University of Bayreuth, Germany
Chair of Environmental Chemistry and Ecotoxicology
&
University of Lorraine, France
Laboratory of Interactions Ecotoxicology Biodiversity Ecosystems

Thesis submitted in fulfillment of the degree of
Doctor in Sciences
Discipline: Ecotoxicology

Effects of copper on calcium metabolism and detoxification mechanisms in freshwater bivalve species of *Anodonta*

Olivier SANTINI

13 June 2012

Supervisors:
Professor Hartmut FRANK
Professor Paule VASSEUR

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List of publications or manuscripts and author's contribution

This Ph.D. dissertation is presented in cumulative form. It comprises four individual manuscripts, of which one has been published, the others are to be submitted. The author's contributions to each manuscript are given below.

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Own contribution: idea 80 %, method development and laboratory work 100 %, data analysis and calculations 100 %, writing 70 %.

List of abbreviations

a.a.m.:	anterior adductor muscle
ACN:	acetonitrile
ADEME-SOGREAH:	agence de l'environnement et de la maîtrise de l'énergie – société Grenobloise d'études et d'applications hydrauliques
ATP:	adenosine-5'-triphosphate
ATSDR:	agency for toxic substances and disease registry
CA:	carbonic anhydrase
Ca^{2+}-ATPase:	calcium dependent adenosine triphosphatase
CAT:	catalase
Cu/Zn SOD:	superoxide dismutase
Cys:	cysteine
DG:	digestive gland
DTPA:	diethylenetriamine-pentaacetic acid
DTT:	1,4-dithiothreitol
γ-ECS:	glutamate-cysteine-ligase
EDTA:	(ethylenedinitrilo)-tetraacetic acid
EGTA:	ethylene glycol-*bis*(β-aminoethylether)-N,N,N',N'-tetraacetic acid
E-PRTR:	the European pollutant release and transfer register
FOREGS:	forum of the European geological surveys
IC_{50}:	half maximal inhibitory concentration
IFEN:	institut Français de l'environnement
INERIS:	institut national de l'environnement industriel et des risques
G:	gills
GFAAS:	graphite furnace atomic absorption spectrometry
Glu:	glutamic acid
γ-GluCys:	γ-glutamylcysteine
Gly:	glycine
GPx:	glutathione peroxidase
GR:	glutathione reductase
GS:	glutathione synthase
GSH:	glutathione

GSSG:	glutathione disulfide
GST:	glutathione transferase
H^+-ATPase:	proton dependent adenosine triphosphatase
HEPES:	N-(2-hydroxyethyl) piperazine N'-(2-ethanesulfonic acid)
HEPPS:	4-(2-hydroxyethyl)-piperazine-1-propane sulfonic acid
HMWC:	high molecular weight complexes
$HO^{\bullet}$:	hydroxyl radicals
HPLC:	high performance liquid chromatography
ICPMS:	inductively coupled plasma mass spectrometry
IUCN:	international union for conservation of nature
K:	kidneys
LIEBE:	interactions ecotoxicology biodiversity ecosystems
LME:	London metal exchange
LOD:	limit of detection
LOQ:	limit of quantification
M:	mantle
mBBr:	monobromobimane
MCE:	mantle cavity epithelium
MDA:	malondialdehyde
Me_4B:	tetramethylbimane
MSA:	methanesulfonic acid
MT:	metallothionein(s)
NAC:	N-acetyl-cysteine
Na^+/K^+-ATPase:	sodium and potassium dependent adenosine triphosphatase
NEM:	N-ethylmaleimide
$NO^{\bullet}$:	nitric oxide radical
$O_2^{-\bullet}$:	superoxide anion radical
OECD:	organisation de coopération et de développement économiques
OME:	outer mantle epithelium
p.a.m.:	posterior adductor muscle
P-ATPase:	P-type adenosine triphosphatase
PC:	phytochelatin(s)
PCS:	phytochelatin synthase
P_i:	inorganic phosphate

PMCA:	plasma membrane Ca^{2+}-ATPases
PMSF:	phenylmethylsulfonic fluoric acid
$RO^{\bullet}$:	alkoxy radical
$ROO^{\bullet}$:	hydroperoxy radical
ROOH:	organic hydroperoxides
ROS:	reactive oxygen species
r^2:	Pearson correlation coefficient
S:	supernatant
SD:	standard error
SDS:	sodium dodecyl sulphate
SE:	standard error
SH:	thiol group
SODs:	superoxide dismutases
TCEP:	tris-(2-carboxyethyl)-phosphine hydrochloride
TFA:	trifluoroacetic acid
Tris:	tris(hydroxymethyl)-aminomethane
Tris-phosphate:	tris(hydroxymethyl)-aminomethane phosphate
w/w:	weight/weight
Xe:	xenobiotic

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Summary

Copper (Cu) is one of the metals contaminating European fresh water ecosystems. Filter feeding bivalves have high bioaccumulation potential for transition metals as Cu. While copper is an essential micronutrient for living organisms, it causes serious metabolic and physiological impairments when in excess.

The objectives of this thesis are to get knowledge on toxic effects and detoxification mechanisms of copper in *Anodonta cygnea* and *Anodonta anatina*, two mussel species widely distributed in continental waters. Because Ca plays a fundamental role in shell formation and in numerous biological processes, Cu^{2+} effects on cellular plasma membrane calcium transport were studied first. In the second step, the investigations focused on Cu^{2+} detoxification mechanism involving cysteine (Cys) rich compounds known to play a major role in homeostasis of essential trace metals and in cellular metal detoxification.

Under our experimental conditions, copper inhibition of Ca^{2+}-ATPase activity was observed in the gills and the kidneys, and inhibition of Na^{+}/K^{+}-ATPase in the gills and the digestive gland (DG) upon 4 d of exposure. At day 7 of exposure to environmental Cu^{2+} concentrations total recoveries was observed in the kidneys and the gills for Ca^{2+}-ATPase activity, and in the DG for Na^{+}/K^{+}-ATPase, but not at high doses. Ca and Na transport inhibition may entail disturbance of osmo-regulation and lead to continuous under-supply of Ca. Recoveries of Na^{+}/K^{+}-ATPase and Ca^{2+}-ATPase enzymes function suggest that metal-detoxification is induced.

Phytochelatins (PC) are Cys-rich oligopeptides synthesised by phytochelatin synthase from glutathione in plants and fungi. Phytochelatin synthase genes have recently been identified in invertebrates; this allows us to hypothesize a role of PC in metal detoxification in animals.

In the second part of this work, PC and their precursors as well as metallothionein were analyzed in the gills and in the DG of *Anodonta cygnea* exposed to Cu^{2+}. Our results showed for the first time the presence of PC_{2-4} in invertebrates. PC were detected in control mussels not exposed to metal, suggesting a role in essential metal homeostasis. Compared to control, PC_2 induction was observed during the first 12 h of Cu^{2+} exposure. Those results confirm the role of PC as a first line detoxification mechanism in *A. cygnea*.

Key words: calcium homeostasis, copper, *Anodonta* freshwater bivalve, phytochelatins

Zusammenfassung

Kupfer ist eines der Übergangs-Metalle, die in Süsswasser-Ökosystemen Europas am weitesten verbreitet sind und in Konzentrationen auftreten, die ökotoxikologische Bedeutung haben können. Die filtrierenden, zweischaligen Muscheln haben die Fähigkeit, solche Übergangs- und Spuren-Metalle wie Kupfer durch Bioakkumulation anzureichern, da Kupfer auch ein wesentliches essentielles Metall für alle lebenden Organismen ist; bei übermässiger Belastung mit diesem Spuren-Metall werden jedoch schwere metabolische und physiologische Störungen hervorgerufen.

Diese Doktorarbeit zielt darauf ab, die Kenntnisse über toxische Wirkungen sowie Entgiftungsmechanismen von Kupfer in *Anodonta cygnea* und *Anodonta anatina* zu erweitern, zwei Arten von zweischaligen Süsswassermuscheln, die in Gewässern Europas weit verbreitet sind. Da Calcium eine fundamentale Rolle für die Zusammensetzung der Muschel-Schalen sowie bei zahlreichen biologischen Prozessen spielt, wurden zuerst die Wirkungen von Cu^{2+} auf den zellulären Transport von Calcium auf der Ebene der Plasmamembran studiert. Dann wurde der Schwerpunkt der Studie auf die Entgiftungsmechanismen des Kupfers gelegt, wobei cysteinreiche Peptide und Proteine im Vordergrund stehen. Cystein ist bekannt für seine Rolle als funktionelles Element für die Homöostase der essenziellen Spurenmetalle sowie für die zelluläre Metallentgiftung.

Unter den gewählten experimentellen Bedingungen wurde nach vier Tagen Cu^{2+}-Exposition bei 0,35 µmol L^{-1} die Hemmung der Ca^{2+}-ATPase in den Kiemen und Nieren und die Hemmung der Na^{+}/K^{+}-ATPase in den Kiemen und der Mitteldarmdrüse beobachtet. Nach sieben Tagen Exposition wurde die Erholung der Enzymtätigkeit in den Nieren und in den Kiemen für die Ca^{2+}-ATPase beobachtet, in der Mitteldarmdrüse für die Na^{+}/K^{+}-ATPase. Die Erholung der Enzymtätigkeiten weist auf die Induktion der Metallentgiftungs-Kapazität hin. Bei doppelt so hoher Cu-Konzentration dauerte die Hemmung aller genannten Enzyme über den gesamten Expositionszeitraum von 15 Tagen an. Die Hemmung des Transports von Calcium und Natrium kann Störungen in der Osmoregulation verursachen und zu einem Defizit an Calcium führen.

Phytochelatine (PC) sind cysteinreiche Polypeptide, die in Pflanzen und Hefen durch PC Synthase aus Glutathion synthetisiert werden. Gene, die zu funktionellen PC Synthasen führen können, wurden in Wirbellosen identifiziert, was uns annehmen ließ, dass PC auch bei Tieren eine Rolle bei der Metallentgiftung spielen könnte. Daher wurden im zweiten Teil der Arbeit PC und deren Vorläufer sowie die Metallothioneine in den Kiemen und in der Mitteldarmdrüse von kupferexponierten *Anodonta cygnea* analysiert. Die Ergebnisse zeigen zum ersten Mal die Präsenz von PC_{2-4} in Wirbellosen. Die PC wurden auch in Kontrollmuscheln gefunden, was auf ihre Rolle bei der Homöostase essenzieller Metalle hinweist. Eine Induktion von PC_2 wurde in den ersten 12 Stunden der Cu^{2+}-Exposition beobachtet, eine Bestätigung seiner Rolle als erster Metallentgiftungsmechanismus in *A. cygnea*.

Schlüsselwörter: Calcium Homöostase, ionisches Kupfer, Zweischalige Süsswasser-Muscheln *Anodonta*, Phytochelatine

Résumé

Le cuivre est l'un des métaux contaminants des écosystèmes d'eau douce le plus représenté en Europe. Les bivalves filtreurs ont une grande capacité de bioaccumulation des métaux de transitions tel que le cuivre. Le cuivre est un oligo-élément essentiel pour les organismes vivants, mais en excès il provoque de graves perturbations métaboliques et physiologiques.

L'objectif de cette thèse est d'acquérir des connaissances sur les effets toxiques et les mécanismes de détoxification du cuivre chez *Anodonta cygnea* et *Anodonta anatina*, deux espèces de bivalves dulcicoles largement distribuées dans les eaux continentales. Parce que le calcium joue un rôle fondamental dans la composition de la coquille et pour de nombreux processus biologiques, les effets du Cu^{2+} ont été étudiés d'abord sur le transport cellulaire du calcium au niveau de la membrane plasmique. Dans un deuxième temps, l'étude a été axée sur les mécanismes de détoxification du Cu^{2+} impliquant des composés riches en cystéine (Cys), connus pour jouer un rôle majeur dans l'homéostasie des métaux traces essentiels et dans la détoxification des métaux dans les cellules.

Dans nos conditions expérimentales (0,35 et 0,64 µmol L^{-1}), l'inhibition de la Ca^{2+}-ATPase par le Cu^{2+} a été observée dans les branchies et les reins, et l'inhibition de la Na^{+}/K^{+}-ATPase dans les branchies et la glande digestive, après 4 jours d'exposition. Au delà de 7 jours d'exposition à la concentration de 0,35 µmol L^{-1} Cu^{2+}, une récupération totale de l'activité enzymatique a été observée dans les reins et les branchies pour Ca^{2+}-ATPase, et dans la glande digestive pour la Na^{+}/K^{+}-ATPase. A dose élevée (0,64 µmol L^{-1}), l'inhibition persiste. L'inhibition du transport du calcium et du sodium peut entraîner des perturbations de l'osmorégulation et conduire à des carences en calcium. La récupération de l'activité enzymatique de la Ca^{2+}-ATPase et de la Na^{+}/K^{+}-ATPase suggère une induction de fonctions de détoxification des métaux.

Les phytochélatines (PC) sont des oligopeptides riches en Cys synthétisés par la phytochélatine synthase à partir du glutathion, chez les plantes et les champignons. Des gènes codant pour des phytochélatine synthases fonctionnelles ont étés identifiés chez des invertébrés tel que le ver de fumier. Ceci nous a incités à rechercher la présence de PC dans les bivalves. Nos résultats ont montré pour la première fois, la présence de PC_{2-4} chez les invertébrés. Les PC ont été détectés dans des moules témoins non exposées aux métaux, ceci suggère une fonction dans l'homéostasie des métaux essentiels. Nous avons donc étudiés leur rôle éventuel dans la détoxification des métaux chez ces organismes et les animaux en général. Jusqu'ici les phytochélatines étaient considérées jouer un rôle uniquement chez les végétaux.

Dans la seconde partie de ce travail, les PC et leurs précurseurs, ont été recherchés dans les branchies et la glande digestive d'*Anodonta cygnea* exposé au Cu^{2+}. Une induction de PC_2 a été observée dés les 12 premières heures d'exposition au Cu^{2+}, comparé aux bivalves témoins. Ces résultats confirment le rôle du PC en tant que mécanisme de première ligne de détoxification des métaux chez *A. cygnea*. Les métallothioneines ont été analysées en parallèle, mais aucune induction n'a été prouvée en présence de cuivre.

Mots clés: homéostasie du calcium, cuivre, bivalves dulcicoles *Anodonta*, phytochélatines

Liste des abréviations

AC :	anhydrase carbonique
ATP :	adenosine-5'-triphosphate
B :	branchies
C :	culot
Ca^{2+}-ATPase :	adénosine triphosphatase calcium dépendante
Cys :	L-cystéine
DTT :	4-dithiothreitol
ERO :	espèces réactives de l'oxygène
GD :	glande digestive
γ-GluCys :	γ -glutamylcystéine
GSH :	glutathion
GSSG :	glutathion disulfide
H^+-ATPase :	adénosine triphosphatase proton dépendante
HPLC :	chromatographie liquide haute performance
INERIS :	institut national de l'environnement industriel et des risques
M :	manteau
mBBr :	monobromobimane
MT :	métallothionéine(s)
Na^+/K^+-ATPase :	adénosine triphosphatase sodium / potassium dépendante
PC :	phytochélatine(s)
PCS :	phytochélatine synthase
P_i :	phosphate inorganique
PMCA :	Ca^{2+}-ATPase de la membrane plasmique
R :	reins
S :	surnageant
SH :	groupement thiol
TCEP :	tris-(2-carboxyethyl)-phosphine hydrochloride

Introduction

L'activité humaine est associée au développement de l'industrie et l'agriculture, qui sont devenus indispensables. Ces secteurs sont responsables de la production et de la diffusion de nombreux polluants. Les propriétés chimiques et physiques et les différents types de transport déterminent la diffusion des polluants dans tous les compartiments des écosystèmes. Le milieu aquatique est le réservoir final pour la plupart des polluants, dont les métaux. Le cuivre appartient aux métaux les plus couramment utilisés du fait de ses propriétés physiques et chimiques (particulièrement pour ses qualités de conductivité électrique et thermique). Comme métal pur, en alliage, ou à l'état ionique, il est utilisé dans un grand nombre de secteurs industriels et agricoles. De ce fait, le cuivre est un métal fréquemment détecté dans les milieux aquatiques continentaux où il est présent dans la colonne d'eau et s'accumule dans les sédiments (INERIS, 2010). Les propriétés chimiques du cuivre en font un élément surtout utilisé en tant que catalyseur biologique des réactions enzymatiques, et un élément essentiel à de nombreux processus biologiques impliquant des fonctions vitales comme la respiration ou la photosynthèse (Tapiero *et al.*, 2003). Les propriétés qui rendent ce métal essentiel (respiration avec le cytochrome C oxydase, formation des tissus connectifs avec la lysyl oxydase) sont aussi à l'origine de sa toxicité lorsqu'il est en excès. Le cuivre est bioaccumulable et peut devenir une menace pour la biocénose. Des mécanismes de régulation de la concentration et de la détoxication du cuivre sont essentiels pour les organismes vivants.

Les mollusques représentent une forte proportion de macroinvertébrés dans les écosystèmes aquatiques. Parmi ce groupe, les bivalves sont particulièrement intéressants. Du fait de leur importante activité de filtration nécéssaire pour satisfaire leur respiration et leur nutrition, les bivalves ont la capacité d'accumuler de nombreux contaminants. Dans l'écosystème, ils jouent un rôle important dans le transfert de matière de la colonne d'eau vers les sédiments. Les excréments et pseudofèces de bivalves rendent le phytoplancton disponible aux détritivores, et peuvent modifier la qualité des sédiments par concentration des polluants. Les bivalves sont en contact étroit avec leur environnement, ils sont largement utilisés pour la surveillance de la pollution dans les écosystèmes aquatiques. Parmi ces animaux, les bivalves Unionidae sont largement utilisés comme organismes indicateurs de la bioaccumulation et des effets toxiques des polluants métalliques et organiques (Winter, 1996; Falfushynska *et al.*, 2009). Pour ces raisons, nous avons choisi comme modèles biologiques pour l'étudier de la toxicité du cuivre, *Anodonta cygnea* et *Anodonta anatina* qui appartiennent aux Unionidae.

Ces deux invertébrés sont autochtones des systèmes hydrologiques européens. Au cours des dernières décennies, une régression des populations d'Unionidae a été observée en Europe.

L'objectif de cette thèse a été d'acquérir des connaissances sur les mécanismes de perturbation par le cuivre du métabolisme du calcium chez *Anodonta anatina*. Le calcium est un élément essentiel dans le fonctionnement des organismes eucaryotes. Il contrôle plusieurs processus vitaux (Ermak et Davies, 2001). L'absorption, le maintien de la concentration intracellulaire de calcium dans l'organisme, et les processus de biominéralisation sont rendus possible par le contrôle de son passage à travers les membranes cellulaires. Ce passage se fait par simple diffusion, mais aussi par des protéines de transport. Le phénomène de biominéralisation nécessite en plus du calcium, des ions carbonate produits en partie par l'anhydrase carbonique (AC). Nous avons étudié les effets du Cu^{2+} sur le transport du calcium chez *Anodonta anatina* par l'évaluation des activités enzymatiques de Ca^{2+}-ATPase, Na^{+}/K^{+}-ATPase et H^{+}-ATPase de la membrane plasmique et de l'activité enzymatique cytosolique de AC, enzymes impliquées dans l'absorption du calcium et dans les processus de biominéralisation. Les organes étudiés ont été les branchies, la glande digestive, le rein, et le manteau, qui jouent un rôle important dans l'absorption du calcium et la synthèse de la coquille (Coimbra *et al.*, 1993). Dans notre étude des inhibitions de l'activité des Ca^{2+}-ATPase et Na^{+}/K^{+}-ATPase ont été observées chez *A. anatina* exposés au Cu^{2+}. Une inhibition suivie d'une reprise totale de l'activité enzymatique, observée à la concentration d'exposition de 0,35 µmol L^{-1} de Cu^{2+} mais non à forte concentration (0.64 µmol L^{-1}) indique un mécanisme de détoxication.

Ces résultats suggérent l'induction de mécanismes de détoxication métallique. La deuxième étape de cette étude a porté spécifiquement sur les composés riches en Cys chélateurs de métaux. Nous avons posé l'hypothèse que des phytochélatines pouvaient être présentes chez les bivalves et jouer un rôle dans la détoxification du cuivre. Les composés riches en Cys sont des polypeptides ou des protéines telles que les phytochélatines ou les métallothionéines, avec une teneur en Cys élevée. Ils jouent un rôle majeur comme chélateur de métaux, dans l'homéostasie des métaux essentiels et pour la détoxication des métaux non-essentiels. Les phytochélatines (PC) sont des polypeptides riches en thiol de formule générale : $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, synthétisés par la phytochélatine synthase (PCS) à partir de glutathion. Les PC complexent les ions métalliques réduisant la concentration intracellulaire en ions métalliques libres chez les plantes, les champignons et les microalgues. L'identification de gènes en mesure de donner des PCS fonctionnelles chez les invertébrés (Clemens *et al.*, 2001; Vatamanuik *et al.*, 2001; Brulle *et al.*, 2008), nous avons fait des

recherches de PC chez les organismes animaux. Des gènes homologues de phytochélatine synthase ont été trouvés répandus chez les invertébrés, (Clemens et Peršoh, 2009). Les invertébrés appartenant aux Unionidae, connus pour leur facilité à bioaccumuler les métaux, pourraient être susceptibles de synthétiser des PC, c'est l'hypothèse que nous émettons dans la deuxiéme partie de la thèse. Leur présence est connue chez les végétaux, non chez les organismes animaux. L'induction de PC, de leurs précurseurs ont été évalués dans les branchies et la glande digestive chez *Anodonta cygnea* exposée au cuivre.

Matériel et méthodes

Acclimatation des bivalves

L'entretien de moules a été décrit en détail dans notre premier article précédent (Santini *et al.*, 2011a). Brièvement *A. cygnea* et *A. anatina* adultes (7,5 ± 0,5 cm de long) ont été placées en aquariums à 20 ± 0,5 ° C. L'eau d'étang artificiel 1,5 L / moule, pH 7,25 ± 0,10 (en mmol L^{-1}: 0.40 Ca^{2+}, 0.20 Mg^{2+}, 0.70 Na^{+}, 0.05 K^{+}, 1.35 Cl^{-}, 0.20 SO_4^{2-}, 0.20 HCO_3^{-}) était renouvelée chaque jour. Les bivalves ont été nourris quotidiennement avec une culture de *Chlorella kessleri* en phase exponentielle de croissance, ajoutée à une densité finale d'algues de 2×10^5 cellules / ml. Les animaux ont été acclimatés à ces conditions pendant deux semaines avant toute expérimentation.

Exposition au cuivre

Les effets du cuivre ont étés évalués chez *A. anatina* sur les activités enzymatiques de Ca^{2+}-ATPase, Na^{+}/K^{+}-ATPase, H^{+}-ATPase de la membrane plasmique et AC cytosolique. Des expériences préliminaires ont été effectuées avec trois bivalves exposés à chacune des concentrations de Cu^{2+} : 0,26, 0,54 et 1,15 µmol L^{-1} pendant quatre jours, pour trouver les concentrations les plus pertinentes. Pour l'étude des effets du cuivre sur ces activités enzymatiques l'exposition a été effectuée à 0,35 et 0,64 µmol L^{-1} de Cu^{2+} sur une durée de 15 jours. Trois moules ont été échantillonnées et disséquées pour chaque traitement : jour 0 (témoin), 4 j, 7 j et 15 j. Les organes de chaque individu ont été préparés et étudiés séparément, ce qui donne 3 réplicats par traitement.

Les effets du cuivre sur les mécanismes de détoxification des métaux ont étés évalués chez *A. cygnea* sur les paramétres suivants : PC et leurs précurseurs, et MT. Les bivalves ont été exposés à 0,35 µmol L^{-1} de Cu^{2+} durant 0 h, 12 h, 48 h, 4 j, 7 j et 21 j, les témoins ont étés maintenus en parallèle, sur la même durée, dans l'eau artificielle. Les milieux tests ont été renouvelés chaque jour, et les bivalves ont été nourris quotidiennement, comme décrit précédemment. Douze moules ont étés échantillonnées par traitement avant d'être disséquées. Les branchies et la glande digestive de deux moules ont été regroupées, donnant 6 réplicats par traitement pour l'analyse.

Analyses statistiques

Comme l'homogénéité des variances et la normalité des données ne se sont pas révélées vérifiées (tests de Bartlett et de Shapiro-Wilk), l'analyse statistique a été réalisée par tests non paramétriques de Kruskal-Wallis et de Mann-Whitney. Les différences ont été considérées comme significatives lorsque $p < 0,05$.

Analyses enzymatiques

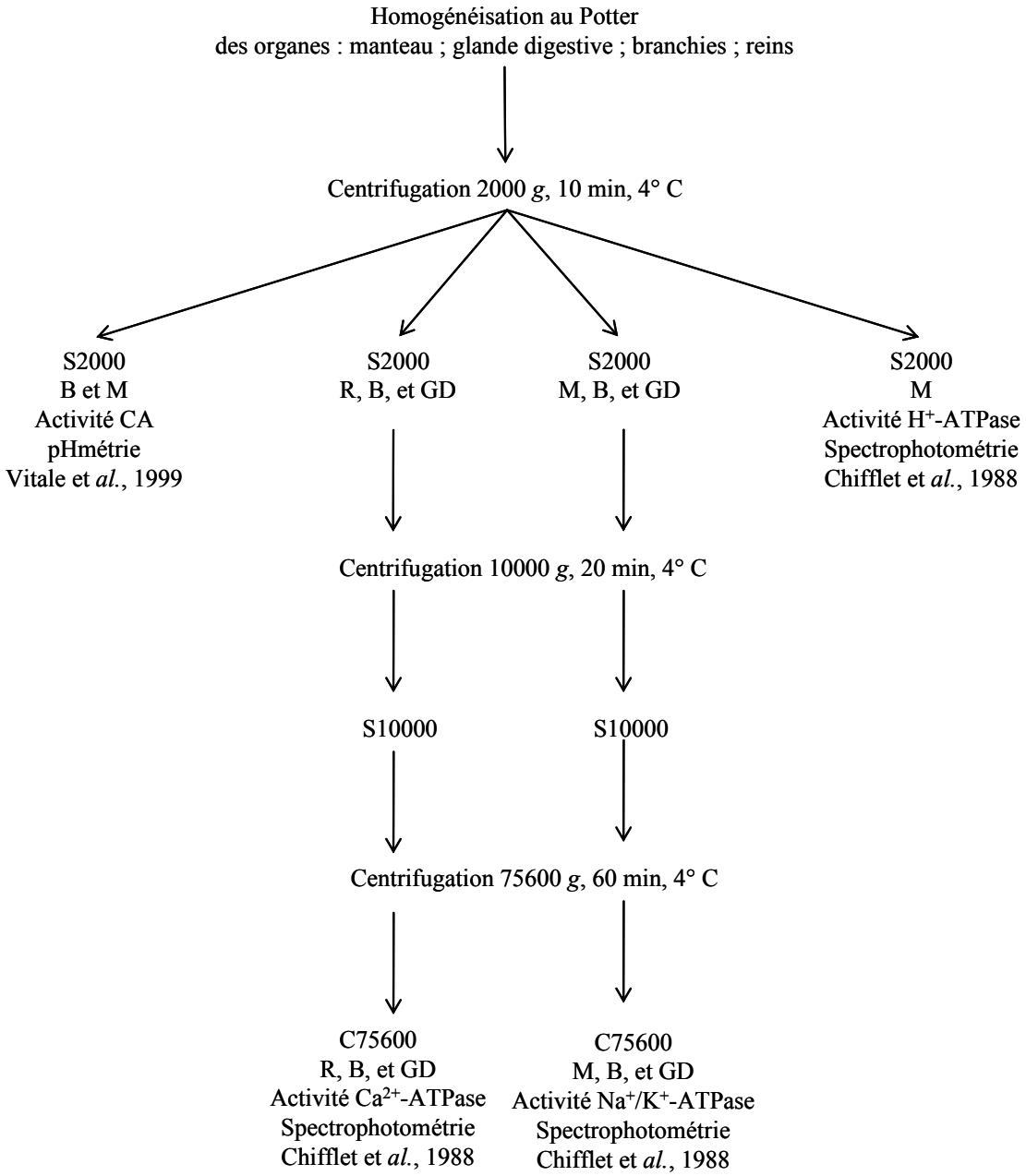

Fig. 1 : Protocole d'analyse enzymatique M : manteau, GD : glande digestive, B : branchies, R : reins, S : surnageant, C : culot.

Les activités des Ca^{2+}-ATPase, Na^{+}/K^{+}-ATPase, et H^{+}-ATPase ont été déterminées dans chacun des organes à partir du culot C75600 remis en suspension (fig. 1). La quantification du phosphate inorganique libéré lors de la dégradation de l'ATP a été réalisée avec la méthode de Chifflet *et al.* (1988) par dosage spectrophotométrique du complexe molybdate d'ammonium à 850 nm. L'activité de CA a été déterminée avec la méthode de Vitale *et al.* (1999) par la mesure de la chute de pH en présence des extraits de tissus et de CO_2.

Analyse des composés riches en thiol

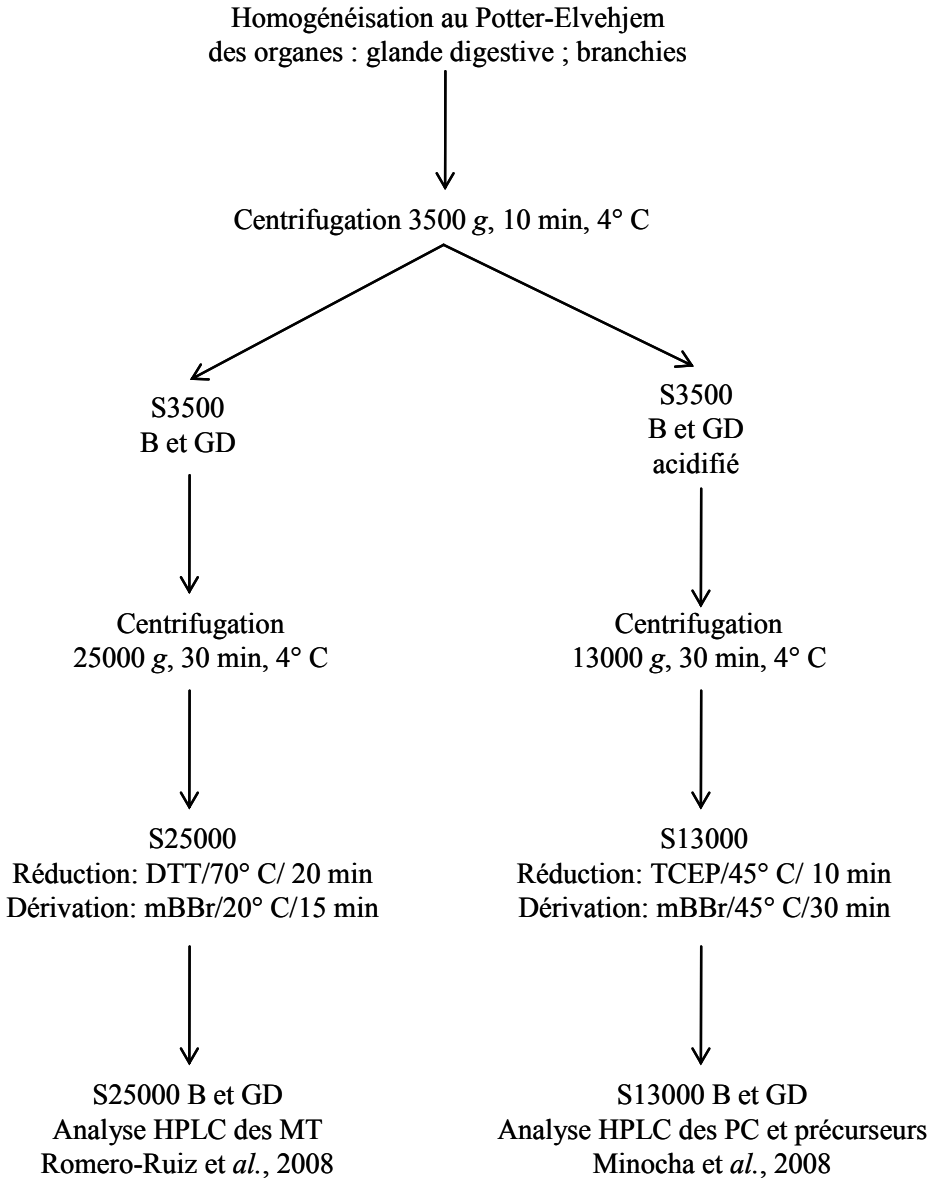

Fig. 2 : Protocole d'analyse des composés riches en thiols GD : glande digestive, B : branchies.

Les composés riches en Cys ont été réduits avec du 4-dithiothréitol (DTT) pour les MT et du tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP) pour les PC et ses précurseurs (fig. 2). Les groupements thiols réduits des MT, PC et précurseurs ont alors étés marqués au monobromobimane (mBBr). L'analyse des composés marqués a été réalisée par HPLC reliée à un détecteur fluorimétrique.

Résultats

Ca^{2+}-ATPase de la membrane plasmique et anhydrase carbonique

L'activité moyenne des Ca^{2+}-ATPase (± écart type) chez les animaux témoins était de 0,087 ± 0,023 µmol P_i/mg de protéine/min dans les reins, 0,45 ± 0,13 dans les branchies, et 0,095 ± 0,03 dans la glande digestive.

Le cuivre a inhibé les activités PMCA dans le rein de façon significative après 4 jours d'exposition à toutes les concentrations testées, soit de 0,26 à 1,15 µmol L^{-1} Cu^{2+}. A 0,35 µmol L^{-1} Cu^{2+}, une récupération de l'activité PMCA a été observée au delà de 7 jours d'exposition. A forte concentration (0,64 µmol L^{-1}), une inhibition de 20 % de l'activité PMCA a été observée sur les 15 jours d'exposition sans aucune récupération. Dans les branchies un profil similaire à celui des reins, mais avec des variations non significatives est observé.

Aucun effet significatif de l'éxposition à 0,35 µmol L^{-1} de Cu^{2+} a été noté sur l'anhydrase carbonique, à l'exception d'une légère inhibition, mais non significative, après 15 jours d'exposition.

Na^+/K^+-ATPase et H^+-ATPase

La moyenne Na^+/K^+-ATPase (± écart type) chez les animaux témoins en septembre était de 0,098 ± 0,006 µmol P_i/mg de protéine/min dans les branchies, 0,045 ± 0,007 dans la glande digestive, et 0,042 ± 0,009 dans le manteau. L'activité de la H^+-ATPase était de 0,002 ± 0,0012 dans le manteau.

Une inhibition significative de l'activité de la Na^+/K^+-ATPase par rapport aux témoins a été observée à 4 jours d'exposition à 0,35 µmol L^{-1} de Cu^{2+} dans les branchies (72 % d'inhibition) et la glande digestive (80 % d'inhibition). Une reprise de l'activité est observée

entre le quatrième et quinzième jour d'exposition à 0,35 µmol L^{-1} de Cu^{2+}, en fin de test une inhibition (54 %) persiste.

Dans le manteau, l'activité de la Na^+/K^+-ATPase baisse de la même façon (26 % d'inhibition à 4 jours) mais la différence n'est pas significative par rapport aux témoins.

Aucun effet significatif du cuivre sur la H^+-ATPase n'a été noté dans le manteau de moules après 15 jours d'exposition.

D'une façon générale et dans tous les tissus, les activités des deux ATPases ont été plus élevées en juillet et septembre par rapport aux valeurs mesurées en janvier, mars et avril.

Métallothionéine, phytochélatines et ses précurseurs

Le temps d'élution moyenne des standards de phytochélatines ont été: PC_2 à 13,09 min, PC_3 à 16,62 min, PC_4 à 18,59 min, et PC_5 à 19,75 min. Des composés marqués au mBBr avec des temps de rétention correspondant aux standards de PC_2, PC_3, PC_4 ont été détectés dans les extraits de glande digestive et de branchies de moules témoins. La PC_5 était au dessous de la limite de détection. La PC_2 présentait la concentration la plus forte avec 2,17 ± 0,59 et 0,88 ± 0,15 mg PC_2 / g de poids frais dans les tissus de la glande digestive et des branchies, respectivement. Dans ces deux organes, l'ordre de grandeur des concentrations en PC_{2-4} était classé de la façon suivante : $PC_2 > PC_3 > PC_4$. Les proportions de PC_2 et de PC_3 sont deux ou trois fois plus élevées dans la glande digestive que dans les branchies, tandis que le niveau de PC_4 est à peu près équivalent dans ces tissus.

Le niveau de PC_2 a nettement et significativement augmenté dans les branchies des moules exposées à 0,35 µmol L^{-1} de Cu^{2+}. Comparé aux bivalves témoins respectifs, une induction de 50 % de PC_2 a été observée dès les 12 premières heures jusqu'à 4 jours d'exposition au cuivre et 30 % d'augmentation à 7 jours d'exposition. Au delà de 7 jours, la PC_2 revient au même niveau que celui mesuré dans les témoins. Dans la glande digestive, aucune variation significative de la PC_2 n'a été mise en évidence, excepté à 12 h d'exposition au Cu^{2+}.

Les concentrations de γ-GluCys sont significativement supérieures dans les branchies des moules exposées au cuivre durant 48 heures et 7 jours. La concentration de γ-GluCys augmente également dans la glande digestive des bivalves à 48 h et 4 j d'exposition au cuivre.

Aucune variation significative du niveau de MT n'a été observée dans les branchies et la glande digestive des moules sur les 21 jours d'exposition au cuivre.

Discussion

Nous avons étudié les effets du cuivre sur les enzymes impliquées dans le transport du calcium et dans les processus de ionorégulation, et les mécanismes de détoxification par phytochélatines et métallothionéines chez les bivalves *Anodonta*.

