalis* (Das and Jana, 1999). Displacement of Zn by Cd at active sites of DNA-binding proteins, MTs and Zn-containing enzymes (Présing et al., 1993; Pruski and Dixon, 2002) might be the main reason. As Zn protects against Cd toxicity (Kaji et al., 1988) and is an essential element in many

enzymes, e.g. carbonic anhydrase being important for the calcification process, Zn-depression may constitute another stress factor involved in Cd toxicity to molluscs.

Overall, this study demonstrates that Cd-uptake via the food and the water at environment-like levels and the mobilization of background Cd by the pulse of incoming Cd may have considerable effects on the steady state levels of divalent metals which are physiologically important. Use of a stable-isotope tracer demonstrates that the biochemical-toxicological reactions upon Cd exposure are not simply related to its total pool, but that different Cd pools are involved. Of these the mobile, free form in the body fluids and the bound form in the kidney may be the most important ones, while the large pool present in the soft body (foot, heart, reproductive gland, adductors) may be relatively inert.

5. Conclusions

Accumulation, distribution, and redistribution of newly incorporated Cd and background Cd between different body compartments are tissue-specific and time-dependent. The digestive gland is active in sequestering incoming Cd while the kidney acts as a sink. The mass balance reveals that the newly incorporated Cd fraction constituted less than 20 % of the total Cd body burden but that the former exerted profound derangements of Ca-homeostasis even at relatively low environment-like levels. Also, it is noteworthy that the load of Cd accumulated over one month of exposure has not been fully eliminated during four months of depuration. Increases of Ca in the body fluids in combination with a decrease in the tissues suggest that Cd is interfering with the uptake of Ca from the outside environment, and with the transfer from the HML and EPF into the cellular endoplasmic and mitochondrial Ca stores. Overall, the difficulty in maintaining the finely tuned Ca balance may constitute an important stress factor; as this affects other physiological functions such as carbohydrate metabolism, the overall vitality and fitness of mussels, and consequently, their development and survival are hampered.

Acknowledgements

We would like to thank Prof. Jorge Machado (Lab. Applied Physiology, University of Porto) for his critical readings and comments, Dr. Gunter Ilgen for assisting in metal analyses.

Financial support by the Oberfranken-Foundation and the Fisheries Department of Upper Franconia, especially Dr. Robert Klupp, is highly appreciated.

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Appendix III:

Subchronic effects of environment-like cadmium levels on the bivalve *Anodonta anatina* (Linnaeus 1758): II. Effects on energy reserves in relation to calcium metabolism

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(Received 24 July 2008; final version received 09 September 2008; accepted 25 September 2008)

Toxicological and Environmental Chemistry, in press

Abstract

Glucose, glycogen and protein levels in freshwater mussels (*Anodonta anatina)* exposed to algae- and water-borne Cd for 35 days and allowed to depurate for posterior 120 days were monitored. In body fluids, glucose was increased while protein levels decreased. Glycogen stores in the tissues generally declined during exposure and remained low until the end of depuration, associated with decreased dry weight and condition index. Tissue protein concentrations remained stable, but subcellular fractions exhibited some alterations. Negative correlations of glycogen to newly incorporated Cd and total Cd were observed in the mantle and digestive gland. Glucose concentrations in the body fluids and glycogen in the mantle and digestive gland were strongly correlated to Ca content.

Keywords: Anodonta anatina; cadmium; condition index; freshwater mussel; glycogen; glucose; protein; energy reserves.

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1. Introduction

Freshwater mussels are declining worldwide regardless of great efforts to elucidate the causes and to rescue populations (Bauer, 1986; Hastie and Young, 2003; Geist, 2005). Therefore, further investigations are necessary to understand the multitude of potential causes in order to save the formerly abundant populations, e.g. *Anodonta anatina* and *Margaritifera margaritifera* (Frank and Gerstmann, 2007). Previous studies have focused on the physical alterations of habitats due to land use, agricultural practices or forest management (Wilcove et al., 1998; Box and Mossa, 1999; Watters, 2000; Allan, 2004); the involvement of organic and inorganic pollutants introduced to limnic ecosystem by human activities have also been addressed (Holwerda et al., 1988; Markich et al., 2001; Perceval et al., 2002; Frank and Gerstmann, 2007). One of these is cadmium (Cd), a metal of pronounced toxicity even at low levels. Once in the aquatic environment, Cd is accumulated via food chains posing a threat to aquatic organisms at higher trophic levels. It is known that Cd exposure may result in depletion of energy reserves in molluscs (Leung and Furness, 2001; Ansaldo et al., 2006) and in changed intermediary metabolism in arthropods (Reddy and Bhagyalakshmi, 1994). Therefore, physiological parameters such as glycogen (Doherty, 1990; Ansaldo et al., 2006) and the body condition index (CI), indicators of the energetic status or the condition of the animals, have been used as biomarkers to monitor environmental stress on molluscs (Lagadic et al., 1994). Body condition is defined by Mann (1978) as the “ability of an animal to withstand an adverse environmental stress, be this physical, chemical or biological”

However, the effects of Cd at environmental levels on energy reserves in freshwater mussels and its linkage to Ca metabolism have not been investigated yet. In a previous paper (Ngo et al. 2008a, in press), the bioaccumulation of Cd and its effects on Ca profiles in the freshwater mussel *Anodonta anatina* exposed to Cd for 5 weeks via food and water and allowed to depurate for another four months were presented. In this study, the effects of Cd on glucose, glycogen and protein in different body compartments were examined, in order to compare them with the alteration of the Ca-levels during both phases.

2. Materials and methods

2.1. Chemicals and labware

Isotopically enriched (96 %) ^{111}Cd metal was used as a tracer (CK GAS Product Ltd, Hampshire, UK). Concentrated nitric acid (65 %) and hydrochloric acid (30 %) were suprapur grade (VWR International, Germany); other chemicals (VWR International, Germany), lyophilized glycogen standard (Type VII, *Mytilus edulis*), Bradford solution, HEPES buffer, and bovine serum albumin (BSA) (Sigma-Aldrich, München, Germany) were of analytical grade. Preparation of a Cd stock solution (100 mg/L) and cleaning of labware were described in a previous paper (Ngo et al. 2008a, in press).

2.2. Food preparation

Control and ^{111}Cd-loaded algae (*Parachlorella kessleri)* were grown in a modified K-medium as previously described (Ngo et al. 2008b, in press). After 7 days of growth and 5 days of incubation, the algal cells were uniformly loaded with Cd (5.9 µg/g dw). They were harvested, centrifuged, freeze-dried (48 h, -20℃), and kept in desiccators in the darkness for later use.

2.3. Animal acclimatization and design of experiment

Mussels 6 – 8 years old (ZOO-Erlebnis Online Shop, Großefehn, Germany) were obtained in May 2006 from a pond with Cd concentrations in the water below the limit of quantification (0.03 µg/L) and in the suspended matter at about 0.03 µg/L. After collection, the mussels were transported to the laboratory in a cool box. Mussel handling, acclimatization and exposure were described elsewhere (Ngo et al. 2008a, in press). Briefly, one week after arrival, 36 mussels were acclimatized for 4 weeks in artificial pond water (APW) (Dietz et al., 1994) under semi-static condition at a light schedule of 12/12 h (13 – 19 µmol/m^2/s); temperature was 17 ± 1℃ and pH was 7.3 ± 0.3.

A group consisting of 18 mussels (44 ± 6 g) was exposed for 35 days to 0.2 µg ^{111}Cd/L in the water plus 1.0 mg/L ^{111}Cd-loaded algae (dry weight) daily, followed by 120 days depuration in Cd-free APW. For the control group of 18 mussels, the conditions were identical except that no Cd was added and the animals were fed with

control algae. During depuration the mussels were fed with freeze-dried control algae. The experiment was carried out under semi-static condition, with two thirds of the water being replaced every second day, and a complete water change every sixth day. The actual Cd concentrations (water + food) were determined every second day.

At given times (exposure: 0.2, 5, 35 d; depuration: 15, 60, 120 d), three mussels were randomly selected before feeding, weighed and anaesthetized by immersion into 2-phenoxyethanol solution (Sigma-Aldrich, Munich, Germany), 4 mL/L, for 20 – 30 min. Hemolymph (HML) and extrapallial fluid (EPF) (7 – 15 mL/mussel) were collected, and the gills, mantle and digestive gland were taken. Tissue samples were transferred into pre-weighed petri dishes (for Cd and Ca analyses) and pre-weighed Eppendorf tubes (for protein and glycogen analyses), and quickly frozen to -80℃ for further processing. After 24 h, aliquots for Cd and Ca determination were lyophilized at -20℃ for 72 h. At other times (exposure: 0.04, 1, 20 d; depuration: 1, 5, 30, 90 d), small volumes (0.3 – 1 mL/mussel) of HML and EPF were sampled without sacrificing the mussels (Gustafson et al., 2005a). Body fluid samples were sonicated (2 x 30 seconds) and centrifuged at 10,000 x g for 5 min to remove particulate matter before assays.

