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Subcellular metal-handling strategies in two groups of aquatic animals (insect, fish): Contributions to the understanding of metal toxicity

Par

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“I don't know anything, but I do know that everything is interesting if you go into it deeply enough”

The Pleasure of Finding Things Out

Richard Phillips Feynman

ABSTRACT

Human-related activities have increased the fluxes of many trace elements from the earth's crust. Trace elements can reach freshwater ecosystems, where they can be readily accumulated, leading to toxic effects. As a result of the need to evaluate the effects of such contaminants on the environment, ecological risk assessments for trace elements have been developed in the last decades. These approaches used to assess the risk posed by trace metal contamination involve three main components: metal availability, metal bioaccumulation and metal toxicity. In this context, significant progress has been made in relating the chemical speciation of trace elements to their bioaccumulation and toxicity for a variety of aquatic organisms, but they have not been completely successful in predicting the toxicity of trace elements. One important element to be refined in such models is the estimation of the subcellular metal concentrations that are likely to be responsible for adverse effects in living cells. Such subcellular estimations will also reveal the metal-handling strategies that aquatic organisms use to cope with these contaminants. Furthermore, detailed characterizations of the biomolecules either involved in metal-detoxification responses or targeted by the trace metals are also required to better understand intracellular metal-trafficking in aquatic organisms chronically exposed in the environment.

To do so, two aquatic animals with differing tolerance to trace metals were collected from metal-contaminated environments, final-instar larvae of the insect *Chaoborus* and North Atlantic yellow eels (*A. anguilla* and *A. rostrata*). A subcellular partitioning procedure using differential centrifugation, NaOH digestion and thermal shock steps was applied to obtain putative metal-sensitive fractions (heat-denatured proteins, mitochondria, microsomes and lysosomes) and detoxified metal fractions (heat-stable proteins and NaOH-resistant granules). To analyse metal complexes with intracellular biomolecules, a metallomic approach based on SEC-ICPMS analysis was applied to the insect.

The subcellular partitioning of trace metals in these aquatic organisms revealed the metal-handling strategies used by these animals to prevent the binding of non-essential metals at physiologically important sites. Metal sequestration by metallothioneins or

metallothionein-like proteins played an important role in metal detoxification (e.g., Cd, Ag). In this respect, the hyphenated analysis of the cytosolic fractions of *Chaoborus* showed that distinct thermostable proteins (or MT isoforms) are involved in Cd sequestration. Incorporation of metals such as Ni, Pb and Tl into mineral inclusions appears to be another important and complementary metal-detoxification strategy. These strategies were not completely successful since significant accumulations in metal-sensitive fractions were observed for all the metals studied; even for animals collected from references sites exposed at the lower end of the metal contamination gradient. These inappropriate bindings of non-essential metals could induce deleterious effects. Among the metal-sensitive fractions, mitochondria appear to be a major binding compartment (for As, Cd, Pb and Tl). The percentage of accumulated trace metals stored in detoxified form varied greatly among metals and animals. Such information should help the development of improved models for predicting metal toxicity, based on the biologically active pools that contribute to toxic responses and not on the total accumulated metal concentrations.

Keywords: subcellular partitioning, trace metals, toxicity, detoxification, *Chaoborus*, *Anguilla*, SEC-ICPMS

RÉSUMÉ

Titre: Les stratégies intracellulaires de gestion de métaux traces chez deux groupes d'animaux aquatiques (insectes, poissons): Contributions à la compréhension de la toxicité des métaux.

Les activités humaines ont augmenté le flux de plusieurs métaux traces. Ces métaux peuvent atteindre des systèmes aquatiques où ils peuvent être accumulés par des organismes pouvant entraîner des effets toxiques. Afin d'évaluer les effets de ces contaminants dans l'environnement, des évaluations de risque environnemental ont été développées. Ces approches ayant comme objectif d'évaluer la probabilité des effets adverses causés par l'exposition de contaminants métalliques ont généralement trois composantes principales : exposition, bioaccumulation et toxicité. Dans ce contexte, différents modèles ont été développés afin de lier la spéciation chimique des éléments traces avec la bioaccumulation et la toxicité. Néanmoins, des modèles ne sont pas capables de déterminer le métal qui est biologiquement actif et donc qui peut être responsable pour les effets adverses. Des mesures de fractionnement subcellulaire de métaux traces qui permettent la séparation des formes détoxiquées et les fractions contribuant aux effets toxiques sont nécessaires pour mieux comprendre la relation entre la bioaccumulation et toxicité des métaux traces. De telles études peuvent aussi révéler les stratégies de gestion de métaux traces utilisées par les organismes aquatiques afin de faire face à ces contaminants. De plus, des caractérisations détaillées des biomolécules qui sont impliquées dans les réponses de détoxication ou sensibles aux métaux traces sont nécessaires pour mieux comprendre la spéciation intracellulaire des métaux traces.