Le calcium joue un rôle fondamental dans de nombreux processus biologiques (production d'énergie, métabolisme cellulaire, contraction musculaire, reproduction) et a d'importantes fonctions mécaniques (coquille, squelette) chez les organismes vivants (Mooren et Kinne, 1998). Contrairement aux mollusques des écosystèmes marins qui sont généralement hypoosmotiques et pour lesquels l'absorption du calcium est facilitée par de plus fortes concentrations ambiantes de calcium, les bivalves d'eau douce sont hyperosmotiques et nécessitent une régulation stricte de leur métabolisme calcique.

La présente étude nous a permis de déterminer des niveaux de base des activités enzymatiques de Ca^{2+}-ATPase, Na^{+}/K^{+}-ATPase et H^{+}-ATPase de la membrane plasmique et de l'anhydrase carbonique cytosolique dans les organes impliqués dans l'homéostasie du calcium. Il y a un manque de données physiologiques chez les bivalves d'eau douce par rapport aux modèles marins tels que *Mytilus edulis* ou *Mytilus galloprovincialis*. Ces données sont nécessaires à la compréhension des mécanismes de transport du calcium puisque les bivalves d'eau douce sont soumis à une osmo-iono-régulation différente des bivalves marins.

Dans notre étude, l'activité de la Ca^{2+}-ATPase de la membrane plasmique (PMCA) des branchies d'*A. anatina* était quatre fois supérieure à celle trouvée chez *Mytilus edulis* (Burlando *et al.*, 2004). Ceci reflète l'importance du transport actif du calcium chez *Anodonta anatina*. Les concentrations de calcium en eau douce sont inférieures à celles trouvées dans l'eau de mer, où l'absorption du calcium est plus facile pour *Mytilus edulis*.

L'activité de la PMCA est également élevée dans les reins et la glande digestive, favorisant l'absorption du calcium provenant des aliments et la réabsorption du calcium dans l'ultrafiltrat rénal. Ces données physiologiques nous ont permis de comprendre dans quelle mesure la PMCA est importante pour l'homéostasie du calcium chez les bivalves d'eau douce par rapport aux organismes marins. En outre, chez les unionidés, le calcium joue un rôle direct dans la reproduction, puisque les glochidies sont incubées dans le marsupium (branchies), il est donc déterminant pour la croissance des populations de moules.

La Na^{+}/K^{+}-ATPase maintient le gradient de sodium cellulaire transmembranaire nécessaire pour la diffusion facilitée du calcium par le Na^{+}/Ca^{2+} antiporteur. L'augmentation

significative de l'activité de la Na^+/K^+-ATPase a été mesurée dans les branchies et la glande digestive en juillet et septembre par rapport au reste de l'année. Ces deux mois correspondent à la période de biominéralisation chez *Anodonta* sp. (Moura *et al.*, 2000).

Les reins ont un rôle essentiel dans la filtration et la réabsorption des ions, de l'eau et des molécules organiques de l'ultrafiltrat. Comme les bivalves d'eau douce sont hyperosmotiques, la pression osmotique résultant du gradient de concentration entre les compartiments internes et l'environnement conduit à l'absorption d'eau par osmose et à une perte par diffusion ionique. L'absorption de l'eau est compensée par la production d'urine et la perte ionique est limitée par la réabsorption ionique. Chez les bivalves d'eau douce, la production journalière d'urine est élevée. Les reins jouent un rôle essentiel dans l'homéostasie du Ca en limitant les pertes ioniques dans l'urine, par réabsorption active des ions Ca^{2+} dans le filtrat (Turquier, 1994). Une inhibition de la réabsorption de calcium peut donc considérablement perturber l'homéostasie calcique.

Notre étude a montré une inhibition de l'activité enzymatique de la PMCA dans les branchies et les reins chez les moules exposées à 0,35 µmol L^{-1} de Cu^{2+}. Une reprise totale de l'activité de la PMCA a été observée à partir de 7 j d'exposition à 0,35 µmol L^{-1} de Cu^{2+}, mais l'inhibition a persisté à concentration plus élevée (0,64 µmol L^{-1}). En raison de problèmes analytiques inhérents à la faible masse des reins, ce tissu a été peu étudié chez les bivalves d'eau douce jusqu'alors. Cet organe joue pourtant un rôle important dans les processus de détoxification (Viarengo et Nott, 1993) ; de plus le rein est essentiel pour la iono-régulation. Nos résultats ont montré la grande sensibilité de cet organe au Cu^{2+} à des concentrations réalistes au plan environnemental (0,35 µmol L^{-1} = 22,3 µg L^{-1}).

Dans le présent travail, une inhibition de la Na^+/K^+-ATPase a été observée dans les branchies et dans la glande digestive. Une telle perturbation de la iono-régulation pourrait conduire à une carence en calcium ; elle peut également affecter les voies de signalisation cellulaires calciques, en plus de perturber la biominéralisation et la formation de la coquille des moules et des glochidies. Une reprise des fonctions enzymatiques (Ca^{2+}-ATPase et Na^+/K^+-ATPase) suggère qu'un mécanisme de détoxification des métaux est induit. Par conséquent, dans la deuxième partie de cette étude, nous nous sommes concentrés sur les mécanismes de détoxification par les composés riches en Cys, chélateurs de métaux par leur groupement thiol.

Les bivalves dulcicoles unionidés sont largement reconnus pour leur capacité à accumuler dans leurs tissus, une grande variété de contaminants de l'environnement, y compris les métaux (Bonneris *et al.*, 2005). Cette tolérance aux métaux est permise par des

stratégies biochimiques impliquant leur séquestration. La séquestration intracellulaire des métaux se fait selon une séquence d'événements en cascade impliquant différents ligands avec une force croissante de liaison vis-à-vis des éléments métalliques. A concentration élevée, les métaux peuvent inhiber ces mécanismes de détoxication. Dans nos résultats, le retour au niveau basal des Ca^{2+}-ATPase et Na^{+}/K^{+}-ATPase observé au-delà de 7 jours d'exposition à 0,35 µmol L^{-1} de Cu^{2+} indique des capacités d'adaptation des bivalves, via des systèmes de détoxification efficace à faible concentration de cuivre. A une concentration plus élevée de Cu^{2+} (0,64 µmol L^{-1}), aucune récupération de l'activité enzymatique n'a été notée. Il est intéressant de remarquer que la récupération a été observée uniquement à faible concentration (0,35 µmol L^{-1}) environnementalement pertinente. Ceci souligne l'importance d'utiliser des concentrations environnementales dans la recherche écotoxicologique; l'extrapolation de résultats observés à des doses élevées pour des situations environnementales peut être critiquée en raison des différents mécanismes de toxicité développés à faibles et fortes doses.

L'étude de phytochélatines chez *Anodonta cygnea*, nous a amené à développer et optimiser un protocole analytique par HPLC pour la quantification des PC dans les tissus animaux, basée sur la méthode de Minocha *et al.* (2008). Le présent travail a confirmé la présence de phytochélatines chez les invertébrés. A notre connaissance, c'est la première fois que les PC ont été trouvés chez les animaux et chez les invertébrés.

Chez les végétaux, les PC sont rapidement induites dans les cellules et les tissus exposés aux métaux de transition. Les PC jouent un rôle important dans la détoxification des métaux. Les PC pourraient également être impliquées dans l'homéostasie des métaux essentiels (Hirata *et al.*, 2005). Dans notre étude, la PC_2, PC_3 et PC_4, ont été détectés dans les branchies et la glande digestive d'*A. cygnea* chez les témoins non exposés. Dans les deux organes, la PC_2 a été trouvée en concentration la plus forte suivie de la PC_3 elle-même en concentration supérieure à celle de la PC_4. Les concentrations de PC_2 et PC_3 étaient environ deux à trois fois plus élevée dans la glande digestive que dans les branchies. Le niveau de base des PC_{2-4} en l'absence de cuivre et d'autres métaux d'exposition suggère leur rôle dans l'homéostasie des métaux essentiels.

A 12 h d'exposition au cuivre, une induction significative de PC_2 a été observée par rapport aux témoins correspondants dans les branchies et à un moindre niveau dans la glande digestive. Ces résultats confirment le rôle des PC comme chélateur de métaux impliqués en première ligne dans les mécanismes de détoxification chez *A.cygnea*. Au delà de 7 jours et jusqu'à 21 j, dans les branchies des moules exposées au cuivre, la PC_2 diminue pour revenir

au niveau basal, identique à celui des témoins. Cette diminution suggère que la détoxification du cuivre a été prise en charge par d'autres mécanismes, sur le long terme. Chez les Unionidae, les métallothionéines et les granules (concretions intracellulaire insolubles le plus souvent minérale, mais également organiques) sont connues pour jouer ce rôle. Bonneris *et al.* (2005) ont montré que les concentrations de cadmium, de zinc et de cuivre dans la fraction de granules des branchies étaient significativement corrélées avec les concentrations de ces métaux sur l'environnement. Les granules sont connues pour être des sites privilégiés pour le stockage de cuivre dans les branchies des unionidés. Environ 65 % du cuivre total dans les branchies a été trouvé séquestré dans les granules chez *Anodonta grandis grandis*, où les concrétions de calcium représentent 51 % du poids sec des branchies. Des valeurs similaires ont été trouvées chez *Anodonta cygnea* (Bonneris *et al.*, 2005).

Aucune variation significative du niveau de MT n'à été observée lors de l'exposition des bivalves au cuivre durant les 21 jours de la présente étude. L'isoforme de MT (<10 kDa) dans l'extrait de moules élué, après séparation dans nos conditions HPLC, n'a pas été induit par le cuivre. Une détoxification par d'autres isoformes de MT non détectées par notre méthode HPLC chez *A. cygnea*, ne peut être exclue. En effet un polymorphisme important des MT est connu chez les invertébrés (Amiard *et al.*, 2006).

Les métallothionéines, les granules et les systèmes antioxydants ont été décrits comme étant impliqués dans les mécanismes de détoxication des bivalves d'eau douce. Cossu-Leguille *et al.* (1997) ont montré le rôle majeur joué chez les Unionidae par les antioxydants et en particulier par le glutathion réduit (GSH) pour la détoxification des métaux. Dans les branchies de *Unio tumidus* une diminution de 45 % du GSH a été observée chez les moules exposées en site contaminés par des métaux, par rapport aux témoins. Cette diminution du niveau de GSH chez les Unionidae exposés au cuivre a été confirmée par Doyotte *et al.* (1997) dans des conditions contrôlées de laboratoire, indiquant la séquestration des métaux directement par le groupe SH du GSH ou de son utilisation comme substrat par les enzymes antioxydantes. En effet, une implication en parallèle des enzymes antioxydantes a été décrite. L'activité de ces enzymes augmente lors d'exposition à faibles concentrations en métaux (Vasseur et Leguille, 2004). Le GSH joue également un rôle dans la synthèse des PC. Ce double rôle dans la détoxification directe des métaux, et comme précurseur de synthèse des PC pourrait expliquer cette diminution.

Les populations d'Unionidae représentent la plus grande partie de la biomasse totale dans de nombreux systèmes aquatiques. Ils prennent une part active dans la purification de l'eau, dans la sédimentation, la modification des communautés de phytoplancton et des

invertébrés détritivores (Aldridge, 2000). Par conséquent la disparition des Unionidae pourrait produire des perturbations structurelles et fonctionnelles dans les écosystèmes aquatiques. Les effets potentiellement négatifs de la compétition entre bivalves, autochtones et invasifs est une question qui fait débat. Les bivalves d'eau douce invasifs *Corbicula fluminea*, *Dreissena polymorpha* n'appartenant pas aux Unionidae colonisent les hydrosystèmes dulcicoles avec des effets préjudiciables pour les autres invertébrés. Ces espèces invasives ne jouent pas le même rôle fonctionnel dans les écosystèmes que les unionidés. Leur présence est l'une des hypothèses expliquant la baisse des populations d'unionidés.

La combinaison de différents facteurs explique la disparition progressive des Unionoidea au profit des espèces invasives. L'appauvrissement en salmonidés qui sont de potentiels poissons hôtes pour les larves d'unionidés est présenté comme une autre hypothèse possible à celle d'une compétition par des éspeces invasives. Les populations de salmonidés sont touchées par la pollution de l'eau, pouvant indirectement renforcer les maladies naturelles tels que le parasitisme connu pour son impact négatif sur les populations de salmonidés (Voutilainen *et al.*, 2009). Les autres principales raisons de ce déclin sont la dégradation physique des ruisseaux par la modification des lits de rivières et canaux, ainsi que la dégradation de la qualité de l'eau. En effet les interactions entre polluants, sont une cause de perturbation même à faible concentration (Vighi *et al.*, 2003). Afin de protéger les populations autochtones d'Unionidae, il est important de déterminer comment et dans quelle mesure ces facteurs sont impliqués dans leur disparition. Les résultats acquis au cours de ce travail de thèse contribuent à la compréhension des effets de la pollution par les métaux sur l'homéostasie du calcium chez les bivalves d'eau douce. La pollution aquatique par les métaux est susceptible d'être l'une des raisons du déclin généralisé des populations d'Unionoidea dans les rivières européennes (Frank et Gerstmann, 2007). Nos résultats sur les effets du cuivre sur *A. anatina* ont confirmé cette hypothèse.

Conclusion

Les communautés du macrobentos sont d'excellents indicateurs de qualité de l'eau (Ippolito *et al.*, 2010). Les objectifs du notre travail ont été l'étude des effets toxiques du cuivre et des mécanismes de détoxification chez les espèces de bivalves dulsicoles du genre *Anodonta* appartenant aux Unionidae. Tout d'abord, l'étude du Cu^{2+} comme un inhibiteur potentiel des protéines enzymatiques jouant un rôle dans le transport du calcium et les processus biominéralisation a été réalisée chez *Anodonta anatina*. Deuxièmement, l'étude s'est orientée sur les mécanismes de détoxification du Cu^{2+} par les composés riches en Cys chélateurs de métaux chez *Anodonta cygnea*.

Dans une première partie de la présente étude, les effets de l'exposition au Cu^{2+} sur les activités enzymatiques de la Ca^{2+}-ATPase, la Na^{+}/K^{+}-ATPase, et la H^{+}-ATPase de la membrane plasmique, et de l'AC cytosolique ont été évalués chez le bivalve dulcicole *Anodonta anatina*.

Dès 4 jours, une inhibition de l'activité enzymatique de la Ca^{2+}-ATPase des branchies et des reins, et de la Na^{+}/K^{+}-ATPase des branchies et la glande digestive, a été observée chez *Anodonta anatina* exposée à 0,35 µmol L^{-1} de Cu^{2+}. Une récupération totale de l'activité de la Ca^{2+}-ATPase a été observée à 7 jours d'exposition à 0,35 µmol L^{-1} de Cu^{2+}, indiquant l'induction de mécanismes de détoxication des métaux. Chez les bivalves exposés à 0,64 µmol L^{-1} de cuivre aucune récupération de l'activité enzymatique n'a été observée sur 15 jours.

Il serait intéressant d'étudier les conséquences à long terme de la perturbation des fonctions osmorégulatrices des reins chez *A. anatina* exposés à 0,64 µmol L^{-1} de Cu^{2+}.

Dans un deuxième temps nous avons recherché la présence de phytochélatines chez les bivalves. Au cours de notre recherche nous avons identifié les phytochélatines PC_2, PC_3 et PC_4. C'est la première fois que la présence de PC est mise en évidence chez des organismes animaux et chez *A. cygnea* en particulier. Chez *Anodonta cygnea* exposés à 0,35 µmol L^{-1} de Cu^{2+}, la PC_2 a été induite par rapport aux témoins à 12 h dans la glande digestive et les branchies, l'induction a persisté après 7 jours dans les branchies. Ces résultats suggèrent un rôle des PC dans l'homéostasie des métaux essentiels, et confirme le rôle des PC en tant que mécanisme de première ligne pour la détoxification des métaux chez *A.cygnea*.

Le cuivre a été signalé comme un faible inducteur des polypeptides riches en thiols chélateur de métaux (Zenk, 1996). L'exposition à un métal non essentiel et puissant inducteur

de PC comme le cadmium pourrait être intéressante pour l'étude de l'induction des PC_{3-4} chez *A. cygnea*. Par ailleurs la comparaison des effets de l'exposition à un métal essentiel comme le cuivre et un métal non essentiel tel que le cadmium pourrait nous permettre de déterminer le rôle joué par les PC dans l'homéostasie des métaux essentiels et les mécanismes de détoxification.

Nos résultats d'analyses de métallothionéine (MT) par HPLC n'ont montré aucune variation de son niveau d'expression après exposition au cuivre. Ceci indique que l'isoforme MT quantifié avec notre méthode n'est pas induit par ce métal. Des analyses complémentaires des MT chez *A. cygnea* exposés au cuivre dans les mêmes conditions, avec une méthode de spectrométrie qui permet la quantification de la totalité des isoformes de MT seraient intéressantes afin de déterminer si d'autres isoformes de MT sont induites chez cette espèce. De plus en parallèle, il serait nécessaire d'optimiser notre protocole d'analyse HPLC de manière à séparer et mesurer les isoformes de MT induites par le cuivre chez *A. cygnea*.

Le cuivre est un inducteur d'espèces réactives de l'oxygène (ERO) à travers la réaction de Fenton. Le rôle antioxydant joué par les PC (Hirata *et al.*, 2005) serait intéressant à étudier pour une meilleure compréhension du mécanisme de détoxification du cuivre par les PC chez *A. cygnea*.

La pollution chimique est l'une cause de dégradation de l'environnement aquatique responsable du déclin des populations de bivalves dulcicoles. La toxicité dépend non seulement de la biodisponibilité des polluants et de leur toxicité intrinsèque, mais également de l'efficacité des systèmes de détoxification dans l'élimination des espèces chimiques réactives.

Cette thèse est une contribution significative à l'étude des systèmes de détoxification des métaux chez les bivalves dulsicoles *A. cygnea* et *A. anatina*.

L'originalité de notre travail est la mise en évidence, pour la première fois, de phytochélatines chez des organismes animaux. Nous avons démontré leur rôle dans la protection vis-à-vis de la toxicité du Cu^{2+}, à des concentrations réalistes et pertinentes au plan environmental.

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Introduction

1 General introduction

Human activity is associated with the development of industry and agriculture which have become indispensable. These sectors are responsible for the production and release of many pollutants. The chemical and physical properties and the different types of transport determine pollutant diffusion in all compartments of ecosystems. The aquatic environment is a sink for most many pollutants including metals. Copper belongs to the metals most commonly used because of its physical (particularly its electrical and thermal conductivity) and chemical properties. As pure metal, as alloy, or in the ionic state it is employed in many industrial and agricultural sectors. Therefore, copper is a metal frequently detected in the aquatic continental environment where it is present in the water column and accumulates in sediments (INERIS, 2010). The chemical properties of copper (including as catalyst) make it essential to many biological processes involving such vital functions as respiration or photosynthesis (Tapiero *et al.*, 2003). The properties that make this metal an essential element are at the same time the reasons for its toxicity when in excess. Copper is bioaccumulative and can become a threat to biocenoses. Mechanisms regulating the copper concentration and detoxification are essential to all living organisms.

Molluscs represent an important group of macroinvertebrates in aquatic ecosystems. Among this group, bivalves are particularly interesting. Through their important filtering activity to satisfy respiratory and nutrition, bivalves have the capacity to accumulate a variety of environmental contaminants. In freshwater ecosystems they play an important role in matter transfer from the water column to the sediment. Faeces and pseudofaeces of bivalves make the plankton fraction available to detrivores and can change the sediment quality by pollutant sedimentation and concentration. In close contact with their environment, they are widely used for pollution monitoring in aquatic ecosystems. Among these animals, freshwater mussels of the Unionidae family are employed for controlling the bioaccumulation and toxic effects of metallic and organic pollutants (Winter, 1996; Falfushynska *et al.*, 2009). For these reasons we chose *Anodonta cygnea* and *Anodonta anatina* which belongs to the Unionidae as biological models to study copper toxicity. These invertebrates are autochtonous species in European hydrosystems, but regression of Unionidae populations, like many other freshwater bivalves, has been observed in the last decades, the main reason for such ecotoxicological studies as this one.

The objective of this thesis was to gain knowledge on the mechanisms of disturbance of calcium metabolism in *Anodonta anatina* by copper. Calcium is a critical element in the functioning of eukaryotic organisms. It controls multiple processes of reproduction, life, and death (Ermak and Davies, 2001). Absorption, biomineralization, and maintenance of intracellular calcium concentrations are effected and controlled by its passage through cell membranes. This takes place by simple diffusion and also by means of transport proteins. In bivalves, calcium is also very important for exo-skeleton synthesis by biomineralization. In addition to calcium, for biomineralization carbonate ions are required, produced by carbonic anhydrase (CA) catalysis. The effects of Cu^{2+} on calcium transport have been tested in *Anodonta anatina* by assessment of the enzymatic activities of Ca^{2+}-ATPase, Na^{+}/K^{+}-ATPase, H^{+}-ATPase of the plasma membrane, and of the cytosolic CA. These enzymes are involved in calcium absorption and in the biomineralization processes. Organs studied were the gills, the digestive gland, the kidney, and the mantle which play an important role in the absorption of calcium and the synthesis of the shell (Coimbra *et al.*, 1993). Enzymatic inhibition in *A. anatina* exposed to Cu^{2+} at concentrations ranging from 0.26 to 1.15 µmol L^{-1} was observed. At low concentration of 0.35 µmol L^{-1}, a total recovery following the inhibition indicated the induction of detoxication mechanisms.

The second part of this study focused specifically on metal binding Cys-rich compounds. These compounds are oligopeptides with high Cys content such as phytochelatins, or proteins such as metallothioneins. They play a major role in animals and plants as metal chelator, in essential metal homeostasis, and in non-essential metal detoxication. Phytochelatins (PC) are thiol rich oligopeptides with $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ as general structure synthesised by phytochelatin synthase from glutathione. PC binds metal ions and forms metal complexes that reduce the intracellular free metal ion concentration in cells of plants, fungi, and microalgae. Since the identification of homologous genes able to give a functional phytochelatin synthase in invertebrates (Clemens *et al.*, 2001; Vatamaniuk *et al.*, 2001; Brulle *et al.*, 2008), PC are strongly suspected to play a wider role in trace metal detoxification in animals. In invertebrates including bivalves, PC synthase genes were found to be widespread (Clemens and Peršoh, 2009). Therefore invertebrates such as Unionidae, known to bioaccumulate metals, are likely to express PC. Induction of PC and their precursors and of MT were assessed in the gills and the digestive gland of *Anodonta cygnea* under Cu^{2+} exposure.

This thesis manuscript is divided into eight chapters. After this general introduction, the second chapter contains a literature review on freshwater bivalves and data on copper, on

its chemical and biological properties, its distribution in aquatic ecosystems, bioaccumulation, toxic effects, and detoxification mechanisms. An extended summary is presented in the third chapter followed by a conclusion (chapter 4). A published article on the inhibition of Ca^{2+}-ATPase and CA activities by Cu^{2+} is presented in the fifth chapter, and the sixth chapter is on the inhibition of Na^{+}/K^{+}-ATPase and the H^{+}-ATPase activities; both dealing with the disruption of calcium transport and biomineralization mechanisms by copper. Detoxification mechanisms by metal binding thiol compounds are presented in the seventh and eighth chapter.

Background

2 Background

2.1 Copper

2.1.1 Origin and use

Copper (symbol Cu, atomic number 29, atomic mass 63.546, two stable isotopes) is a transition metal belonging to the group 11 IB (silver, gold) of the periodic table of elements. Copper is a ductile metal with good electrical and thermal conductivities. It occurs naturally in oceans, lakes, rivers, and soils. The average natural levels of copper in the Earth's crust vary from 40 to 70 mg kg^{-1}. Values in ores range from 80 to 150 mg kg^{-1} (IFEN, 2011). Copper is found mainly as the sulfides CuS and Cu_2S in tetrahedrite ($Cu_{12}Sb_4S_{13}$) and enargite (Cu_3AsS_4) and as the oxide Cu_2O (cuprite). The most important ore is chalcopyrite (Cu_2S, Fe_2S_3). It is also found in malachite ($CuCO_3(OH)_2$), azurite $Cu_3(CO_3)_2(OH)_2$, chalcocite (Cu_2S), and bornite (Cu_5FeS_4). Since 1980, an increase in global mining production has been observed due to the growing demand for copper in the fields of renewable energy, construction, and transport (LME, 2011). Primary production of copper increased from 9.6 million in 1980 to 16.9 million in 2006 (INERIS, 2010). In 2007, according to INERIS (2010), 35 % of the world copper consumption came from recycled copper. In Europe, in 2007 the recycling rate of copper was 41 %, and in 2006 copper consumption was about 4.7 million tons of copper (21 % of global demand). The demand for copper in Europe was estimated in 2007 at 3.85 million tons (European Copper Institute, 2009).

Copper is commonly used because of its physical properties, particularly its electrical and thermal conductivity and its resistance to corrosion. It is mainly used as metal in electronics (42 %), construction (28 %), and in vehicles (12 %). Seventy percent of pure copper are used for electrical wire laminates, and pipes. In alloys, the main families are the brasses (copper-zinc), the bronzes (copper-tin), copper-aluminium, and copper-nickel blends. Many other alloys are also in use for special purposes, such as copper-nickel-zinc, copper-silicon, copper-lead, copper-silver, copper-gold, copper-zinc-aluminium-magnesium, as well as copper alloyed with cadmium, tellurium, chromium, or beryllium (Blazy and Jdid, 2002). Salts of copper at different levels of oxidation are also used for their chemical and biochemical properties.

Copper is encountered in many fields of technical application:

- Electricity, used pure or alloyed in wires of electrical equipment (coils, generators, transformers, connectors) (Blazy and Jdid, 2002).
- Electronics and communication: pure or alloyed it plays an important role in communications technology, computers, and mobile phones (Blazy and Jdid, 2002), in printed circuit boards, in solar cells of solar panels, and in the semiconductor industry.
- Building: copper and brass are used for pipes and plumbing fixtures, in heating and guttering, as copper sheets for cover.
- Transportation: alloys of copper and nickel are used in boat hulls and in anti-fooling paint to reduce hull fouling by algae. It is also in motors, radiators, connectors, and brakes; an automobile contains about 22.5 kilograms of copper. Trains can contain between 1 and 4 tons (INERIS 2010).
- In various industrial equipments, copper and copper alloys are used in the manufacture of turbine blades, gears and bearings, heat exchangers, tanks and pressure equipment, marine equipment, pressed steel, and smelters.

Some commercial preparations are particularly polluting for the aquatic compartment since they contain copper in ionic form directly soluble in water. Salts of copper are used in many preparations and in industrial quantities:
- As, copper acetate, cupric chloride, and copper sulphate for colouring of textiles, glass, ceramics, paints, and varnishes, and for tanning (OECD, 1995). Copper acetate, cuprous and cupric oxide, cuprous and cupric chloride are used as catalysts in petrochemical organic synthesis and for rubber production. They are also used in solder pastes, in electroplating, as polishing agent for optical glass, and for refining of copper, silver, and gold.
- Pyrotechnics, oxides and cupric copper to colour firework, brass in ammunition.
- General consumer products, pool algicides, decorative objects made in copper and its alloys (buttons, zippers, jewelry), coins.
- In the agriculture, the farming of cattle, pigs, and poultry is a large sector, using copper as a dietary supplement (INERIS, 2010). Copper acetate, copper sulphate, copper hydroxide, cuprous oxide, and tetracupric oxychloride are used in fungicides, herbicides, insecticides, molluscicides, and as antiseptics. Copper treatment is practiced in vinyards and on fruit trees with average doses from 1000 to 2500 g Cu / ha / year and from 3750 to 5000 g Cu / ha / year respectively (ADEME-SOGREAH, 2007).
- In wood preservation, copper sulphate, cupric oxide, and cupric chloride are used (INERIS, 2005). Copper is also used as a substitute for other substances such as lead, arsenic, and tributyltin. This enumeration illustrates the widespread use of copper.

2.1.2 Sources of emissions and environmental levels

Natural inputs in the order of decreasing importance are volcanic eruptions, plant decomposition, forest fires, and marine aerosols (INERIS, 2010). Water and soil surrounding sites of agricultural and industrial activities are most heavily exposed to copper and, to a lesser extent, the air contaminated by road and rail traffic. In the European Union, some countries represent a large part of copper emission such as France with 43% in water, 15% in soil, and 13% in air. According to IFEN (2011), anthropogenic inputs of copper originating from industrial activities are mostly into waters and soils, while urban and agricultural activities as well as road traffic emit mostly into the air. In 2007, the emissions of copper in-to the environment in Europe were estimated as 371,000 kg $year^{-1}$ to water, 146,000 kg $year^{-1}$ to air, and 139,000 kg $year^{-1}$ to soil (INERIS, 2010).

Air emissions are mainly non-industrial. The road transport sector is a major issue representing 51 % of emissions in France in 2004. These are mainly caused by wear of brake pads containing copper. The railway transport sector represents 32 % of total air emissions of copper which is caused by wear of overhead lines (ADEME-SOGREAH, 2007). Emissions of copper-related road transport are increasing with the development of this sector. Industrial discharges of copper are mainly from the synthesis of organic chemicals, in the production of nonferrous metals from ore, ferrous metal smelting, and the production of iron and steel. In the atmosphere, copper metal oxidizes slowly to Cu_2O which covers the metal with a protective layer against corrosion. Copper is released into the atmosphere as particulate oxide, sulphate, or carbonate as particulate matter.

Releases to the aquatic environment are mainly due to corrosion of equipment made from copper or brass, and urban waste is another important source of release. One of the most significant non-industrial discharges of copper is the treatment of urban wastewater. This sector represents 28 % (15617 kg $year^{-1}$) of the total French emissions of copper and its derivatives to the aquatic environment (INERIS, 2010). Copper is one of the compounds always detected in the input and output of secondary sewage treatment plants (but not always in sewage treatment plants with tertiary treatment). Copper is one of the highest concentrated compounds in sewage treatment plant inflow with concentrations generally greater than 10 µg L^{-1}. At the outlet it is usually found at concentrations between 1 and 10 µg L^{-1}. In Europe in 2007, the main emitters of industrial origin are the United Kingdom, France, Germany, and Romania. They represent respectively 35 % (129000 kg), 15 % (55700 kg), 11 % (39800 kg) and 7 % (24500 kg) of the European industrial emissions to water (INERIS, 2010). In the

European Union, the most significant sectors are thermal power plants and other combustion plants. In France, another important sector is the production of nonferrous metals from ore from concentrates and from secondary materials.

Speciation and behaviour of copper in the environment will directly influence its bioavailability. In aquatic environments, the fate of copper is influenced by many processes and factors such as chelation by organic ligands (especially on NH_2 and SH groups, to a lesser extent on the OH group). Adsorption phenomena can also occur on metal oxides, clays, or particulate organic matter, bioaccumulation, presence of competing cations (Ca^{2+}, Fe^{2+}, Mg^{2+}) or of anions (OH, S^{2-}, PO_4^{3-}, CO_3^{2-}) plays also a role in copper behaviour (INERIS, 2010). The great portion of copper released into the water is in the particulate form and tends to settle, to precipitate, or adsorb to organic matter, hydrous iron, manganese oxides, or clay particles (ATSDR, 1990; INERIS, 2005). In hard water (carbonate concentration up to 1 mg L^{-1}), the largest fraction of copper is precipitated as insoluble compounds. Cuprous oxide Cu_2O is insoluble in water. Except in the presence of a stabilizing ligand such as sulfides, cyanide, or fluoride the oxidation state Cu(I) is easily oxidized to Cu(II) as $CuSO_4$, $Cu(OH)_2$ and $CuCl_2$ more soluble in water. The Cu^{2+} ion forms many stable complexes with inorganic ligands such as chloride or ammonium, or organic ligands. When copper enters the aquatic environment, the chemical equilibrium of oxidation states and of soluble and insoluble species is usually reached within 24 hours (INERIS, 2005). In aquatic environments, copper is mainly absorbed on particles, and suspended solids are often heavily loaded. In Europe, according to the forum of the European geological surveys (FOREGS, 2010), copper concentrations up to 3 µg L^{-1} are found in continental water. In regions with intensive human activities (agricultural, urban), copper concentrations of 40 µg L^{-1} and even 100 µg L^{-1} can be found seasonally (Neal and Robson, 2000; Falfushynska *et al.*, 2009). Trace metals as copper are mainly transported to the marine environment by rivers through estuaries. The magnitude of metal input to the marine environment depends on the levels in the river waters and on the physico-chemical processes that take place in the estuaries (Waeles *et al.*, 2004). In sea water, copper is found in concentrations ranging from 0.1 to 4 µg L^{-1} (Waeles *et al.*, 2005; Levet *et al.*, 2009).

Depositions of copper into soils are from different sources. In respect to industrial activities in Europe, in 2007 the major industrial emitters to soil are the United Kingdom, France, and Germany with respectively 74 tons, 60 tons, and 5 tons. The most significant activities are landfilling or recycling of non-hazardous waste (45 %), abattoirs (21 %) and sewage sludge from urban waste water treatment plants (12 %) (E -PRTR, 2010). The main

agricultural sources identified (ADEME-SOGREAH, 2007) for the release of copper are animal wastes (faeces and manure), sewage sludge from water treatment plants, compost, mineral fertilizers, lime and magnesium soil amendment. Animal waste represents an important input into soils (53 %). This reflects the fact that the feed of cattle, pig and poultry production is supplemented with copper for promotion of growth and prevention of diseases. As copper is poorly absorbed, it is added in great quantities to the feed (sometimes added at levels 30 times in excess of the needs of the animal), and the surplus is then found in the droppings. The spreading of animal manure is important for agricultural soils (Jondreville *et al.*, 2002). Thirty four % of the copper amount found in agricultural soils comes from phytosanitary treatment (ADEME-SOGREAH, 2007). Furthermore, mineral fertilizers and lime and magnesium soil amendments contribute to copper inputs to agricultural soils. The burden of copper to soil by urban activities is mainly through spreading and composting of sludge from wastewater treatment plants. For example, the average concentration of copper in sewage sludge of the Rhone Mediterranean basin was about 350 mg kg^{-1} in 2004 (Agence de l'eau Rhône Méditerranée Corse, 2004). In soils, copper is in the oxidation states I or II in the form of sulfides, sulphates, carbonates, and oxides. The behavior of copper in soils depends on many factors such as soil pH, redox potential, cation exchange capacity, type and distribution of organic matter, presence of oxides, rate of decomposition of the organic matter, proportions of clay, silt, and sand, climate, and type of vegetation (INERIS, 2010). Acidification for instance causes a decrease of the metal bound to solids and thus release from solid matter (Hlavackova, 2005). Copper binds preferentially to organic matter representing between 25 to 50 % of copper, to iron oxides, manganese oxides, carbonates, and to clays. This characteristic makes the majority of copper strongly adsorbed in the upper few centimeters of soil, especially on organic matter. Copper does not migrate deeply, except under special circumstances of drainage or in highly acidic environment (INERIS, 2010). In the European Union, in 2010 the most polluted solid matters are the sediments of alluvial plains with 25 mg kg^{-1}, the sediments of rivers with 22 mg kg^{-1}, soil with 17 mg kg^{-1}. The maximum concentration determined in a river sediment was found to be 877 mg kg^{-1}, more than 40 times the average (FOREGS, 2010).

In France, concentrations of copper in the surface layers of soil can reach 46 mg kg^{-1}. The levels of copper in surface and deep layers of the most polluted soils are found mainly in the South of France and the West of Bretagne.

2.1.3 Copper metabolism and its physiological role in animals (generalities)

Copper is an essential trace element in microorganisms, plants, and animals. It plays a basic role being in the active centre of enzymes involved in connective tissue formation with lysyl oxidase, in respiration with cytochrome C oxidase (López de Romana, 2011), in photosynthesis with plastocyanin (Grotz and Guerinot, 2006), and in controlling the level of oxygen radicals with Cu / Zn-superoxide dismutase (Table 1). It allows also the transport of oxygen in the haemolymph of many invertebrates. Copper is used in several cell compartments, and the intracellular distribution of copper is regulated in response to metabolic demands and changes in the cell environment (Tapiero *et al.*, 2003). The same properties that make transition metal ions indispensable for life at low exposure level are also the ones that are responsible for toxicity when present in excess. Copper metabolism must be tightly regulated, ensuring a sufficient supply without toxic accumulation. Copper homeostasis involves a balance between absorption, distribution, use, storage, and detoxification.

Table 1: Functions of the main cuproproteins (Tapiero *et al.*, 2003)

Metalloenzymes	**Functions**
Superoxide dismutase (Cu/Zn SOD)	Dismutation of superoxide anions
Cytochrome C oxidase	Energy production
Histaminase	Histamine degradation
Lysyl oxidase	Binding between collagen fibrillar and elastane
Dopamine β-hydroxylase	Catecholamines production
Tyrosinase	Melanin production
Amine oxidase	Oxidation of primary amines
Peptidylglycine monooxygenase	Peptidic hormones bioactivation
Ceruloplasmin	Ferroxidase and copper transport
Angiogenin	Angiogenesis
Peptidylglycine amidating monoxygenase	Synthesis of neuroendocrine hormones
Plastocyanin	Photosynthesis

In the mammalian intestine where low pH and the presence of ligands promote its solubility, copper passes by the enterocytes. Copper is absorbed by saturable active transport through membrane transporters. In the cytosol of enterocytes, copper binds to metallothioneins which play a role in copper sequestration and transport. Once absorbed, copper is transported to the liver, predominantly by albumin, but also by transcuprein and free amino acids including histidine. In the liver, depending on the status of the animal, copper is bound to metallothioneins to be stored, incorporated into ceruloplasmin (Cousins, 1985), and then transported to other organs, or secreted into the intestine via the bile. In monogastric animals, copper homeostasis is largely maintained by increased excretion avoiding excessive accumulation in the liver (fig. 1). Biliary excretion is the major route, losses via urine, skin, or via cellular desquamation in the gut are minor (Arredondo and Núñez, 2005).