2.4. Glycogen analysis

Glycogen in the tissues and glucose in the HML and EPF were determined by a modified phenol-sulphuric acid method (Dubois et al., 1956; Naimo et al., 1998). Briefly, seven standard concentrations (0, 100, 200, 400, 600, 800 and 1,000 µg/mL) were prepared using a glycogen stock solution of 2 mg/mL (Type VII, Sigma-Aldrich, Munich, Germany). For sample digestion, three volumes (v/v or v/w) of 30 % aqueous KOH were added to each standard, body fluid, and tissue sample in 2-mL polypropylene cryovials (Simport Plastic, Quebec, Canada). The vials were placed in a shaking water bath at 100℃ for 20 min. After vor texing and cooling on ice, 200 µL digestate and 200 µL absolute ethanol were pipetted into a new cryovial, vortexed, and shaken in a water bath at 100℃ for 15 min. Bid istilled water, 1 mL, was added to each sample which was vortexed and kept at room temperature for 5 min. Subsequently, glycogen and glucose concentrations were determined at 492 nm in triplicate using a microtiter plate reader (340-ATTC, Easy software, SLT Labinstruments, Crailsheim, Germany), reference filter 600 nm.

2.5. Protein determination

2.5.1. Subcellular fractionation

Tissues were subjected to subcellular fractionation according to Henry et al. (2003) and Depierre and Dallner (1976) with some modifications. The frozen samples (50 – 100 mg) were immersed in 300 µL ice-cold isotonic extraction buffer (buffer A) containing 10 mM HEPES, pH 7.5, with 250 mM sucrose, 1 mM EGTA, 25 mM KCl, and protease inhibitors (1 µM leupeptin, 200 µM Pefabloc® SC, 800 µM benzamidine, 1 µM Pepstatin) in 1.5-mL Eppendorf tubes, vortexed and placed on ice for 45 min. All subsequent operations were performed at 0 – 4°C. The samples were homogenized by sonication (6 x 30 seconds) at 20 kHz, acoustic power 50 W (Labsonic U tip sonicator, B. Braun Biotech International, Melsungen, Germany). Homogenates were centrifuged at 1,000 x g for 10 min, the pellet was resuspended in 200 µL buffer A, the suspension was centrifuged again at the same speed to yield a pellet (P1) and a supernatant (S1). S1 was then subjected to further differential centrifugation: 10,000 x g for 15 min (Micro 200 R, Hettich Zentrifugen, Tuttlingen, Germany) to produce a mitochondrial pellet (P2), and at 100,000 x g for 60 min (Beckman Optima^{TM} TL Ultracentrifuge, TLA-45) for preparation of a microsomal pellet (P3) and cytoplasmic fraction (S3). The pellets were resuspended in 300 µL buffer A.

2.5.2. Protein assay

Aliquots of 50 µL body fluids, tissue homogenates, cell fractions or standard were diluted with 450 µL 0.01 N NaOH in Eppendorf vials. The mixtures were vortexed and incubated at 60°C for 30 min. The samp les were diluted appropriately and total protein was determined (Bradford, 1976). In summary, 30 µL of each sample was dispensed into a 96-well microtiter plate (3 replicates); to each well, 150 µL Bradford solution, 3x diluted, was quickly added and the samples were measured at 600 nm, reference filter 492 nm, after 5 min up to 1 h, using a microtiter plate reader (340-ATTC, Easy software, SLT Labinstruments, Crailsheim, Germany).

2.6. Determination of cadmium and calcium

Sample preparations and determinations of total Cd and $^{114}Cd/^{111}Cd$ ratio by inductively-coupled plasma mass spectrometry (ICP-MS) and of Ca by ICP-atomic emission spectrometry were done as described elsewhere (Ngo et al. 2008a, in press). Newly incorporated Cd was calculated according to Rodríguez-Cea et al. (2006).

2.7. Data analyses

At each sampling time, shell length, thickness and width, total body weight (total wet tissue + shells), tissue wet and dry weight were determined. The CI was calculated based on the tissue dry weight and shell length according to Day et al. (1990).

The data are presented as means ± SD (standard deviation). Two-way analysis of variance was used to determine whether differences in glycogen and protein concentrations between groups and sampling times were significant. If significant differences were found, the post-hoc test (paired t-test and Newman-Keuls test) was used to identify time points when Cd exposure had a significant effect (GraphPad Software, San Diego, CA). Linear regression between tissue-Cd and glycogen concentrations as well as Spearman's rank correlations between energy reserves (glucose, glycogen and protein) and Ca levels were tested. Statistical significance was assigned at $p < 0.05$, $p < 0.01$, and $p < 0.001$.

3. Results

During the experiment with a total length of 5 months, differences in dry weight between exposed and control animals became apparent at the end of Cd-exposure, becoming even more pronounced during depuration (Table 1). In the end, the relative dry weight had decreased from 14.4 down to 11.3 % in the Cd-exposed group, in the control group from 14.4 to 13 %. The CI fell by almost 50 % in the exposed group, in the control group by 18 %.

In the Cd-exposed animals, glucose (Table 2) in the body fluids (HML and EPF) rose by about 50 % during exposure, an effect which started already during the first hours. During depuration, the levels in the HML and EPF fell to control values within a

few days, in the EPF decreasing even further. In control mussels, the glucose concentrations in the HML were slightly higher than in the EPF; they tended to decrease during the course of the experiment but not significantly.

Table 1

Dry weight in percentage of wet weight and condition index (CI) over five weeks Cd-exposure followed by four months depuration (mean ± SD, n = 3)

	Dry weight [%]		**CI**	
[d]	Exposed	Control	Exposed	Control
Exposure				
0.2 (5 h)	14.4 ± 0.4^{a}	14.4 ± 0.3^{a}	1.7 ± 0.1^{a}	1.7 ± 0.1^{a}
5	14.1 ± 0.3^{a}	14.4 ± 0.3^{a}	1.7 ± 0.1^{a}	1.7 ± 0.1^{a}
35	13.8 ± 0.3^{a}	14.4 ± 0.5^{a}	1.6 ± 0.1*^{a}	1.7 ± 0.1^{a}
Depuration				
15	13.4 ± 0.5*^{ab}	14.2 ± 0.2^{a}	1.4 ± 0.2*^{b}	1.6 ± 0.1^{ab}
60	12.5 ± 0.2*^{c}	13.6 ± 0.2^{b}	1.2 ± 0.1**^{b}	1.5 ± 0.1^{ab}
120	11.3 ± 0.3*^{d}	12.9 ± 0.3^{c}	0.9 ± 0.1**^{c}	1.4 ± 0.2^{b}

Asterisks denote statistically significant differences from control values (* $p < 0.05$; ** $p < 0.01$). Superscript letters (a-d) indicate that values are significantly different ($p < 0.05$; ANOVA followed by Student-Newman-Keuls test).

Glycogen levels (Fig. 1) in the mantle and digestive gland started to decrease during Cd-exposure and declined strongly during depuration ($p < 0.05$); in the end, the levels were about one-fifth (20 %) of the initial values. In the control group, glycogen in both organs fell to about half. In the gills, glycogen levels were generally lower but remained stable in both groups.

For the mantle and digestive gland, highly significant correlations (Fig. 2) of glycogen contents to newly incorporated Cd, i.e. Cd newly taken up during exposure, and to total Cd, i.e. the Cd-burden the animals had accumulated during their whole life-span including the experiment, have been found, but not for the gills (data not shown).

Highly significant correlations of glucose in the body fluids and of glycogen in the mantle and digestive gland (Table 3) to Ca levels were found. Protein contents

were weakly correlated to Ca levels in the body fluids ($p < 0.05$, HML: $r_s = 0.35$; EPF: $r_s = 0.45$) (not shown), and not at all in the tissues.

Table 2

Glucose (mg/L) in hemolymph (HML) and extrapallial fluid (EPF) of *A. anatina* exposed to Cd for five weeks followed by four months depuration (mean ± SD, n = 3)

	HML		**EPF**	
[d]	Exposed	Control	Exposed	Control
Exposure				
0.04 (1 h)	177 ± 17^{bcd}	156 ± 19^{a}	151 ± 19^{cd}	146 ± 17^{a}
0.2 (5h)	198 ± 19*^{bc}	155 ± 20^{a}	226 ± 39**^{b}	146 ± 18^{a}
1	221 ± 24**^{ab}	154 ± 16^{a}	278 ± 18***^{a}	146 ± 12^{a}
5	248 ± 24**^{a}	150 ± 15^{a}	239 ± 26***^{ab}	144 ± 22^{a}
20	255 ± 20***^{a}	147 ± 18^{a}	223 ± 21**^{b}	142 ± 25^{a}
35	259 ± 31**^{a}	145 ± 19^{a}	218 ± 20**^{b}	136 ± 21^{a}
Depuration				
1 (24 h)	200 ± 26*^{bc}	149 ± 18^{a}	181 ± 19*^{c}	137 ± 18^{a}
5	158 ± 8^{cde}	142 ± 23^{a}	158 ± 19^{cd}	136 ± 29^{a}
15	139 ± 16de	137 ± 18^{a}	128 ± 19de	131 ± 23^{a}
30	130 ± 30de	133 ± 14^{a}	116 ± 15^{e}	127 ± 19^{a}
60	123 ± 26de	125 ± 21^{a}	109 ± 10^{e}	120 ± 15^{a}
90	124 ± 13de	122 ± 22^{a}	105 ± 14^{e}	117 ± 19^{a}
120	113 ± 21^{e}	116 ± 17^{a}	88 ± 10^{e}	111 ± 18^{a}

Asterisks denote statistically significant differences from the control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Superscript letters (a-e) indicate that values are significantly different ($p < 0.05$; ANOVA followed by Student-Newman-Keuls test).

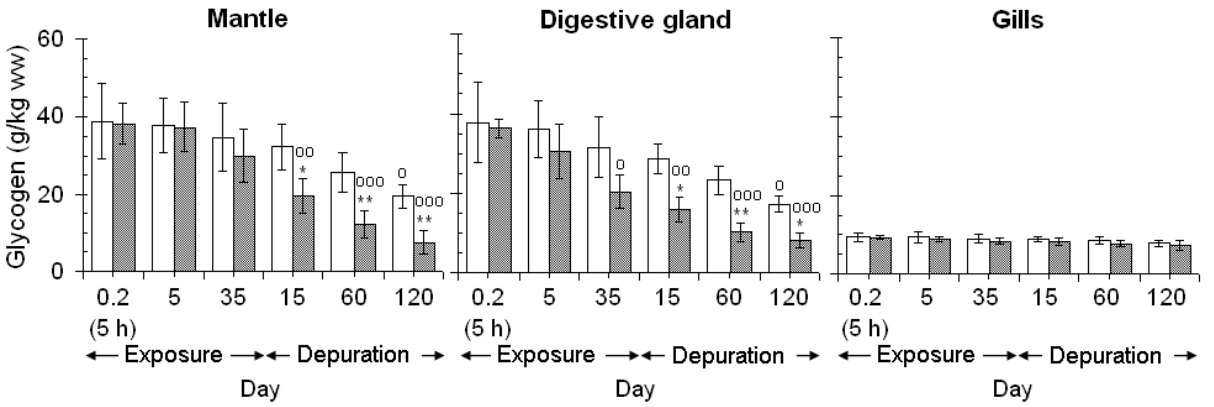

Fig. 1. Glycogen (g/kg ww) in the mantle, digestive gland and gills of control (open bars) and Cd-exposed (shaded bars) animals (mean ± SD, n = 3). Statistically significant differences from control values (* $p < 0.05$; ** $p < 0.01$) and in comparison to the same group in the beginning of experiment ($^{o}p < 0.05$; $^{oo}p < 0.01$; $^{ooo}p < 0.001$) are shown.

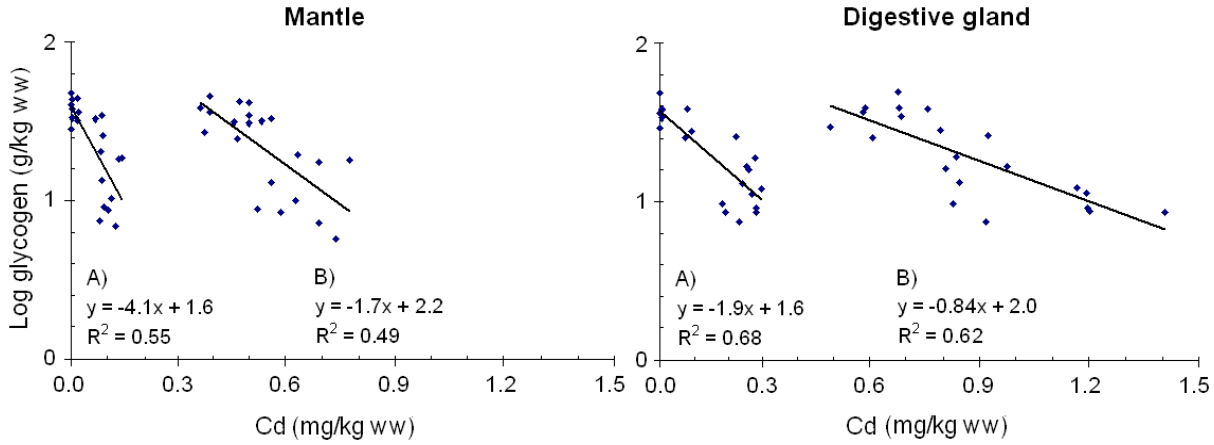

Fig. 2. Newly incorporated Cd (A) and total Cd (B) *versus* glycogen concentrations in the mantle and digestive gland of Cd-exposed *A. anatina* over the whole experimental period (n = 21).

Table 3

Correlation factors (Spearman r or r_s) of glucose or glycogen concentrations to Ca levels in different body compartment of *A. anatina* over five weeks exposed to algae- and water-borne Cd followed by four months depuration

	Glucose *vs.* Ca	**Glycogen *vs.* Ca**
HML	0.73***	n/a
EPF	0.66***	n/a
Mantle	n/a	-0.55**
Digestive gland	n/a	-0.57**
Gills	n/a	0.21

Significant correlations are indicated (body fluids: n = 42; tissues: n = 21). ** $p < 0.01$; *** $p < 0.001$; n/a: not applicable.

Protein levels (Table 4) in the HML started to fall from the first hour of Cd-exposure and continued to decline during the depuration phase by 77 % until the end. Over the same period, protein levels in the EPF decreased by about 60 % ($p < 0.05$). In the control group, total proteins in both HML and EPF fell by 45 %. In the tissues, total proteins in control mussels (data not shown) were highest in the digestive gland (45 – 50 g/kg ww), slightly lower (30 – 35 g/kg ww) in the mantle and gills; these levels diminished only slightly ($p > 0.05$) towards the end of depuration.

Protein profiles in subcellular fractions (Fig. 3) showed some notable alterations. Nuclear proteins (Fig. 3A) increased in the mantle and gills of exposed animals, being significantly higher than in controls at the end of exposure and in the beginning of depuration. In the digestive gland, the trend was similar but not significant. Mitochondrial proteins (Fig. 3B) were significantly higher in the mantle of Cd-exposed animals during depuration, an effect which was also observed in the digestive gland in the beginning of depuration, but not in the gills. Cytoplasmic proteins (Fig. 3C) in the three organs tended to increase during Cd-exposure ($p < 0.05$). Microsomal proteins (Fig. 3D) in the gills, mantle and digestive gland decreased slowly during depuration, both in exposed and control animals, being significantly lower in exposed animals only in the gills towards the end of depuration.

Table 4

Protein (mg/L) in hemolymph (HML) and extrapallial fluid (EPF) of *A. anatina* exposed to Cd for five weeks followed by four months depuration (mean ± SD, n = 3)

	HML		**EPF**	
[d]	Exposed	Control	Exposed	Control
Exposure				
0.04 (1 h)	539 ± 39*^{a}	620 ± 50^{a}	394 ± 26^{ab}	399 ± 49^{a}
0.2 (5h)	506 ± 48*^{a}	623 ± 32^{a}	408 ± 42^{a}	404 ± 29^{a}
1	485 ± 38**^{a}	618 ± 41^{a}	390 ± 25^{ab}	396 ± 36^{a}
5	422 ± 36**^{b}	613 ± 45^{a}	380 ± 40^{ab}	393 ± 33^{a}
20	371 ± 28***^{c}	606 ± 43^{a}	348 ± 31^{abc}	385 ± 23^{a}
35	337 ± 35***^{c}	591 ± 40^{ab}	329 ± 26^{bcd}	375 ± 64^{ab}
Depuration				
1 (24 h)	334 ± 23***^{c}	583 ± 26^{ab}	325 ± 30^{bcd}	365 ± 31^{ab}
5	254 ± 20***^{d}	571 ± 27^{ab}	289 ± 33*^{cde}	352 ± 21^{ab}
15	193 ± 23***^{e}	554 ± 32^{ab}	267 ± 43*^{def}	331 ± 31^{ab}
30	161 ± 20***^{e}	513 ± 28^{bc}	232 ± 25**^{efg}	300 ± 18^{bc}
60	152 ± 24***^{e}	460 ± 30^{cd}	212 ± 22*^{fgh}	260 ± 15^{cd}
90	142 ± 14***^{e}	402 ± 39^{d}	182 ± 35^{gh}	231 ± 25^{d}
120	144 ± 24** ^{e}	321 ± 35^{e}	155 ± 17*^{h}	216 ± 20^{d}

Asterisks denote statistically significant differences from the control (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Superscript letters (a-h) indicate that values are significantly different ($p < 0.05$; ANOVA followed by Student-Newman-Keuls test).