In aquatic organisms, especially bivalves, copper is ingested and internalized after solubilization in the gastrointestinal tract and absorbed by the intestinal tract (associated with the particulate phase or in solution). Copper in the dissolved phase can also be directly absorbed by the tissues (gills, mantle, etc.) in contact with the surrounding water (Viarengo and Nott, 1993).

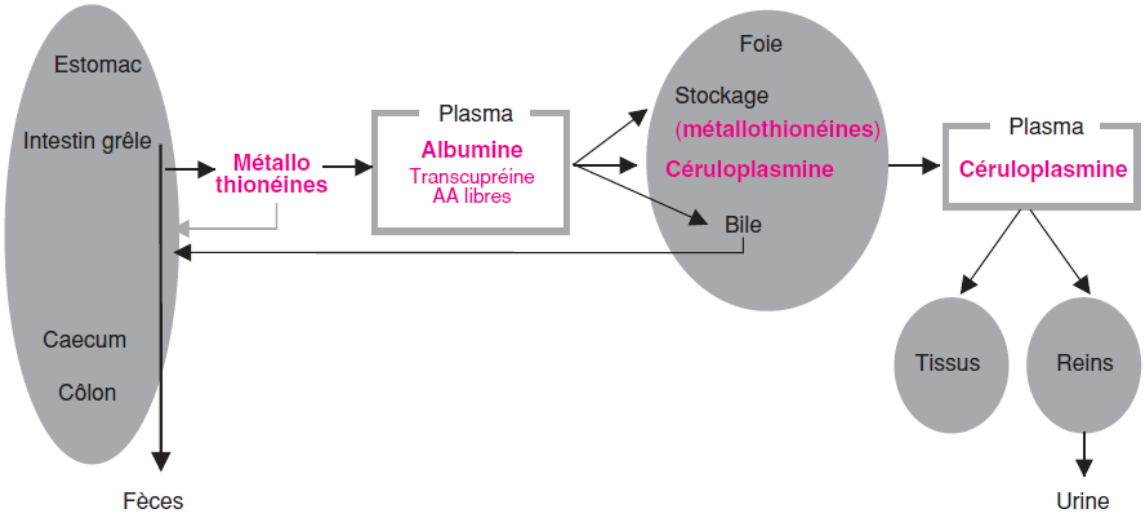


Fig. 1: Copper metabolism in mammals (Jondreville *et al.*, 2002).

The entry of copper into the cell occurs mainly by mechanisms that depend on copper transporting membrane channels. In this process, Cu^{2+} ion is first reduced to Cu^{+} by reductases associated with the membrane to facilitate its entry. Once inside the cytoplasm, it is likely that reduced glutathione (GSH) and metallothioneins (MT) bind the copper, serving as intracellular stores. Copper appears to be interchanged between MT forming a stable

complex and its bound state with GSH. As copper bound to GSH turns over more rapidly than when bound to MT, copper becomes available for other uses and for its transport. The delivery of copper ions to their specific pathways into the cell is mediated by metallochaperones that protect the metal from intracellular scavengers and delivers it directly to the respective target proteins and cellular compartments (Kozlowski *et al.*, 2009). The copper chaperone for Cu/Zn superoxide dismutase guides copper to superoxide dismutase (Cu/Zn SOD) which participates in the defence against oxidant stress within the cytoplasm. Cytochrome c oxidase copper chaperone is another protein that channels copper to cytochrome c oxidase in the inner mitochondrial membrane which plays a critical role in the electron transport chain for cellular respiration. Antioxidant protein 1 presents copper to the ATPase of type P, ATP7A of ATP7B (Lutsenko *et al.*, 2010). These three transporters play an essential role in copper homeostasis. They perform distinct functions depending on their cellular localization: when at the Golgi apparatus they enable the management of copper by ceruloplasmin and incorporation of copper into enzymes which require it as cofactor. When localized in vesicular compartments, they allow the removal of copper from the cell and thus participate in copper homeostasis (Arredondo and Núñez, 2005; Hejl *et al.*, 2009).

2.1.4 Toxicology of copper (general)

In living organisms, cupric ion (Cu^{2+}) is fairly soluble whereas cuprous (Cu^{+}) solubility is in the sub-micromolar range. Cu is present mainly as Cu^{2+} since in the presence of oxygen or other electron acceptors, Cu^{+} is readily oxidized. Strong reductants such as ascorbate or reduced glutathione can reduce Cu^{2+} back to Cu^{+} (Arredondo and Núñez, 2005). As in the case of iron through the Haber-Weiss and Fenton reactions, free copper ions can catalyse the production of hydroxyl radicals ($HO^{\bullet}$). Copper toxicity results also from nonspecific binding which can inactivate important regulatory enzymes by displacing other essential metal ions from catalytic sites, by binding to catalytic Cys groups or by allosterically altering the functional conformation of proteins (Mason and Jenkins, 1996).

Thus, the mechanisms of toxicity are associated both with oxidative stress and direct interactions with cellular compounds.

Free copper ions have high affinity to sulfur-, nitrogen-, and oxygen-containing functional groups in biological molecules which can inactivate and damage them. Cytotoxicity observed in copper poisoning results from inhibition of the pyruvate oxidase system by competing for the protein's sulfhydryl groups. Glucose-6-phospho-dehydrogenase

and GR are also inhibited (competitive inhibition) proportionally to the concentration of intracellular copper (Barceloux, 1999). The same applies to some transporters as the ATPase(s) which are also inhibited by copper, causing disruption of homeostasis of the respective transported entities. Toxic effects of copper can also result from its affinity to DNA (Agarwal *et al.*, 1989; Bremner, 1998; Sagripanti *et al.*, 1991). Another mechanism of toxicity of excessive concentrations of copper is the modification of the zinc finger structures of transcriptional factors which cannot any longer bind to DNA (Pena *et al.*, 1999). Copper in excess can also promote apoptosis (Kozlowski *et al.*, 2009) wile copper deficiency may be the cause of many diseases due to cuproprotein and copper dependent reaction inhibition.

In mammals, copper homeostasis is primordial. Wilson's and Menkes diseases are caused by genetic mutations in copper transporter proteins. The former results from accumulation of copper in several organs and tissues. There are different varieties, the most common being liver disease and anaemia (Hejl *et al.*, 2009). The accumulation of copper arises from a defect in the P-type Cu-protein ATP7B (called Wilson protein), a specific transporter of copper. The gene which encodes this protein is located on autosome number 13 in humans. It also allows the incorporation of copper in cuproproteins and excretion of copper into the bile. Accumulation of copper leads to liver cirrhosis and neurodegeneration.

Menkes disease is a neurodegenerative disease. Copper, after ingestion, accumulates in the intestine and absorption by other organs and tissues (blood, liver, brain) is defective. Menkes syndrome is caused by a mutation in the ATP7A gene located on chromosome X which encodes a protein ATP7A Cu-type P. This membrane protein is the first specific transporter of copper found in eukaryotes.

Copper (Cu), besides cadmium, is one of the major metals causing environmental problems in fresh water ecosystems. Since it is highly toxic to fish, it is also used as piscicide (Manzl *et al.*, 2004).

2.2 Biological models studied: *Anodonta anatina* and *Anodonta cygnea*

2.2.1 Taxonomy, description, and distribution

Taxonomy

The two biological models studied are *Anodonta cygnea* and *Anodonta anatina*. Both belong to the Unionoida order which represent a taxonomic group comprising 842 species, exclusively freshwater, spread all over the globe (except in artic regions). The Unionoida taxon is 200 millions years old, its origin is probably from the Triassic. The taxonomy of these two mussels is:

Phylum: Mollusca (Cuvier 1795)
Class: Bivalvia (Linnaeus 1758)
Subclass: Eulamellibranchia (Pelseneer 1889)
Superorder: Palaeheterodonta (Newell 1965)
Order: Unionoida (Stoliczka 1870)
Superfamilia: Unionoidea (Rafinesque 1820)
Familia: Unionidae (Fleming 1828)
Genus: *Anodonta* (Lamarck, 1799)
Species: *A. cygnea* (Linnaeus 1758)
Species: *A. anatina* (Linnaeus 1758)

For *Anodonta cygnea* common names are: swan mussel (English), anodonte des cygnes (French), Große Teichmuschel (German); for *Anodonta anatina*: duck mussel (English), mulette des cannards (French), Gemeine Teichmuschel (German).

Description:

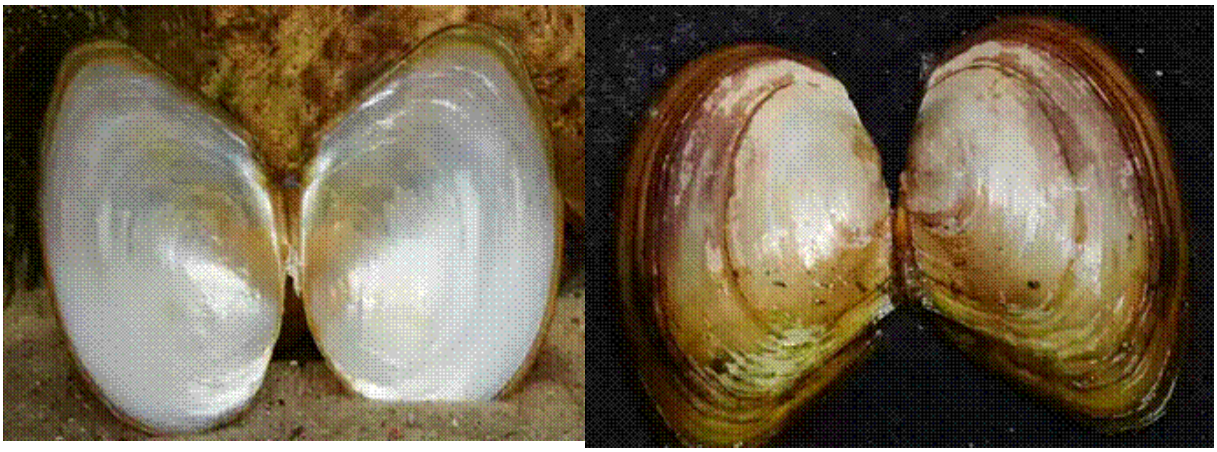
In both species, the shell is nacreous, and there are no teeth on the ligament.

Anodonta anatina (fig. 2A): Shell slightly elongated, triangular because of the presence of a large rear wing or crest, the top and bottom form an angle of greater or lesser extent, the upper edge rises in an almost straight line towards the back to the highest point of

the crest, then descends in a more concave line towards the posterior end; the anterior is broadly rounded, rather short, the posterior is longer, ending posteriorly by an obtuse rostrum; the ligament is rather long and prominent; the top is a little bulging, covered with fine wrinkles, a little curved, obliquely cutting the growth lines; the shell is rather thick, solid, shiny, greenish gray, or brownish. (Length: 14 cm, height: 10.5 cm, thickness: 6 cm) (Vrignaud, 2005).

Anodonta cygnea (fig. 2B): Shell oval, more or less elongated with the top and bottom edges roughly parallel or convex, the upper edge is more straight than the lower, the posterior area being much longer; the ligament is rather long, and prominent; the top is a little bulging, covered with fine wrinkles parallel to growth lines; the shell is thin, not strong, fairly shiny, greenish-yellow. (Length: 20 cm, height: 10 cm, thickness: 6 cm) (Vrignaud, 2005).

A



B

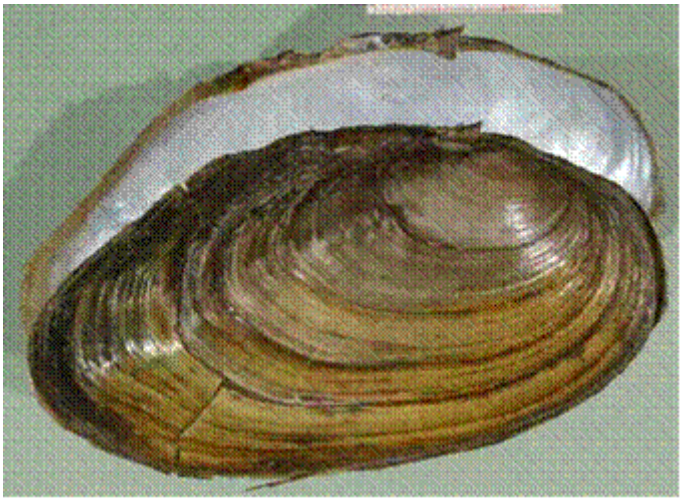


Fig. 2: A : *Anodonta anatina*, B: *Anodonta cygnea* (www.biopix.eu)

Geographic distribution

Anodonta cygnea and *Anodonta anatina* are autochthonous bivalves, widely distributed under the polar circle in European continental hydrosystems. The two mussels have a similar distribution, but wider for *Anodonta cygnea*. The geographical distribution (fig. 3) of *Anodonta anatina* extends from the British Isles to the eastern limit of Europe and from Sweden to Spain. *Anodonta cygnea* is present from the British Isles to Siberia and from Sweden to North Africa (Başçınar and Düzgüneş, 2009; www.discoverlife.org).

Fig. 3: Geographical distribution (enclosed by the red line) of *Anodonta anatina*, and distribution of *Anodonta cygnea* in European continental hydrosystems (Başçınar and Düzgüneş, 2009; www.discoverlife.org)

Life Cycle

Of the unionids, the individual animals are usually gonochoric. No age limit is known for gametogenesis. The spermatozoids are released into the water through the exhalant siphon. They are filtered by mussels located downstream. After fertilization, the eggs are incubated in the marsupium which is a modification of the gills of the mussel where the larvae or glochidia will hatch (fig. 4). They are produced in large quantities, from 50000 to 2 million (Başçınar and Düzgüneş, 2009), some of them attaching themselves with hooks at the end of their valves on the gills of a fish host, and then live as encysted parasites. After a few weeks, the cyst bursts and releases a small bivalve similar in its anatomy to an adult mussel. This juvenile will grow buried in the substratum before becoming adult and gradually rising to the surface. The expulsion of glochidia begins at the end of winter and may continue until September (Mouthon, 1982). The Unionidae produce only one generation per year. It is estimated that mussels of the genus *Anodonta* can live very long, about fifteen to twenty years (Taskinen and Valtonen, 1995).

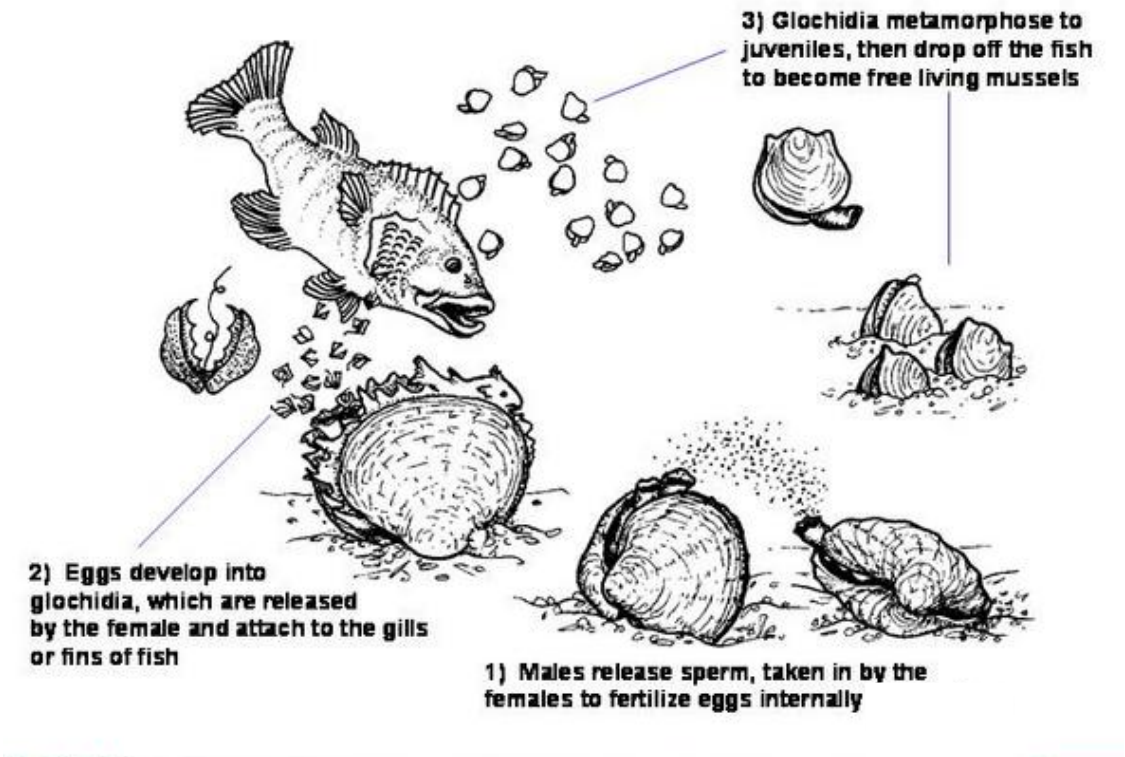


Fig. 4: Biological cycle of Unionidae (http://biodidac.bio.uottawa.ca)

Diet

According to the conventional nomenclature, among the freshwater molluscs one distinguishes vegetarians, detritivores, and more rarely omnivores; there is no true carnivor. Most bivalves have a mixed diet with detritivore and vegetarian dominance throughout the year, as for the Unionidae. Except during their larval stage when the glochidium parasites a host fish and feeds on his plasma (Uthaiwan *et al.*, 2001), *Anodonta* mussels are filter feeders, although their diet is not precisely known. They feed on seston (phytoplankton, filamentous algae, detritus, protists, epipelic) suspended in water. Water enters the mantle cavity through the inhalant siphon, passes through the gills and is exhaled through the exhalant siphon. Food particles in the water are intercepted by rows of cilia or cirri which extend between the gill filaments. The particles are either transferred and incorporated into mucus strings or carried in a concentrated suspension as they are transported to the labial palps. Particles selected for ingestion are transferred to the mouth, while rejected particles are bound up into a mucus ball and expelled from the inhalant siphon or pedal gap (gap between the valves) as pseudofaeces.

2.2.2 Anatomy and ecology

Anatomy

The mantle cavity is divided by the gills into a ventral inhalant chamber and a dorsal exhalant chamber. Water passes through the inhalant siphon to enter the branchial chamber, flows across the gills and then into the exhalant suprabranchial chamber. As the water crosses the gills, food particles are filtered from it and oxygen is removed. From the suprabranchial chamber the water flows out of the exhalant siphon. The gills consist of a sheet of coalescent filaments folded into a “W” shape and attached to the dorsal wall of the mantle. This sheet divides the mantle cavity into the ventral chamber and the dorsal suprabranchial chamber. To get from the ventral chamber to the dorsal, water must pass through pores in the gills. The three major functions of the gills are: homeostasis of blood, the capture of food particles, and eggs incubation. The intensity of the filtering activity depends on many factors, external such as temperature, particle load, and internal control factors. The pericardial cavity and the heart are located on the dorsal edge of the visceral mass immediately ventral to the middle of the posterior lateral tooth (fig. 5). A lobe of the nephridium which composes kidneys is present just posterior to the pericardial cavity. The heart is in the pericardial cavity. The digestive

gland is pigmented in brown or yellow and composed by a part of the oesophagus, stomach, and part of the intestine. It is located in the visceral mass at the base of the foot used for locomotion of the bivalve.

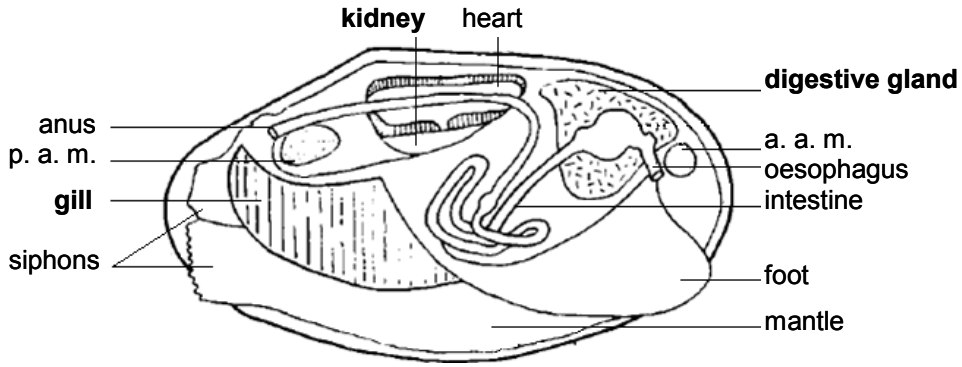


Fig. 5: Longitudinal-section of an Unionidae (Mouthon, 1982), p.a.m.: posterior adductor muscle, a.a.m.: anterior adductor muscle

Ecology

Anodonta cygnea and *Anodonta anatina* are species that frequent ponds, oxbow lakes, slow rivers, and canals with weak stream which often have high trophic level. They prefer bottom with fine granulometry (silt, sand, gravel), often with accumulations of organic matter. These species are very tolerant to average temperature of the water as evidenced by their presence in Spain to Sweden. They are localised in bed areas, out of strong stream undergoing some erosion, in general in a depth range between 0.2 and 2.5 m. These are burrowing organisms, living vertically partially embedded in the substrate. These bivalves are generally sedentary organisms. In Spain, a displacement of about ten meters by year was found for mussels *A. cygnea* and *A. anatina* marked by Aldridge (2000). However, flooding can cause passive dispersal from upstream to downstream. The most mobile stage is the larval stage, which is capable of moving long distances through the host fish.

Threats

Over the past 50 years, a general decline of freshwater mussel has-been observed. It seems that some factors are combined to cause the gradual disappearance of these species: among the potential causes, human activities leading to chemical pollution and habitat exchange have been suggested (Aldridge, 2000; Kádár *et al.*, 2001). Given the lack of ecological and ecotoxicological knowledge for these bivalves (including the tolerance to

pollution of adults, but especially of larvae and juveniles), little is available to define the main threats and causes of extinction:

- The virtual disappearance or severe depletion of potential host-fish.
- The physical degradation of streams and reworks of the beds of rivers and canals (Aldridge, 2000), rectification, dams and channel dredging, but also the impact of intensive agriculture, quality and quantity of sediments and their transit.
- Degradation of water quality, including eutrophication, as well as pollutants from human activity.
- The fragmentation of populations, one of the main causes of biodiversity loss.
- Introduced species, the potential effects of competition in bivalves such as *Corbicula fluminea*, *Sinanodonta woodiana* as well as *Dreissena polymorpha* are poorly documented. Zebra mussels appear to have a negative impact by binding to the valves of certain freshwater mussels, hampering their opening. *S. woodiana* also seems to have a negative impact on native unionid populations (Adam, 2010).

2.2.3 Ecotoxicological interest

Molluscs are common, highly visible, ecologically and commercially important on a global scale as food and as non-food resources (Rittschof and McClellan-Green, 2005). In some aquatic ecosystems (lakes, slow streams) molluscs can represent up to 80% of the total biomass of the benthic macroinvertebrates, so their impact can become major. Populations of bivalves filter large amounts of water (Unionidae 300ml/individual/h) and take an active part in sedimentation and water purification. Faeces and pseudofaeces concentrate sometimes a large fraction of planktonic microorganisms not used, making them accessible to detritivorous invertebrates such as oligochaetes, and many diptera. But they also change the quality of sediment by concentration and excretion of many substances (metals, pesticides, radionuclides). Because of their sedentary lifestyle, their filtration capacity, and their wide distribution, molluscs and bivalves are excellent sentinels for monitoring the fraction of bioavailable pollutants in their environment (Hayer and Pihan, 1996). In close contact with water-suspended particles and sediment, they are widely used for controlling the bioaccumulation and toxic effects of metallic and organic pollutants in aquatic ecosystems (Viarengo *et al.*, 2000; Rittschof and McClellan-Green, 2005). Several Unionidae mussels, especially from the *Anodonta* genus have since been used as biomonitor organisms in toxicity assessment of numerous compounds released in continental water. The anatomy and

physiology of these animals has been studied for a long time, allowing the study of toxic effects of compounds but also the mechanisms of detoxification. *Anodonta cygnea* and *Anodonta anatina* are biological models widely used in ecotoxicology (Falfushynska *et al.*, 2009). They are present in large quantities, sedentary, easy to collect and to acclimatize in aquaria.

2.3 Effects of copper exposure and detoxification mechanisms

2.3.1 Copper exposure effects

2.3.1.1 Copper bioaccumulation

Trace elements are known to be highly accumulated by aquatic molluscs. Bivalves are in close contact with sediments which constitute a major environmental sink for metals, with an important filtering activity to satisfy respiration and nutrition, and tolerance mechanisms that involve metal sequestration rather than metal exclusion or elimination. They provide accurate and integrated information about the environmental impact and bioavailability of chemicals. They are therefore extensively applied in marine environments using mussels and oysters, but are also implemented in freshwater systems using other bivalve species such as *Anodonta* sp., *Dreissena polymorpha, Elliptio complanata,* and Asiatic clams. Among freshwater organisms, unionid molluscs are widely recognised for their capacity to accumulate a variety of environmental contaminants including metals in their tissues (Winter, 1996; Bilos *et al.*, 1998; Kádár *et al.*, 2001; Falfushynska *et al.*, 2009). The widespread recent decline in the species diversity and population density of freshwater mussels (Lydeard *et al.*, 2004) may be partly related to chronic, low-level exposure to toxic metals (Frank and Gerstmann, 2007). Freshwater mussels are exposed to metals that are dissolved in water, associated with suspended particles, and deposited in bottom sediments. Thus, freshwater mussels can bioaccumulate certain metals to concentrations that greatly exceed those dissolved in water. In adult mussels, the most common site of metal uptake is the gills, followed by the digestive gland, the mantle, and the kidneys (Pagliarani *et al.*, 1996; Bonneris *et al.*, 2005). Bioaccumulation of metals varies strongly according the water chemistry conditions, pH and water hardness being important parameters. Aqueous concentrations of calcium probably enhance the bioavailability and toxicity of metal cations, because the permeability of membranes is inversely related to aqueous calcium concentration; calcium

ions apparently compete with other metal cations for binding sites on the gill surface, decreasing the direct uptake of other cationic metals. The pH influences both the chemistry of metals and macromolecules of surface structures. A modification of membrane permeability causes an alteration in metal diffusion. Additionally, changes in membrane potential modify the transport of polar metal species (Winter, 1996). In bivalves, the biological barriers are the gill epithelium, the wall of the digestive tract, and the shell (which is often reported as a site of bioaccumulation). Metals or metalloids in solution are more easily absorbed by the surfaces in direct contact with the external environment, while those associated with the particulate phase are rather ingested and internalized after solubilization in the digestive tract, or transferred by endocytosis to then undergo lysosomal digestion (Wang and Rainbow, 2005). Once past the first barrier, the mechanisms of transfer of metals or metalloids into the cell involve intracellular diffusion (passive or facilitated), active transport, and endocytosis (phagocytosis and pinocytosis). The oxygen concentration of the water or the density of microalgae are parameters that will directly influence the ventilatory activity of the bivalve and so its exposure to metals in solution or particulate (Tran *et al.*, 2000; Tran *et al.*, 2004). Copper and metal bioaccumulation can change between individuals of the same species with the difference in sizes, or between different species mussels due to different physiology (breathing, eating) (Winter, 1996; Bilos *et al.*, 1998; Gundacker, 2000; Hédouin *et al.*, 2006; Falfushynska *et al.*, 2009). In bivalves, the determination of trace metal concentrations in whole individuals presents little interest, since determination of bioconcentration factors in various tissues suggested that the principal accumulating organs are: the gills, the digestive gland, the kidney, and the mantle, also the shell acting as a storage matrix (Viarengo and Nott, 1993; Roméo and Gnassia-Barelli, 1995; Das and Jana, 1999; Bonneris *et al.*, 2005). The intracellular sequestration of metals is based on a sequence of cellular events involving a cascade of different ligands with increasing metal binding strengths. *Anodonta* sp. and other molluscs accumulate metals to high levels in their tissues (Falfushynska *et al.*, 2009). Metal tolerance in such accumulator organisms involves sequestration of metals in non-toxic forms. Among the best studied intracellular sites involved in the sequestration of essential and non-essential metals in aquatic invertebrates are lysosomes, granules, and soluble Cys-rich ligands as metal-binding peptide and proteins. Unionids also lay down calcium microspherule concretions, particularly in the connective tissue of the gills, in the mantle, and in the digestive gland (Pynnönen *et al.*, 1987; Moura *et al.*, 2000; Lopes-Lima *et al.*, 2005). Dissimilar mechanisms for copper and metal uptake, storage, mobilisation, and excretion

performed by different cell types in different organs explain the pattern of metal accumulation and tissue distribution (Soto *et al.*, 1997).

2.3.1.2 Oxidative stress

Reactive oxygen species comprise a variety of unstable oxygen derivatives. Some ROS are free radicals, presenting unpaired electrons (hydroxyl radical $HO^{\bullet}$), others are non-radical species such as hydrogen peroxide H_2O_2. To reach a better level of stability, these radicals will capture electrons from reductor molecules, ROS reduction causing oxidations in chains. All the bio-molecules of the cell (nucleic acids, lipids, proteins, polysaccharides) are potential reductor substrates of ROS. The level of ROS instability characterized their diffusion capacity. A low-reactive form tends to act far from its site of production as it has a significant diffusion radius. On the contrary, a very reactive species acts very quickly and its diffusion is limited. ROS include the superoxide anion radical ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), hydroperoxy ($ROO^{\bullet}$), and alkoxy ($RO^{\bullet}$) radicals, nitric oxide ($NO^{\bullet}$), and hydroxyl radical ($HO^{\bullet}$). Molecular oxygen O_2 can also be regarded as a radical species since it has two single electrons. The superoxide anion is produced during various reactions with transition metals, and enzymes are implied in its formation. H_2O_2 reacts weakly, diffuses freely, and has a long lifetime. The $ROO^{\bullet}$ and $RO^{\bullet}$ radicals arise from the peroxidation of lipids. These radicals allow the gradual propagation of lipid peroxidation. Free radicals are produced physiologically during normal cell metabolism. They can also be formed in response to a wide range of exogenous agents including radiation, metal ions, solvents, particulate matter, nitrogen oxides, and ozone. $NO^{\bullet}$ plays at the same time a role in the destruction and the production of radicals. It is not very reactive with cellular components and reacts with radicals generating less reactive species. Combined with the superoxide anion radical, it may be involved in the formation of peroxynitrite, a highly toxic species. Due to its high reactivity, $HO^{\bullet}$ is quite non-specific in its targets for oxidation, whereas ROS with lower rate constants react more specifically. ROS are at the origin of lipid peroxidation. Copper ions are involved in redox reactions which result in the production of ROS. In the Fenton reaction, cuprous ions react with H_2O_2 giving rise to the extremely reactive $HO^{\bullet}$ (Labieniec and Gabryelak, 2007). In the case of organic hydroperoxides (ROOH), a homologous reaction is thought to occur leading to the formation of $ROO^{\bullet}$ and of the more reactive $RO^{\bullet}$. Copper ions may participate both in the initiation and the propagation steps of lipid peroxidation, thus stimulating the *in*

vivo degradation of membrane lipids (Viarengo *et al.*, 1990; Géret *et al.*, 2002a; Sheehan and McDonagh, 2008). An excess of ROS causes a risk of oxidative stress to the cell.

Free radicals are naturally produced and may also have a physiological role. The major role of ROS is an activity of regulation of molecules containing sulfhydryl groups. This type of regulation can in particular affect molecules implied in the mechanisms of signal transduction like protein kinase C (Lander 1997, Dalton *et al.*, 1999). Significant sources of free oxygenated radicals are redox cycles and oxidations catalysed by cytochrome P450 monooxygenases implicated in detoxification. Like many exogenous compounds that can stimulate the production of ROS, copper is known as hydroxyl radical inducer (Company *et al.*, 2008). Metal deficiency can also lead to oxidative stress. Indeed, copper belong to the molecular composition of antioxydent enzyme such as Cu / Zn-SOD. Thus copper deficiency disrupts in this case the dismutation of the superoxide anion.

ROS can potentially react with every cellular component. They influence in particular the thiol groups of proteins, leading to the formation of intra- or inter-molecular disulphide bridges. The most widely studied action of ROS is lipid peroxidation, mainly carried out by $HO^{\bullet}$. After rearrangement and addition of oxygen, $ROO^{\bullet}$ and $RO^{\bullet}$ radicals are generated. Oxidation of phospholipids modifies the membranes with loss in permeability and membrane potential, leading to inactivation of membrane receptors and enzymes. In a general way, during this reaction, various compounds are produced such malondialdehyde (MDA) and 4-hydroxynonenal, both able to bind to proteins and to form DNA adducts (Blair, 2001). Thus, lipid peroxidation may trigger endogenous DNA damage as nuclear DNA and mitochondrial DNA are the targets of ROS. Four main classes of damage can be noted: single and double strand breaks, modified bases, DNA-DNA and DNA-protein bridges, and abasic sites. Proteins are essential structural and functional cellular components which may undergo oxidative modification, thus inducing their aggregation or digestion by proteases (Davies, 2005). Oxidation of amino acids, particularly of sulphur-containing and aromatic amino acids, result in structural modifications of proteins. $HO^{\bullet}$ is the main initiator of the oxidation of polypeptide chains producing free radicals. In the absence of oxygen, two radicals can react together to form intra- or interchain cross-links. In the presence of oxygen, an addition reaction may take place to yield a peroxyl radical. A series of reactions follows, leading to the formation of alkoxyl radicals, precursors to the fragmentation of polypeptide chains. The oxidation of glucose can be performed in the presence of metal ions, leading to the release of aldehydes and hydrogen peroxide. This leads to glycation of proteins by attachment of aldehydes, often entailing the cleavage of protein chains (Wolff *et al.*, 1989). Glycation of

proteins promotes their oxidizability. The structural modifications induce functional changes, in particular of cellular metabolism. The oxidation by ROS can disturb ionic transport, enzymatic activities, and calcium homeostasis. Antioxidant defense systems can directly inhibit the production of ROS, limit their propagation, or destroy them. These antioxidant systems may act by reducing ROS species or by trapping them to form stable compounds. Two categories of antioxidant systems are generally defined, i.e. antioxidant enzymes, and molecules without enzymatic activity. Antioxidant enzymes are superoxide dismutases (SODs) and catalase (CAT): SODs catalyze the dismutation of the superoxide anion radical under formation of molecular oxygen and H_2O_2, the latter detoxified by CAT. SOD and CAT are localised in peroxysomes, and also in mitochondria and cytosol. Isoenzymes of SOD are found in various compartments of the cell.

In the antioxydent system glutathione peroxidases (GPxs) / glutathione reductase (GR), GPxs are also able to reduce H_2O_2 and other peroxides. Their enzymatic activities of GPxs are coupled with the oxidation of glutathione (GSH). Oxidized glutathione (GSSG) will be reduced by GR using NADPH. These enzymes are localised in the cytoplasm and the mitochondrial matrix (Lushchak, 2011).

Antioxidant molecules or free radical scavengers such as Cys thiol rich compounds, principally GSH and the metallothioneins, will be discussed later. Their antioxidant capacity is conferred by the thiol group of the Cys residue. Vitamin E is also known for its powerful anti-radical activity operating in lipid membranes. Other scavenger molecules include vitamin C, carotenoids, and uric acid.

2.3.1.3 Calcium transport and perturbation of bio-mineralization

Calcium transport perturbation

Normally, intracellular Ca homeostasis is maintained by a balance between extrusion and compartmentation systems (Mooren and Kinne, 1998). Alteration of these processes during cell injury can result in inhibition of Ca^{2+} extrusion or intracellular compartmentation mechanisms, as well as in enhanced Ca^{2+} influx and release of Ca^{2+} from intracellular stores such as the endoplasmic reticulum and mitochondria (Marchi *et al.,* 2004). This can lead to uncontrolled rises in cytosolic Ca^{2+} concentration. The biological functions of Ca^{2+} are versatile; they control multiple processes of birth, life and death. Location, duration, and amplitude of calcium signals form a complex code that can control vital physiological

processes. Any disturbance in these signals would change the "Ca^{2+} code" and modify multiple life processes which are usually associated with loss of cell viability (Ermak and Davies, 2001). Filter feeding bivalves have high bioaccumulation potential for trace metals which at higher concentration can cause serious metabolic, physiological, and structural impairments. Continuous decline of freshwater mussels during the past decades, could be attributed to calcium homeostasis perturbation by trace metals (Frank and Gerstmann, 2007). Slight deregulations of Ca^{2+} homeostasis like those deriving from low-dose trace metal contamination can affect the cellular ability to maintain and modulate Ca^{2+} signaling. Copper is known to increase Ca^{2+} intracellular concentration by release of the Calcium stock of the endoplasmic reticulum, leading to lysosomal membrane destabilisation in the mussels (Marchi *et al.*, 2004) and apoptosis or cytotoxicity and necrosis (Ermak and Davies, 2001). Cell Ca^{2+} extrusion systems allowing physiological calcium concentration maintenance appear to be essential for cell viability.

The absorption and release of calcium in the environment, biomineralization, and the maintenance of intracellular calcium concentration in involved its passage through cell membranes. Ca^{2+} passively enters the apical membrane down to its concentration gradient through carrier-mediated facilitated diffusion Na^{+}/Ca^{2+} antiporters and via simple diffusion through Ca^{2+} channels. Intracellular Ca^{2+} gradients are maintained by membrane Ca^{2+}-ATPases across the plasma membrane (PMCA) and the membranes of intracellular stores. In *Mytilus edulis* copper is known to inhibit PMCA. Thiol groups of PMCA proteins are directly oxidized or they bind copper because of high affinity to the metal. Hydroxyl radicals $OH^{\bullet}$ are induced by copper which indirectly causes PMCA protein impairment (Viarengo and Nicotera, 1991; Viarengo *et al.*, 1996; Burlando *et al.*, 2004). Besides the Ca^{2+}-ATPases, another important system for the maintenance of calcium homeostasis is the plasma membrane Na^{+}/Ca^{2+} antiporter, the activity of which is based upon the transmembrane Na^{+} electrochemical gradient. Inhibition of the Na^{+}/K^{+}-ATPase modifies the Na^{+} gradient, and therefore it can also affect the activity of the Na^{+}/Ca^{2+} antiporter. Na^{+}/K^{+}-ATPase inhibition was observed in *Mytilus edulis* and *Perna viridis* after Ag, Cr, and copper exposure (Viarengo *et al.*, 1996; Vijayavel *et al.*, 2007).