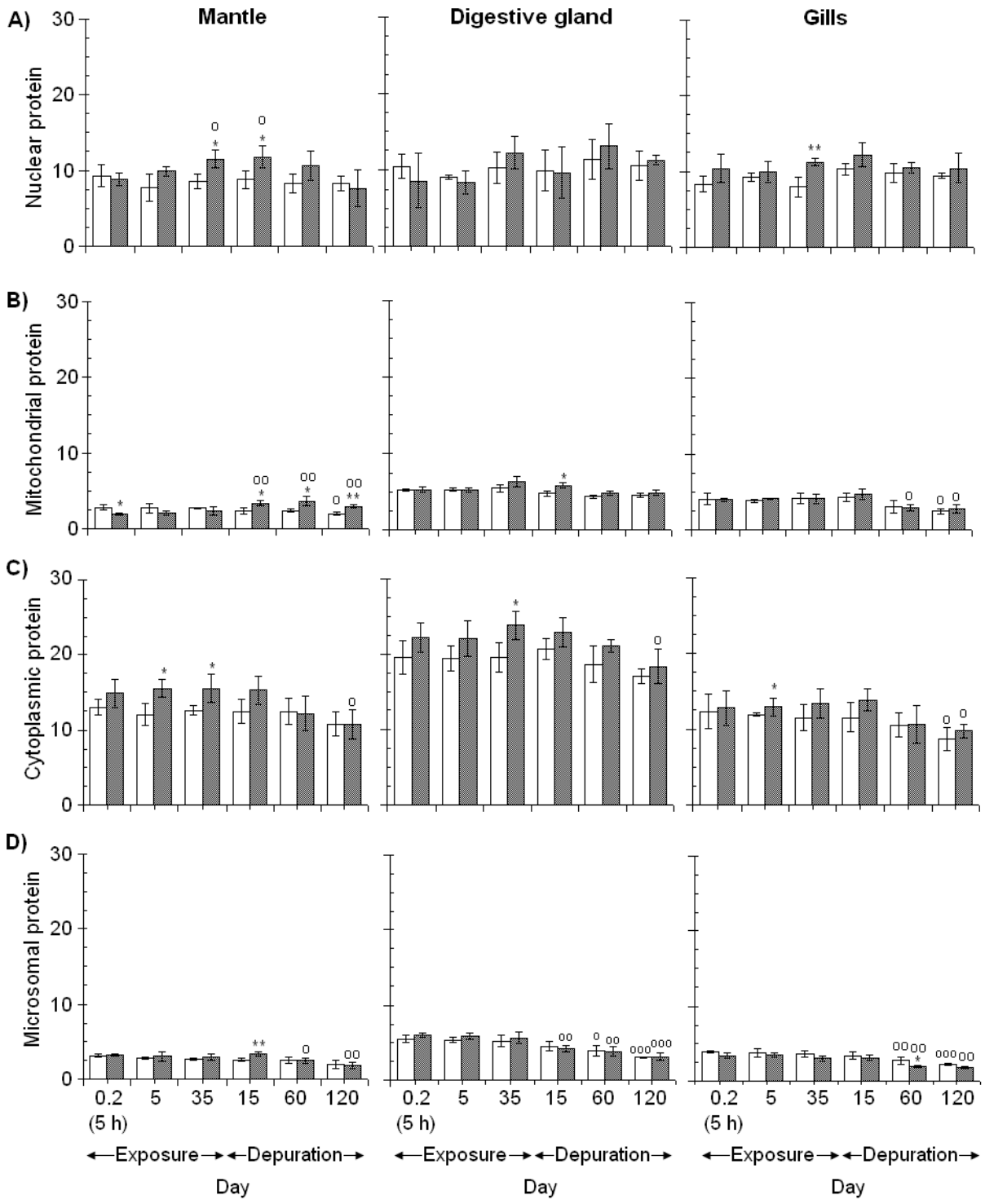

Fig. 3. Nuclear (A), mitochondrial (B), cytoplasmic (C) and microsomal proteins (D) (g/kg ww) in the mantle, digestive gland and gills of control (open bars) and Cd-exposed (shaded bars) animals (mean ± SD, n = 3). Statistically significant differences from control values (* $p < 0.05$; ** $p < 0.01$), and in comparison to the same group in the beginning of experiment (^{o} $p < 0.05$; ^{oo} $p < 0.01$; ^{ooo} $p < 0.001$) are shown.

4. Discussion

The time-dependent decrease in dry weight of *A. anatina,* usually observed under artificial laboratory conditions (Mäkelä and Oikari, 1995; Naimo and Monroe, 1999), is aggravated in Cd-exposed animals during and after exposure. Under Cd stress, mussels utilize more energy reserves by breaking down more glycogen; thus, the relative tissue water content becomes higher and dry weight decreases. The same is reflected in the decline of the CI of exposed animals from the end of exposure and during depuration, coinciding with low glycogen contents in the mantle and digestive gland. Other authors reported that the CI was not altered in *Mytilus edulis* exposed to Cd under semi-field conditions (Veldhuizen-Tsoerkan et al., 1991). These differences are likely due to the differences in species and experimental conditions.

Obviously, the Cd-exposed animals have a high metabolic demand to cope with toxicity and to maintain normal functions. The alterations in glucose levels in body fluids and glycogen budgets (Table 2; Fig. 1) under sub-chronic low-level Cd exposure are sensitive toxicological endpoints and signs of indirectly induced starvation. Changes in glycogen concentrations are long-lasting and not sensitive to non-toxicant stress (Lagadic et al., 1994), and therefore a useful biomarker. The observed decrease in glycogen in the mantle and digestive gland but not in the gills is similar to *A. cygnea* exposed to 50 µg Cd/L for 8 weeks (Hemelraad et al., 1990).

The depletion in energy reserves indicates a high activity of glycogenolysis. Breakdown of glycogen in the tissues leads to a rise in glucose contents in the body fluids (Table 2), a response of mussels to immediate nutritional need when handling stress (Pekkarinen and Suoranta, 1995). Glycogen mobilization in molluscs is known to be regulated by a neurohormonal factor in the cerebral ganglia, the so called hyperglycemic factor or glycogen mobilizing hormone (Hemminga et al., 1985; Robbins et al., 1990; 1991; Abdraba and Saleuddin, 2000) whose secretion is induced by Cd (Reddy et al., 1989). At the beginning of exposure to stressors, mussels tend to minimize their energy use, a defense mechanism often observed in animals. However, in the long run the animals must break down more glycogen which finally must affect growth, reproduction and population development.

Linear regressions of glycogen on newly incorporated Cd and on total Cd (Fig. 2) were significant for the mantle and digestive gland, but not for the gills ($p > 0.05$). The latter may be due to the fact that at low concentrations Cd is trapped in Ca concretions, accounting for more than 60 % of the Cd burden in the gills of freshwater mussels (Bonneris et al., 2005). Thus, the gills may be better protected against Cd toxicity. In the mantle and digestive gland with much less Ca concretions than the gills, newly incorporated Cd and total Cd were high (Ngo et al. 2008a, in press). It seems that more biologically active Cd is available for exerting toxic effects and, as a consequence, more glycogen is broken down.

Shells do not grow normally in stressed mussels as they cannot maintain Ca uptake, transport and regulation due to lack of high metabolic energy in the form of ATP which require for those processes (Schatzmann, 1973). Thus, shortage in Ca for normal shell growth and repair may arise. Furthermore, proteins and polysaccharides are important components of the calcifying organic matrix which control $CaCO_3$ polymorphism, size and shape of the crystallites, and shell texture (Marin and Luquet, 2004).

Ca has many other physiological functions including energy metabolism and glycogen turnover (Goans et al., 1996). The present study showed a positive correlation between glucose and Ca concentrations in both HML and EPF, and a negative correlation between Ca and glycogen levels in the mantle and digestive gland. Gustafson et al. (2005b) also reported higher glucose and Ca levels in HML of *Epilliptio complanata* in a heavy-metal polluted region. Due to acidosis caused by Cd stress (Moura et al., 2000), Ca ions are perhaps mobilized from concretions in the gills, leading to a rise of its levels in body fluids and being circulated to the other organs, e.g. the mantle and digestive gland. There, it plays a role in the activation of glycogen phosphorylase, a key enzyme in glycogenolysis (Goans et al., 1996). As a consequence, lower glycogen in these organs and higher glucose levels in body fluids are observed.

Opposite to glucose, protein levels tended to decrease in the HML and EPF, but total tissue proteins remained largely unchanged. At higher Cd-exposure levels, tissue protein contents were significantly lowered on day 4 in *Mytilopsis sallei* exposed to 143 µg Cd/L (Uma Devi, 1996) or in *Mytilus edulis* exposed to 200 µg Cd/L for 21 days (Geret and Cosson, 2002). A more detailed analysis of proteins in

the subcellular fractions showed interesting changes. An increase of nuclear proteins (Fig. 3A) in the gills and the mantle at the end of exposure and in the beginning of depuration may be indicative of Cd-induced nuclear protein kinase C activity, a key enzyme family known to control cell growth and differentiation (Block et al., 1992). At the same time, nuclear translocation of MTF-1, a six-zinc finger protein important in activating metallothionein (MT) expression is known to take place (Smirnova et al., 2000). A fluctuating mitochondrial protein contents in the mantle of exposed animals (Fig. 3B), and the trend of increasing protein levels in the cytoplasmic fraction in all tissues during exposure (Fig. 3C) may be associated with MT production; Cd is known to alter mitochondrial metabolism (Viarengo et al., 1980). Effects on protein synthesis by Cd by interfering with RNA synthesis and attachment of polyribosomes to the rough endoplasmic reticulum membrane (Viarengo, 1985) might explain why microsomal proteins tend to decrease in exposed mussels, especially in the gills (Fig. 3C).

5. Conclusions

A. anatina exposed to environment-like Cd concentration (~0.2 µg/L), i.e. within the level (0.07 – 0.53 µg Cd/L) recommended by ECE (Economic Commission for Europe) for the maintenance of freshwater aquatic life, class II (40 – 50 mg $CaCO_3$/L) (UNECE, 1994), exhibit dramatic decreases in CI, in relative dry weight of soft tissues, and in glycogen in the mantle and digestive gland. The alteration of carbohydrate metabolism under Cd stress is tissue-specific and time-dependent. Correlations of glycogen and glucose with Ca concentrations in the respective compartment suggest that Cd exerts adverse effects on glycogen metabolism which sequentially disturbs Ca homeostasis. Protein levels in both HML and EPF are strongly suppressed, but not in the soft tissues. The present findings are relevant in respect to the potential effects of environmental Cd exposure on the physiological status and population development of freshwater mussels.

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Appendix IV:

Subchronic effects of environment-like cadmium levels on the bivalve *Anodonta anatina* (Linnaeus 1758):

III. Effects on carbonic anhydrase activity in relation to calcium metabolism

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(Received 24 July 2008; final version received 09 September 2008; accepted 25 September 2008)

Toxicological and Environmental Chemistry, in press

Abstract

The effects of sub-chronic cadmium (Cd) exposure at environment-like levels (water- and algae-borne Cd) on carbonic anhydrase (CA), its distribution in various body compartments and subcellular fractions, and its correlation to Cd and Ca concentrations in the freshwater mussel *Anodonta anatina* were investigated. CA activity in the hemolymph and extrapallial fluid were strongly affected in both directions, i.e. inhibition and then induction depending on the length of exposure and depuration. Cd also lowered total and subcellular CA activity in all tested tissues, especially in the digestive gland and gills. These effects were confirmed by negative correlation of both total and cytoplasmic CA activity relative to newly incorporated Cd. The specific activity of total CA was correlated to Ca in the hemolymph and in the mantle but not in other compartments. These observations suggest that Cd exerted indirect effects on ion regulation and Ca homeostasis through its effects on CA, perhaps constituting the physiological basis of Cd-toxicity in this freshwater bivalve.

Keywords: Anodonta anatina; cadmium; carbonic anhydrase; subcellular distribution of carbonic anhydrase; freshwater mussel; calcium.

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1. Introduction

Carbonic anhydrase (CA) (E.C. 4-2-1-1) is a zinc-containing enzyme which catalyzes reversibly the hydration of carbon dioxide (CO_2) to bicarbonate. CA is present in many tissues and is involved in a wide range of physiological and biochemical processes including respiration, pH regulation, maintenance of CO_2-balance, and Ca homeostasis (Henry, 1996; Chegwidden and Carter, 2000). In bivalve molluscs, CA is involved in the mobilization of $CaCO_3$ reserves (Istin and Girard, 1970), in calcification, and in shell formation (Freeman, 1960; Wilbur and Saleuddin, 1983; Medakovic, 2000). There are at least two types of CA in molluscs, i.e. in the cytoplasm and plasma membrane (Henry, 1987). Subcellular distribution of CA of various tissues of freshwater mussels, and the effects of cadmium (Cd) on their activities has not been determined so far. Digestive gland, gills and mantle constitute the interfaces of a bivalve with the outside environment; therefore, they are likely to be the primary targets for the action of environmental pollutants such as the heavy metal Cd. The subcellular CA distribution and the effects of Cd at environment-like levels on total and subcellular CA activities in the digestive gland, gills and mantle as well as in body fluids of the freshwater mussel *Anodonta anatina* are reported. Correlations of Cd *versus* CA activities and of CA *versus* Ca levels were tested. This may provide insights how a Cd-impact translates into effects on Ca homeostasis and energy reserves, as reported in previous publications (Ngo et al. 2008a, c, in press).

2. Materials and methods

2.1. Chemicals and labware

Cd metal, 96 % ^{111}Cd isotope enriched, was purchased from CK GAS Product Ltd (Hampshire, UK). HNO_3 (65 %) and HCl (30 %) were suprapur grade; other chemicals used to prepare experimental water were analytical grade (VWR International, Darmstadt, Germany). HEPES buffer, protease inhibitors, Bradford solution, bovine serum albumin (BSA) and other chemicals were of analytical grade (Sigma-Aldrich, Munich, Germany). Preparation of Cd stock solution (100 mg/L) and cleaning of labware were described in a previous publication (Ngo et al. 2008a, in press).

2.2. Food preparation

Control and ^{111}Cd-loaded algae (*Parachlorella kessleri)* were grown for 7 days in a modified K-medium as described elsewhere (Ngo et al. 2008b, in press). After 7 days of growth, the algae were harvested, centrifuged, freeze-dried (48 h, -20℃), and kept in desiccators in the darkness for later use. The Cd concentration in the freeze-dried ^{111}Cd-loaded algae was 5.9 µg/g dry weight (dw).

2.3. Design of experiment and sampling procedures

Freshwater mussels, about 6 – 8 years old (ZOO-Erlebnis Online Shop, Großefehn, Germany), obtained in May 2006 were acclimatized, exposed to Cd, and sampled as described previously (Ngo et al. 2008a, in press). Briefly, after 4 weeks of acclimatization, the animals were exposed to Cd via water (0.2 µg ^{111}Cd/L) and food (1.0 mg dry weight ^{111}Cd-loaded algae per L and day) for 35 days, followed by depuration in Cd-free artificial pond water (APW) for 120 days under semi-static conditions. The experiment involved a group of 18 exposed mussels and 18 control animals, the latter being kept under the same condition as the exposed group, except that Cd was not added and the mussels were fed with control algae. During the whole experiment, two thirds of the water was replaced every second day, and a complete change was carried out every sixth day. The actual Cd concentrations (water + food) were determined every second day.

At various time intervals (exposure: 0.2, 5, 35 d; depuration: 15, 60, 120 d), three mussels were randomly selected before feeding for taking body fluids and tissue samples. They were immersed in 4 mL/L of 2-phenoxyethanol solution (Sigma-Aldrich, Munich, Germany) for 20 – 30 min and dissected on ice into gills, mantle, and digestive gland. Samples were transferred into pre-weighed petri dishes for Cd and Ca analyses, and into pre-weighed Eppendorf tubes for CA activity determination. Tissue samples for CA determination were washed with phosphate-buffered saline (PBS), weighed and frozen immediately at -80℃ for further processing. Small samples of HML and EPF (0.3 – 1 mL/mussel) were also taken in between (exposure: 0.04, 1, 20 d; depuration: 1, 5, 30, 90 d) by a nonlethal method (Gustafson et al., 2005).

2.4. CA analyses

2.4.1. Preparation of subcellular fractions

Subcellular fractions were prepared as described elsewhere (Ngo et al. 2008c, in press) employing previously published methods (DePierre and Dallner, 1976; Henry et al., 2003) with the following modifications, i.e. the crude homogenates were centrifuged twice at 1,000 x g for 10 min to yield nuclei and cell debris (P1) and the supernatants combined as S1, and protease inhibitors were used (see below). The following fractions were obtained by centrifugation of S1 at 10,000 x g for 15 min to yield mitochondria and lysosomes (P2) and supernatant S2, which was then centrifuged at 100,000 x g for 60 min to yield microsomes (P3) and cytoplasm (S3). All procedures were conducted at 0 – 4℃.

2.4.2. CA activity determination

The subcellular pellets (P1, P2 and P3) were sonicated in 300 µL ice-cold buffer A containing 10 mM HEPES, pH 7.5, with 250 mM sucrose, 1 mM EGTA, 25 mM KCl and protease inhibitors (1 µM leupeptin, 200 µM Pefabloc® SC, 800 µM benzamidine, 1 µM Pepstatin) before assays. Body fluid samples were sonicated (2 x 30 seconds) and centrifuged at 10,000 x g for 5 min to remove cells and particulate matter. For CA assays, the pH method (Vitale et al., 1999) was employed. A known volume (0.05 – 0.1 mL) of tissue homogenate, subcellular fraction, or body fluids was added to 7.5 mL ice-cold reaction medium containing 10 mM Tris/phosphate, 225 mM mannitol, 75 mM sucrose, pH 7.4. The reaction was started by adding 1 mL ice-cold CO_2-saturated bidistilled water under stirring. The pH was determined every 15 seconds until 75 seconds. A linear regression of pH *versus* time was fitted, with the slope representing the catalyzed reaction rate ($b_{\text{catalysed}}$). Before and after each series of seven samples, the non-catalyzed reaction rate ($b_{\text{non-catalysed}}$) was determined from the pH decrease in the same reaction medium with 0.1 mL buffer A being added instead of sample. All assays were performed in triplicate. Total protein content in each fraction was determined spectrophotometrically with bovine serum albumin (BSA) as standard (Bradford, 1976). Specific CA activity was calculated as units per mg protein (U/mg protein) (Vitale et al., 1999).

Total CA of each fraction (P1, P2, P3 and S3) was calculated as

$$\textit{fractional CA} = CA_f \times V_f \qquad (1)$$

(fractional CA is the total CA activity in the respective fraction; CA_f is the specific CA activity (U/mL); V_f (mL) is the total volume of the respective fraction), the percentage of fractional CA relative to the total CA of the respective tissue was calculated as follows:

Relative fractional CA (%) = [fractional CA x 100]/total homogenate CA *(2)*

2.5. Cadmium and calcium determination

Sample preparation and determination of Cd (ICP-MS) and Ca (ICP-AES) was done as described elsewhere (Ngo et al. 2008a, in press). In summary, the samples (10 – 100 mg dw) were digested in 5 mL acid (HNO_3 65 %/ HCl 30 %, 4/1) at 40°C for 1 h and at 120°C for 3 h. A blank (HNO_3 65 %/ HCl 30 %, 4/1) was included for every 10 samples. Each digestate was diluted to 10 mL with bidistilled water and filtered through a 0.45 µm cellulose syringe filter. Body fluid samples (200 – 1000 µL) were acidified with 500 µL HNO_3 and diluted with bidistilled water to 10 mL before analysis.

2.6. Statistical analyses

Data are presented as means ± standard deviation (SD). Two-way analysis of variance was used to detect differences in CA activities between groups and sampling times. If significant differences were found, the post-hoc test (paired t-test and Newman-Keuls test) was used to find at which times the Cd had significant effects (GraphPad Software, San Diego, CA).