Due to their physiological function for respiratorion, nutrient absorption and excretion, the gills, the digestive gland, and the kidneys are preferential sites of metal uptake and bioaccumulation in bivalves. These organs play important roles in iono-regulation, in particular for calcium homeostasis in freshwater bivalves (Coimbra *et al.*, 1993). Effects on

the Ca^{2+} transport systems in these tissues leads to perturbation of calcium homeostasis in the whole organism.

Perturbation of biomineralization

Biomineralization is a complex process; in bivalves principally it enables the formation, growth, and repair of the shell. In addition to the shell, mineral concretions are produced which also play a role in detoxification mechanisms (Viarengo and Nott, 1993). In freshwater molluscs such as *Anodonta*, several internal tissues, bathed by haemolymph, also produce calcified structures, namely microspherules (Lopes-Lima *et al.*, 2005). Microspherules are usually present between both epithelia of the mantle under $CaCO_3$ and / or $Ca_3(PO_4)_2$ deposits. These calcium transitory reserves are devoted to shell growth or glochidia larvae. The mantle of lamellibranchs is a leaflet that covers the internal surface of the shell and surrounds the body of the animal. It consists of two epithelia: internal in contact with the external medium, and external, the outer mantle epithelium (OME) facing the shell. The mantle is the tissue responsible for shell synthesis (Coimbra *et al.*, 1993). Shell growth and maintenance of its mineral content are thus dynamic equilibrium involving a continuous exchange of Ca^{2+} between the shell and the OME through the extrapalleal fluid dividing the two. The dynamic calcium exchange may result in a net accumulation of Ca^{2+} in the shell, or its re-absorption, depending on the developmental stage and the metabolic state of the animal. The biomineralization mechanism depends on pH changes or its tendency (Lopes-Lima *et al.*, 2005). H^+-ATPases in cells of the OME regulate the deposition of calcium or its reuptake into the haemolymph by pH control. H^+-ATPases probably play a role equivalent to those of the two bone cell lines, the osteoblasts and the osteoclasts (Machado *et al.*, 1988). H^+-ATPases of the OME induce calcium mobilisation from the shell mainly by pumping protons into the extrapalleal fluid. H^+-ATPase inhibition under bis(tributyltin)-oxide exposure was observed (Machado *et al.,* 1989). CA is a ubiquitous enzyme; in the mantle of bivalves it controls bicarbonate balance between haemolymph and extrapalleal fluid for $CaCO_3$ formation. CA is a bioindicator of biomineralization already studied in ecotoxicology of metals (Vitale *et al.*, 1999, Rousseau *et al.*, 2003). So, if some alteration of the ion transport mechanisms across the mantle occurred in bivalves*,* it may depend on a direct inhibition of biomineralization. In freshwater bivalves, trace metals and in particular copper, seem to interfere with the shell calcification conditions by direct action on the proton pump and CA. Perturbation of biomineralization could cause subsequent shell thickening (Machado *et al.,* 1989) or thinning.

2.3.2 Detoxification mechanisms

2.3.2.1 Cysteine thiol rich compounds

Glutathione

The tripeptide glutathione (GSH, γ-glutamyl-cysteinylglycine) is a non-protein thiol. The GSH/glutathione disulfide (GSSG) system is the most abundant redox system in eukaryotic cells, playing a fundamental role in cell homeostasis and metal detoxification, and being involved in signalling processes associated with programmed cell death termed apoptosis (Canesi *et al.*, 1999; Camera and Picardo, 2002). It is present mainly in its reduced form, GSH, and represents the most significant thiol in eukaryotic cells (0.2 to 10 mM). GSH biosynthesis requires two enzymes (fig. 6): first, glutamate-cysteine-ligase (γ-ECS) catalyzing the fusion of glutamic acid (Glu) and Cys to γ-glutamyl-cysteine (γ-GluCys), which in turn is converted into GSH by addition of glycine (Gly) by glutathione synthase (GS). γ-ECS is inhibited in feedback by GSH (Monostori *et al.*, 2009). GSH exerts many functions in the cell. It intervenes in processes of reduction such as the synthesis and degradation of proteins, and the formation of deoxyribonucleotides. GSH plays a role as co-enzyme of various reactions, and it is also used for being combined with either endogenous (oestrogens, prostaglandins, and leucotrienes) or exogenous compounds (drugs and xenobiotics), thus taking part in their metabolism. GSH is thus regarded as a central component in antioxidant defense (Cossu *et al.*, 2000; Manduzio *et al.*, 2005). The biologically active site of GSH is represented by the thiol group of the Cys residue. The high nucleophilicity of the thiol function facilitates the role of GSH as a free radical scavenger both under physiological conditions and in xenobiotic toxicity. GSH also helps in the regeneration of other antioxidants such as vitamin E, ascorbic acid, and metallothionein (Knapen *et al.*, 1999). GSH is a cofactor for glutathione peroxidase in the decomposition of hydrogen peroxide or organic peroxides (fig. 6), for glyoxalase 1 in the detoxification of methylglyoxal and other oxo-aldehydes, and for maleylacetoacetate isomerase in the conversion of maleylacetoacetate and maleylpyruvate to the corresponding fumaryl derivatives (Monostori *et al.*, 2009). GS-conjugates of endogenous compounds are involved in metabolism, transport, and storage in cell. In addition, conjugation of xenobiotics to GSH initiates a detoxification pathway that generally leads to excretion or

compartmentation of the biotransformed compound. Although some adducts can be formed directly, glutathione-*S*-transferases (GST) mediated reactions generally predominate. GST belong to cellular mechanisms of detoxification and elimination of molecules (Wünschmann *et al.*, 2009). In animals, GS-conjugates are hydrolyzed and degraded by c-glutamyl-transferases, followed by carboxypeptidation of the glycine residue. GR reduces GSSG to GSH which, with synthesis of new GSH, maintains the cellular GSH stock (fig. 6).

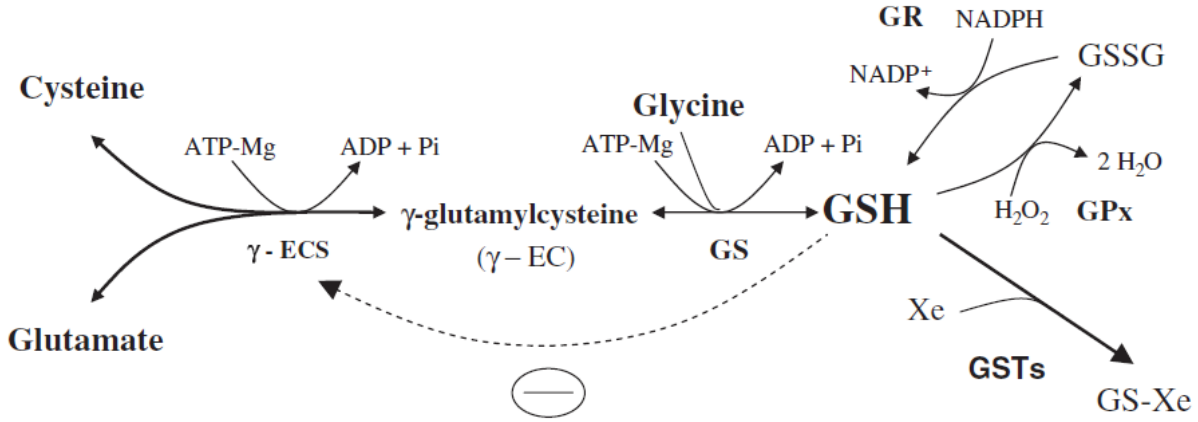


Fig. 6: GSH metabolism (Mendoza-Cózatl and Moreno-Sánchez, 2006), γ-ECS: glutamate-cysteine-ligase, GS: glutathione synthase, GR: glutathione reductase, GPx: glutathione peroxidase, GST: glutathione transferase, Xe: xenobiotic, GSH: glutathione, GSSG: glutathione disulfide

Metallothioneins

Metallothionein (MT) is a generic name for a superfamily of ubiquitous low molecular weight cytosolic Cys-rich metalbinding proteins possessing a unique type of sulfur-based metal clusters (Vašák, 2005). The designation MT reflects the extremely high thiolate sulfur and metal content, both of the order of 10% (*w/w*). MT have been identified in a wide range of organisms, from bacteria to mammals, in many fish and aquatic invertebrates, mainly molluscs. Classically these extremely heterogeneous polypeptides were grouped into three classes of MT (Fowler *et al.*, 1987; Viarengo and Nott, 1993): Class I, including the MT which show biochemical homology and close elution time by chromatography with horse MT, Class II, including the rest of the MT with no homology with horse MT, and Class III, which includes phytochelatins, Cys-rich enzymatically synthesised peptides. A second classification

was performed (Binz and Kägi, 1999; http://www.bioc.uzh.ch/mtpage/classif.html) and takes into account taxonomic parameters and the patterns of distribution of Cys residues along the MT sequence. Cysteine (Cys) residues are distributed in typical motifs consisting of Cys-Cys, Cys-X-Cys or Cys-X-X-Cys sequences (X denoting amino-acid residues other than Cys). It results in a classification of 15 families for proteinaceous MT. Mollusc MT belong to family 2, divided in two subfamilies.

Metallothioneins are playing a role in the homeostatic control of essential metals (Cu, Zn) as they can act as essential metal stores ready to fulfil enzymatic and other metabolic demands (Amiard *et al.*, 2006). Their involvement in metal metabolism is based on their capacity to complex metals, effectively buffering free-metal ion concentrations in the intracellular environment. Additionally, the biosynthesis of these metalloproteins may be induced by exposure to essential and non-essential metals (Bonneris *et al.*, 2005). Vital roles for this pleiotropic protein result in its involvement in homeostasis of essential trace metals, zinc and copper, or sequestration of the environmental non essential metals. Moreover, MT could protect cells from oxidative stress (Géret *et al.*, 2002b). In the presence of ROS, zinc can be removed (Vašák, 2005). The release of zinc is accompanied by the formation of the MT-disulfide (or thionin, oxidized form of the protein) which in turn can be reduced by the ratio GSH / GSSG to restore the ability of the protein to bind zinc. This redox cycle of MT (fig. 7) plays a crucial role in maintaining physiological homeostasis of metals, detoxification of toxic metals and protection against oxidative stress (Kang, 2006). MT plays an essential role in other metabolic processes, since MT expression is rapidly induced by a variety of factors such as cold, heat, hormones, cytokines (Monserrat *et al.*, 2007), in some molluscs important seasonal variations in MT levels also correlate with the gametogenesis process. MT, like other proteins, are degraded in lysosomes (Ng *et al.*, 2007).

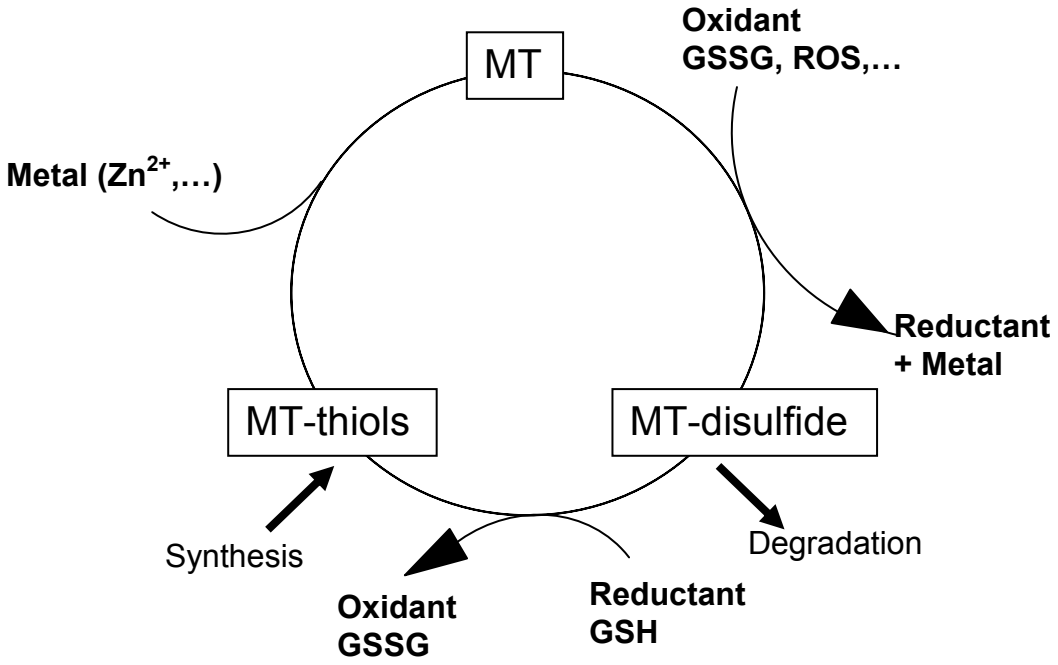


Fig. 7: Redox cycle of MT (Kang, 2006), MT: metallothionein, ROS: reactive oxygen species, GSH: glutathione, GSSG: glutathione disulfide

Phytochelatins

Phytochelatins (PC) are small metal-binding thiol rich peptides with the structure PCn, (γ-Glu-Cys)n-Gly, where n = 2 to 11. In plants glycine residues can be replaced by alanine, serine, glutamic acid, or glutamine. PC have been characterized in a wide range of species in plants, fungi, and microalgae. They play a major role in metal detoxification but are also implicated in the regulation of intracellular essential metal concentrations. They are capable of binding trace metals such as Cd, Hg, Cu, Zn, Ni, and Ag via thiolate coordination and have higher metal-binding capacity (on a per Cys basis) than MT (Grill *et al.*, 1985). An antioxidant activity assay has shown that PC molecules have strong scavenging activity *in vitro* towards ROS such as hydrogen peroxide (H_2O_2) and the superoxide radical anion ($O_2^{-\bullet}$) (Hirata *et al.*, 2005). These results suggest that PC play important roles not only in the chelation of trace metals but also as antioxidant defence. PC are synthesized from glutathione, homo-glutathione, hydroxymethyl-glutathione or γ-glutamylcysteine, catalysed by a transpeptidase, named phytochelatin synthase (fig. 8), which is a constitutive enzyme requiring post-translational activation by trensition metals (Grill *et al.*, 1989; Clemens, 2006). Phytochelatin synthase has been shown to be activated by a broad range of metals and

metalloids, in particular Cd, Ag, Pb, Cu, Hg, Zn, Sn, Au, and As, both *in vivo* and *in vitro*. After the completion of the full genome sequence of the nematode *Caenorhabditis elegans* two publications independently described a functional PC synthase enable to synthesize PC in this model invertebrate (Clemens *et al.*, 2001; Vatamaniuk *et al.*, 2001). In addition sequences similar to PC synthase gene have been identified from the aquatic midge, *Chironomus*, and a species of earthworm (Brulle *et al.*, 2008; Cobbett, 1999). There is, yet, no evidence that these animal genes encode PC-synthase activity, however it seems likely that they to encode PC synthase. It became clear that PC could play a wider role in trace metal detoxification than has previously been thought. Organisms with an aquatic or soil habitat are more likely to express PC (Cobbett, 2000). Clemens *et al.* (2001) hypothesized that PC may be ubiquitously involved in the tolerance and homeostasis of metals in all eukaryotic organisms. Clemens and Peršoh (2009) suggest how PC synthase genes are far more widespread than anticipated, homologous sequences are found through the entire animal kingdom including in bivalves.

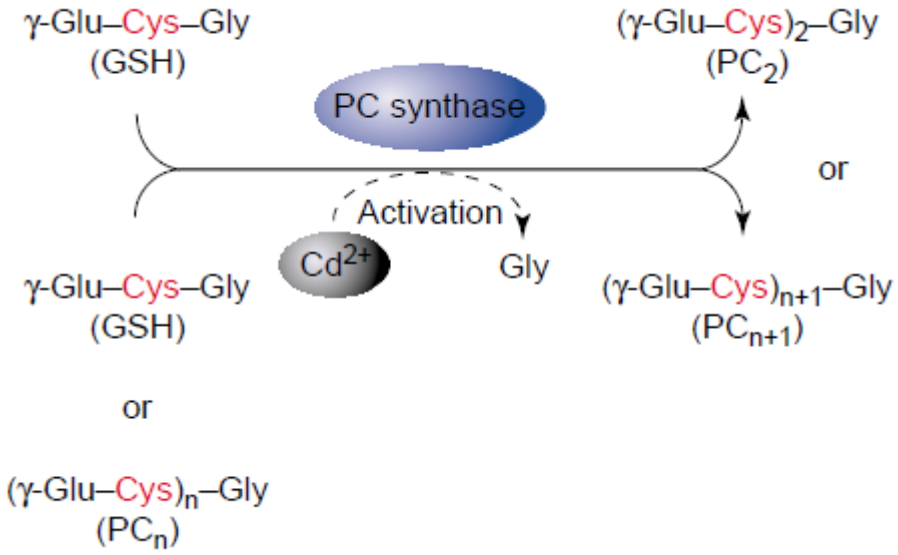


Fig. 8: PC synthesis (Vatamaniuk *et al.*, 2002)

2.3.2.2 Metal detoxification mechanisms in bivalves

Bivalves and particularly freshwater mussel unionids are widely recognised for their capacity to accumulate a variety of environmental contaminants, including metals, in their tissues and yet survive in these polluted environments (Winter, 1996; Cossu *et al.*, 1997; Das and Jana, 1999; Bonneris *et al.*, 2005). Such tolerance depends on the ability of these animals to regulate the essential metal concentration and detoxify nonessential metal. In molluscs

three physiological and biochemical ways allow metal regulations: by binding to specific, soluble, Cys-rich ligand; by compartmentalization within organelle; and by formation of insoluble non-toxic precipitates (Viarengo and Nott, 1993; Wang and Rainbow, 2005). Metal sub-cellular partitioning depending of organs and metal nature. For example in mussels, copper bound to granules are dominant in the gills, in the digestive gland copper is principally bound to soluble Cys-rich compounds (Bonneris *et al.*, 2005). For a long period of exposure, metals might be displaced from soluble metal-binding ligands to granules (Roméo and Gnassia-Barelli, 1995).

Insolubles storage, compartmentalization and elimination of metals

In mollusc an important storage or detoxification system is carried out by sequestration of metals in intracellular cytosolic and compartmentalized precipitated structures named granules (Howard *et al.*, 1981; Viarengo and Nott, 1993). Granules are observed in different tissues of bivalves, mainly in the digestive gland, gills, and kidneys which are implicated in metal homeostasis and detoxification (Wang and Rainbow, 2005). Lysosome is mainly involved in the catabolism of both endogenous and exogenous molecules. This organelle accumulate high concentrations of trace metals in non-toxic granules forms and thus represent an important detoxification way. The ferritin-rich and copper-sulphur granules are related to Fe and Cu metabolism in the respiratory pigment and also in copper detoxification. Lipofuscins are mainly lipid peroxidation end-products which are accumulated in the lysosomes as insoluble lipoprotein granules. Metals such as Cu, Cd, and Zn are trapped by the lipofuscin and sterically prevented from moving in or out of the granule. In mollusc intracellular granules composed to calcium / magnesium orthophosphate ($Ca_3(PO_4)_2$, $Mg_3(PO_4)_2$) and pyrophosphate ($Ca_2P_2O_7$, $Mg_2P_2O_7$) can contain Mn, Zn, Cu, Fe, Co, Cd and Ni and appear to be linked with metal detoxification. These insoluble granules are often present in cells from which they can be eliminated by exocytosis (Howard *et al.*, 1981; Viarengo and Nott, 1993). Granules alone or with the lysosome are finally eliminated, principally by exocytosis in urine, haemolymph, and faeces.

In addition, a specificity of unionids is calcium extracellular concretions named microspherules. They are found in extrapalial fluid and haemolymph particularly in the gills, and in the mantle. The calcium of these microspherules is usually bound to phosphate and carbonate, either as orthophosphate $Ca_3(PO_4)_2$, and carbonate calcium $CaCO_3$ (Moura *et al.*, 2000; Lopes-Lima *et al.*, 2005). These microspherules appear to act as a calcium reservoir,

serving as a source of calcium for embryonic shell development, but can also play a role in the detoxification of metals (Pynnönen *et al.*, 1995; Bonneris *et al.*, 2005).

An other site of metal storage in mollusc is the shell which may act as safe storage matrix for toxic contaminants resistant to soft tissue detoxification mechanisms (Das and Jana, 1999). In shell of bivalves and unionids, substantial bioaccumulation of copper and other metals was shown (Gundacker, 2000).

Cysteine rich metal binding compounds detoxification mechanisms

A strategy for cells to detoxify non-essential metal ions and essential metal ions excess is the synthesis of high-affinity binding sites to suppress blockage of physiologically important functional groups (Clemens, 2006). Metal ions have high reactivity with thiol, amino or hydroxyl groups making molecules carrying these functional groups candidates for metal detoxification processes (Viarengo and Nott, 1993). Therefore, the best-known and presumably the first line to Cu, Zn and non-essential metal chelation are the Cys-containing peptides glutathione and phytochelatins and small Cys-rich proteins metallothioneins.

Metal cation with a high affinity for SH residues displace Zn^{2+} from a metallothionein physiological pool always present in the cells. An excess of trace metal cations, including Zn^{2+} released from pre-existing metallothioneins, induces in the nucleus the synthesis of the mRNA coding for metallothioneins, consequently increasing MT on the cytosolic compartment. These MT chelate the trace metal cations, thus reducing their cytotoxic effects (Viarengo and Nott, 1993). The metal-thiolate clusters within the MT molecules allow rapid exchanges of metallic ions between clusters and with other MT molecules (Monserrat *et al.*, 2007). MT are usually not saturated by a single metal but contain several atoms of Cu, Zn, Cd, or Hg and Ag when present (Amiard *et al.*, 2006). In Zn-thioneins the seven metal atoms of Zn or Cd are distributed between two clusters: α cluster of 3 Zn and β cluster of 4 Zn (Maret, 2009). Functionally, the two clusters show different affinities for metal cations. At pH 7 or below, cluster α is the first to be saturated and cluster β the first to release the metal. Data for Zn/Cu-thionein indicate that copper is present in the form of Cu(I). Unlike to Zn, Cu is arranged differently in the MT clusters. Cu(I) atoms are binding by one bivalent connection which allows 10 copper atoms to bind per MT protein. The relative affinities of each metal ion is different for MT (Géret *et al.*, 2002b). Indeed, the stability constant for copper is 100 times higher than for cadmium and 1000 times higher than for zinc. Due to the high affinity of Cu(I) for the SH residues, the complex is stable and the metal is not easily released. It is

important to note that the Cu-thionein has distinct chemical characteristics, including the capacity to produce oxidized insoluble polymers. In the digestive gland of bivalve, the metal is detoxified into the lysosomes trapped in the form of oxidized insoluble Cu-thioneins, which is subsequently eliminated by exocytosis of residual bodies. Nevertheless, literature data indicate that MT synthesis is not always induced in freshwater mussel, studies carried out in laboratory in *A. cygnea* by Tallandini *et al.* (1986) and in *Dreissena polymorpha* by Lecoeur *et al.* (2004) showed no induction of MT after Cu^{2+} exposure. MT in response to a metal exposure might be reflected in increased turnover (synthesis and breakdown) of the protein, but not necessarily in changes in MT concentration (Mouneyrac *et al.*, 2002).

The Cys-containing peptides PC and GSH have been shown to form complexes with various metals, through their thiolate sulfur atom function. In aquatic organisms, glutathione is believed to play a fundamental role in detoxifying metals. The soluble tripeptide GSH complexes and detoxifies trace metal cations soon after their entrance in the cells, thus representing a first line of defence against trace metal cytotoxicity (Canesi *et al.*, 1999). In particular, reduction of Cu(II) by GSH produces a stable Cu(I)-SG complex which is also a physiological donor of Cu(I) to copper apoproteins in both mammalian and marine invertebrate cells. Methylmercury has also a high affinity for SH groups of GSH, methylmercury–SG complexes have been identified in different animals tissues. Glutathione metal complexes are transported across the plasma membranes and, therefore represent a carrier for the elimination of the metal from the cells (Viarengo and Nott, 1993). Conjugation of glutathione to metals prevents them from interacting deleteriously with other cellular components. Enhanced cadmium toxicity after glutathione depletion has been observed in both in vitro and *in vivo* mammalian studies. There is also evidence that glutathione depletion enhances metal toxicity in aquatic organisms. Glutathione depletion by inhibitor of glutathione synthesis (buthionine sulfoximine) enhances copper toxicity in the oysters *Crassostrea virginica* (Conners and Ringwood, 2000).

Biochemical and genetic studies have confirmed that GSH is the substrate for PC biosynthesis. PC have been assumed to function in the cellular homeostasis of essential transition metal nutrients, particularly Cu and Zn (Schat *et al.*, 2002). PC are high-affinity chelators of metals, and play major roles in the detoxification. Unequivocal evidence was shown for the involvement of PC synthesis in metal detoxification (Courbot *et al.*, 2004; Morelli and Scano, 2004; Thangavel and Minocha, 2007). Induction of PC is triggered by exposure to various physiological and non-physiological metal ions. Some of these: Cd, Ag, Pb, Cu, Hg, Zn, Sn, Au, and As form complexes with PC *in vivo* in algae, plants, and fungi

(Clemens, 2006). They are suspected to play a role in animals as well as in plant (Cobbett, 2000; Vatamaniuk *et al.*, 2002; Clemens and Peršoh, 2009). In the structural model of a PC-Cd complex, for example, the Cd coordinately binds one, two, three or, at maximum capacity, four sulfur atoms from either single or multiple PC molecules, resulting in amorphous complexes. *In vivo* the size of the PC chain molecules, and the pH stability are essential and determined the metal binding capacity per molecule of PC (Hirata *et al.*, 2005). Konishi *et al.* (2006), introduced into the early embryos of zebrafish, mRNA coding for PC synthase from *Arabidopsis thaliana*. As a result, the heterogeneous expression of PC synthase and the synthesis of PC from GSH in embryos could be detected. The developing embryos expressing PC synthase and became more tolerant to Cd exposure.

Extended summary

3 Extended summary

The present work was undertaken in order to investigate some potential causes involved in the Europe-wide observed phenomenon of the decline of various freshwater bivalve species. As stated in the IUCN Red List of Threatened Species, 44 % of all freshwater molluscs are under threat (http://www.iucnredlist.org/news/european-red-list-press-release). One of the potential causes is the increasing use and corresponding local or regional release of industrially and technologically important metals, besides many other potential factors involved in such a complex eco-toxicologically sequence of events. Clearly, such limited toxicological-pathobiochemical study as presented in the few investigations described here is far too limited to come to a distinct and exclusive delineation of one single, individual explanation for the widespread decline of freshwater molluscs including the bivalves, one class of this important, threatened phylum. On the other hand, without such detailed and necessarily limited studies of concentrating on the potential patho-biochemical and toxicological mechanisms of a single likely pollutant, the delineation of the multitude of facets of such ecotoxicological effects will remain blurred. Therefore, the overall conclusions derived from the investigations presented in more detail in the following is not meant as proof of the only relevance of the increasingly used, technologically important metal copper, but as a demonstration of its potential relevance when considering various possibilities which can have an impact on the stability of bivalve populations, including the most spectacular and most strongly threatened, the European pearl mussel.

3.1 Optimization of analytical protocols

Bivalve maintenance and copper exposure

Particular attention was paid to mussel maintenance in order to carry out the tests under stable, homogenous, and reproducible conditions (detailed in article 1). The bottom of the tank was covered with a layer of glass beads of 10 mm of diameter, so the mussels could find conditions for burying. This was important for giving the animals a substrate for close-to-nature behaviour, at the same avoiding the use of natural river sediment as substrate; thus, unpredictable or unaccountable metal accumulations by adsorption onto sediment particles or interferences of microorganisms by metal species conversion could be avoided without

excessively disturbing the behaviour of the animals, as indicated by their regular ventilation activity. Moreover, the large beads facilitated a deep daily cleaning of the tank by suction. The water, artificially reconstituted from deionised water, was renewed daily in order to ensure equal conditions during the whole experimental course for all replicates and avoid any uncontrolled metal accumulation or cross-contamination. Under these conditions, the copper concentrations could be maintained within close limits of the target concentration during the whole exposure period, as verified by graphite furnace atomic absorption spectrometry (GFASS) or inductively coupled plasma mass spectrometry (ICPMS).

Enzymatic analyses

The enzymatic analyses were carried out as described in article 1 and 2. The activities of Ca^{2+}-ATPase and Na^{+}/K^{+}-ATPase were determined in the suspension of the microsomal pellet (as shown in fig. 1) obtained by homogenization of the mantle, digestive gland, gills, and kidney, and by centrifugation at 75600 *g*, the activity of H^{+}-ATPase was determined in the supernatant of the first centrifugation step at 2000 *g* as shown in (fig. 1). Inorganic phosphate released by the ATPases was quantified by spectrophotometry of the ammonium molybdate complex according to Chifflet *et al.* (1988). The CA activity was evaluated in the supernatants (S 2000, fig. 1) by measuring the pH decrease according to Vitale *et al.* (1999).

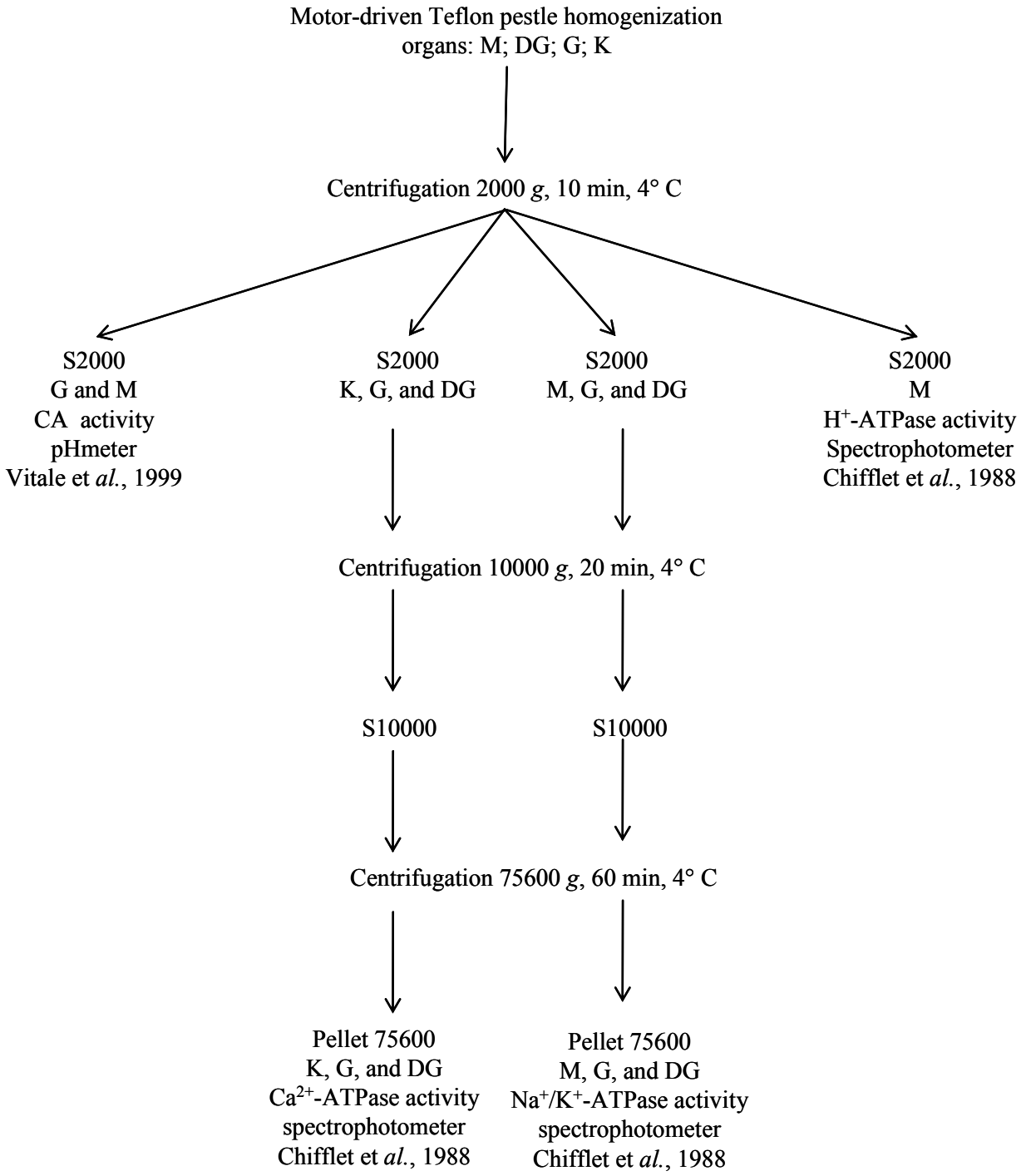


Fig. 1: Protocol of tissue fraction preparation for enzymes analysis of Ca^{2+}-ATPase, Na^{+}/K^{+}-ATPase, H^{+}-ATPase, and carbonic anhydrase (CA), in the mantle (M), digestive gland (DG), gills (G), and kidneys (K) of the fresh water mussel *A. anatina*.

Thiol rich compound analyses

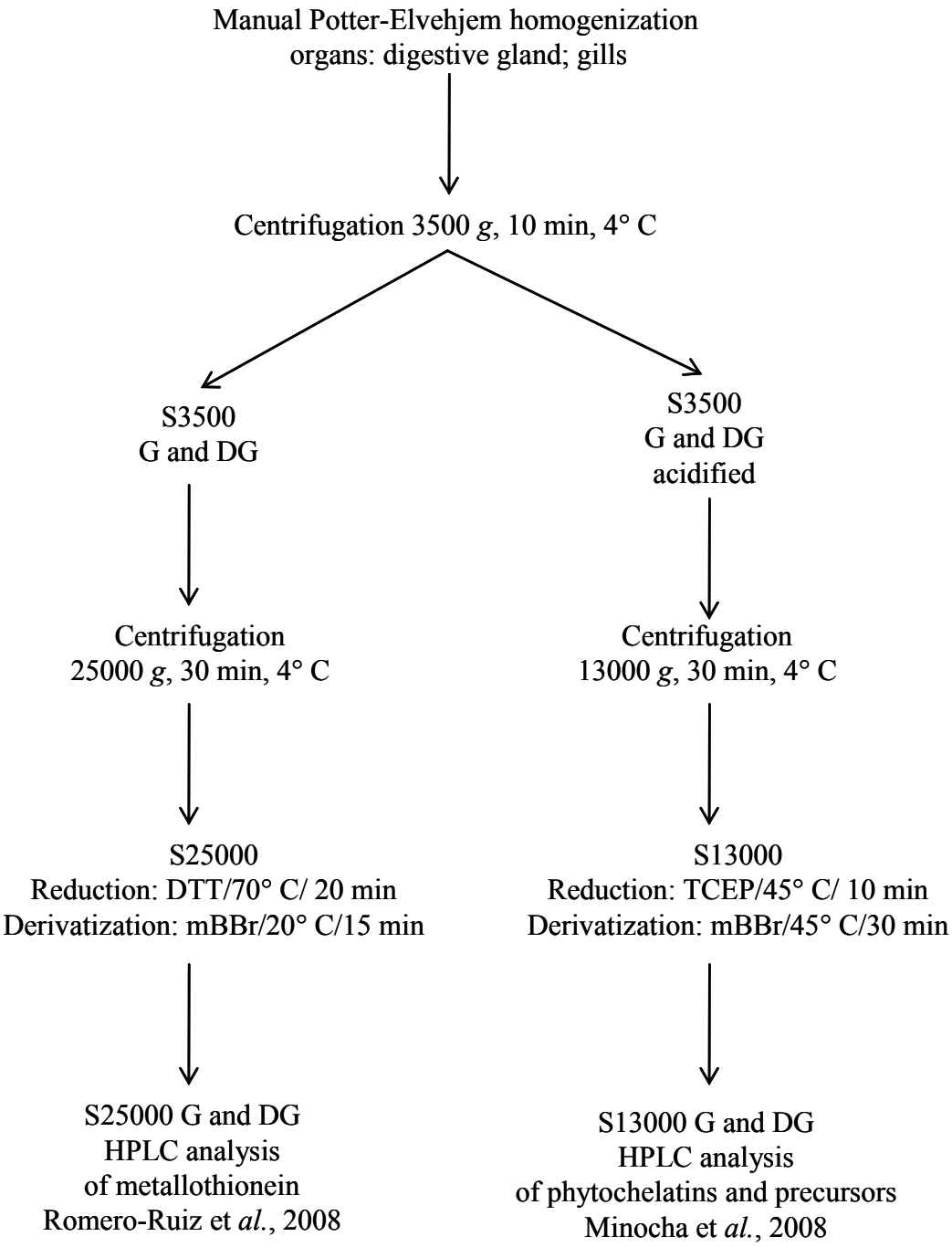


Fig. 2: Protocol of tissue fraction preparation for thiol rich compound analysis, i.e. of metallothionein (MT), phytochelatins (PC) and precursors in the digestive gland (DG), and gills (G) of the fresh water mussel *A. anatina*.