Correlations of Cd *versus* CA activity as well as of CA activity *versus* Ca in all body compartments were tested with the nonparametric Spearman's rank correlation test. Statistical significance was assigned at $p < 0.05$, $p < 0.01$ and $p < 0.001$.

3. Results

CA activities in the body fluids (Table 1) varied greatly upon Cd exposure; it decreased in HML to become significantly lower than in controls at the end of exposure, i.e. about 20 % of the initial value or 40 % of the control group. Immediately upon depuration, CA increased strongly, being up to ten-fold higher than in controls at day 5 of depuration. Subsequently it declined returning to initial values at day 90 of depuration. In control animals, CA in HML decreased continuously over the whole time period, becoming undetectable after two months of depuration. In EPF, the Cd-

elicited decrease was very fast, becoming significantly lower than control within one day. Similar to HML, although earlier, i.e. after three weeks of Cd-exposure, CA activities increased strongly to become almost double that of control in the first week of depuration ($p < 0.05$), and then declining. In control mussels, CA activity in the EPF slowly fell over the whole experimental period to about 75 % of its initial value ($p > 0.05$).

Table 1

CA activity (U/mg protein) in hemolymph (HML) and extrapallial fluid (EPF) of *A. anatina* during Cd-exposure and depuration (mean ± SD, n = 3)

	HML		**EPF**	
[d]	Exposed	Control	Exposed	Control
Exposure				
0.04 (1 h)	7.5 ± 1.5^{ab}	8.0 ± 1.5^{b}	7.3 ± 2.2^{ab}	7.0 ± 1.5^{a}
0.2 (5 h)	6.9 ± 1.8^{ab}	8.4 ± 0.7^{b}	5.6 ± 1.6 ^{a}	7.1 ± 0.7^{a}
1	5.7 ± 2.2^{ab}	7.3 ± 2.5^{ab}	2.1 ± 1.4*^{a}	6.3 ± 1.1^{a}
5	5.5 ± 0.6^{ab}	7.1 ± 2.1^{ab}	n.d.	6.5 ± 0.8^{a}
20	4.0 ± 1.9^{ab}	5.6 ± 1.7^{ab}	11.7 ± 6.5^{ab}	6.6 ± 1.3^{a}
35	1.6 ± 2.2*^{a}	4.0 ± 0.9^{ab}	11.0 ± 3.3^{ab}	6.6 ± 2.2^{a}
Depuration				
1 (24 h)	17.3 ± 2.3**^{cd}	3.5 ± 2.2^{ab}	15.6 ± 3.6*^{b}	6.9 ± 1.9^{a}
5	21.3 ± 4.5**^{d}	2.2 ± 0.9^{a}	15.3 ± 5.6*^{b}	6.3 ± 2.5^{a}
15	19.4 ± 1.7**^{cd}	3.1 ± 0.6^{ab}	11.2 ± 4.6^{ab}	6.4 ± 2.3^{a}
30	18.3 ± 3.3***^{cd}	2.0 ± 1.5^{a}	5.2 ± 1.2^{a}	6.0 ± 1.5^{a}
60	15.1 ± 4.7^{bc}	n.d.	4.5 ± 3.0^{a}	6.3 ± 2.0^{a}
90	9.1 ± 2.0^{ab}	n.d.	3.2 ± 2.1^{a}	4.5 ± 2.8^{a}
120	3.8 ± 1.2^{ab}	n.d.	n.d.	5.2 ± 1.6^{a}

n.d.: not detectable. Asterisks denote statistically significant differences from control values (* $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$). Superscript letters (a-d) indicate that values are significantly different ($p < 0.05$; ANOVA followed by Student-Newman-Keuls test).

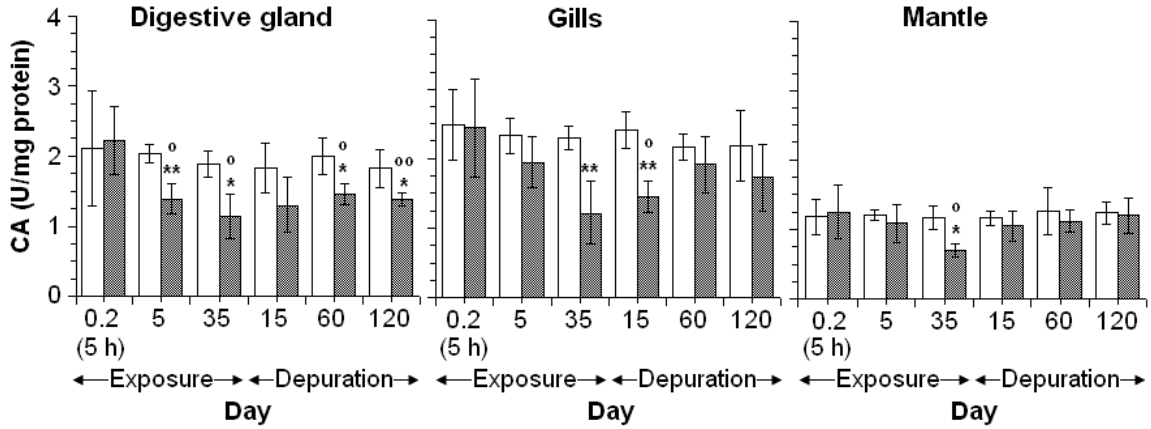

Figure 1. CA activities in the digestive gland, gills and mantle of control (open bars) and Cd-exposed (shaded bars) animals (mean ± SD, n = 3). Asterisks denote statistically significant differences from control values (* $p < 0.05$; ** $p < 0.01$). Statistically significant differences compared to 5 h from the same group are indicated by ^{o} (^{o} $p < 0.05$; ^{oo} $p < 0.05$).

In all tested tissues, CA activities were affected by Cd (Fig. 1) although delayed compared to the body fluids. In the digestive gland, the decrease was significant at day 5, reaching about 50 % at the end of exposure (35 d) and remaining lower than in controls ($p < 0.05$) until the end of depuration. For the gills this was similar, reaching about 50 % of controls at day 35 of exposure and then slowly but incompletely recovering during depuration. In the mantle, a significantly lower CA activity, i.e. about 60 % of control, was observed in exposed mussels at day 35 of exposure.

Relative distributions of CA in the subcellular fractions were determined. In control animals (Fig. 2), cytoplasmic activity represented the largest fraction, i.e. about 60 % of the total enzyme activity. The nuclear activity was about 16 %, followed by the microsomal (10 – 15 %) and the mitochondrial (10 – 13 %) fraction. In the mantle, the microsomal CA was higher (15 %) than in the other two organs. Cd caused some alterations in this pattern (data not shown): cytoplasmic CA activity increased in the gills (from 61 % to 65 %) and in the digestive gland (from 62 % to 65 %) but decreased in the mantle (from 59 % to 54 %); microsomal CA was enhanced in the digestive gland (15 % to 20 %) but unaltered in other organs; nuclear CA declined in all three organs (from 16 % to 11 %); mitochondrial CA remained similar to control levels.

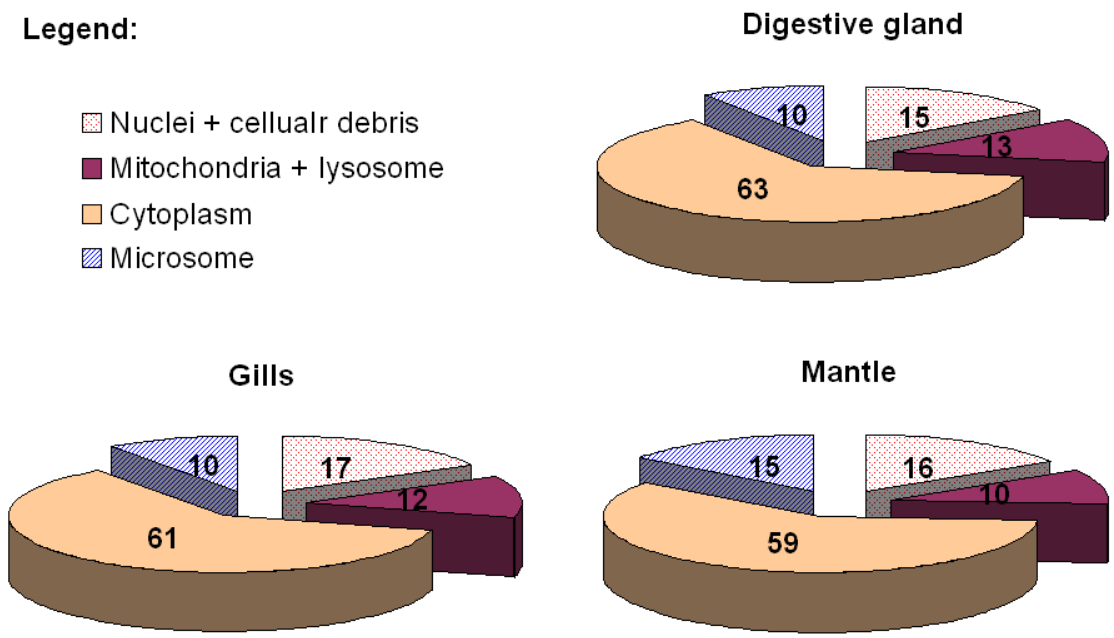

Figure 2. Relative distribution of subcellular CA activities (%) in the digestive gland, gills and mantle of control mussels.

Most pronounced was the Cd-induced inhibition of cytoplasmic and nuclear CA (Fig. 3A, B) while for mitochondrial and microsomal CA (Fig. 3C, D) Cd-effects were minor and non-significant. Cytoplasmic CA activities (Fig. 3A) in the digestive gland, gills and mantle decreased towards the end of exposure, being only 40 – 50 % of control levels; it slightly recovered in the mantle and gills after two weeks of depuration, and in the digestive gland after two months. An even stronger effect was observed for nuclear CA activities (Fig. 3B), with a strong decrease to only 30 – 40 % of control towards the end of exposure, gradually increasing during depuration but remaining lower than control levels, especially in the digestive gland ($p < 0.05$). For mitochondrial CA activities (Fig. 3C), lower levels were observed in the gills and mantle at the end of exposure ($p < 0.05$), being non-significant in the digestive gland. Microsomal, membrane-bound CA activity (Fig. 3D) was not notably affected by Cd, except for slight effects in the digestive gland.

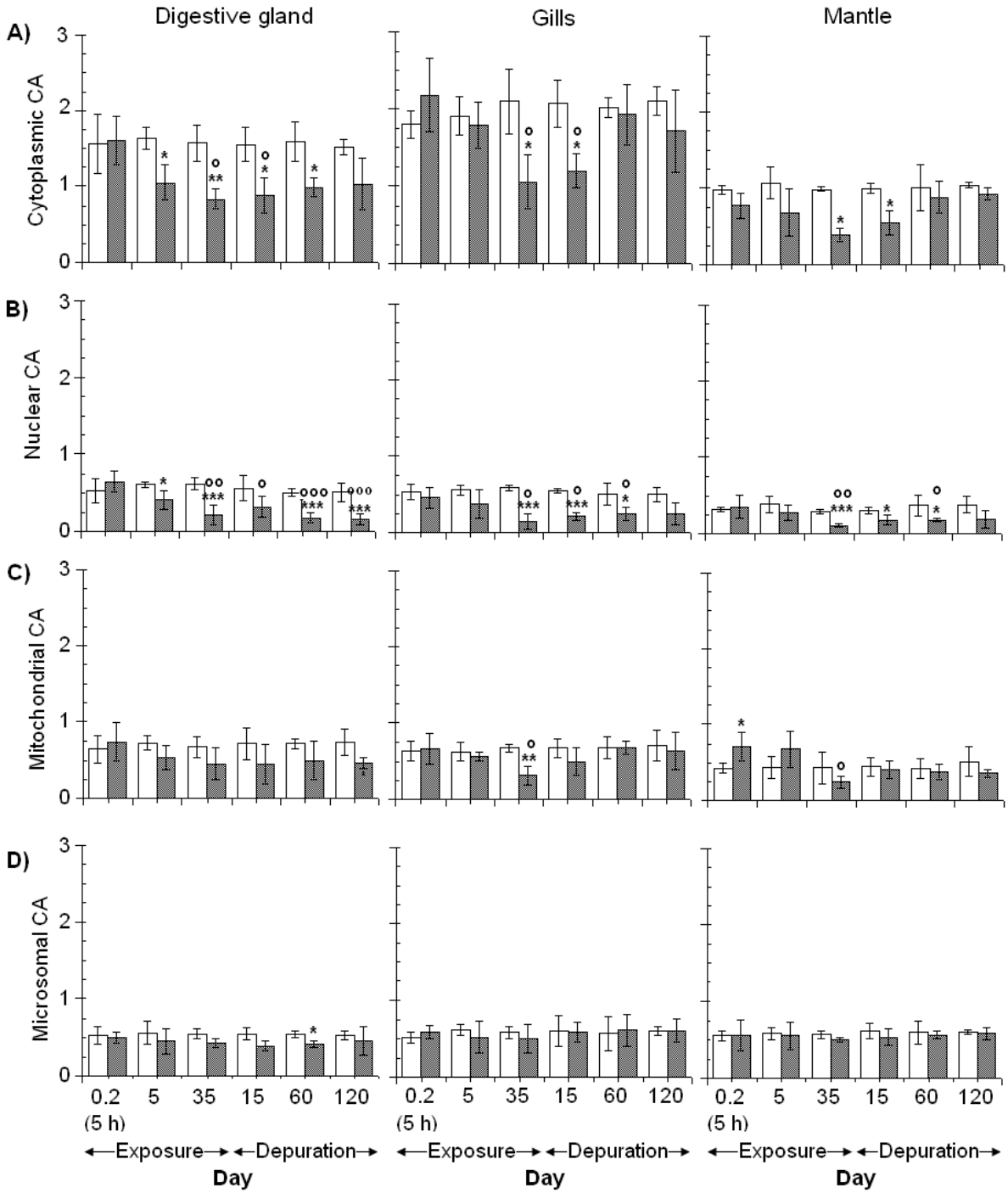

Figure 3. CA activity (U/mg protein) in the cytoplasmic (A), nuclear (B), mitochondrial (C) and microsomal fraction (D) of the digestive gland, gills and mantle of control (open bars) and Cd-exposed (shaded bars) mussels (mean ± SD, n = 3). Statistically significant differences from control values (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$), and in comparison to the same group in the beginning of experiment ($^{o}p < 0.05$; $^{oo}p < 0.01$; $^{ooo}p < 0.001$) are shown.

With the Spearman's rank correlation test (Table 2), tight correlations of total CA and of cytoplasmic CA activity in the gills and in the digestive gland to newly incorporated Cd ($p < 0.05$) were detected; correlations of CA were lower to total Cd concentration, i.e. the Cd the animals had taken up during the life before the experiment plus the Cd taken up during exposure ($p > 0.05$).

Positive correlation between specific activity of total CA and Ca level (data not shown) were found ($p < 0.05$) for the HML ($r_s = 0.38$) and mantle ($r_s = 0.45$), but not for the EPF, gills and digestive gland ($p > 0.05$).

Table 2

Correlation factors (Spearman r or r_s) of Cd levels *versus* CA activity in different tissues of *A. anatina* over five weeks exposed to algae- and water-borne Cd, followed by four months depuration

Organs	**Newly incorporated Cd *vs.***		**Total Cd *vs.***	
	Total CA	Cytoplasmic CA	Total CA	Cytoplasmic CA
Digestive gland	-0.55**	-0.59**	-0.44*	-0.44*
Gills	-0. 61**	-0.49*	-0.37	-0.28
Mantle	-0.18	-0.10	-0.30	-0.05

Significant correlations are indicated (n = 21). * $p < 0.05$; ** $p < 0.01$; CA: carbonic anhydrase.

4. Discussion

This is the first time that the effects of Cd-exposure on CA activity in freshwater mussel are reported, including its activity in the HML and EPF. CA activities are higher in the body fluids than in the tissues, although it has been speculated that in molluscs CA is present only in the gills, the organ for gas exchange and ion uptake, transport and regulation (Henry, 1984). However, the enzyme has been found previously in HML of marine snails, in the clam *Mercenaria campechiensis* and in the oysters *Crassostrea virginica,* and at very high levels in *Crassostrea equestris* (128 U/mg protein) (Nielsen and Frieden, 1972).

In our study, CA activities in the HML and EPF decreased at the beginning of Cd-exposure but were strongly induced (Table 1) towards the end of exposure and in

the beginning of depuration, right after the increase in glucose levels in these body compartments (Ngo et al. 2008c, in press). This increased activity was specific to the body fluids and may be due to its involvement in glycogen re-synthesis and glucose metabolism, which does not occur in tissues, as suggested for trout and other vertebrates (Henry, 1996; Wang et al., 1998) to avoid hyperglycemic condition. Freshwater mussels live in environments poor in Ca and other ions; in order to maintain Ca concentrations in their HML and EPF about 10 fold higher than in the ambient water (Van der Broght and Van Puymbroeck, 1966), CA plays an important role in maintaining high ionic strength and stabilizing pH-regulation (Nielsen and Frieden, 1972). This is also important in the EPF for calcification as it is involved in regulating the solubility and concentration of Ca at saturation conditions (Coimbra et al., 1993).

The total activity of CA (Fig. 1) was found to be highest in the gills (~2.5 U/mg protein), followed by the digestive gland (~2.1 U/mg protein) and the mantle (~1.2 U/mg protein). A similar pattern was reported for crustacean gills with a character of CA similar to Na^+/K^+-ATPase (Henry and Cameron, 1982). Most marine gastropods have from 1 to 3 U/mg protein in the mantle and gills, slightly higher in oysters (Nielsen and Frieden, 1972).

In general, CA activities in the digestive gland and gills appear to be more sensitive to Cd-exposure than in the mantle (Fig. 1). In the digestive gland of exposed mussels, significantly lower activities from day 5 of exposure coincide with an increase of newly incorporated Cd (Ngo et al. 2008a, in press). Depression of CA activity upon exposure to water-borne Cd, although at much higher concentration than in this study (200 µg/L), was found in the mantle of *Mytilus galloprovincialis* (Lionetto et al., 2006) and in the gills of estuarine crabs (Vitale et al., 1999). The delayed effects observed here for the mantle and gills, and the recovery during the last two months of depuration indicate that these organs are less affected than the digestive gland. This shows that CA in different tissues have variable sensitivities to Cd, similar to estuarine crabs exposed to heavy metals (Skaggs and Henry, 2002) or eel exposed to 6 – 60 µM Cd *in vitro* (Lionetto et al., 1998).