The study of phytochelatins in *Anodonta cygnea* lead us to develop and optimise an HPLC analytical protocol for phytochelatin (PC) quantification in animal tissues, based on the method for plants developed by Minocha *et al.* (2008). A protein removal step by acidification and centrifugation was added (fig. 2) and the mobile phase gradient profile was modified as described in articles 3 and 4. PC were quantified in the cytosolic fractions of deproteinized tissue homogenates and metallothionein (MT) in the non-deproteinized cytosolic fractions, after thiol reduction with tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP) and 1,4-dithiothreitol (DTT), respectively. Monobromobimane (mBBr) was used as a fluorescent tag. Initially non-fluorescent, the dye mBBr became fluorescent after its binding to thiol groups under dehalogenation. It is selective for thiol groups and allows their quantification with high sensitivity by fluorimetry. Each sample was spiked with 0.6 µmol L^{-1} of each standard PC_{2-5} to certify PC identity. The quantification limits per 20 µL sample injected into the HPLC column were 1.5 pmol for PC_{2-3}, 2.7 pmol for PC_4, 5 pmol for PC_5.

3.2 Effects of copper on calcium transport in *Anodonta anatina*

Physiological data

A good physiological knowledge of the biomarkers is important for their ecotoxicological interpretation.

The present study (article 1 and article 2) allowed us to determine basal levels of the enzymatic activities of Ca^{2+}-ATPase, Na^+/K^+-ATPase, and H^+-ATPase of the plasma membrane. These enzymes were studied in gills, digestive gland, kidneys, and mantle, organs playing an important role in calcium input. The Ca^{2+}-ATPase controls the cellular calcium concentration by active transport. The Na^+/K^+-ATPase and the H^+-ATPase are maintaining sodium and proton gradients necessary for calcium Na^+/Ca^{2+} and H^+/Ca^{2+} transporters. H^+-ATPase and cytoplasmic CA were also studied for their implication in the biomineralization process of the shell and in the maturation of glochidia. There is a lack of physiological data in freshwater bivalves compared to marine models such as *Mytilus edulis* or *M. galloprovincialis*. These data are useful in understanding calcium transport mechanisms since freshwater bivalves are subject to osmo- and iono-regulations different from marine mussels. Enzymatic activity found in the gills of *A. anatina* (table 1) were consistent to those reported in the gills of *A. cygnea* for Na^+/K^+-ATPase and Ca^{2+}-ATPase 0.11 µmol P_i/mg protein/min,

(Lagerspetz and Senius, 1979) and 0.58 µmol P_i/mg protein/min (Pivovarova and Lagerspetz, 1996) respectively, and in the gills of *A. anatina* for CA 2.1 U/mg protein (Ngo *et al.*, 2011).

Table 1: Basal enzymatic activities of plasma membrane ATPase(s) (µmol P_i/mg protein/min) and cytoplasmic CA (U/mg protein) in *Anodonta anatina*.

	G	DG	M	K	Reference
Ca^{2+}-ATPase	0.45	0.095		0.087	Article 1
Na^+/K^+-ATPase	0.098	0.015	0.042		Article 2
H^+-ATPase			0.002		Article 2
CA	2.4		1.76		Article 1

G: gills; DG: digestive gland; M: mantle; K: kidneys, CA: carbonic anhydrase; ─: not determined

The plasma membrane Ca^{2+}-ATPase (PMCA) activity in the gills (article 1) was four-fold higher than in the marine bivalve *Mytilus edulis* (Burlando *et al.*, 2004). This reflects how important calcium active transport is to the freshwater mussel *Anodonta anatina*. Calcium concentrations in seawaters are higher than in freshwaters which explains that calcium absorption is easier for *Mytilus edulis*. PMCA activity is also high in the kidneys and the digestive gland, favouring calcium absorption from food and calcium reuptake from renal filtrate. These physiologic data allowed us to understand how PMCA is crucial for calcium homeostasis in freshwater bivalves compared to marine organisms. Moreover in Unionidae, calcium plays a direct role in reproduction since glochidia are incubated in the marsupium (gills) and is therefore important for population growth of mussels. Na^+/K^+-ATPase maintained the sodium transmembrane cellular gradient necessary for calcium facilitated-diffusion by Na^+/Ca^{2+} antiporters. In *Anodonta anatina*, Na^+/K^+-ATPase activity varied in a consistent manner as function of the seasons (article 2). The enzymatic activities of Na^+/K^+-ATPase and H^+-ATPase were found to be maximum in summer, the season of shell and glochidia calcification in *Anodonta* mussels (Taskinen, 1998; Moura *et al.*, 2000). This seasonal variation is an important aspect which is imperative to consider for data interpretation in ecotoxicological investigations.

Copper and enzymatic perturbation

The enzymatic activity of the plasma membrane Ca^{2+}-ATPase was significantly inhibited in the kidneys of *A. anatina* upon 4 days of exposure at all concentrations of Cu^{2+} tested in the rage of 0.26 to 1.15 µmol L^{-1} (article 1). In the kidneys and the gills, a significant inhibition of Ca^{2+}-ATPase activity was observed upon 4 d of exposure at 0.35 µmol L^{-1} followed by a recovery at 7 d of exposure. Significant Ca^{2+}-ATPase activity inhibition with no recovery was observed upon 15 d in the kidneys at the higher concentration of 0.64 µmol L^{-1} of Cu^{2+}. No significant effect was noted on CA activity in gills and digestive gland of *A. anatina* exposed to Cu^{2+} (article 1).

Compared to controls mussels, the Na^{+}/K^{+}-ATPase activity was significantly inhibited in the gills (72 % inhibition) and the digestive gland (80 % inhibition) of *A. anatina* upon 4 days of exposure at 0.35 µmol L^{-1} of Cu^{2+} (article 2). The Na^{+}/K^{+}-ATPase activity was inhibited by 26 % in the mantle of mussels exposed 4 d to the same conditions, but not significantly relative to controls.

No recovery was observed upon the 15 days of exposure in the gills which still showed 54 % of inhibition at the end of test, and a partial recovery was observed at day 7 in the digestive gland. In the mantle, the H^{+}-ATPase activity declined continuously but the decline was not statistically significant due to high variability between mussels (article 2).

Calcium plays a fundamental role in numerous biological processes (energy production, cellular metabolism, muscle contraction, reproduction) and has important mechanical functions (shell, skeleton) in many organisms (Mooren & Kinne, 1998). Contrary to molluscs from marine ecosystem which are generally hyposmotic and for which calcium uptake is easy, freshwater bivalves are hyperosmotic and require tight regulation of their calcium metabolism.

Despite some recovery beyond 4 days in the digestive gland, the Na^{+}/K^{+}-ATPase activity in the gills of *A. anatina* was still significantly inhibited upon 15 days of experiment; an inhibition of Ca^{2+}-ATPase activity was also observed in the gills and the kidneys under the same conditions (article 1 and 2). Inhibition of these enzymes was consistent with the results obtained with *Mytillus galloprovincialis* exposed to copper by Viarengo *et al.* (1996) and Burlendo *et al.* (2004). Inhibition of Na^{+}/K^{+}-ATPase modified the cellular Na^{+} gradient which could result in a reduced activity of Ca^{2+}/Na^{+} antiporter; this, in addition to direct inhibition of Ca^{2+}-ATPase, may lead to a disturbance of calcium homeostasis. Such a disturbance of iono-regulations could lead to continuous under-supply of calcium which may also affect Ca^{2+}

signalling pathways in addition to biomineralization and shell formation in mussels and in glochidia.

The kidneys have an essential role in filtration and reabsorption of ions, water, and organic molecules from the ultrafiltrate. As freshwater organisms are hyperosmotic, the osmotic pressure resulting from the gradient of concentrations between internal and surrounding compartments leads to water uptake by osmosis and ionic loss by diffusion. The water uptake is compensated by urine production and ionic loss is limited by active ion reabsorption. In freshwater bivalves, the daily output of urine is high. The kidneys play an essential role in calcium homeostasis by limiting ionic loss in urine through active Ca^{2+} reuptake from the filtrate (Turquier, 1994). An inhibition of calcium reabsorption may therefore dramatically disturb calcium homeostasis. Because of analytic problems inherent to the small biomass of kidneys, this tissue has been poorly studied in freshwater bivalves. This organ plays a role in detoxification (Viarengo and Nott, 1993) and is essential to iono-regulation. Our results (article 1) showed the high sensitivity of Ca^{2+}-ATPase to Cu^{2+} in this organ.

The Unionidae freshwater bivalves are widely recognized for their capacity to accumulate a variety of environmental contaminants in their tissues including metals (Bonneris *et al.*, 2005). This marked metal tolerance is effected by biochemical strategies that involve metal sequestration. The intracellular sequestration of metals is based on a sequence of cellular events involving a cascade of different ligands with increasing metal binding strengths. High concentration of metal could also inhibit detoxification mechanisms. In our results (article 1 and 2), recovery of Ca^{2+}-ATPase and Na^{+}/K^{+}-ATPase activity within 7 days at the low Cu^{2+} concentration (0.35 µmol L^{-1}) indicated adaptive ability. This suggested the mobilisation of detoxification systems efficient at low Cu^{2+} concentrations. At higher Cu^{2+} concentration (0.64 µmol L^{-1}), no recovery was noted. It is interesting to note that recovery was observed only at 0.35 µmol L^{-1}, a concentration environmentally more relevant than the higher concentration studied (article 1). Therefore, it is important to use environmental concentrations in ecotoxicological research; extrapolation of the results observed at high doses to environmental situations may be critical and not pertinent due to different mechanisms of adaptation and toxicity at high and low doses.

3.3 Metal detoxification mechanism in *Anodonta cygnea*

Aquatic molluscs have a number of properties that make them one of the most popular sentinels for monitoring water quality. The molluscs take up and accumulate high levels of trace metals, although the body concentrations show wide variability across metals and invertebrate taxa. The study of the capacity of bivalves for assessment of water quality is primarily linked to their tendency to accumulate trace metals even at high concentrations (Doyotte *et al.*, 1997). Tolerance depends on the ability to regulate the metal concentrations in the cells and to accumulate excess metals in non-toxic forms (Viarengo and Nott, 1993).

In our study, the recovery of Ca^{2+}-ATPase (article 1) and Na^{+}/K^{+}-ATPase (article 2) activity observed within 7 days of exposure indicate an induction of detoxification mechanisms. Copper belongs to the transition metals most of which are known to show various degrees of affinity for thiol group. This property makes chelating ligands carrying thiol functions the first mechanism of metal detoxification. In articles 3 and 4, the study was focused on the mechanisms of metal detoxification by thiol rich compounds. In most animals, tolerance to trace metals depends on the induction of MT, a family of thiol-rich proteins of low molecular weight. These metallo-proteins are known to play an important role in the homeostatic control of essential metals such as Cu and Zn but also in the detoxification of excessive amounts of essential and non-essential trace metals. In the tissues of metal-exposed mussels is a rapid increase of metallothioneins, the soluble proteins involved in transition metal detoxification which is synergetic with lysosome compartmentalization (Amiard *et al.*, 2006).

Phytochelatins (PC) are thiol rich oligopeptides which have been characterized in a wide range of plant species (Grill *et al.*, 1985). PC plays a major role in the detoxification of trace metals in plants by chelating metals with high affinity. In 2001, two publications independently described a functional phytochelatin synthase in the invertebrate nematode *Caenorhabditis elegans* (Clemens *et al.*, 2001; Vatamaniuk *et al.*, 2001). Brulle *et al.* (2008) provided the first evidence of a phytochelatin synthase from the earthworm *Eisenia fetida* implicated in dose-dependent cadmium detoxification. Neither study furnished evidence of the existence of the peptide PC in the invertebrates and animals. Recent cloning of the gene encoding phytochelatin synthase in *E. fetida* (Brulle *et al.*, 2008; Bernard *et al.*, 2010) suggested the existence of this detoxification pathway in this species. A superficial view of the limited selection of species in which such sequences have been identified might suggest

that invertebrates with an aquatic or soil habitat are more likely to express PC (Cobbett, 2000). The objectives of our study were to determine the ability of *A. cygnea* to synthesize PC (article 3), and to study the possible role played by PC in copper detoxification in this freshwater bivalve (article 4).

In plants, PC are rapidly induced in cells and tissues exposed to a range of transition metals and play an important role in detoxification. The results obtained in the present work (articles 3 and 4) showed the presence of phytochelatins in invertebrates; to our knowledge this is the first time. Basal levels were determined for PC_{2-4}, γ-GluCys, and MT in the gills and the digestive gland (table 2). Besides the interest of these data for freshwater bivalve studies, the detection of PC_{2-4} in the absence of excessive copper and other metals suggests their role in essential metal homeostasis. In the gills and the digestive gland, PC_2 was found in higher concentrations, followed by PC_3 which again was higher in concentration than PC_4. PC_2 and PC_3 were found to be two or three times higher in the digestive gland than in the gills.

Table 2: Basal levels of phytochelatines$_{2-4}$ (µg PC/g tissue wet weight), γ-GluCys (µg γ-GluCys/g tissue wet weight), and metallothionein (mg MT/g protein) in *Anodonta cygnea*.

	PC_2	PC_3	PC_4	γ-GluCys	MT
G	0.88	0.72	0.40	0.52	10.10
DG	2.17	1.10	0.47	0.93	15.11
Reference	Article 3	Article 3	Article 3	Article 4	Article 4

G: gills; DG: digestive gland; PC: phytochelatin; MT: metallothionein

In the European continental hydrosystem affected by agricultural and / or urban activities, levels of up to 0.6 µmol L^{-1} of Cu^{2+} are seasonally found (Neal and Robson, 2000; Falfushynska *et al.*, 2009). In our work (article 1 and 2), inhibition followed by recovery of Ca^{2+}-ATPase and Na^{+}/K^{+}-ATPase activities were observed in mussels exposed to 0.35 µmol L^{-1} Cu^{2+}, an environmentally relevant concentration. Therefore, in our studies on the detoxification mechanisms by metal binding Cys-rich compounds (articles 3 and 4), the mussels were exposed to the same Cu^{2+} concentration.

A statistical significant PC_2 induction in the gills of *A. cygnea* exposed to of 0.35 µmol L^{-1} Cu^{2+} was observed (article 4), i.e. 50 % from 12 h to 4 d exposure, and 30 % upon 7 d. In

the digestive gland, significant PC_2 induction was observed only at 12 h of Cu^{2+} exposure. Relative to the respective control, γ-GluCys increased significantly in the gills within 48 h and 7 d exposure and within 48 h and 4 d in the digestive gland.

The induction of PC_2 in *A. cygnea* exposed to Cu^{2+} suggests its key role as metal chelator in a first line of detoxification, together with other compounds such as GSH. Higher sensitivity of PC_2 induction in the gills could be explained by the water route of Cu-exposure. The gills may play a more important role than the digestive gland in the uptake of copper dissolved in the test media. However, beyond 7 d of exposure, PC_2 declined to control values within 21 d of exposure. This decrease suggests that long-term Cu^{2+} detoxification was shifting to other mechanisms.

In Unionidae, MT and insoluble granules are known to play a role in metal detoxification in the long term. Bonneris *et al.* (2005) showed that cadmium, zinc, and copper concentrations in the gill granule fraction were significantly correlated with environmental concentrations of these metals. The granules are known to be a preferential site for copper storage in the gills of Unionidae. Around 65 % of the total copper in the gills was found to be bound to granules in *Anodonta grandis grandis*, were the calcium concretion represented 51 % of the gills dry weight. Similar values were found in *Anodonta cygnea* (Bonneris *et al.*, 2005).

No increase of MT was observed in the present study upon Cu^{2+} exposure for 21 d. The MT isoform (< 10 kDa) in the mussel extract identified by HPLC separation in our study was not induced by Cu^{2+}. Detoxification in *A. cygnea* by other MT isoforms not detected by the HPLC method cannot be excluded. Indeed, MT polymorphism is known to be important in invertebrates (Géret *et al.*, 2002b; Amiard *et al.*, 2006). A good example of such polymorphism and functional divergence is found for MT in the snail *Helix pomatia*. These snails can tolerate exceptionally high concentrations of cadmium. Additionally, they accumulate relatively high amounts of copper, needed for the biosynthesis of the oxygen carrier hemocyanin. The specific metal accumulation is paralleled by the presence MT forms which bind specifically one type of metal (Cd or Cu), both containing 18 conserved Cys residues but differing in other amino acids (Vašák, 2005).

Metallothioneins, granules, and antioxidant systems have been described to be involved in detoxification mechanisms in freshwater bivalves. Cossu *et al.* (1997) have shown that in Unionidae antioxidants and especially GSH play major roles in metal detoxification. In the gills of *Unio tumidus*, a decrease by 45 % of GSH was found in mussels exposed at a metal-contaminated site. A decrease in GSH level in Unionidae exposed to copper was

confirmed by Doyotte *et al.* (1997) under controlled laboratory conditions, indicating either metal blockage by SH groups or the use of GSH as substrate of antioxidant enzymes. Indeed, a parallel involvement of antioxidant enzymes had been described with increased activity at low metal concentration (Vasseur and Leguille, 2004). GSH also plays a role in PC synthesis. This double role in direct metal detoxification and as a PC precursor could explain this decrease (fig. 1).

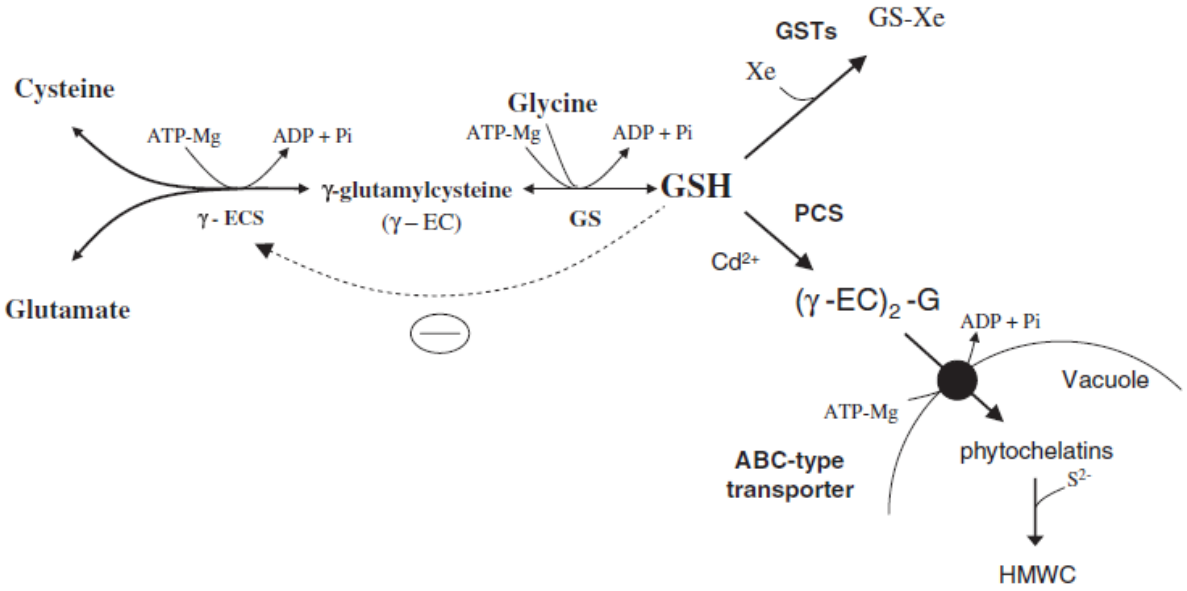


Fig. 3: Phytochelatin and precursor biosynthesis in plants (Mendoza-Cózatl and Moreno-Sánchez, 2006), PCS: phytochelatin synthase, γ-ECS: glutamate-cysteine-ligase, GS: glutathione synthase, GST: glutathione transferase, Xe: xenobiotic, GSH: glutathione, GSSG: glutathione disulfide, HMWC: high molecular weight complexes.

3.4 Decline of the Unionidae populations

Over the past 50 years, a world-wide decline of autochthonous freshwater molluscs has been observed (Lydeard *et al.*, 2004). Among the different species, the Unionoida taxon seems to be particularly endangered. Species such as the pearl mussels *Margaritifera margaritifera* and *Margaritifera auricularia* are declining dramatically in European rivers. Other species belonging to the Unionidae family are included in the red list of threatened species established by the World Conservation Union and is the case for *Anodonta anatina* and *Anodonta cygnea*.

The Unionidae populations represented the largest part of the total biomass in many aquatic systems. They take an active part in sedimentation and water purification, modifying the phytoplankton community, and are detritivorous invertebrates (Aldridge, 2000; Vaughn *et al.*, 2008). Therefore, the disappearance of Unionidae may result in structural and functional perturbations of aquatic ecosystems. The competition with invasive bivalves is also a matter of debate. Invasive freshwater bivalves as *Corbicula fluminea* or *Dreissena polymorpha* not belonging to the Unionidae colonise freshwater hydrosystems with detrimental effects to other invertebrates. These invasive species do not play the same functional role as Unionidae in ecosystems. Their presence is hypothesized to contribute to the decline of the Unionidae.

A combination of various factors may explain the gradual disappearance of Unionoidea in their competition with invasive species. Depletion of salmonids as host-fish has also been mentioned as a possible cause. Salmonid fish populations are affected by water pollution; moreover, pollutants may indirectly promote natural disease factors such as parasitism which is known to endanger salmonids (Voutilainen *et al.*, 2009), and bivalves; trematode parasitism of *Anodonta* population entails complete infertility (Taskinen and Valtonen, 1995). Other major reasons for the decline are the physical degradation of streams and the reworking of river beds and canals as well as the degradation of water quality. Indeed, interactions between pollutants may disturb these biocoenoses even at low concentrations (Vighi *et al.*, 2003). In order to protect the autochthonous Unionidae it is important to determine how and to which extent those factors contribute to their disappearance.

The present work is meant as a contribution to understand the role of metal pollution in affecting calcium homeostasis in fresh water mussels, likely to be one of the reasons for the widespread decline of mussel populations in European rivers (Frank and Gerstmann 2007).

Conclusion

4 Conclusion

A macrobentos community is an excellent indicator of water quality (Ippolito *et al.*, 2010). The objectives of the present work were to study some toxic effects of ionic copper and its detoxification mechanisms in the freshwater mussel *Anodonta* species belonging to the Unionidae. First, Cu^{2+} as a potential inhibitor of proteins playing a role in calcium transport and biomineralization was assessed with *A. anatina*. Secondly, mechanisms of copper detoxification by MT, PC, and γ-GluCys were studied in *A. cygnea*.

In the present study, the effects of Cu^{2+} exposure on the activities of Ca^{2+}-ATPase, Na^{+}/K^{+}-ATPase, and H^{+}-ATPase of the plasma membrane, and of cytosolic CA have been evaluated in the freshwater bivalve *A. anatina*.

Upon 4 d exposure, inhibition of Ca^{2+}-ATPase activity in the gills and the kidneys, and of the Na^{+}/K^{+}-ATPase activity in the gills and the digestive gland was observed in *A. anatina* exposed to 0.35 µmol L^{-1} Cu^{2+}. A total recovery of Ca^{2+}-ATPase was observed upon 7 d of exposure, indicating that detoxication mechanisms are activated, except for the higher Cu^{2+} concentration of 0.64 µmol L^{-1}.

Ca^{2+}-ATPase inhibition was particularly sensitive in the kidney, the organ playing an important role in calcium reuptake and iono-regultion. It would be interesting to study the long-term consequences on disturbances of osmoregulatory functions in the kidney of *A. anatina* exposed to 0.64 µmol L^{-1} Cu^{2+}.

The present work is the first to identify the phytochelatins, i.e. PC_2, PC_3, and PC_4, in freshwater mussels. In *A. cygnea* exposed to 0.35 µmol L^{-1} of Cu^{2+}, PC_2 was induced in the digestive gland and the gills within 12 h. The induction in the gills persisted for 7 days. These results suggest a role of PC in essential metal homeostasis and their involvement in first-line detoxification.

Our HPLC results showed no variation of MT levels after Cu^{2+} exposure, at least the MT isoform quantified with our method. Additional MT experiments with *A. cygnea* exposed to Cu^{2+} at the same conditions and employing a spectrometric method which allows the quantification of the total MT isoforms would be interesting, in order to compare our results with those found in *A. anatina* by Nugroho and Frank (2012). In parallel the HPLC method should be optimized to allow the detection of other MT isoforms induced by copper.

Copper has been reported as a weak inducer of metal binding thiol peptides (Zenk, 1996). Exposure to a non-essential metal being a strong inducer of PC, such as cadmium,

could be interesting. Comparison of effects of an essential metal like copper and a non essential metal like cadmium could allow to determine the role played by PC in homeostasis of essential metal and detoxfication mechanisms.

Copper is an inducer of reactive oxygen species (ROS) through Fenton type reactions. The activity of PC in scavenging ROS in *A. cygnea* exposed to copper would be interesting to study.

Chemical pollution is one of the environmental factors that may impact bivalve populations. Toxicity depends not only on the bioavailability of pollutants and their intrinsic toxicity, but also on the efficiency of detoxifying systems in eliminating reactive chemical species.

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Effects of low-level copper exposure on Ca^{2+}-ATPase and carbonic anhydrase in the freshwater bivalve *Anodonta anatina*

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Calcium (Ca^{2+}) in body fluids and cellular compartments is maintained within narrow concentration ranges by various regulatory processes and enzymes including plasma membrane Ca^{2+}-ATPase (PMCA) and carbonic anhydrase (CA). Ca^{2+} has key regulatory functions in cell physiology and biomineralization, and therefore any disturbance of its homeostasis may have severe pathobiochemical consequences. Some bivalent heavy metals may interfere with Ca^{2+}-homeostasis, one of them being copper. Therefore, in the freshwater bivalve *Anodonta anatina* the effects of Cu(II) at environmentally relevant concentrations were assessed on PMCA activities in kidney, gills, and digestive gland, and on CA in gills and mantle. During the first day of Cu(II) exposure at 0.35 µmol L^{-1}, PMCA in the kidney became strongly inhibited, recovering during the following days of exposure; at 0.64 µmol L^{-1}, the inhibition persisted for more than 15 days. Recovery of the enzyme function suggests that metal-detoxification is induced. In the digestive gland, inhibition was also observed though non-significantly. CA activities in mantle and gills were not significantly affected, except in the gills upon 15 days of exposure at 0.35 µmol L^{-1} Cu(II). The high accumulation of Cu(II) in the kidney and the ensuing sensitivity of PMCA in this organ may entail disturbance of the renal electrolyte- and osmo-regulations and Ca^{2+} re-uptake in *A. anatina*. This suggests that disruption of Ca^{2+}-homeostasis by Cu(II) may be involved in the general decline of freshwater mussel populations.

Keywords: freshwater bivalve; calcium homeostasis; copper; plasma membrane Ca^{2+}-ATPase; carbonic anhydrase; kidney; *Anodonta anatina*

Introduction

Over the past 50 years, a world-wide decline of freshwater mussels has been observed. Among the potential causes, human activities leading to habitat changes and chemical pollution have been suggested (Aldridge 2000; Kádár et al. 2001). Bivalves are known to accumulate persistent organic pollutants and metals which may result in biochemical-toxicological and pathophysiological effects (Rittschof and McClellan-Green 2005); several of them disturb calcium (Ca^{2+}) homeostasis

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which has been hypothesized as a possible cause in the decline of pearl mussel populations (Frank and Gerstmann 2007). Ca^{2+} plays an important role in the formation of bivalve shells representing about 80% of the total body store (Wheatly, Zanotto, and Hubbard 2002). Any disturbance in intracellular Ca^{2+} levels will impair cellular and organelle functions resulting in metabolic stress and eventual cell death (Ermak and Davies 2001). Ca^{2+} is also essential for development of glochidia and for shell regeneration.

In the cytosol, Ca^{2+} is maintained at low concentrations by tight regulation of its transport through the plasmalemma and of its release from the endoplasmic and sarcoplasmic cisterns. The plasma membranes of the epithelial cells of the gills and the digestive gland are the first barriers involved in Ca^{2+}-uptake and homeostasis (Lopes-Lima et al. 2008) (Figure 1). Ca^{2+} may diffuse passively through the plasma membrane and is actively transported against the electrochemical gradient by means of plasma membrane Ca^{2+}-ATPase (PMCA) (Ermak and Davies 2001), contributing to the maintenance of low Ca^{2+}-concentrations in the cytosol. PMCA drives the active Ca^{2+} absorption from the external surrounding (Senius and Lagerspetz 1978; Viarengo et al. 1991) and its re-uptake from the primary ultrafiltrate in the kidney (Figure 1).

Carbonic anhydrase (CA) is a ubiquitous enzyme catalyzing the reversible hydration of carbon dioxide to bicarbonate. It plays important roles in respiration, acid–base regulation, and mineralization (Henry 1996). In bivalves, it is involved in the formation of $CaCO_3$ for shell formation (Wilbur 1964).

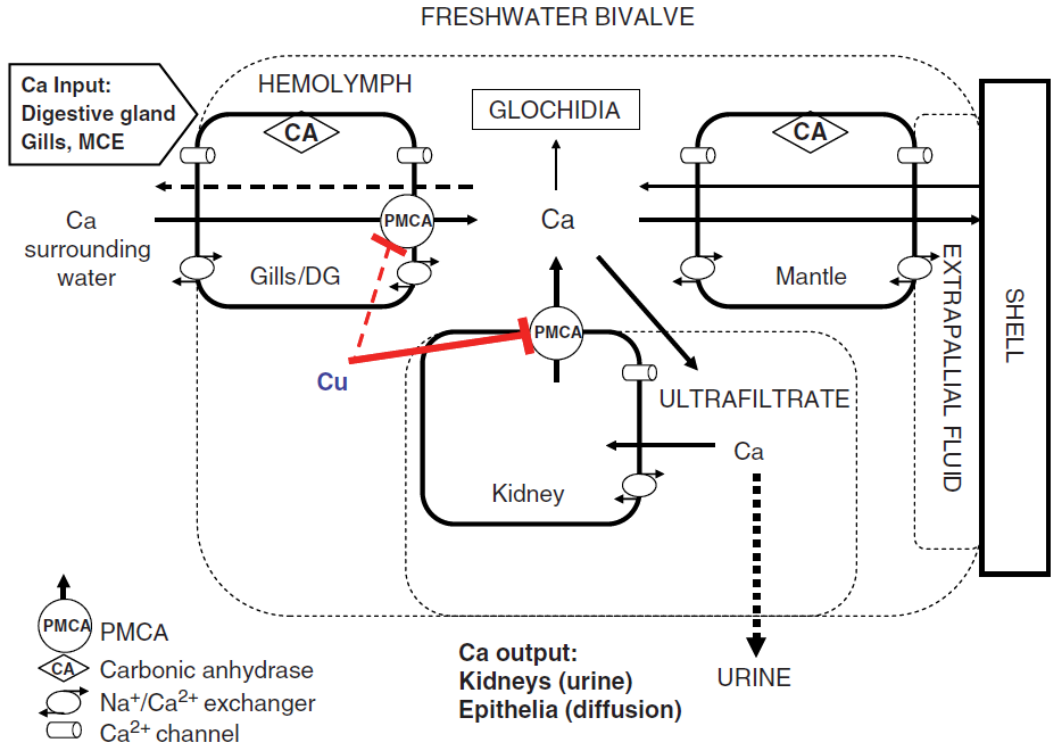

Figure 1. Schematic presentation of the Ca flow through the different compartments of a freshwater bivalve, and inhibition of PMCA by Cu in kidney and gills. MCE: mantle cavity epithelium, DG: digestive gland, PMCA: plasma membrane Ca^{2+} ATPase.

These two enzymes have been studied in bivalves and their activities were shown to be inhibited by heavy metals, such as Cu(II) and cadmium (Viarengo et al. 1993; Pattnaik, Chainy, and Jena 2007; Ngo, Gerstmann, and Frank 2010), but mostly at Cu(II) levels higher than typical for surface waters.

Cu(II) is introduced to the biosphere in large quantities by industrial activities including galvanic industries, from agricultural use as fungicide, and by corrosive release from copper roofing, rain water drainage pipes, and all kinds of electrical equipments including overland high-voltage power lines. In some countries, its release may also derive from copper mining or aeolic dispersion with dust from mine tailings. Therefore, the Cu(II) burden of the aquatic environment can be substantial; concentrations as high as 100 $\mu g\,L^{-1}$ (1.5 $\mu mol\,L^{-1}$) have been found in freshwaters affected by agriculture (Neal and Robson 2000) and up to 160 $\mu g\,L^{-1}$ (2.5 $\mu mol\,L^{-1}$) in interstitial waters of river sediments (Kalbitz and Wennrich 1998).

In the present study, the effects on the activities of PMCA and CA have been evaluated with the freshwater bivalve *Anodonta anatina* exposed to environmentally relevant copper concentrations. As freshwater bivalves are hyperosmotic, processes of osmo- and iono-regulations are more critical than for marine bivalves (Turquier 1994) and therefore the former may be more sensitive to disruption of Ca^{2+}-homeostasis. *Anodonta anatina* belongs to the Unionidae, a family widely distributed in European continental waters; it is a filter-feeding and burrowing species living at the water/sediment interface. As most other freshwater bivalves (Aldridge 2000), it is included in the red list of threatened species established in 2008 by the World Conservation Union (IUCN). The dose-effect-time response was investigated over 15 days at copper concentrations ranging from 0.2 to 1.2 $\mu mol\,L^{-1}$ (10–75 $\mu g\,L^{-1}$).

PMCA activities were determined in kidney, gills, and digestive gland, organs mainly involved in calcium uptake and homeostasis (Coimbra et al. 1993), at the same time prone to heavy metals accumulation (Duquesne and Coll 1995; Winter 1996; Kádár et al. 2001). CA activities were determined in gills and mantle.

Material and methods

Chemicals

All chemicals used for maintenance and exposure of bivalves, for sample preparation, and for biochemical assays were of analytical grade or supra pure.

Ethylene glycol-bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), ouabain, phenylmethylsulfonic fluoric acid (PMSF), sodium azide, sodium dodecyl sulfate (SDS), and tris(hydroxymethyl)-aminomethane phosphate (Tris-phosphate) were from Fluka (Schnelldorf, Germany). Adenosine-5′-triphosphate (ATP), N-(2-hydroxyethyl) piperazine N′-(2-ethanesulfonic acid) (HEPES), and tris(hydroxy-methyl)-aminomethane (Tris) were from Carl Roth (Karlsruhe, Germany), and nitric acid 65% Merck Suprapur was from VWR (Ismaning, Germany).

Animal maintenance

Adult bivalves (*A. anatina*) with shell lengths of 6.5–7.5 cm were provided by a commercial supplier (Zoo-Erlebnis, Grossefehn, Germany). The mussels were scrubbed clean of epibionts and kept at 17°C in a glass aquarium containing 1.5 L

per animal of filtered aerated artificial pond water (in mmol L^{-1}: 0.40 Ca^{2+}, 0.20 Mg^{2+}, 0.70 Na^{+}, 0.40 K^{+}, 1.35 Cl^{-}, 0.40 SO_4^{2-}, 0.20 HCO_3^{-}), prepared according to Smith et al. (2002) and renewed every second day. The bottom of the tank was covered with a 5 cm layer of glass beads (10 mm diameter) so mussels could find conditions for burying. Bivalves were fed daily with unicellular algae *Chlorella kessleri* from a culture on exponential growth phase and added to a final algal density of 2×10^5 cells mL^{-1} (1 mL of an algal suspension of 2×10^8 cells mL^{-1} L^{-1} of the tanks). Bivalves were allowed to acclimatize to laboratory conditions for a period of 3 weeks.

Exposure

The experiments were performed in 20 L glass aquaria lined with dye- and pigment-free high-density polyethylene foils, with a glass beads layer and filled with 1.5 L of artificial pond water per mussel. The bivalves were fed daily as described above. Air was bubbled continuously to ensure aeration and water column homogeneity. The following parameters were controlled daily: pH 7.2 ± 0.1, dissolved oxygen: 8.4 mg L^{-1}, water conductivity: 230 µS cm^{-1}, temperature: 17 ± 0.5°C.

The test media were renewed every day. For exposure, Cu(II) was added to the tank water using a $CuSO_4$ stock solution (31.3 mmol L^{-1}). The Cu(II) concentrations were regularly determined in water samples collected just after renewal and 24 h later. Three 50 mL water samples were taken in low-density polyethylene bottles previously acid-washed (10% nitric acid) for 10 days and rinsed with bi-distilled water. The samples were acidified to pH 3 with concentrated HNO_3 (65%, Merck Suprapur) and frozen at −20°C. Cu(II) concentrations were determined by inductively coupled plasma mass spectrometry (detection limit 0.5 µg L^{-1}, 0.008 µmol L^{-1}).

Preliminary experiments were performed with three bivalves each exposed to Cu(II) at concentrations of 0.26, 0.54 and 1.15 µmol L^{-1} for 4 days, to find the relevant concentration range. The main experiment was conducted at 0.35 and 0.64 µmol $L^{-1}$with groups of 12 randomly assigned bivalves each over a total length of 15 days. On days 0 (control), 4, 7, and 15, three mussels were randomly taken and dissected for tissue sample preparation.

Tissue sample preparation for enzymatic analysis

Tissue samples of gills, mantle, and digestive gland were prepared separately for each mussel, while the kidney samples were pooled due to the low mass of the organ. The dissected tissues were suspended in 6 vol ice-cold HEPES buffer (10 mmol L^{-1} HEPES, 250 mmol L^{-1} sucrose, 1 mmol L^{-1} EDTA, 1 mmol L^{-1} phenylmethylsulfonic fluoric acid (PMSF) as protease inhibitor, adjusted to pH 7.4 with HCl, 1 mol L^{-1}). Tissues were homogenized by means of a motor-driven Teflon pestle homogeniser with 30 up-and-down strokes. The resulting homogenates were centrifuged at 2000 *g* (10 min, 4°C), the supernatants (S2000) were diluted (12 mL ice-cold HEPES buffer per gram tissue wet weight) and centrifuged at 10,000 *g* (20 min, 4°C). The supernatants (S10000) were ultracentrifuged at 75,600 *g* (60 min, 4°C), and the final pellets were suspended in 6 vol ice-cold Tris-HCl buffer (25 mmol L^{-1} Tris, 1 mmol L^{-1} PMSF, adjusted to pH 7.4 with HCl, 1 mol L^{-1}) for PMCA determination. CA activities were determined in the S2000 supernatant of

gills and mantle. All samples were frozen in liquid nitrogen and stored at −80°C until analyses were carried out, not later than a week after sampling.