Most of the CA activity is present in the cytoplasmic fraction, being about 60 % of the total enzyme activity (Fig. 2). This percentage is similar to that in the intestines of eels (about 61 %) (Maffia et al., 1996) but much higher than in estuarine crabs

(about 10 %) (Henry et al., 2003). The difference may be partially due to different centrifugation methods as in the present study the homogenates were centrifuged twice, so that most of the soluble cytoplasmic CA could be extracted. The high microsomal CA activity in the mantle in comparison to other organs may be due to its importance in the active transport of CO_2 into and out of the mantle cavity (Wilbur and Saleuddin, 1983) owing to its role in catalyzing the reversible hydration of CO_2. This may facilitate the dissolution of Ca stores in vesicular compartments in the outer mantle epithelial (OME) cells (Lopes-Lima et al., 2007) and favor the movement of Ca^{2+} and HCO_3^- towards the shell compartment (Moura et al., 2004). A higher proportion of this more stable membrane-bound CA may explain the lower Cd-effects on the mantle (Fig. 1).

Cytoplasmic CA activities show the highest sensitivity to Cd (Fig. 3A). Their depression from the end of exposure until the first two weeks (gills and mantle) or the first half of depuration (digestive gland) are concomitant with an increase in newly incorporated Cd (Ngo et al. 2008a, in press). Cytoplasmic CA in invertebrates is considered to be kinetically similar to mammalian Type II CA with high turnover and sensitivity to inhibitors (Henry, 1987). Inhibition of this isozyme might entail a build-up of CO_2 in the intracellular fluid resulting in respiratory acidosis which would disrupt normal physiological functions (Henry, 1996).

Nuclear CA has not yet been investigated in freshwater mussels. In this study it was strongly inhibited by Cd in all examined tissues (Fig. 3B). This isozyme was suggested to be involved in DNA transcription and in the maintenance of pH in the nucleus of interstitial testis cells (Karhumaa et al., 2000). Mitochondrial CA (Fig. 3C) has not been characterized in molluscs so far; in vertebrates it has a role in cell metabolism (Henry, 1996). It tends to be lowered during Cd exposure in the digestive gland and gills, but mostly non-significantly. Membrane-associated microsomal CA (Fig. 3D) is less sensitive to Cd exposure. This is compliant with the findings in intestines of eels exposed to Cd *in vitro* (Lionetto et al., 2000) or in the gills of euryhaline crabs exposed to heavy metals (Skaggs and Henry, 2002). Since it is tightly bound to the basolateral membrane, a lipid-rich environment, its active site may be inaccessible to Cd. Further, it has been reported to contain many disulfide bonds (Whitney and Briggle, 1982) which may be important for protection from Cd toxicity.

Both total and cytoplasmic CA activity in the digestive gland and gills are correlated to newly incorporated Cd level ($p < 0.05$) (Table 2). The reason for the weak correlation in the mantle may be due to the low extent of newly incorporated Cd (Ngo et al. 2008a, in press); in addition, in this organ the proportion of membrane-associated CA is relatively high. The digestive gland and gills are more active in taking up Cd (Ngo et al. 2008a, in press), which might exceed its rates of excretion and detoxification of metabolically available Cd, so toxicity may be unavoidable (Rainbow, 2002). It is also possible that Zn is displaced by Cd from the active site of the enzyme (Garmer and Krauss, 1992), or that the strong affinity of Zn to metallothionein (MT) induced by Cd (Vitale et al., 1999) tends to lower the availability of this essential metal. The strong correlations of CA activities in the digestive gland and gills to newly incorporated Cd, less to total Cd, indicates that the former is biologically active whereas most of the total Cd is inactive (Rainbow, 2002), perhaps immobilized in MT-complexes.

Shell growth involves the deposition of $CaCO_3$ which is driven by the mantle epithelium where Ca passes from the HML across the outer epithelial layer to the inner shell surface (Simkiss and Wilbur, 1989). In the mantle cavity, bicarbonate is needed to form $CaCO_3$, facilitated by CA. Correlation between its total activity and total Ca concentration found in the HML and mantle (data not shown) suggests that this enzyme is involved in both osmoregulation and Ca metabolism.

5. Conclusions

The study shows that Cd at a surprisingly low, environmentally relevant level has strong effects on CA, a family of enzymes centrally important in a variety of osmoregulatory, acid-base balance and metabolic processes by generating H^+ and HCO_3^- from CO_2 or from $CaCO_3$ depending on the pH balance; this influences transport and regulation of other ions (Henry and Saintsing, 1983). CA in the mantle is less sensitive to Cd than those in the digestive gland and gills. The time-dependent alteration of CA activity in the HML and EPF, and the inhibition of total, nuclear, and cytoplasmic CA activities in all tested organs indicate the complexity of physiological disturbances induced in Cd-exposed freshwater mussels.

Acknowledgements

Financial support by the Oberfranken-Foundation and the Regional Administration of Upper Franconia, Fisheries Department, is highly appreciated. The work is dedicated to Dr. Robert Klupp on the occasions of his 60th birthday.

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Appendix V:

Tables a.

Total Cd, newly incorporated Cd and background Cd concentrations relative to wet weight (µg/kg ww) and total Ca (g/kg ww) during the whole period of time (mean ± SD, n = 3); values are not corrected for weight loss of the soft body mass during the experiment

	Exposure			**Depuration**		
	0.2 d (5 h)	5 d	35 d	15 d	60 d	120 d
Digestive gland:						
- Total Cd	670 ± 96	690 ± 56	860 ± 76	1000 ± 166	1200 ± 62	1050 ± 241
- Newly accumulated Cd	7.1 ± 0.6	83 ± 12	258 ± 20	260 ± 36	280 ± 29	170 ± 20
- Background Cd	660 ± 95	610 ± 45	600 ± 55	730 ± 135	920 ± 45	880 ± 175
- Total Ca	3.6 ± 0.7	2.9 ± 0.3	3.8 ± 0.6	4.3 ± 0.9	4.4 ± 0.5	4.0 ± 0.5
Gills:						
- Total Cd	580 ± 85	710 ± 117	780 ± 123	630 ± 95	570 ± 80	570 ± 35
- Newly accumulated Cd	2.4 ± 0.2	34 ± 8	123 ± 8	104 ± 14	96 ± 20	95 ± 6
- Background Cd	575 ± 85	680 ± 109	660 ± 116	530 ± 81	470 ± 62	480 ± 35
- Total Ca	21 ± 5	21 ± 2	22 ± 2	17 ± 1	16 ± 2	18 ± 2
Mantle:						
- Total Cd	380 ± 76	490 ± 36	550 ± 86	600 ± 106	640 ± 52	650 ± 126
- Newly accumulated Cd	2.5 ± 0.6	18 ± 2	80 ± 10	107 ± 26	120 ± 19	94 ± 30
- Background Cd	370 ± 75	470 ± 15	470 ± 85	500 ± 105	520 ± 35	560 ± 115
- Total Ca	2.7 ± 0.2	2.0 ± 0.5	2.9 ± 0.4	3.0 ± 0.5	3.3 ± 0.5	3.3 ± 0.3

Tables b.

Total Cd, newly incorporated Cd and background Cd concentrations relative to protein (mg/kg protein) and total Ca (g/kg protein) during the whole period of time (mean ± SD, n = 3); values are not corrected for weight loss of the soft body mass during the experiment

	Exposure			**Depuration**		
	0.2 d (5 h)	5 d	35 d	15 d	60 d	120 d
Digestive gland:						
- Total Cd	14.1 ± 2.1	14.9 ± 1.8	17.2 ± 2.5	22.5 ± 1.7	29.7 ± 2.2	28.6 ± 5.2
- Newly accumulated Cd	0.1 ± 0.02	1.8 ± 0.2	5.0 ± 0.4	6.0 ± 0.6	6.8 ± 0.6	5.3 ± 0.4
- Background Cd	13.8 ± 2.1	13.2 ± 0.3	11.9 ± 0.8	16.5 ± 2.6	22.8 ± 0.9	23.5 ± 3.1
- Total Ca	80 ± 16	65 ± 15	75 ± 11	100 ± 26	110 ± 15	110 ± 11
Gills:						
- Total Cd	17.8 ± 1.6	22.4 ± 2.1	22.5 ± 4.5	18.5 ± 1.7	19.8 ± 3.2	22.2 ± 2.3
- Newly accumulated Cd	0.1 ± 0.01	1.1 ± 0.2	3.5 ± 0.4	3.0 ± 0.4	3.4 ± 0.8	3.7 ± 0.4
- Background Cd	17.7 ± 0.6	21.3 ± 1.9	19.0 ± 4.2	15.5 ± 1.6	16.5 ± 2.2	18.5 ± 2.1
- Total Ca	670 ± 174	660 ± 29	630 ± 88	540 ± 75	580 ± 68	680 ± 44
Mantle:						
- Total Cd	14.7 ± 2.6	15.1 ± 1.8	17.4 ± 1.6	18.1 ± 2.7	20.8 ± 2.8	24.5 ± 5.1
- Newly accumulated Cd	0.1 ± 0.02	0.5 ± 0.1	2.5 ± 0.2	3.2 ± 0.7	3.9 ± 0.8	3.5 ± 1.0
- Background Cd	14.6 ± 2.3	14.5 ± 1.6	14.9 ± 1.6	14.8 ± 2.7	16.9 ± 2.1	21.0 ± 5.1
- Total Ca	85 ± 10	60 ± 12	90 ± 10	95 ± 23	110 ± 19	125 ± 12

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 27 November 2008 _____

Ngo Thi Thuy Huong