Protein was determined according to Bradford (1976), using bovine serum albumin as standard.

PMCA activity assay

PMCA activity was determined as released inorganic phosphate (Chifflet et al. 1988), quantified by spectrophotometry of the ammonium molybdate complex at 850 nm (Spectrophotometer Unicon, Kontron 930). The reaction medium contained 100 mmol L^{-1} NaCl, 20 mmol L^{-1} KCl, 0.2 mmol L^{-1} ouabain, 25 mmol L^{-1} Tris, 0.5 mmol L^{-1} sodium azide as inhibitor of mitochondrial ATPase activities, 4 mmol L^{-1} Na_2ATP, adjusted to pH 7.4. Samples of the membrane fractions (suspensions of the 75,600 *g* pellet) of gills, kidney, and digestive gland (20, 70, and 60 µg protein, respectively) were incubated at 1 mL final volume for 20 min in a shaking water bath at 39°C, with or without addition of Ca^{2+} and EGTA as Ca-chelating agent (Senius and Lagerspetz 1978). The reaction was stopped by addition of 400 µL sample to 400 µL of a 12% solution of SDS. Blanks were prepared in the same way except that the tissue samples were added after the SDS-dilution step. PMCA activity, expressed in micromoles of P_i released per milligram protein and minute, was determined as the difference between the ATPase activity in the presence of 10 mmol L^{-1} $CaCl_2$, and the ATPase activity without Ca and in the presence of EGTA (2 mmol L^{-1}).

CA assay

CA activity was determined in the S2000 supernatant of gills and mantle by measuring the decrease in pH (Vitale et al. 1999). The reaction medium consisted of 10 mmol L^{-1} Tris-phosphate, 75 mmol L^{-1} sucrose, 225 mmol L^{-1} mannitol, adjusted to pH 7.4 with HCl (1 mol L^{-1}). Enzyme activity was assessed with 7.5 mL of reaction medium, 0.1–0.2 mL of gill and mantle tissue homogenates, and 1 mL CO_2-saturated distilled water at 2.5°C. The pH drop was followed over 60 s using a pH-meter (Multi 340i, Wissenschaftlich Technische Werkstätten, Weilheim, Germany). Linear regressions of pH *versus* time were plotted, the slope representing the catalyzed reaction rate (b_{cat}). The non-catalyzed reaction rate ($b_{non\text{-}cat}$) was determined in the same way except that 0.1–0.2 mL HEPES buffer was used instead of tissue homogenate. The specific CA activity was calculated as $(b_{cat}/b_{non\text{-}cat}-1)$ mg^{-1} protein.

Statistical analyses

Data distributions were not normal, so statistical analysis for comparison of the enzymatic activities between treated and control mussels was done by the non-parametric Mann-Whitney U test (Statistica, StatSoft France 2001, data analysis software, version 6, Maison-Alfort, France). All data are reported as mean ($n = 3$) ± standard error (SE. Differences were considered as significant when $p < 0.05$.

Results

The mean PMCA activity (±SE) in control animals (Figure 2) was 0.087 ± 0.023 µmol P_i mg^{-1} protein min^{-1} in kidney, 0.45 ± 0.13 in gills, and 0.095 ± 0.03 in digestive gland. Cu(II) inhibited the PMCA activities in the kidney significantly ($p < 0.05$) upon 4 days of exposure at all concentrations tested, i.e. 0.26–1.15 µmol L^{-1}. At 0.35 µmol L^{-1}, recovery was observed at day 7 and significant elevation at day 15 (Figure 2a). At 0.64 µmol L^{-1}, PMCA activity was inhibited ($p < 0.05$) by about 20% over the whole exposure period (Figure 2b).

In the gills, a similar profile of response was observed at 0.35 µmol L^{-1} Cu(II) (Figure 2a), with a significant decrease upon 4 days of exposure and recovery thereafter. In the digestive gland, PMCA behaved similarly but recovery was faster.

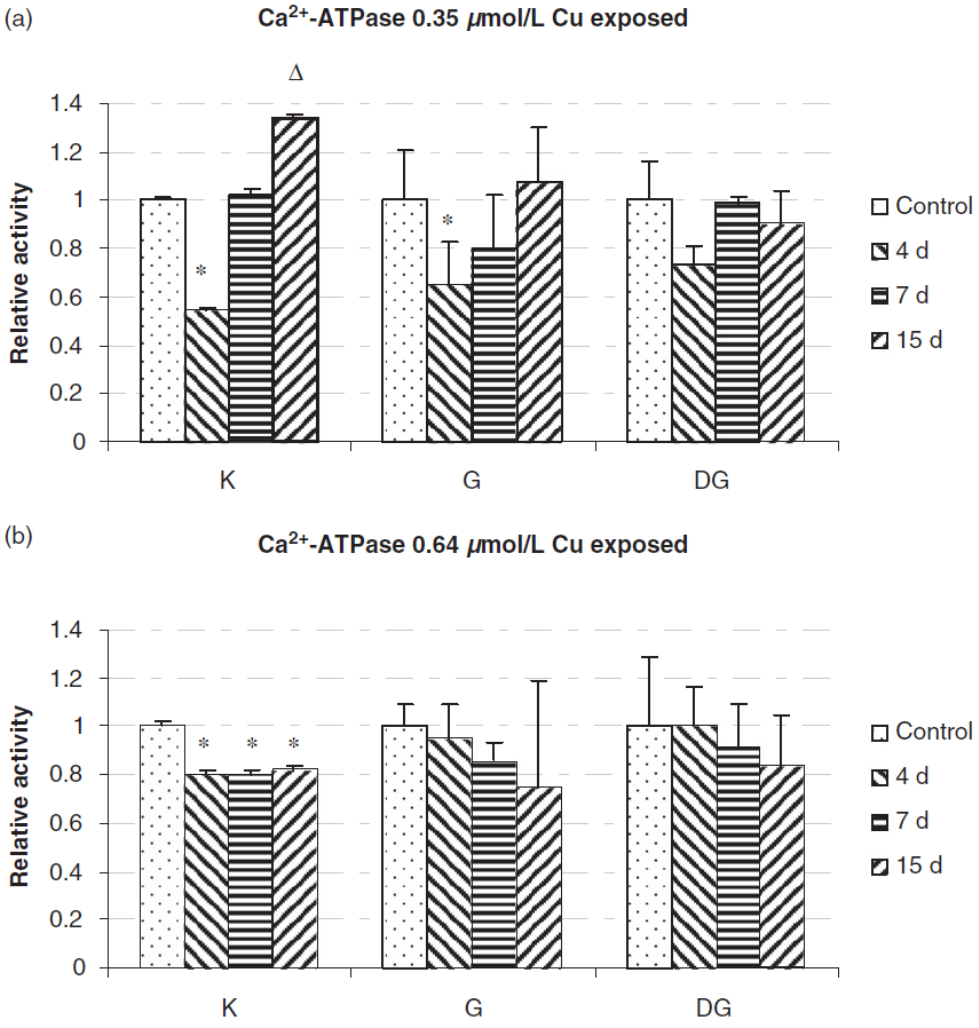

Figure 2. PMCA activity in kidneys (K), gills (G), and digestive gland (DG) of *A. anatina* upon 0 (control), 4, 7, 15 days of exposure to 0.35 µmol L^{-1} (a) and 0.64 µmol L^{-1} Cu (b). Means of results, ($n = 3$) ± SE, are presented as ratios of enzymatic activity at the respective Cu concentration and time *vs.* control (mentioned on the graph).
Notes: For K, the error bars reflect the variability of the analytical determination of the pooled tissue sample, for G and DG the biological variation between animals.
* = significantly lower than control, Δ = significantly higher than control (U test $p < 0.05$).

At 0.64 µmol L^{-1} Cu(II), the PMCA activities both in the gills and the digestive gland exhibited a continuously declining trend, although statistically not significant.

CA activity in the gills of controls averaged 2.4 units (U) mg^{-1} protein, and 1.8 U mg^{-1} protein in the mantle. No significant effect of Cu(II) was noted, except for a slight but significant inhibition at 0.35 µmol L^{-1} Cu upon 15 days of exposure (data not shown).

Overall, PMCA of the kidney was affected by Cu(II) at all concentrations tested, strongest upon 4 days of exposure. Return to basal level within 7 days at the lower Cu(II) concentration indicated considerable adaptive ability, most likely by means of a detoxification system efficient at low copper concentrations. At higher Cu(II) concentration (0.64 µmol L^{-1}), no recovery was noted.

Discussion

The basal activities of PMCA determined in the gills of *A. anatina* control animals were of the same order of magnitude as found for other freshwater bivalves, albeit being much higher than in marine bivalves (Table 1). Freshwater organisms are hyperosmotic, and therefore the osmotic pressure resulting from the concentrations gradient between hemolymph and surrounding aqueous compartment may lead to water uptake by osmosis ion loss by diffusion (Turquier 1994) which is compensated by active uptake and renal re-absorption of electrolytes. So far, no data have been

Table 1. PMCA activities (µmol P_i L^{-1} mg^{-1} protein min^{-1}) in different tissues of freshwater and marine organisms.

Plasma membrane Ca^{2+}-ATPase			
Species	Activity µmol P_i L^{-1} mg^{-1} protein min^{-1}	Tissues	References
Freshwater bivalve			
Lamellidens marginalis	0.41	G	Pattnaik, Chainy, and Jena 2007
Anodonta cygnea	0.58	G	Pivovarova and Lagerspetz 1996
Anodonta anatina	0.45	G	Present study
	0.095	DG	
	0.087	K	
Marine bivalve			
Mytilus galloprovincialis	0.093	G	Viarengo et al. 1993
	0.12	G	Burlando et al. 2004
	0.1	G	Viarengo et al. 1996
	0.1	G	Viarengo et al. 1991
Freshwater Fish			
Oreochromis niloticus	0.08	G	Üner, Oruç, and Sevgiler 2005
	0.21	Muscle	
Anguilla rostrata	0.76	G	Flik, Wendelaar Bonga, and Fenwick 1983

Note: G: gills, DG: digestive gland, K: kidneys.

published on PMCA activities in the kidney and in the digestive gland of *A. anatina* and other species; it is noteworthy that the basic activities in these organs are only about one-fifth relative to the gills. Kidneys play an essential role in filtration and re-absorption of electrolytes, water, and low-molecular weight organic molecules from the primary ultrafiltrate, as indicated in the scheme (Figure 1). In *Anodonta*, the water uptake is compensated by a daily output of urine corresponding to about 65% of extracellular fluid, i.e. 24% of mussel weight. Kidneys take a major position in Ca^{2+} homeostasis by active re-uptake from the filtrate (Robertson 1964).

In *A. anatina*, CA activities are about 1.5 to two-fold higher in the gills than in the mantle, as found by Ngo, Gerstmann, and Frank (2010) (Table 2). They are lower as compared to marine invertebrates and appear to be particularly active in fish.

The inhibitory effect of Cu(II) on PMCA activity observed in this study is consistent with the results of others with marine bivalves. Inhibition of the enzyme activity in gills and in the digestive gland of *Mytilus galloprovincialis* by Cu(II) at 0.6 µmol L^{-1} has been described by Viarengo et al. (1996) and Burlando et al. (2004), followed by a return to baseline level within 7 and 6 days respectively, similar to our findings.

The Ca^{2+}-ATPase activity was shown to be inhibited *in vitro* by thiol-binding compounds (Viarengo et al. 1991). Cu(II) has high affinity to thiol groups, and its

Table 2. CA activities in freshwater and marine organisms evaluated by measurement of pH decrease caused by CO_2 enzymatic hydration.

Carbonic Anhydrase				
Species	Activity U mg^{-1} protein	Tissues	Fractions	References
Freshwater bivalve				
	2.1	G	Cytoplasmic	Ngo, Gerstmann, and Frank 2009
Anodonta anatina	1.0	M	Cytoplasmic	
	2.4	G	S2000 *g*	Present study
	1.76	M	S2000 *g*	
Marine bivalve				
Pinctada margaritifera	4.5 < G < 17.3	G	S750 *g*	Rousseau et al. 2003
	3	M		
Marine crustacean				
Chasmagnathus granulatus	11.8	G posterior	S2000 *g*	Vitale et al. 1999
	0.5	G anterior	Cytosolic	Genovese et al. 2005
	2	G anterior	Microsomal	
	3.5	G posterior	Cytosolic	
	2	G posterior	Microsomal	
Freshwater fish				
Anguilla anguilla	186	G	Cytoplasmic	Lionetto et al.1998
	55	Intestine	Cytoplasmic	
	15	Intestine	Membrane	
Marine cnidarian				
Aiptasia pulchella	1.8	Whole body	S900g	Weis 1991

Note: G: gills, M: mantle; S: supernatant.

binding to thiol functions in PMCA may be responsible for the inhibition. Yet, oxidative stress has also been hypothesized as causative mechanism (Viarengo et al. 1996). Cu(II) can generate reactive oxygen species, and inhibition of PMCA by free radical damage has been demonstrated (Rohn, Hinds, and Vincenzi 1993). With *Mytilus* sp., 3 days exposure to 40 µg L^{-1} Cu(II) (0.63 µmol L^{-1}) resulted in 65% metal-binding to cytosolic proteins, such as metallothioneins, the synthesis of which increased in response to Cu(II) (Viarengo and Nott 1993). Besides direct PMCA inhibition, a mechanism which should also be considered to affect enzymatic activity is gene down-regulation of PMCA expression. The present study design does not allow for discrimination between the two mechanisms, but gene down-regulation of PMCA of the digestive gland of *M. galloprovincialis* was not observed upon 4 days of Cu(II) exposure at 0.6 µmol L^{-1} (Burlando et al. 2004).

Small effects on CA activity were observed in the mantle and the gills of *A. anatina* exposed to Cu(II) between 0.26 and 1.15 µmol L^{-1}. With cytosolic fractions of marine invertebrates inhibition of CA activities takes place only at much higher Cu(II) concentrations: the IC_{50} for CA in cytosolic fraction of gills was 3.8 µmol L^{-1} in *Chasmagnathus granulate* (Vitale et al. 1999), no effect being registered at 0.5 µmol L^{-1} Cu(II). In the cytosol from gills of *Callinectes sapidus* and *Carcinus maenas*, 95% inhibition was noted in the range between 16.7 µmol L^{-1} and 5 mmol L^{-1} Cu(II) (Skaggs and Henry 2002). It can be expected that also *in vivo* exposure of *A. anatina* to such high Cu(II) concentrations will have an inhibitory effect on CA activity. On the other hand, an increase in CA activity in the mantle of *Anodonta cygnea* exposed *in vitro* to 100 µmol L^{-1} Cu (II) was shown, considered as secondary interference resulting from direct inhibition of the transport system (Antunes et al. 2002).

In bivalves including *Anodonta*, the kidney is the main site for accumulation of the metals Cd, Zn, and Cu (Duquesne and Coll 1995; Winter 1996; Kádár et al. 2001), to reach critical concentrations which may induce injury of epithelial cells. Metals are first bound to cytosolic proteins and then sequestered into the lysosomal system. In the kidneys of mussels, lysosomes have a long half life and can accumulate high concentrations of heavy metals (Viarengo and Nott 1993). This explains the particular susceptibility of the organ to metal toxicity and alteration of PMCA, in addition to the relatively low basal activity of the enzyme in the kidneys being only one-fourth compared to the gills.

A decrease in Ca^{2+} resorption in the gills and the gastrointestinal tract and of renal re-uptake due to inhibition of PMCA by Cu(II) may lead to continuous under-supply of Ca, which may also affect muscle contraction and nerve conduction, in addition to biomineralization and shell formation in mussels and glochidia. Such derangement of homeostasis may also affect other Ca^{2+} signaling pathways.

To conclude, the present study points out that PMCA of the kidney of the freshwater mussel *A. anatina* is inhibited by Cu(II) at environmental levels, in the gills to a lesser extent. At low level (0.35 µmol L^{-1}) this disturbance of the PMCA may be overcome after a few days, but at concentrations of 0.64 µmol L^{-1} and above, inhibition persists.

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6 Article 2: Copper effects on Na^+/K^+-ATPase and H^+-ATPase in the freshwater bivalve *Anodonta anatina*

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ABSTRACT

Effects of copper on the activities of the cell plasma membrane H^+-ATPase and Na^+/K^+-ATPase of the freshwater mussel *Anodonta anatina* were assessed after 4, 7, and 15 days of exposure to Cu^{2+} at the environmentally relevant concentration of 0.35 µmol L^{-1}. The H^+-ATPase was measured in the mantle, and the Na^+/K^+-ATPase in the gills, the digestive gland and the mantle. The Na^+/K^+-ATPase activities showed significant inhibition upon 4 days in the gills (72 %) and in the digestive gland (80 %) relative to control mussels: in the mantle, no inhibition of the enzyme activities was noted. Incipient recovery of the Na^+/K^+-ATPase activity was registered after 7 and 15 days in the gills and the digestive gland yet, in the gills not returning to basal level, within 15 days. H^+-ATPase activity remained unaffected by Cu^{2+} at the test concentration.

Key words: calcium homeostasis, copper, Na^+/K^+-ATPase, H^+-ATPase, *Anodonta anatina*

INTRODUCTION

Na^+/K^+-ATPase and H^+-ATPase are important enzymes mainly involved in osmoregulation and acid-base balance. H^+-ATPase plays as proton pump also an essential role in shell synthesis by pH control, a decisive factor in the biomineralization process (Machado *et al.*, 1989). The plasma membrane Na^+/K^+-ATPase and H^+-ATPase maintain the sodium and

proton gradients necessary for the calcium antiporter system. Although calcium uptake is mainly achieved by active flux generated by Ca^{2+}-ATPase. Facilitated diffusion through Ca^{2+}/Na^{+} or Ca^{2+}/H^{+} antiporters are other important ways of entry of calcium into the cell (Wheatly *et al.*, 2002).

Perturbation of calcium homeostasis has been hypothesized as a possible cause in the decline of the pearl mussel populations (Frank and Gerstmann 2007). Another freshwater mussel belonging to the Unionoida order known to bioaccumulate copper is *Anodonta anatina* (Cossu *et al.*, 1997; Nugroho and Frank, 2011). Inhibition of Ca^{2+}-ATPase activity in the plasma membrane was shown in *A. anatina* exposed to 0.35 µmol L^{-1} of copper (Santini *et al.*, 2011a).

Both H^{+}-ATPase and Na^{+}/K^{+}-ATPase of the plasma membrane are metal-sensitive enzymes with functional SH groups. Due to its thiol affinity, copper is likely to affect the activity of these enzymes (Viarengo *et al.*, 1991) and calcium homeostasis could thus be indirectly affected by copper; alteration of Na^{+} and H^{+} gradients will entail decreased efficacy of Ca^{2+}/Na^{+} or Ca^{2+}/H^{+} antiporters. Therefore, it seemed interesting to evaluate the activities of both enzymes in mussels in order to determine their sensitivity to this transition metal which nowadays is widely found in the environment at elevated levels.

Therefore, in the present study the effects on the activities of Na^{+}/K^{+}-ATPase and H^{+}-ATPase have been evaluated with *A. anatina* mussels exposed to copper ions (Cu^{2+}) at 0.35 µmol L^{-1}. The H^{+}-ATPase activity was measured in the mantle, which plays the dominant role in the generation, growth and repair of the shell. Na^{+}/K^{+}-ATPase activity was determined in the mantle, the gills, and the digestive gland, organs which are mainly involved in calcium uptake and homeostasis (Coimbra *et al.*, 1993).

MATERIAL AND METHODS

Chemicals

All chemicals used for maintenance and exposure of bivalves, for sample preparation, and the biochemical assays were of analytical grade.

Ethylene glycol-*bis*(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), ouabain, phenylmethylsulfonic fluoric acid (PMSF), sodium azide, sodium dodecyl sulphate (SDS), N-ethylmaleimide (NEM), and sodium orthovanadate were from Fluka (Schnelldorf, Germany). Adenosine-5'-triphosphate (ATP), N-(2-hydroxyethyl) piperazine N'-(2-ethanesulfonic acid)

(HEPES), and tris-(hydroxymethyl)-aminomethane (Tris) were from Carl Roth (Karlsruhe, Germany).

Animal maintenance and copper exposure

Mussel maintenance was described in detail in a previous article (Santini *et al.,* 2011a). Briefly, adult mussels *A. anatina* with shell lengths of 6.5 - 7.5 cm were kept at 17° C in a glass aquarium containing 1.5 L per animal of filtered and aerated artificial pond water (in mmol L^{-1}: 0.40 Ca^{2+}, 0.20 Mg^{2+}, 0.70 Na^{+}, 0.05 K^{+}, 1.35 Cl^{-}, 0.20 SO_4^{2-}, 0.20 HCO_3^{-}). The water was renewed every 48 h. The bottom of the tank was covered with a 5-cm layer of glass beads (about 10 mm diameter), so the mussels could find conditions for burying. They were fed daily with unicellular algae *Chlorella kessleri* from a culture in the exponential growth phase, added to a final algal density of 2×10^5 cells mL^{-1}. Animals were acclimatized for 3 weeks in September before any experiment.

For the copper (Cu^{2+}) exposure, animals were placed in a 20 L aquarium lined with dye- and pigment-free high-density polyethylene foils, with a glass beads layer and filled with 1.5 L per mussel of experimental media (pH 7.2 temperature: 17 ± 0.5° C). The bivalves were fed daily as described above and the test media were renewed every day. Concentration of 0.35 µmol L^{-1} Cu^{2+} was chosen for exposure test. The concentration was controlled by inductively-coupled plasma mass spectrometry, with a limit of Cu detection of 0.5 µg L^{-1} = 8 nmol L^{-1}. The test was conducted with 12 mussels randomly assigned by groups of 3 to each treatment. At the beginning (time 0) a group of 3 mussels unexposed to Cu^{2+} was used as control. A group of 3 mussels was taken from the aquarium after 4 d, 7 d, and 15 d of Cu^{2+} exposure. Mussels used on each treatment were dissected separately for biochemical analyses, i.e. 3 replicates per treatment.

Tissue sample preparation for enzymatic analysis

Tissue samples of the gills, the mantle, and the digestive gland were prepared. The samples were suspended in 6 vol. ice-cold HEPES buffer (10 mmol L^{-1} HEPES, 250 mmol L^{-1} sucrose, 1 mmol L^{-1} EDTA, 1 mmol L^{-1} phenylmethylsulfonic fluoric acid (PMSF) as protease inhibitor, adjusted to pH 7.4 with HCl, 1 mol L^{-1}) and homogenized by means of a motor-driven Teflon pestle homogeniser with 30 up-and-down strokes. The resulting homogenates were centrifuged at 2000 *g* (10 min, 4° C), the supernatants (S2000) were

diluted with 12 mL ice-cold HEPES buffer per g tissue wet weight and centrifuged at 10000 *g* (20 min, 4° C). The supernatants (S10000) were ultracentrifuged at 75600 *g* (60 min, 4° C), and the final pellets were suspended in 6 vol. ice-cold Tris-HCl buffer (25 mmol L^{-1} Tris, 1 mmol L^{-1} PMSF, adjusted to pH 7.4 with HCl, 1 mol L^{-1}) for plasma membrane Na^+/K^+-ATPase test. Plasma membrane H^+-ATPase activity was determined in the S2000 supernatant used as mantle extract. All samples were frozen in liquid nitrogen and stored at –80° C until analyses were carried out, not later than one week after sampling.

Protein concentrations were determined according to Bradford (1976), using bovine serum albumin as standard.

Plasma membrane H^+-ATPase and Na^+/K^+-ATPase activity assay

Plasma membrane H^+-ATPase and Na^+/K^+-ATPase activities were determined by measurement of inorganic phosphate released (Chifflet *et al.*, 1988) and quantified by spectrophotometry of the ammonium molybdate complex at 850 nm (Spectrophotometer Unicon, Kontron 930).

The H^+-ATPase reaction medium contained (final concentrations) 6 mmol L^{-1} $MgSO_4$, 50 mmol L^{-1} hepes, 0.5 mmol L^{-1} sodium orthovanadate (P-ATPase inhibitor), 0.5 mmol L^{-1} sodium azide as inhibitor of mitochondrial ATPase activities, 4 mmol L^{-1} Na_2ATP, adjusted to pH 7.4. The mantle extract (160 µg protein) was incubated at 1 mL final volume for 60 min in a shaking water bath at 25° C, with or without addition of NEM as H^+-ATPase inhibitor (Lin and Randall, 1993). The reaction was stopped by addition of 400 µL sample to 400 µL of a 12 % solution of sodium dodecyl sulphate (SDS). Blanks were prepared in the same way except that the tissue samples were added after the SDS-dilution step. H^+-ATPase activity, expressed in micromoles of P_i released per mg protein and min, was determined as the difference between the ATPase activity in the presence of 10 mmol L^{-1} NEM and the ATPase activity without NEM.

The Na^+/K^+-ATPase reaction medium contained (final concentrations) 100 mmol L^{-1} NaCl, 0.5 mmol L^{-1} EGTA, 5 mmol L^{-1} $MgCl_2$, 25 mmol L^{-1} Tris, 0.5 mmol L^{-1} sodium azide as inhibitor of mitochondrial ATPase activities, 4 mmol L^{-1} Na_2ATP, adjusted to pH 7.4. Samples of the membrane fractions (suspensions of the 75600 g pellet) of the gills, the digestive gland, and the mantle (20, 70, 60 µg protein, respectively) were incubated at 1 mL final volume for 20 min in a shaking water bath at 37° C, with or without addition of K^+ and ouabain as Na^+/K^+-ATPase inhibitor (Lionetto *et al.*, 1998). The reaction was stopped by

addition of 400 µL sample to 400 µL of a 12 % solution of sodium dodecyl sulphate (SDS). Blanks were prepared in the same way except that the tissue samples were added after the SDS-dilution step. Na^+/K^+-ATPase activity, expressed in micromoles of P_i released per mg protein per min, was determined as the difference between the ATPase activity in the presence of 20 mmol L^{-1} KCl, and the ATPase activity without KCl and in the presence of ouabain (1 mmol L^{-1}).

Statistical analyses

Data distributions were not normal, so statistical analysis for comparison of the enzymatic activities between treated and control mussels, was done using the non-parametric Mann-Whitney test (Statistica, StatSoft France 2001, data analysis software, version 6, Maison-Alfort, France). All data are reported as mean (n = 3) ± standard deviation (S.D.). Differences were considered as significant when $p < 0.05$.

RESULTS

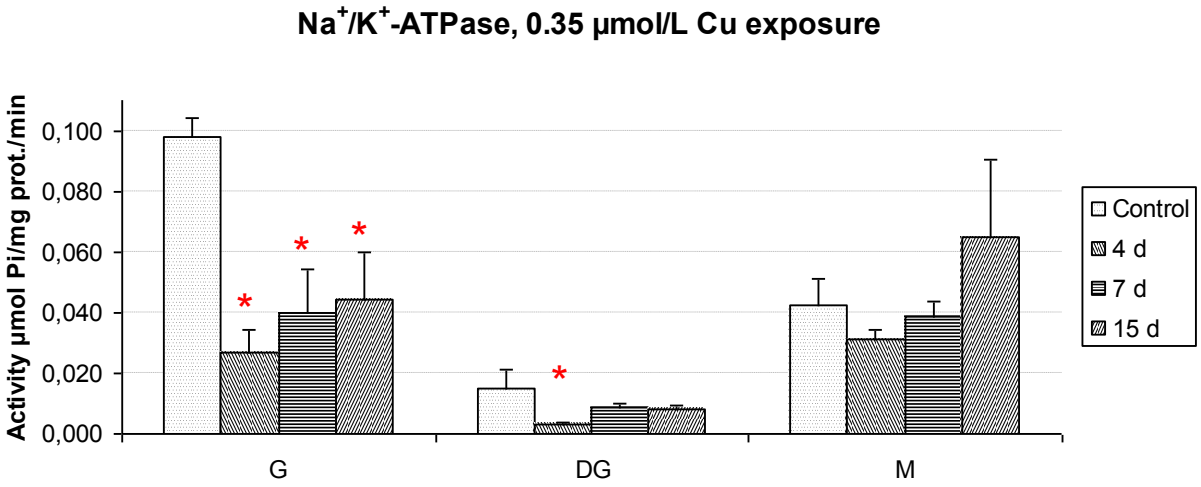

Fig. 1: Na^+/K^+-ATPase activity determination in September in the gills (G), the digestive gland (DG), and the mantle (M) of *A. anatina* upon 0 (control), 4, 7, 15 days of exposure to 0.35 µmol L^{-1} Cu^{2+}. Means of results, n = 3 ± SD, are presented as enzymatic activity in µmol P_i/mg protein/min. *= significantly lower than control (Mann-Whitney test two sided test α = 0.05).

ATPase activities were determined in September for copper exposure test. The mean Na^+/K^+-ATPase activity (± SD) in control animals (fig. 1) was 0.098 ± 0.006 µmol P_i/mg protein/min in the gills, 0.015 ± 0.007 in the digestive gland, and 0.042 ± 0.009 in the mantle, and the activity of H^+-ATPase 0.002 ± 0.0012 in the mantle (fig. 2).

A significant inhibition of Na^+/K^+-ATPase activities (fig. 1) compared to controls was observed upon 4 days of exposure to 0.35 µmol L^{-1} of Cu^{2+} in the gills (72 % inhibition) and the digestive gland (80 % inhibition). A same pattern of decrease was observed in the mantle, but the inhibition recorded at day 4 (26 % inhibition) was not significant compared to controls. A partial recovery was observed at day 7 in the digestive gland. Despite a trend of increased activities from 4 d to 15 d, no complete recovery was observed upon the 15 days of exposure in the gills, which still showed 54 % of inhibition at the end of test.

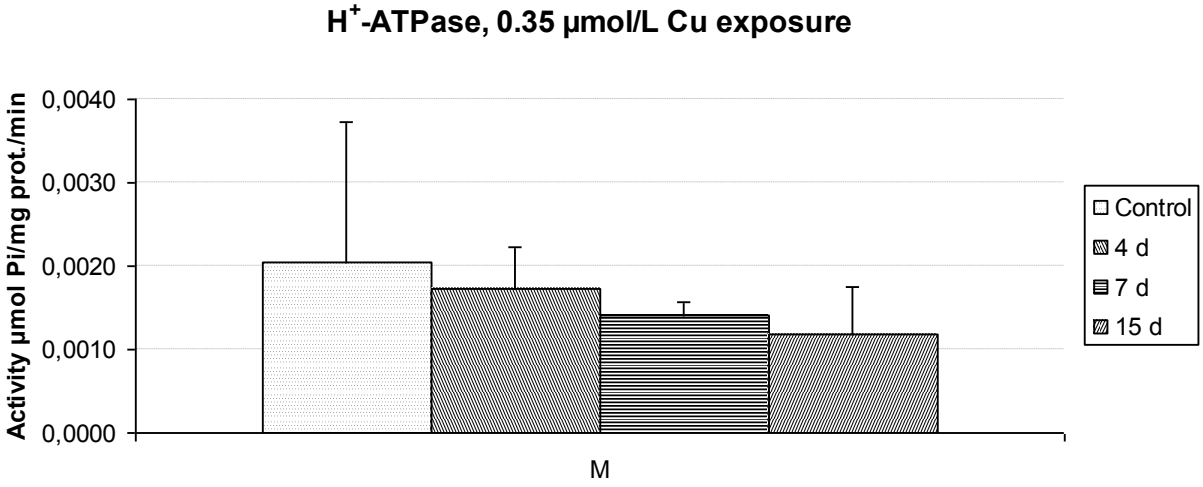

Fig. 2: H^+-ATPase activity determination in September in mantle (M) of *A. anatina* upon 0 (control), 4, 7, 15 days of exposure to 0.35 µmol L^{-1} Cu^{2+}. The Means of results, n = 3 ± SD, are presented as enzymatic activity in µmol P_i/mg protein/min. *= significantly lower than control (Mann-Whitney test two sided test $\alpha = 0.05$).

No significant effect of copper on H^+-ATPase activity was noted (fig. 2) in the mantle of mussels upon 15 days of exposure. The H^+-ATPase activities exhibited a continuously declining trend, although statistically not significant due to high variability between mussels.

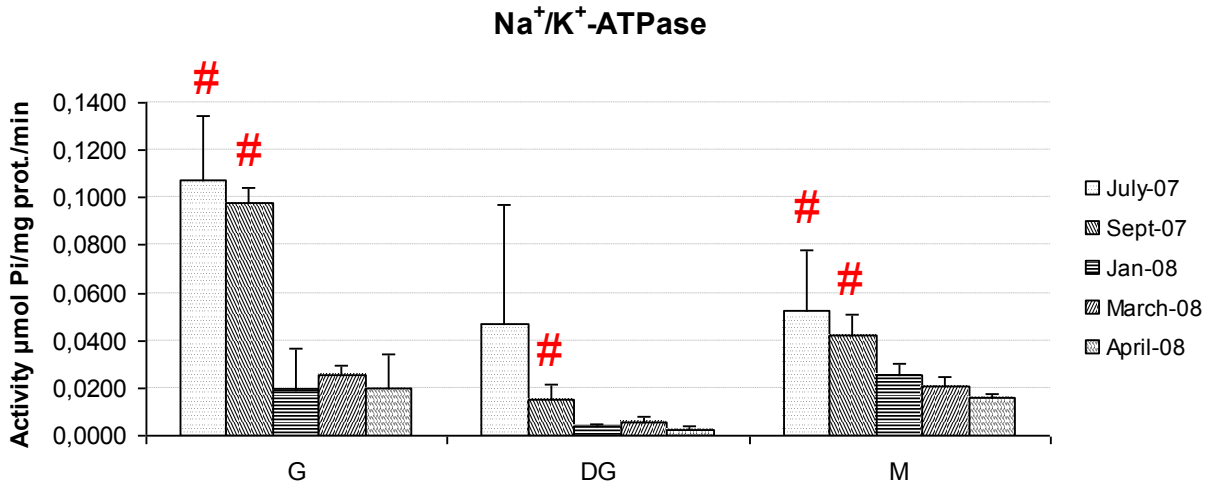

Fig. 3: Na^+/K^+-ATPase basal activity in gills (G), the digestive gland (DG), and mantle (M) of *A. anatina* in July, September, January, March, and April 2007 / 2008. Means of results, n = 3 ± SD, are presented as enzymatic activities in µmol P_i/mg protein/min. # = significantly higher than in January, March, and April (Mann-Whitney test two sided test $\alpha = 0.05$).

These basal activities (expressed in micromoles of P_i released per mg protein per min) varied in consistent manner in function of seasons. The activities were the highest in July and September compared to the values measured in January, March and April (fig. 3). In July, the Na^+/K^+-ATPase activity expressed as µmol P_i mg/protein/min was 0.107 ± 0.027 in the gills, 0.047 ± 0.050 in the digestive gland, and 0.053 ± 0.025 in the mantle (fig. 3). The H^+-ATPase of the mantle did not significantly differed over the study period (from July to April) with 0.0025 ± 0.001 µmol P_i/mg protein/min in July (fig. 4). Minimal values of Na^+/K^+-ATPase and H^+-ATPase activities were observed in April in every tissue with a strong decrease by half and ten of the July values, depending on organ.

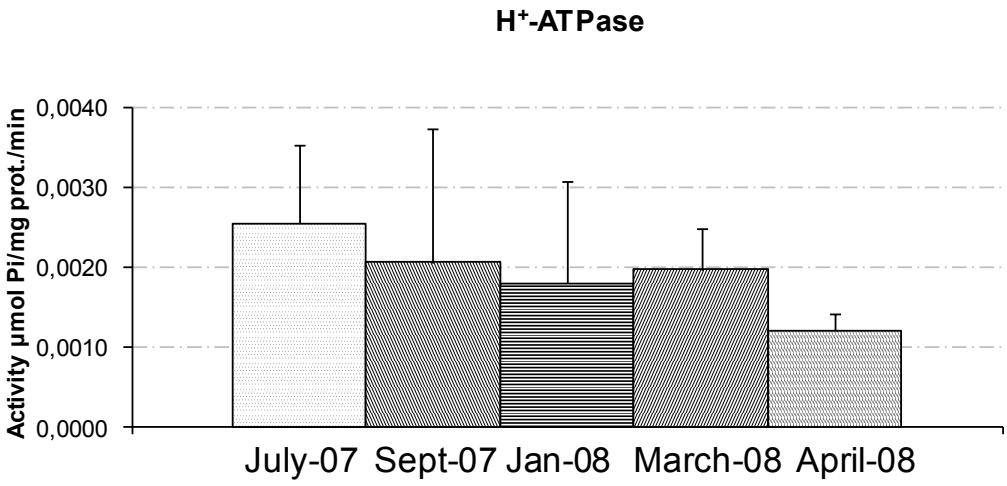

Fig. 4: H^+-ATPase basal activity in mantle of *A. anatina* in July, September, January, March, and April 2007 / 2008. Means of results, n = 3 ± SD, are presented as enzymatic activity in µmol P_i/mg protein/min. # = significantly higher than in January, March, and April (Mann-Whitney test two sided test $\alpha = 0.05$).

DISCUSSION

In the general decline of freshwater bivalve observed (Lydeard *et al.*, 2004), the Unionoida taxon is particularly endangered. Alteration of ionic transport systems as Na^+/K^+-ATPase and H^+-ATPase by copper is poorly investigated in the freshwater mussel *Anodonta anatina* belonging to this taxon. Copper, a metal extensively used in industry, building sector, public energy supply and transportation systems, and agriculture was studied in the present work as a potential factor implicated in this complex phenomenon of decline.

The magnitude of mean basal activities of Na^+/K^+-ATPase in the different organs studies were gills > mantle > digestive gland. The high activity measured in the gills reflects the specialization and implication of this organ in ionoregulation. The gills are known to play a major role in active uptake of mineral ions from surrounding water (Turquier, 1994). The mantle assumes the ionic balance between haemolymph and the extrapallial fluid necessary for shell growth or calcium body reabsorption (Coimbra *et al.*, 1993).

The basal activities of Na^+/K^+-ATPase determined in the gills of control *A. anatina* mussels were in the same order of magnitude as found by Lagerspetz and Senius (1979) in *A. cygnea*, and in the freshwater fish *Bidyanus bidyanus* (Alam and Frankel, 2006) (Table 1). The hyperosmotic status of *A. anatina* and *A. cygnea* is expressed by the high basal activities

of the enzyme. In comparison, the marine mussel *Mytilus galloprovincialis* showed a Na^+/K^+-ATPase activity threefold lower than *A. anatina* (Viarengo *et al.*, 1996).

There is little information in the scientific literature on the H^+-ATPase activity in the mantle of mussels. Investigations on the enzyme activity expressed as µmol P_i mg/protein/min (table 1) were carried out mostly in the gills of crustacean 0.023 and fish 0.003 to 0.041. In the present work the mean H^+-ATPase activity 0.0025 ± 0.001 found in the mantle of *A. anatina* was lower.

In this study, seasonal fluctuations of Na^+/K^+-ATPase basal activity were observed with clear and significantly elevated levels in July and September. It is known that the freshwater mussel shows seasonal changes in calcification (Taskinen, 1998). Our results correspond to the cycle of calcification found in *Anodonta cygnea* a close species. The growth of clam shells and the level of glycosaminoglycans know to be important for biomineralisation, increased both in summer with a maximum in July and August (Taskinen, 1998; Moura *et al.*, 2000). These fluctuations, parallel to the calcification cycle, suggest an indirect implication of Na^+/K^+-ATPase in the calcium transport by the Ca^{2+}/Na^+ antiporter. H^+-ATPase activities showed not statistically significant comparable seasonal fluctuations.

Na^+/K^+-ATPase of the gills and the digestive gland were strongly affected by exposure to 0.35 µmol L^{-1} of Cu^{2+} upon 4 days of exposure, to a lesser extent in the mantle. Therefore, even though our results do not provide direct evidence of the Ca^{2+}/Na^+ antiporter inhibition, a decrease of calcium ionic transport can be assumed. This is consistent with the results observed by Viarengo *et al.* (1996) in *M. galloprovincialis* exposed 4 d to 0.6 µmol L^{-1} of Cu^{2+}. In *A. anatina,* inhibition of Na^+/K^+-ATPase modified the cellular Na^+ gradient, which could result in a reduced activity of Ca^{2+}/Na^+ antiporter and disturbance of calcium homeostasis. A similar profile of inhibition of the H^+-ATPase activity was observed in the mantle, but inter-individual variations were too high to be statistically significant.

Table 1: Plasma membrane Na^+/K^+-ATPase and H^+-ATPase activities (µmol/L P_i/mg protein/min) in different tissues of freshwater and marine organisms.

Plasma membrane ATPase activity µmol L^{-1} Pi/mg protein/min				
Species	**Na^+/K^+-ATPase**	**H^+-ATPase**	**Tissues**	**References**
Freshwater bivalve				
Anodonta anatina	0.098		G	The present study
	0.015		DG	
	0.042	0.002	M	
Anodonta cygnea	0.109		G	Lagerspetz and Senius, 1979
Asellus aquaticus	0.018		G	Bouskill *et al.*, 2006
Dreissena polymorpha	0.006		G	
Marine bivalve				
Mytilus galloprovincialis	0.033		G	Viarengo *et al.*, 1996
Freshwater crustacean				
Dilocarcinus pagei		0.023	G	Firmino *et al.*, 2011
Brackishwater crustacean				
Acartia tonsa	0.014		total	Pedroso *et al.*, 2007
Freshwater fish				
Oncorhynchus mykiss		0.025	G	Lin and Randall, 1993
Trichogaster microlepis	0.015	0.003	G	Huang *et al.*, 2010
Bidyanus bidyanus	0.124	0.041	G	Alam and Frankel, 2006
Macquaria ambigua	0.052	0.032	G	
Perca flavescens	0.064		G	Packer and Garvin, 1998

G: gills, DG: digestive gland, M: mantle.

Some recovery of the Na^+/K^+-ATPase was observed in the digestive gland of *A. anatina* within 7 days of Cu^{2+} exposure. Viarengo *et al.* (1996) also showed a return to basal level of the Na^+/K^+-ATPase activity in *M. galloprovincialis* exposed during 7 d to 0.6 µmol L^{-1} of Cu^{2+}. This may indicate the occurrence of detoxification systems mobilized in the first times of exposure to cope with stressors through metal sequestration or binding to metallothioneins.

Despite a trend indicating partial recovery beyond 4 days the Na^+/K^+-ATPase activity in the gills of *A. anatina* was still significantly inhibited upon 15 days of experiment. An inhibition of Ca^{2+}-ATPase activity was also observed in the gills and the kidneys of *A. anatina* exposed to Cu^{2+} in same conditions as in the present study (Santini *et al.*, 2011a). These whole results indicate an inhibition of ionic transport by Cu^{2+}, which may perturb calcium homeostasis and ionoregulation more generally. In the long term, this may lead to

biomineralization perturbation (shell slimmer, glochidia viability decrease), and physiologic impairments.

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7 Article 3: Phytochelatins in the freshwater bivalve *Anodonta cygnea*

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ABSTRACT

Phytochelatins (PC_n) are metal binding polypeptide with $(\gamma\text{-Glu-Cys})_n$-Gly as general structure, synthesised by phytochelatin synthase (PCS) from glutathione. PC_n belongs to metal detoxification and homeostasis mechanisms well studied in plants, microalgae, some fungi, and yeast. PCS was found in the invertebrates *Caenorhabditis elegans*, *Eisenia fetida*. Phytochelatin synthase-coding sequences were found in the genomes of several other invertebrates including bivalves. The results obtained in the present study show, for the first time, the ability of the freshwater bivalve *Anodonta cygnea* to synthesize PC_n. PC_n were analyzed by HPLC with precolumn monobromobimane derivatization. The magnitude of PC levels was: $PC_2 > PC_3 > PC_4$. Proportions of PC_2 and PC_3 are two or three times higher in the digestive gland than in the gills. These results suggest that PC_n could play a wider role in heavy-metal detoxification in bivalves than previously expected.

Key words: metal homeostasis, freshwater bivalve, phytochelatins, *Anodonta cygnea*

INTRODUCTION

Several transition metals (Cr, Mn, Fe, Co Cu, Zn, Mo) have chemical properties that make them essential for biological systems but toxic in excess. Essential and non-essential metals pose the problem of being toxic in the micromolar concentration range. For homeostasis and detoxification of such metals, bacteria, plants, and animals employ a strategy by synthesizing cysteine-rich peptides and proteins with high-affinity metal binding sites in

the form of thiol groups (Clemens, 2006), *viz*. glutathione, phytochelatins, and metallothioneins.

Until recently, in animal studies on trace metal detoxification, only reduced glutathione (GSH) and the family of metallothioneins (MT) were taken into consideration. The ubiquitous tripeptide GSH (γ-GluCysGly) is present in most eucaryotic cells at concentrations of about 0.2 to 10 mmol L^{-1}. Metallothioneins are cysteine-rich, ubiquitous cytosolic proteins of low molecular weights (~ 4 - 14 kDa). In addition to GSH and MT, in plants, some fungi, and yeasts, phytochelatins (PC_n) represent a third type of thiol-bearing entity (Grill *et al.*, 1985; Clemens, 2006). PC_n are synthesised by phytochelatin synthase (PCS) which catalyzes the transpeptidation of the γ-Glu-Cys moiety of GSH onto a second GSH moiety or with PC_n $(\gamma\text{-Glu-Cys})_n$-Gly (n=2-11) to form PC_{n+1}. PC_n are rapidly induced in cells and tissues when exposed to a range of transition metal ions, including the cations Cd, Ni, Cu, Zn, Ag, Hg, and Pb, and the metalloid arsenic (Clemens, 2006). Homologous genes enabling to give a functional PCS were identified in the nematode *Caenorhabditis elegans* (Clemens *et al.*, 2001; Vatamaniuk *et al.*, 2001) and more recently in the oligochaete *Eisenia fetida* (Brulle *et al.*, 2008). These findings suggest that PC_n may play a role in metal homeostasis also in animals.

Bivalves are known to bio-accumulate persistent organic pollutants and metals, which is one of the reasons suspected of the freshwater bivalve general decline (Franck and Gerstmann, 2007). According to Clemens and Peršoh (2009), bivalves also present a homologous gene of PCS. *Anodonta cygnea* is a freshwater bivalve belonging to the Unionidae, a filter-feeding and burrowing species living at the water/sediment interface. As bivalves are in close contact with the aquatic environment, they are known to bioaccumulate transition metals (Gundacker, 2000; Nugroho and Frank, 2011). The aim of the present study was to determine whether PC_n are eventually synthesised by *A. cygnea* in the gills and the digestive gland. Indeed the presence of PC_n in animals has been established for the first time.

MATERIALS AND METHODS

Chemicals

Phytochelatins standards (PC_2, PC_3, PC_4, and PC_5) [PC_n, $(\gamma\text{-Glu-Cys})_n$-Gly, where n = 2-5], and monobromobimane (mBBr) were from Anaspec (San Jose, CA, USA). Acetonitrile

(ACN) was from Carl Roth (Lauterbourg, France). Tris-(hydroxymethyl)-aminomethane (Tris), 4-(2-hydroxyethyl)-piperazine-1-propane sulfonic acid (HEPPS), methanesulfonic acid (MSA), diethylenetriamine-pentaacetic acid (DTPA), γ-glutamylcysteine (γ-GluCys), and cysteine (Cys) were from Sigma-Aldrich (St. Quentin Fallavier, France). Trifluoroacetic acid (TFA), tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP), N-acetyl-cysteine (NAC), glutathione (GSH), and perchloric acid ($HClO_4$) were from VWR (Strasbourg, France). All chemicals and solvents were of analytical or HPLC reagent grade.

All solvents were filtered through a 0.2 µm polypropylene filter (Polypro, Pall Corp. VWR), Water was purified (18.2 MΩ) by a Milli-Q system (Millipore, France).

Animal maintenance

Mussel maintenance is described in detail in a previous article (Santini *et al.*, 2010a). Adult mussel *A. cygnea* with shell lengths of 7.5 ± 0.5 cm were provided by a commercial supplier (Amazon fish, Pfaffenhoffen, France). The mussels were kept in 10 L aquaria under a photoperiod of 16 h illumination and 8 h darkness. The bottom of the tank was covered with a layer of glass beads (10 mm diameter) so mussels could find conditions for burying. The artificial pond water (1.3 L/mussel), composition 0.40 Ca^{2+}, 0.20 Mg^{2+}, 0.70 Na^{+}, 0.05 K^{+}, 1.35 Cl^{-}, 0.20 SO_4^{2-}, 0.20 HCO_3^{-} mmol L^{-1}, was renewed every day. Bivalves were fed daily with unicellular algae *Chlorella kessleri* from a culture in the exponential growth phase, which were added to a final algal density of 2×10^5 cells mL^{-1}. Air was bubbled continuously to ensure aeration and water column homogeneity. Animals were acclimatized to these conditions for 2 weeks before any experiment.

Determination of PC_n and other cysteine-rich metal-binding peptides

PC_ns were determined according Minocha *et al.* (2008) with a slight modification in the protein removal step and in the gradient profile of the mobile phase in HPLC. Cysteine-rich peptides levels were studied in the cytosolic fractions of deproteinized tissue homogenates after reduction.

Extraction:

All dissection, extraction, and centrifugation steps were carried out at 4°C. The gills and the digestive gland were carefully dissected, digestive content was removed, and washed in Tris buffer (Tris 25 mmol L^{-1}, NaCl 50 mmol L^{-1}, pH 8.0). Organs from two mussels were

pooled in order to obtain sufficient mass for analysis. The samples were immediately analysed after sampling or frozen in liquid N_2 and stored at – 80°C for no more than 4 weeks until analysis. To avoid enzymatic degradation or PC_n oxidation, tissues were homogenised in acid buffer (6.3 mmol L^{-1} DTPA, 0.1% TFA) with a manual Potter-Elvehjem homogenizer with glass pestle. The homogenization was performed with 500 mg tissues in 1 mL buffer. The homogenate was centrifuged at 3500 *g* for 10 min (model 1-15PK, Sigma-Aldrich, St. Quentin Fallavier, France). The resulting supernatant (500 µL) was deproteinized by addition of 125 µL 5 mol L^{-1} perchloric acid, and centrifuged again at 13000 *g* for 30 min. The supernatant (500 µL) was neutralised with 100 µL 5 mol L^{-1} NaOH, and used for PC_n analyses.

Reduction and derivatization:

Just after extraction, 99 µL of tissue extract was mixed with 244 µL of HEPPS buffer (200 mmol L^{-1} HEPPS, 6.3 mmol L^{-1} DTPA, pH 8.2), 10 µL TCEP solution (20 mmol L^{-1} TCEP in HEPPS buffer, pH 8.2) as disulfide reductant, and 4 µL of a solution of 0.5 mmol L^{-1} NAC as internal standard. Reduction was conducted in a water-bath at 45°C for 10 min. Derivatization was carried out by addition of 4 µL of a solution of 50 mmol L^{-1} mBBr acetonitrile and incubation in a water-bath at 45°C for 30 min in the dark. The reaction was stopped by addition of 40 µL of an aqueous solution of MSA 1 mol L^{-1}.

The high-performance liquid chromatographic analysis was performed with an HPLC instrument (Gilson, Roissy, France) equipped with a dual solvent pump (model 322), autosampler (model 234), a 100 µL injection loop, and fluorescence detector (model 122). The sample injection volume was 20 µL. Fluorescence of mBBr-labeled compounds was monitored at an excitation wave length of 382 nm and 470 nm of emission. Derivatized PC_n were separated in a reversed-phase column (Phenomenex-Synergi-Hydro RP C18), 100 mm × 4.6 mm, 4 µm particle size, protected by a C18 guard column, 4 mm × 3 mm, 5 µm (Phenomenex Security guard cartridge). The temperature of the column oven was 40°C. Peak areas were integrated using proper software (UniPoint system, version 1.90, Gilson, Villier-le-Bel, France). The bimane derivatives were separated using a gradient of mobile phase A (99.9 vol-% ACN, 0.1 vol-% TFA) and B (89.9 vol-% water, 10 vol-% ACN, 0.1 vol-% TFA). The gradient profile was a linear gradient of mobile phase A from 0 to 10.6% run for 11.2 min at 1 mL min^{-1}. Further, the linear gradient of solvent A was raised from 10.6 to 28.6% in 13.6 min. Before injecting a new sample, the column was rinsed with 100% of solvent A for 5.5 min at a flow rate of 2.5 mL min^{-1}. The column was equilibrated with 100% of solvent B for a total

of 10 min at 1 mL min^{-1}. Total run time for each sample was 40.3 min including column rinsing and re-equilibration.

Purified standards at seven increasing concentrations ranging from 0.2 to 2 µmol L^{-1} for PC_{2-5}, and 1 to 10 µmol L^{-1} for Cys, GSH, and γ-GluCys, were used to plot calibration curve. In samples thiol compound concentration were determined using equations of calibration curve. PC content was expressed in µg of PC per g of tissue wet weight. Each sample was spiked with 0.6 µmol L^{-1} of each standard PC_{2-5} to certify PC identity.

Statistical analyses

Homogeneity of variances and normality of data were not verified (Bartlett and Shapiro-Wilk tests), so statistical analysis was done using the non parametric Kruskal-Wallis and Mann-Whitney two sided tests (R Development Core Team, 2010. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org). All data means were reported with standard deviations (SD). Differences were considered as significant when $p < 0.05$.

RESULTS AND DISCUSSION

Table 1: Average retention time in minutes (n = 20) ± SD, limit of detection (LOD), and limit of quantification (LOQ) in pmol per 20 µL injected for cysteine-rich metal-binding peptides standards. Standard curves were run with 7 concentrations: 1 to 10 µmol L^{-1} for Cys, GSH, γ-GluCys, and 0.2 to 2 µmol L^{-1} for PC_{2-5}.

Component name	Retention time (min)	LOD pmol / 20 µl	LOQ pmol / 20 µl	r^2
Cys	3.14 ± 0.08	0.21	1.09	0.999
GSH	5.99 ± 0.12	0.24	1.40	0.999
γ-GluCys	6.43 ± 0.13	0.37	1.46	0.999
NAC	9.16 ± 0.23	–	–	–
PC_2	13.09 ± 0.33	0.59	1.39	0.999
PC_3	16.62 ± 0.28	0.79	1.52	0.998
PC_4	18.59 ± 0.25	1.93	2.70	0.991
PC_5	19.75 ± 0.18	4.71	5.09	0.961

– Not determined for the NAC used as an internal standard
r^2: Pearson correlation coefficients of standard curves

In this study, with precolumn mBBr derivatization and reversed-phase HPLC analyses, an excellent linearity was obtained for the calibration curves as shown by the Pearson coefficients (table 1). The quantification limits per 20 µL sample injected into the HPLC column were 1.5 pmol for PC_{2-3}, GSH, γ-GluCys, 2.7 pmol for PC_4, 5 pmol for PC_5, and 1 pmol for Cys. In the samples a part of mBBr underwent reductive dehalogenation with TCEP to give tetramethylbimane (Me_4B) (Graham *et al.*, 2003).

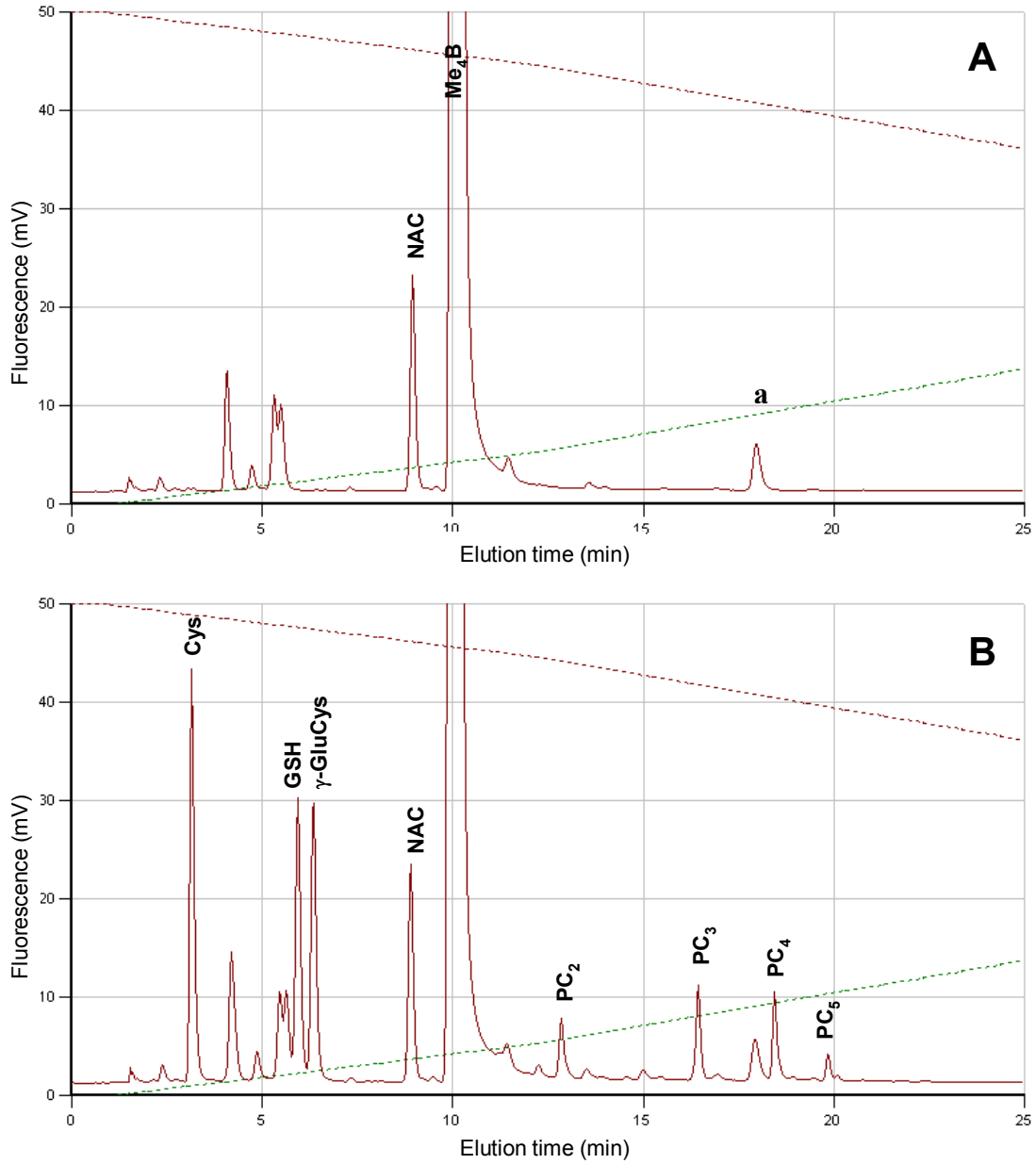

Fig. 1: Chromatograms of (A) reagents blank with homogenisation buffer, and (B) mix of the eight cysteine-rich peptide standards. The broad peak is tetramethylbimane (Me_4B). Peak "a" is an unidentified compound originating from the derivatization reaction with reagent. Standard concentration was 10 µmol L^{-1} for cysteine (Cys), glutathione (GSH), γ-glutamylcysteine (γ-GluCys), 5 µmol L^{-1} for the internal standard N-acetyl-cysteine (NAC), and 2 µmol L^{-1} for phytochelatins 2-5 (PC_{2-5}).

The broad reagent peak observed at approximately 10.5 min (fig. 1 A) in every sample was identified as Me_4B by Kawakami *et al.* (2006). An unidentified compound originating from the derivatization reagent (peak a, fig. 1 A) eluted at 17.96 min, just before the elution of PC_4.

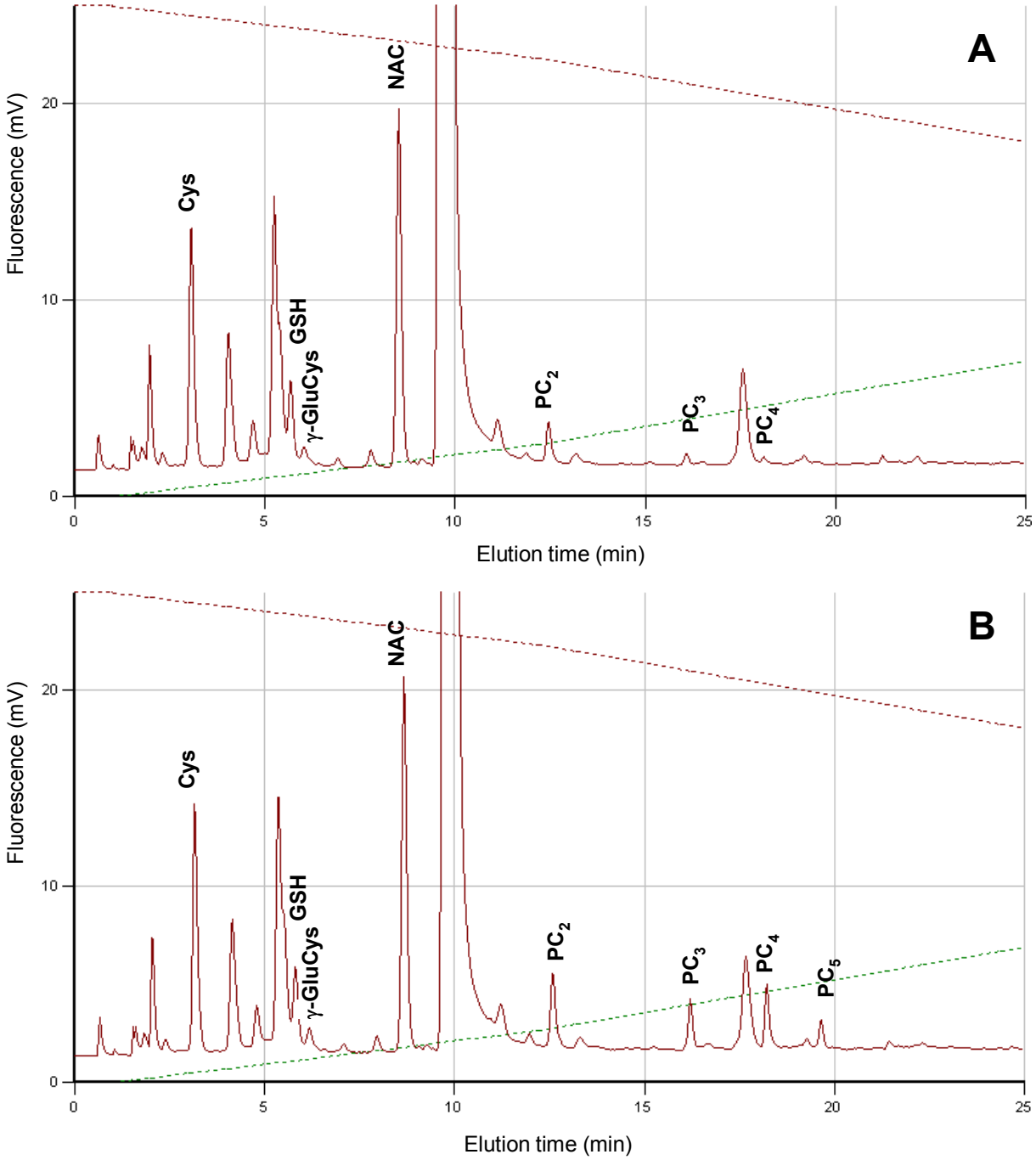

Fig. 2: Chromatograms of digestive gland samples obtained with the same extract: alone (A), and spiked with 0.6 µmol L^{-1} of each standard PC_{2-5} (B).

Chromatograms of extracts of the digestive gland (fig. 2 A) and the gills (fig. 3 A) revealed bimane-labelled compounds with retention times corresponding to PC_2, PC_3, and PC_4 standards. Duplicate samples were spiked at 0.6 µmol L^{-1} of each $PC_{2-5}$with a solution of standard to confirm the identity of the peaks. Concordances of peaks PC_{2-4} were observed (figs. 2 B, 3 B); PC_5 was not detected.

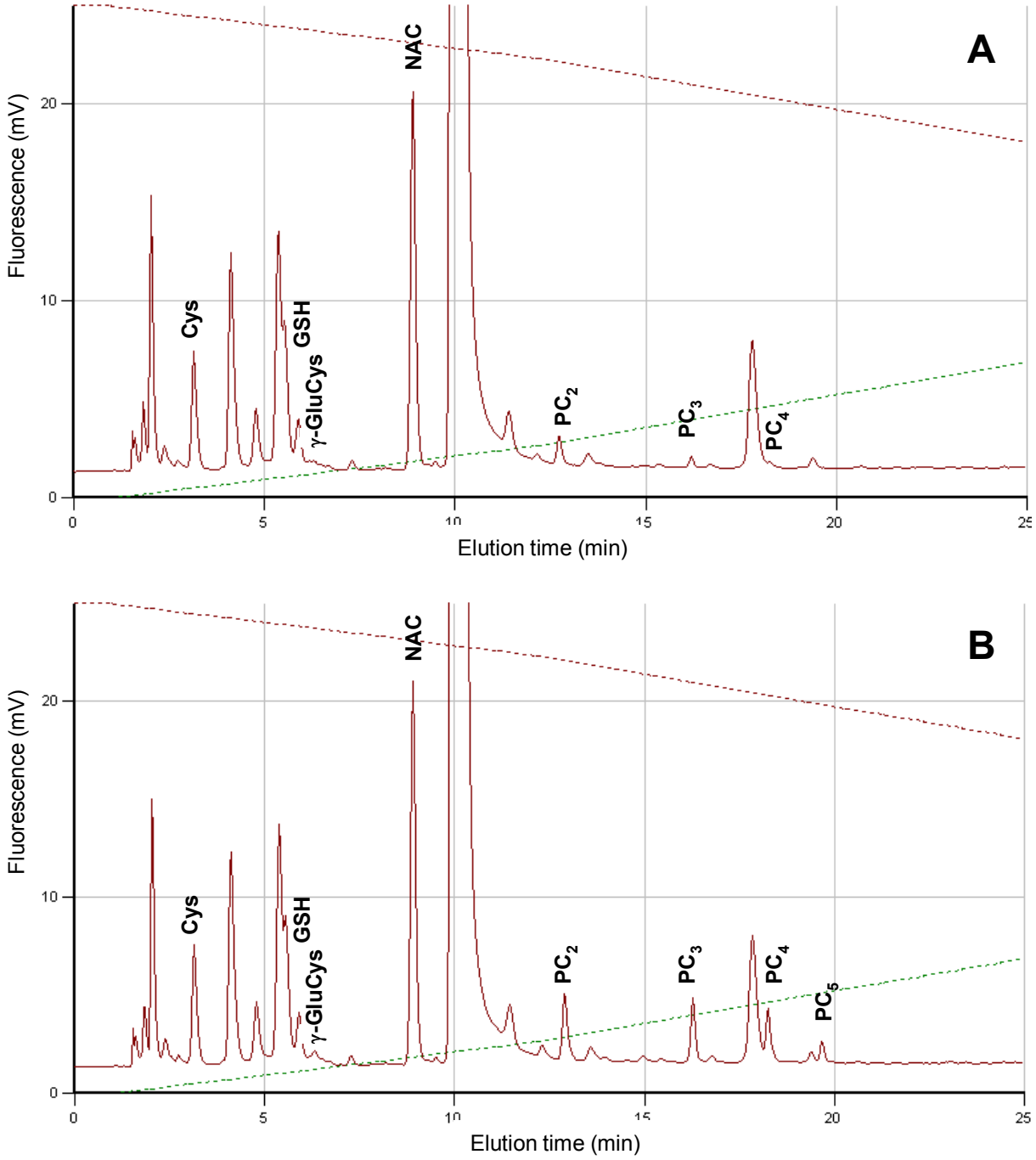

Fig. 3: Chromatograms of gills samples made with the same extract: alone (A), and spiked with 0.6 µmol L^{-1} of each standard PC_{2-5} (B).

Table 2: PC content [µg PC/g tissue wet weight] in the digestive gland and the gills of *Anodonta cygnea*, means (n = 6) ± SD.

	PC_2	PC_3	PC_4	PC_5
Digestive gland	2.17 ± 0.59	1.10 ± 0.12	0.47 ± 0.52	< LOD
Gills	0.88 ± 0.15	0.72 ± 0.57	0.40 ± 0.44	< LOD

< LOD: below limit of detection

Table 2 shows the PC_n content in the gills and the digestive gland in µg per g tissue wet weight. The concentrations in the digestive gland and the gills were the highest for PC_2, i.e. 2.2 ± 0.6 and 0.9 ± 0.2 µg g^{-1} wet weights respectively. In both organs, the PC_n levels decreased in the order $PC_2 > PC_3 > PC_4$. The concentrations of PC_2 and PC_3 were two to three times higher in the digestive gland than in the gills, whereas the PC_4 levels were nearly equivalent in both organs. Wilcoxon, Mann-Whitney test showed no significant difference in PC_2 content (fig. 4) and $PC_{3\text{-}4}$ (data not shown) between fresh and frozen tissues.

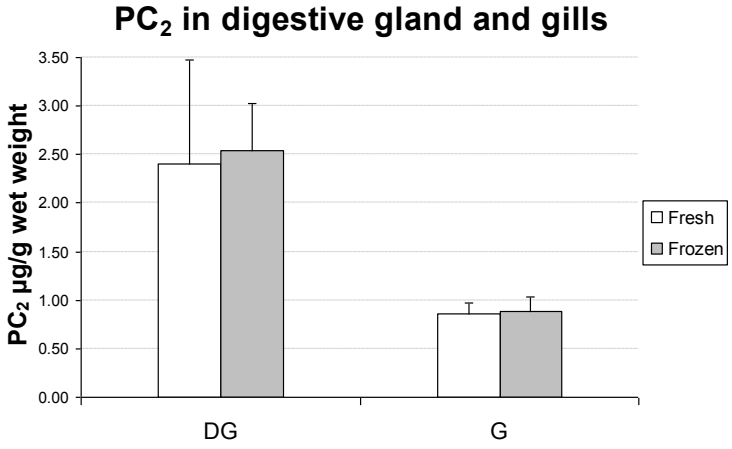

Fig. 4: PC_2 content in the digestive gland (DG) and the gills (G) of *Anodonta cygnea*, means (n = 6) ± SD. No significant difference was found between fresh (white bars) and frozen (grey bars) tissues (Wilcoxon, Mann-Whitney test).

PC are important for detoxification of the non-essential metal Cd in plants and fungi. Clemens and Peršoh (2009) and Gonzalez-Mendoza *et al.* (2007) support the hypothesis that PC have dual functions: metal detoxification and essential metal homeostasis (Zn, Cu). In our work, before analyses the mussels were kept at least for 2 weeks in artificial pond water (see animal maintenance) without exposure to non-essential metals like Cd or the like, but PC_{2-4} could be detected anyway. The presence of PC_{2-4} without the animals being exposed to Cd or other transition metals suggests that PC could have functions comparable to other cysteine-rich compounds, i.e. for homeostasis of essential trace metals or as reducing agent. The evidence of homologous genes for functional PCS in animals suggests that PC_n play a wider role in heavy-metal detoxification than previously thought. The results obtained in this study highlight, for the first time, the ability of the freshwater bivalve *Anodonta cygnea* to synthesize PC_{2-4}.

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8 Article 4: Phytochelatins, a group of metal-binding peptides induced by copper exposure in the bivalve *Anodonta cygnea*

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ABSTRACT

Phytochelatins (PC) are thiol-containing peptides with general structure (γ -Glu-Cys)n-Gly synthesised by plants and algae and fungi in response to metal exposure. Recently the freshwater bivalve *Anodonta cygnea* revealed the ability to make PC synthesis. The aim of this study was to evaluate effect of copper exposure on PC and metallothionein (MT) synthesise in *A. cygnea*. A clear and significant increase of PC_2 was observed in the gills upon 7 d and in the digestive gland at 12 h of 0.35 µmol L^{-1} Cu^{2+} exposure. Thus, phytochelatins appears play a role for metal homeostasis and detoxification also in certain animals as *A. cygnea*. The γ-GluCys increased significantly in the gills at 48 h and 7 d, and in digestive gland at 48 h and 4 d exposure. No significant variation in MT level was observed.

Key words: copper, freshwater bivalve, phytochelatins, *Anodonta cygnea,* metal tolerance

INTRODUCTION

Copper is extensively used for various technical applications, especially conducting metal for electro-technical equipment, electrical power lines, as catalyst in the chemical industry, and in the building industry for water pipes and roofings; a small amount goes into the use as fungicide by which way it is directly emitted into the environment. Annual copper consumption in Europe alone is 3.5×10^6 tons, worldwide about 17×10^6 tons (INERIS,

2010). Another possible source is fly ash from coal combustion, containing up to 20 g Cu per ton coal. Copper is easily found in aquatic ecosystems (Waeles *et al.*, 2004) since they are the ultimate way for numerous contaminants.

Copper is an essential trace element for the function of many cellular enzymes and proteins. However, copper become toxic when excessive intracellular accumulation occurs (Viarengo and Nott, 1993). Copper toxicity results both from non-specific metal binding to proteins, from is involvement in Fenton reactions leading to formation of reactive oxygen species (ROS) and oxidative stress. Through their important filtering activity they satisfy their respiratory and nutrition needs, by which bivalves have the capacity to accumulate a variety of environmental contaminants. The freshwater bivalve *Anodonta cygnea* belongs to Unionidae family, and is a species well distributed in continental waters. Unionidae are widely recognised for metal bioaccumulation including copper (Cossu *et al.*, 1997). The level up to which mussels can tolerate transition metals depends on their ability to regulate the metal cation concentration in cells.

The proteins of the metallothionein (MT) group, the polypeptides glutathione (GSH) and phytochelatins (PC) are protective compounds rich in the amino acid cysteine containing a thiol group (SH). Cu, as other transitions metals have high affinity for SH groups, making cysteine rich peptides the principal biological reagents for transition metal sequestration (Viarengo and Nott, 1993, Clemens, 2006). PC bind transition metals with high thiol complexation constants, reducing the intracellular concentration of free ions of such metals in plants, fungi and microalgae. The general structure of PC is $(\gamma$ -Glu-Cys$)_n$-Gly (n = 2 to 11), synthesized by the constitutive enzyme phytochelatin synthase, which is activated by the presence of metal ions with GSH as substrate. (Grill *et al.*, 1985). A PC synthase homologous sequence has been found in the genomes of the invertebrates *Caenorhabditis elegans* (Clemens *et al.*, 2001; Vatamaniuk *et al.*, 2001), *Eisenia fetida* (Brulle *et al.*, 2008) and *Chironomus* (Cobbett, 2000) genomes and more generally throughout the invertebrates (Clemens and Peršoh, 2009). In our previous article (Santini *et al.*, 2011b) we have shown the presence of PC in *Anodonta cygnea*. It became clear that PC could play a wider role in trace metal detoxification in animals and are not restricted to plant. The objective in the present work was to elucidate the implication of PC in metal detoxification mechanisms in *Anodonta cygnea* exposed to copper (Cu^{2+}).

MATERIAL AND METHODS

Chemicals

Phytochelatin standards (PC_2, PC_3, PC_4 and PC_5) [PC_n, $(\gamma\text{-Glu-Cys})_n$-Gly, where n = 2-5], and monobromobimane (mBBr) were from Anaspec (San Jose, CA, USA). HPLC-grade acetonitrile (ACN), and 1,4-dithiothreitol (DTT) were from Carl Roth (Lauterbourg, France). Tris-(hydroxymethyl)-aminomethane (Tris), 4-(2-hydroxyethyl)-piperazine-1-propane sulfonic acid (HEPPS), methanesulfonic acid (MSA), diethylenetriamine-pentaacetic acid (DTPA), γ-glutamylcysteine (γ-GluCys), cysteine (Cys), phenylmethylsulfonyl fluoride (PMSF), leupeptin, sodium dodecyl sulphate (SDS), and (ethylenedinitrilo)-tetraacetic acid (EDTA) were from Sigma-Aldrich (St. Quentin Fallavier, France). Trifluoroacetic acid (TFA), tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP), N-acetyl-cysteine (NAC), glutathione (GSH), and perchloric acid ($HClO_4$) were from VWR (Strasbourg, France). Rabbit liver metallothionein 1 (MT-I) was from Enzo Life Sciences (Villeurbanne, France).

Chemicals and solvents were of analytical or HPLC reagent grade. All solvents were filtered through a 0.2 µm polypropylene filter (Polypro, Pall Corp. VWR), water was purified (18.2 MΩ) by a Milli-Q system (Millipore, France).

Animal maintenance and copper exposure

Mussel maintenance was described in detail in our previous article (Santini *et al.*, 2011a). Briefly adult individuals of *A. cygnea* with shell lengths of 7.5±0.5 cm were kept in aquaria at a temperature of 20 ± 0.5° C. The bottom of the tank was covered with a layer of glass beads so the mussels could find conditions for burying. Artificial pond water 1.5 L/mussel, pH 7.25 ± 0.10 (in mmol L^{-1}: 0.40 Ca^{2+}, 0.20 Mg^{2+}, 0.70 Na^{+}, 0.05 K^{+}, 1.35 Cl^{-}, 0.20 SO_4^{2-}, 0.20 HCO_3^{-}) was renewed every day. The bivalves were fed daily with *Chlorella kessleri* from a culture in the exponential growth phase and added to a final algal density of 2×10^5 cells mL^{-1}. The animals were acclimatized to these conditions for two weeks before any experiment.

Previous results (Santini *et al.*, 2011a) showed inhibition of enzymes involved in osmoregulationin *A. cygnea* exposed to 0.35 µmol L^{-1} of Cu^{2+}. A total recovery of Ca^{2+}-ATPase upon 7 d followed, indicating the induction of detoxication mechanisms. In the

present work 0.35 µmol L^{-1} Cu^{2+} (controlled by graphite furnace atomic absorption spectrometry GFAAS, limit of detection for Cu = 0.5 µg L^{-1} = 8 nmol L^{-1}) was chosen as concentration for exposure test. The experiments were performed in aquaria lined with dye- and pigment free high-density polyethylene foil, filled with 1.5 L of experimental medium (pH 7.25) per mussel. The mussels were kept at 20° C in a thermo-regulated room with a photoperiod of 16 h light and 8 h darkness. The animals were divided in groups of 12 mussels. These groups were exposed to 0.35 µmol L^{-1} Cu^{2+} for 0 h, 12 h, 48 h, 4 d, 7 d, and 21 d, or kept for the same duration in artificial pond water as corresponding time controls. Test media were renewed every day, and the bivalves were fed daily as previously. For sampling, the 12 mussels used on each treatment were dissected. The gills and digestive glands of two mussels were pooled, which gives 6 replicates per treatment for analysis.

Determination of PC and other cysteine-rich metal-binding peptides

PC were determined according to Minocha and *al.* (2008) with a slight modification in the protein removal step and the HPLC mobile phase gradient profile (Santini *et al.*, 2011b). Cysteine-rich peptides were determined in the cytosolic fractions of the deproteinized tissue homogenates after reduction.

Extraction:

All dissection, extraction, and centrifugation steps were carried out at 4°C. The gills and digestive gland were carefully dissected, the digestive content was removed, and the tissues were washed in Tris buffer (Tris 25 mmol L^{-1}, NaCl 50 mmol L^{-1}, pH 8.0). The organs from two mussels were pooled in order to obtain sufficient mass for analysis. The samples were immediately analysed after sampling, or frozen in liquid N_2 and stored at – 80°C until analysis. For extraction to avoid enzymatic degradation or PC oxidation, the tissues were homogenised in acid buffer (6,3 mM DTPA, 0,1% TFA) with a manual Potter-Elvehjem homogenizer with glass pestle. The homogenization was performed with 500 mg of tissues in 1 mL of buffer. The homogenate was centrifuged at 3500 *g* for 10 min (model 1-15PK, Sigma-Aldrich, St. Quentin Fallavier, France). The resulting supernatant (500 µL) was deproteinized by addition of 125 µL perchloric acid 5 mol L^{-1}, and centrifuged again at 13000 *g* for 30 min. The supernatant, 500 µL, was neutralised with 100 µL aqueous NaOH 5 mol L^{-1}, and used as tissues extract for PC analyses.

Reduction and derivatization:

Just after extraction, 244 µL HEPPS buffer (HEPPS 200 mmol L^{-1}, DTPA 6.3 mmol L^{-1}, pH 8.2) was mixed with 10 µL TCEP (TCEP 20 mmol L^{-1} in HEPPS buffer, pH 8.2) used as disulfide reductant, 4 µL of NAC 0.5 mmol L^{-1} as an internal standard, and 99 µL of tissue extract. Disulfide reduction was conducted in a water-bath at 45°C for 10 min. Derivatization was carried out by addition of 4 µL of mBBr (50 mmol L^{-1} in acetonitrile), and incubated in a water-bath at 45°C for 30 min in the dark. The reaction was stopped by addition of 40 µL of an aqueous solution of MSA 1 M.

Analyses were performed using HPLC instrument (Gilson, Roissy, France) equipped with a dual solvent pump (model 322), an autosampler (model 234), a 100 µL injection loop, and a fluorescence detector (model 122). The fluorescence of mBBr-labeled molecules was monitored with an excitation wave length of 382 nm and emission at 470 nm. The injection volume was 20 µL. The derivatized PC were separated on a reversed-phase column (Phenomenex-Synergi-Hydro RP C18), 100 mm × 4.6 mm, 4 µm particle size, fitted with a C-18 guard column (Phenomenex Securityguard cartridge) 4 mm × 3 mm, 5 µm. The temperature of the column oven was 40°C. Peak areas were integrated using proper software (UniPoint system version 1.90, Gilson, Villier-le-Bel, France). The bimane derivatives were separated using a gradient of mobile phase A (99.9 vol-% ACN, 0.1 vol-% TFA) and B (89.9 vol-% water, 10 vol-% ACN, 0.1 vol-% TFA). The gradient profile was a linear gradient of mobile phase A from 0 to 10.6 vol-% was run for 11.2 min at 1 mL min^{-1}. Further, the linear gradient of solvent A was raised from10.6 to 28.6 vol-% in 13.6 min at 1 mL min^{-1}. Before injecting a new sample, the column was rinsed with 100 % of solvent A for 5.5 min at a flow rate of 2.5 mL min^{-1}. The column was equilibrated with 100 % of solvent B for a total of 10 min at 1 mL min^{-1}. Total run time for each sample was 40.3 min, including column rinsing and re-equilibration.

Purified standards at seven increasing concentrations ranging from 0.2 to 2 µmol L^{-1} for PC_{2-5}, and 1 to 10 µmol L^{-1} for Cys, GSH, and γ-GluCys, were used to plot calibration curve. In samples thiol compound concentration were determinate using equations of calibration curve. PC content was expressed in µg of PC per g of tissue wet weight. Each sample was spiked with 0.6 µmol L^{-1} of each standard PC_{2-5} to certify PC identity. The quantification limits per 20 µL sample injected into the HPLC column were 1.5 pmol for PC_{2-3}, 2.7 pmol for PC_4, 5 pmol for PC_5.

Determination of metallothionein

The MT content was determined by the method of Romero-Ruiz *et al.* (2008). All steps were carried out at 4°C. Organs from two mussels were pooled in order to obtain sufficient mass for analysis. The samples were immediately frozen in liquid N_2 and stored at –80°C until analysis. The gills and digestive gland were homogenised in buffer (0.1 mol L^{-1} Tris, 1 mmol L^{-1} DTT, 50 mmol L^{-1} PMSF, 6 mmol L^{-1} leupeptin, pH 9.5) at a ratio of 5 mL g^{-1} with a manual Potter-Elvehjem homogenizer with glass pestle. The homogenate was centrifuged at 3500 *g* for 10 min. The resulting supernatant was centrifuged again at 25000 *g* for 30 min, and the supernatant was used as extract for MT analyses.

Tissue extract, 125 µL was mixed with 30 µl of Tris buffer, 0.23 mol L^{-1}, pH 9.5, 10 µL, DTT 0.3 M, added to 5 µL EDTA, 0.1 mol L^{-1}, pH 7, and 63 µL of 12 % SDS aqueous solution. Reduction and denaturation was conducted in a water-bath at 70°C for 20 min. For derivatization, 16.7 µL of mBBr solution in ACN, 0.18 mol L^{-1}, was added and the sample was incubated for 15 min at room temperature away from light.

The derivatized samples were chromatographically analyzed on VWR reversed-phase column (LiChrocart LiChrosphere RP C18, Strasbourg, France) 250 mm × 4 mm, 5 µm particle size, fitted to a C-18 guard column, 4 mm × 3 mm, 5 µm (Phenomenex Securityguard cartridge). The fluorescence detector parameters were the same than for PC analyses.

MT was separated using a gradient of mobile phase A (99.9 vol-% ACN, 0.1 vol-% TFA) and B (99.9 vol-% water, 0.1 vol-% TFA). After injection, the mobile phase A was kept for 10 min at 30 %, and changed by 1 min linear rise to 70 % of phase A. These conditions were maintained for 10 min, before the initial conditions (30 % phase A) were re-established by 1min linear decrease. Before injecting a new sample, the column was rinsed and equilibrated at the initial conditions for 8 min. T flow was kept at 1 mL min^{-1} throughout analysis. The injection volume was 20 µL.

A plot of peak area versus purified standard content was established using seven increasing concentrations from 0.4 to 1 µmol L^{-1} rabbit liver MT-I. The equation obtained from this calibration line was used for MT quantification in the samples. The MT content was expressed in mg per g of protein. Protein was determined according to Bradford (1976) using bovine serum albumin as standard.

Statistical analyses

As homogeneity of variances and normality of data were not verified (Bartlett and Shapiro-Wilk tests), statistical analysis was done using the non parametric Kruskal-Wallis and Mann-Whitney two sided tests (R Development Core Team (2010). R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org). All data were reported as means ± standard deviations (SD). Differences were considered as significant when $p < 0.05$.

RESULTS

A broad peak of tetramethylbimane resulting from the reaction between mBBr and TCEP eluted approximately at 10.5 min (fig. 1 A). At 17.96 min an unidentified compound (peak "a") from reagents eluted. In standards profile (fig. 1 B), the phytochelatin mean elution times, were: PC_2 at 13.09 min, PC_3 at 16.62 min, PC_4 at 18.59 min, and PC_5 at 19.75 min.

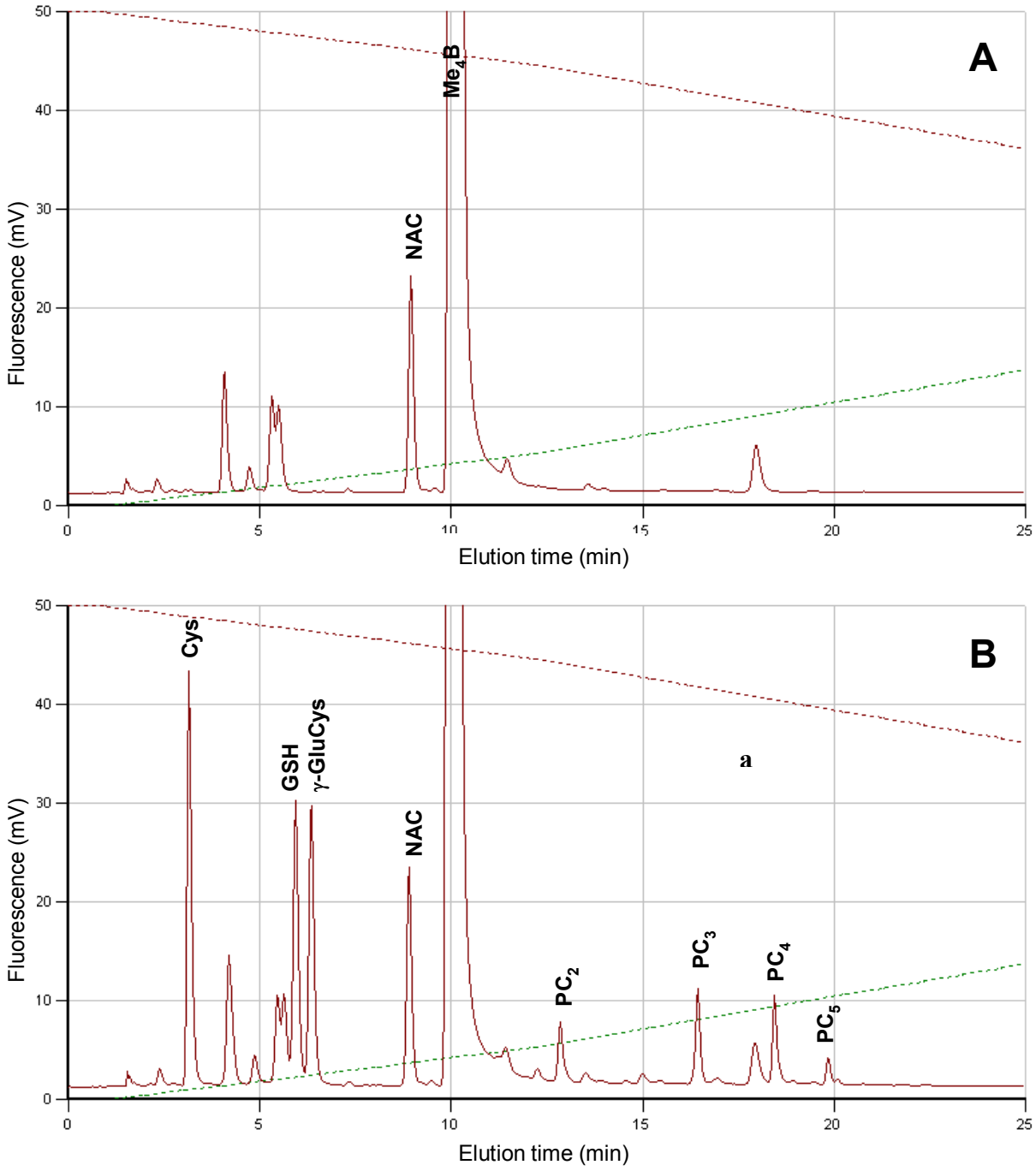

Fig. 1: Chromatograms of (A) reagent blank with homogenisation buffer, and (B) mix of the eight cysteine-rich peptide standards. The broad peak is tetramethylbimane (Me_4B). Peak "a" is an unidentified compound originating from the derivatization reaction with reagent. Standard concentration was 10 µmol L^{-1} for cysteine (Cys), glutathione (GSH), γ-glutamylcysteine (γ-GluCys), 5 µmol L^{1} for the internal standard N-acetyl-cysteine (NAC), and 2 µmol L^{-1} for phytochelatins 2-5 (PC_{2-5}).

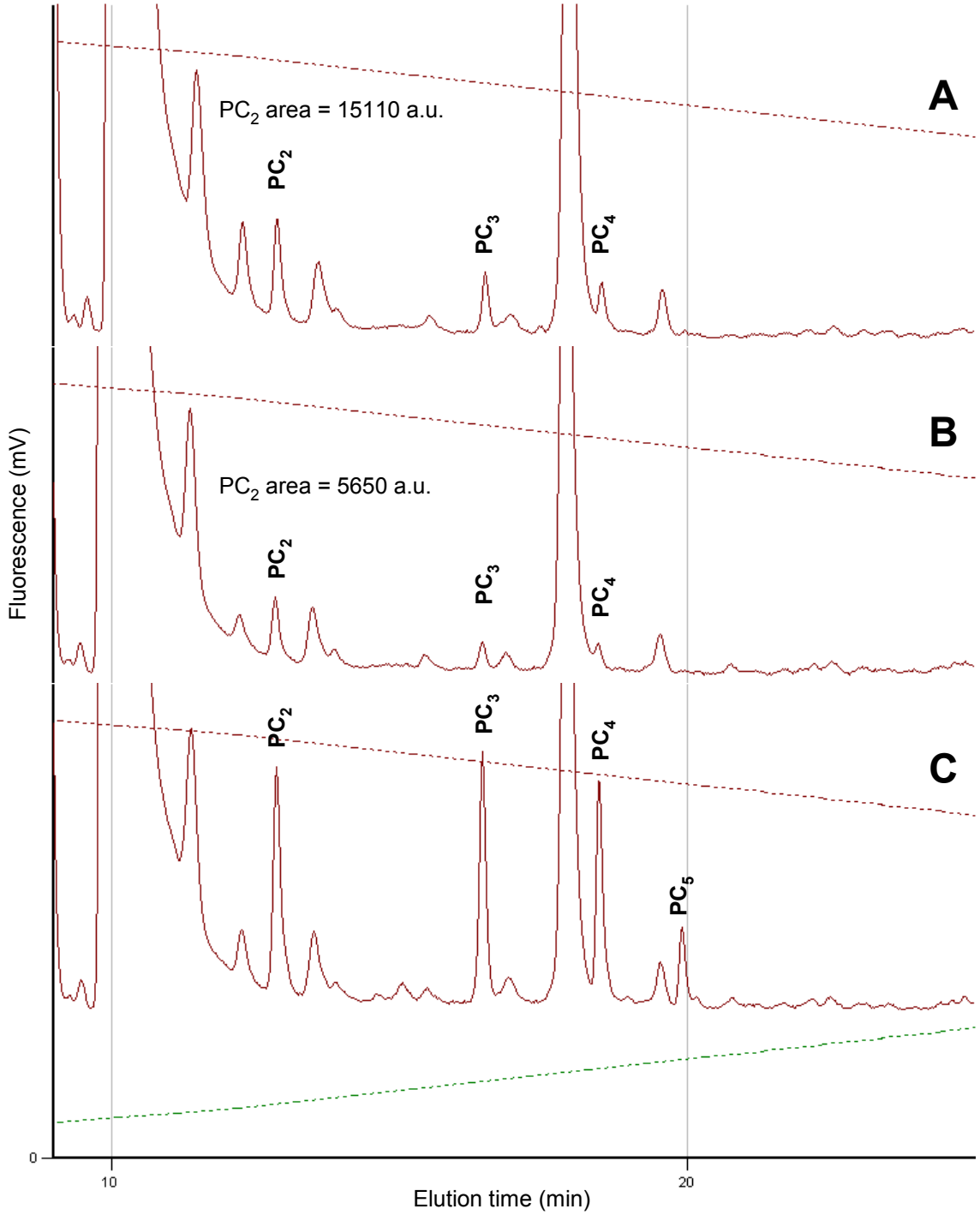

Fig. 2: Chromatograms of gills samples: mussels exposed for 4 days to 0.35 µmol L^{-1} Cu^{2+} (A), mussel controls 4 days (B), mussel control 4 days spiked with 0.6 µmol L^{-1} of each standard PC_{2-5} (C), a.u.: area unit.

Exact co-elution of the peaks in the non-spiked samples and those spiked with PC standards were observed, allowing there tentative identification in the gills and digestive gland; PC_5 was under detection limit. Concordance of PC_{2-4} peaks is showed (fig. 2) in the chromatograms of samples obtained from the gills between: mussels exposed to copper for 4

days (fig. 2 A), control mussel (fig. 2 B), and control mussel spiked with PC_{2-5} standards (fig. 2 C).

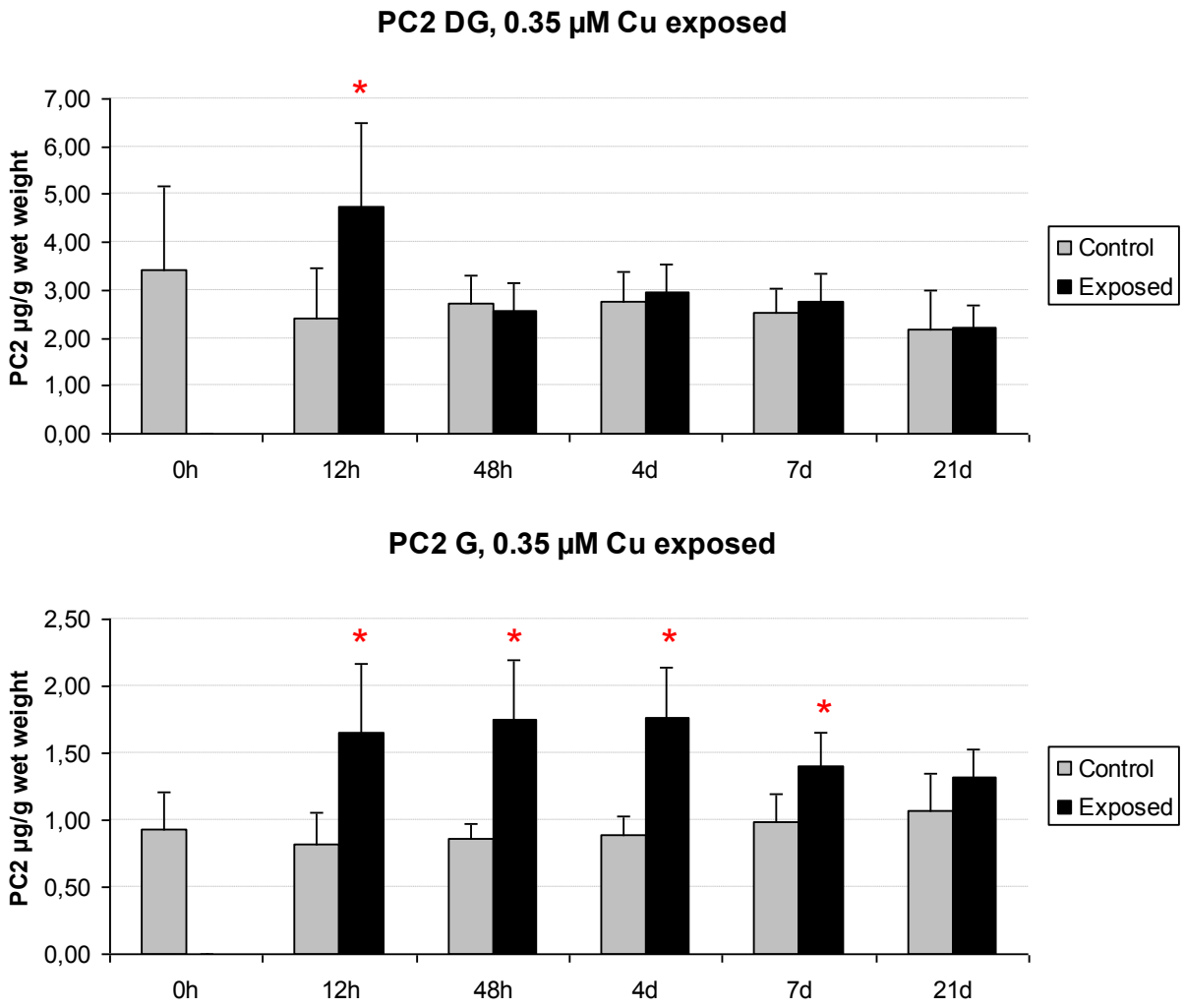

Fig. 3 PC_2 content in the digestive gland (DG) and the gills (G) of *A. cygnea* exposed to 0.35 µmol L^{-1} Cu^{2+} for 0 h, 12 h, 48 h, 4 d, 7 d, and 21 d. Means of results (n = 6) ± SD. *= significant difference between exposed group and its respective control (Kruskal-Wallis and Mann-Whitney two sided test (n = 6, $\alpha = 0.05$))

PC_2 levels were clearly and significantly increased in the gills of mussels exposed to 0.35 µmol L^{-1} of Cu^{2+} (fig. 3). Relative to the respective controls a 50 % increase was observed from 12 h to 4 d of Cu^{2+} exposure, and a 30 % increase upon 7 d. Upon 21 d PC_2 declined back to control values. In digestive gland (fig. 3), except for the 12 h of Cu exposure, no significant variation of PC_2 was seen. The mean PC_2 level in control mussels was nearly three times higher in the digestive gland than in the gills. The PC levels determinate in both

organs varied as follows: $PC_2 > PC_3 > PC_4$, no significant induction of PC_{3-4} was observed. The quantities of PC_{2-4} found in digestive gland were higher than in gills (data not shown).

In the gills of mussels exposed to Cu^{2+} upon 48 h and 7 d, γ-GluCys levels (table 1) are significantly superior to the respective controls. γ-GluCys increase as well, in the digestive gland of bivalves at 48 h and 4 d of exposure.

Table 1: γ-GluCys and MT contents in the digestive gland and the gills of *A. cygnea* exposed to 0.35 µmol L^{-1} Cu^{2+} for 0 h, 12 h, 48 h, 4 d, 7 d, and 21 d. Means of results (n = 6) ± SD. *= significant difference between exposed groups and their respective control (Kruskal-Wallis and Mann-Whitney two sided test (n = 6, $\alpha = 0.05$))

Time	γ-GluCys µg/g wet weight				MT mg/g protein			
Exposure	Digestive gland		Gills		Digestive gland		Gills	
	Control	Exposed	Control	Exposed	Control	Exposed	Control	Exposed
0 h	0.77 ± 0.09	-	0.52 ± 0.12	-	15.11 ± 3.85	-	10.10 ± 3.58	-
12 h	1.06 ± 0.07	0.99 ± 0.22	0.48 ± 0.05	0.48 ± 0.01	15.47 ± 3.97	15.47 ± 3.85	11.33 ± 1.90	9.75 ± 2.70
48 h	0.93 ± 0.15	1.14 ± 0.08 *	0.39 ± 0.01	0.47 ± 0.04 *	13.75 ± 1.55	12.73 ± 2.61	8.89 ± 3.15	9.41 ± 1.79
4 d	0.85 ± 0.09	1.15 ± 0.22 *	0.90 ± 0.45	0.81 ± 0.23	13.34 ± 2.10	13.46 ± 3.82	11.61 ± 2.41	9.40 ± 2.95
7 d	0.93 ± 0.13	0.97 ± 0.04	0.62 ± 0.04	0.70 ± 0.08 *	13.12 ± 3.73	13.93 ± 2.91	11.49 ± 2.43	9.84 ± 1.47
21 d	0.99 ± 0.11	0.98 ± 0.23	0.66 ± 0.05	0.65 ± 0.03	14.86 ± 2.83	15.12 ± 2.54	8.92 ± 2.07	11.66 ± 2.05

- Not determinate

No significant variations of MT levels were observed in the gills and the digestive gland of mussels upon 21 d of copper exposure.

DISCUSSION

Anodonta cygnea was used to investigate whether phytochelatins were induced upon water exposure to 0.35 µmol L^{-1} Cu^{2+}. γ-GluCys, the precursor of glutathione and indirectly of the PC, increased from 48 h to 7 d Cu^{2+} exposure in comparison to the control, and in parallel to PC_2 increases. This γ-GluCys increase could be caused by neo-synthesis necessary as indirect requirement for PC synthesis.

No induction of MT was observed upon Cu^{2+} exposure for 21 d, a result similarly to the one found in *A. cygnea* (Amiard *et al.*, 2006). MT polymorphism appears to be particularly important in invertebrates compared to mammals. Different isoforms of MT play

different physiological roles (Amiard *et al.*, 2006). In the present work, MT identification and quantification was carried out with purified rabbit liver MT-I standard. After HPLC separation, the MT in the mussel extract eluted at the same time as the rabbit liver standard, i.e. have a molecular weight and cysteine content similar to MT-I of mammals (Alhama *et al.*, 2006). This MT isoform from *A. cygnea* does not appear to be induced by copper. Cu^{2+} induction of MT in *A. anatina* was shown using a spectrophotometric method which allowed total MT isoform quantification (Nugroho and Frank, 2012).

Since the discovery of the PC synthase gene, able to give a functional PC synthase in invertebrates, the question of the implication of PC in metal detoxification and homeostasis in animals has arisen. The present study shows a clear PC_2 induction in freshwater mussels under Cu^{2+} exposure, indicating a key role of this cysteine rich polypeptide as metal chelator. PC_2 increased in the gills from 12 h to 7 d, so it may serve as first line defense, with other compounds such as GSH. The PC_2 decline to control values upon 21 d suggests that at later time copper is taken in charge by other detoxication mechanisms such as MT and insoluble granules (Viarengo and Nott, 1993). Sequestration of 65 % of the intracellular copper in granules was shown in the gills of Unionidae bivalves (Bonneris *et al.*, 2005).

In the digestive gland, PC_2 increased significantly only up to 12 h of Cu^{2+} exposure. The water media route of Cu-exposure could be the reason of higher sensitivity of PC_2 induction in the gills, which may play a more important role than digestive gland, in the uptake of copper dissolved in the surrounding water. Moreover, PC induction depends on the metal species, Cu having been reported as a weak inducer compared to Cd (Zenk, 1996). Further studies with Cd would be interesting, in order to determin whether $PC_{2\text{-}4}$ may be efficient in detoxification of such a non-essential metal. Additional investigations are necessary to assess the relevance of PC as potential biomarker for metal exposure of molluscs.

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Annexe

Annexe

Annexe 1: Copper quantification in water media by ICPMS and GFAAS

Concentration of copper determinated by ICPMS in water media		
Nominal in µg L^{-1}	Nominal in µmol L^{-1}	Mesured in µmol L^{-1}
0.00	0.00	0.017 ± 0.007
16.00	0.25	0.262 ± 0.008
22.24	0.35	0.351 ± 0.003
35.00	0.55	0.537 ± 0.001
41.30	0.65	0.638 ± 0.027
76.25	1.20	1.146 ± 0.026

Controlled by inductively coupled plasma mass spectrometry (ICPMS) (detection limit: Cu = 0.5 µg L^{-1} = 0.008 nmol L^{-1}), means (n = 3) ± standard deviations (SD)

Concentration of copper determinated by GFAAS in water media		
Nominal in µg L^{-1}	Nominal in µmol L^{-1}	Mesured in µmol L^{-1}
0.00	0.00	0.016 ± 0.001
22.24	0.35	0.347 ± 0.009

Controlled by graphite furnace atomic absorption spectrometry (GFAAS) (detection limit Cu = 0.5 µg L^{-1} = 8 nmol L^{-1}), means (n = 3) ± standard deviations (SD)

Annexe 2: Algae culture medium

Solution 1:	
KNO_3	101.1 g
NaH_2PO_4 (H_2O)	62.1 g
Na_2HPO_4 ($2H_2O$)	8.9 g
Distillate water until	1000 ml
Solution 2:	
$MgSO_4$ ($7H_2O$)	49.2 g
Distillate water until	100 ml
Solution 3:	
$CaCl_2$ ($2H_2O$)	7.35 g
Distillate water until	100 ml
Solution 4:	
H_3BO_3	0.0610 g
$MnSO_4$ (H_2O)	0.1690 g
$CuSO_4$ ($5H_2O$)	0.0024 g
$ZnSO_4$ ($7H_2O$)	0.2870 g
$(NH_4)_6Mo_7O_{24}$ ($4H_2O$)	0.0123 g
Distillate water until	100 ml
Solution 5:	
Na_2EDTA ($2H_2O$)	0.930 g
$FeSO_4$ ($7H_2O$)	0.690 g
Distillate water until	100 ml
Culture medium:	
Solution 1	5 ml
Solution 2	0.5 ml
Solution 3	0.5 ml
Solution 4	0.5 ml
Solution 5	0.5 ml
Distillate water until	1000 ml

Annexe 3: Pictures of micro-algae (*Chlorella kessleri*) culture

Bach culture under CO_2 rich aeration and continuous stirring.

Annexe 4: Pictures of mussel maintenance and exposure

Anodonta cygnea in acclimatization aquarium

Anodonta cygnea copper exposure in aquaria lined with polyethylene foil, in a thermo-regulated room

Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass ich die Arbeit selbständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe.
Ferner erkläre ich, dass ich anderweitig mit order ohne Erfolg nicht versucht habe, diese Dissertation einzureichen. Ich habe keine gleichartige Doktorprüfung an einer anderen Hochschule endgültig nicht bestanden.

Olivier Santini, 28/01/2013

Summary: Copper (Cu) is one of the metals contaminating European fresh water ecosystems. Filter feeding bivalves have high bioaccumulation potential for transition metals as Cu. While Cu is an essential micronutrient for living organisms, it causes serious metabolic and physiological impairments when in excess. The objectives of this thesis are to get knowledge on toxic effects and detoxification mechanisms of copper in *Anodonta cygnea* and *Anodonta anatina*, two mussel species widely distributed in continental waters. Because Calcium (Ca) plays a fundamental role in shell formation and in numerous biological processes, Cu^{2+} effects on cellular plasma membrane Ca transport were studied first. In the second step, the investigations focused on Cu^{2+} detoxification mechanisms involving Cys-rich compounds known to play a major role in homeostasis of essential trace metals and in cellular metal detoxification. Under our experimental conditions, Cu^{2+} inhibition of Ca^{2+}-ATPase activity was observed in the gills and the kidneys, and inhibition of Na^{+}/K^{+}-ATPase in the gills and the digestive gland upon 4 d of exposure. At day 7 of exposure to 0,35 µmol L^{-1} Cu^{2+}: total recovery was observed in the kidneys and the gills for Ca^{2+}-ATPase activity, and in the digestive gland for Na^{+}/K^{+}-ATPase, but not at high doses. Ca and Na transport inhibition may entail disturbance of osmo-regulation and lead to continuous under-supply of Ca. Recoveries of Na^{+}/K^{+}-ATPase and Ca^{2+}-ATPase enzymes function suggest that metal-detoxification is induced. Phytochelatins (PC) are Cys-rich oligopeptides synthesised by phytochelatin synthase from glutathione in plants and fungi. Phytochelatin synthase genes have recently been identified in invertebrates; this allows us to hypothesize a role of PC in metal detoxification in animals. In the second part of this work, PC and their precursors as well as metallothionein were analyzed in the gills and in the digestive gland of *A. cygnea* exposed to Cu^{2+}. Our results showed for the first time the presence of PC_{2-4} in invertebrates. PC were detected in control mussels not exposed to metal, suggesting a role in essential metal homeostasis. Compared to control, PC_2 induction was observed during the first 12 h of Cu^{2+} exposure. Those results confirm the role of PC as a first line detoxification mechanism in *A. cygnea*.

Key words: calcium homeostasis, copper, *Anodonta* freshwater bivalve, phytochelatins

Résumé : Le cuivre (Cu) est l'un des métaux contaminants les écosystèmes dulcicoles Européens. Les bivalves filtreurs ont une grande capacité de bioaccumulation des métaux de transitions tel que le Cu. Le Cu est un oligo-élément essentiel pour les organismes vivants, mais en excès il provoque de graves perturbations métaboliques et physiologiques. L'objectif de cette thèse est d'acquérir des connaissances sur les effets toxiques et les mécanismes de détoxification du cuivre chez *Anodonta cygnea* et *Anodonta anatina*, deux espèces de bivalves largement distribuées dans les eaux continentales. Parce que le Calcium (Ca) joue un rôle fondamental dans la composition de la coquille et pour de nombreux processus biologiques, les effets du Cu^{2+} ont été étudiés d'abord sur le transport cellulaire du Ca au niveau de la membrane plasmique. Dans un deuxième temps, l'étude a été axée sur les mécanismes de détoxification du Cu^{2+} impliquant des composés riches en Cys, connus pour jouer un rôle majeur dans l'homéostasie des métaux traces essentiels et dans la détoxification des métaux dans les cellules. Dans nos conditions expérimentales, l'inhibition de la Ca^{2+}-ATPase par le Cu^{2+} a été observée dans les branchies et les reins, et l'inhibition de la Na^{+}/K^{+}-ATPase dans les branchies et la glande digestive, après 4 jours d'exposition à concentrations environnementales. Au delà de 7 jours d'exposition à 0,35 µmol L^{-1} Cu, une récupération totale de l'activité enzymatique a été observée dans les reins et les branchies pour Ca^{2+}-ATPase, et dans la glande digestive pour la Na^{+}/K^{+}-ATPase. A dose élevée l'inhibition persiste. L'inhibition du transport du Ca et du Na peut entraîner des perturbations de l'osmorégulation et conduire à des carences en Ca. La récupération de l'activité enzymatique de la Ca^{2+}-ATPase et de la Na^{+}/K^{+}-ATPase suggère une induction de fonctions de détoxification des métaux. Les phytochélatines (PC) sont des oligopeptides riches en Cys synthétisés par la phytochélatine synthase à partir du glutathion, chez les plantes et les champignons. Des gènes codant pour des phytochélatine synthases fonctionnelles ont étés identifiés chez des invertébrés. Dans la seconde partie de ce travail, les PC et leurs précurseurs, ainsi que les métallothioneines ont été étudiés dans les branchies et la glande digestive d'*A. cygnea* exposé au Cu^{2+}. Nos résultats ont montré pour la première fois, la présence de PC_{2-4} chez les invertébrés. Les PC ont été détectés dans des moules témoins non exposées aux métaux, ceci suggère une fonction dans l'homéostasie des métaux essentiels. Une induction de PC_2 a été observée dés les 12 premières heures d'exposition au Cu^{2+}, comparé aux bivalves témoins. Ces résultats confirment le rôle du PC en tant que mécanisme de première ligne de détoxification des métaux chez *A. cygnea*.

Mots clés : homéostasie du calcium, cuivre, bivalves dulcicoles *Anodonta*, phytochélatines