Pour ce faire, deux organismes aquatiques ayant des tolérances différentes à ces contaminants ont été récoltés dans le milieu naturel : des larves de *Chaoborus* et des anguilles nord-atlantiques (*A. anguilla* et *A. rostrata*). Un protocole de répartition subcellulaire incluant des étapes de centrifugation différentielle, digestion avec hydroxyde de sodium et traitement thermique a été appliqué pour déterminer les concentrations de métaux trouvés dans le compartiment de métaux détoxiqués de celui dites sensible telles que dans les fractions de protéines thermosensibles, les mitochondries et d'autres organites. Pour analyser des complexes métalliques avec des biomolécules

intracellulaires, une approche métallomique basée sur l'analyse *SEC-ICPMS* a été aussi appliquée chez les larves de l'insecte.

La répartition subcellulaire de métaux traces chez les organismes aquatiques étudiés a révélé des stratégies intracellulaires de gestions de métaux par ces animaux afin de prévenir la liaison de ces métaux non essentiels avec des sites physiologiquement importants. La séquestration de métaux par les métallothionéines ou par des protéines semblables aux métallothionéines joue un rôle important dans la détoxication des métaux (par exemple Cd, Ag). À cet égard, l'analyse métallomique des fractions du cytosol de *Chaoborus* a montré que différentes protéines thermostables (ou isoformes de métallothionéines) sont impliquées dans la séquestration du Cd. L'incorporation de métaux tels que Ni, Pb et Tl en inclusions minérales semble être une autre stratégie importante et complémentaire de détoxication de ces métaux. Cependant, ces stratégies de détoxication n'ont pas été complètement efficaces pour éviter des accumulations de métaux traces dans les fractions sensibles, même chez des organismes exposés à des faibles concentrations de métaux traces. Ces liaisons de métaux avec des sites inappropriés peuvent induire des effets délétères chez les organismes étudies. Parmi les sites sensibles étudiés, les mitochondries, où des processus biochimiques importants ont lieu, se sont révélés comme la fraction la plus importante en concentrations de ces contaminants potentiellement toxiques (par exemple, pour l'As, le Cd, le Pb et le Tl). Le pourcentage d'un métal trace qui est détoxiquée a varié entre les métaux et les organismes étudiés. L'ensemble des informations peuvent être utiles pour raffiner les évaluations de risque en précisant les concentrations de métaux accumulées qui sont susceptibles de causer des effets néfastes.

Mots-clés : distribution subcellulaire, métaux traces, détoxication, *Chaoborus*, *Anguilla*, séparation chromatographique

FOREWARD

This thesis is presented in two parts. The first part is a synthesis of my doctoral research that comprises a *Résumé récapitulatif*, an introduction, the objectives and hypotheses, the methods, the results and discussion, the conclusions and future research directions. The second part is composed of five publications (as listed below) that have resulted from this research; four of which have been published, whereas the fifth is in preparation.

The contribution of each author was as follows:

1- Rosabal, M., Hare, L., Campbell, P.G.C., 2012. Subcellular metal partitioning in larvae of the insect *Chaoborus* collected along an environmental metal exposure gradient (Cd, Cu, Ni and Zn). *Aquatic Toxicology* 120-121, 67-78.

M. Rosabal: Conception and completion of the research project (sampling, analysis, data treatment and interpretation); writing article.

L. Hare: Conception of the research project, contribution to article writing

P.G.C. Campbell: Conception of the research project, contribution to article writing

2- Rosabal, M., Hare, L., Campbell, P.G.C., 2014a. Assessment of a subcellular metal partitioning protocol for aquatic invertebrates: Preservation, homogenization, and subcellular fractionation. *Limnology and Oceanography: Methods* 12, 507-518.

M. Rosabal: Conception and completion of the research project (sampling, analysis, data treatment and interpretation); writing article.

L. Hare: Conception of the research project, contribution to article writing.

P.G.C. Campbell: Conception of the research project, contribution to article writing, to data interpretation.

3- Rosabal, M., Ponton, D.E., Campbell, P.G.C., Hare, L., 2014b. Uptake and subcellular distributions of cadmium and selenium in transplanted aquatic insect larvae. *Environmental Science and Technology* 48, 12654-12661.

M. Rosabal: Conception and completion of the research project (sampling, analysis, data treatment and interpretation); writing article.

D.E. Ponton: Conception and completion of the research project (sampling, analysis, data treatment and interpretation); writing article.

L. Hare: Conception of the research project, contribution to article writing.

P.G.C. Campbell: Contribution to article writing.

4- Rosabal, M., Pierron, F., Couture, P., Baudrimont, M., Hare, L., Campbell, P.G.C., 2015a. Subcellular partitioning of non-essential trace metals (Ag, As, Cd, Ni, Pb, and Tl) in livers of American (*Anguilla rostrata*) and European (*Anguilla anguilla*) yellow eels. *Aquatic Toxicology* 160, 128-141.

M. Rosabal: Conception and completion of the research project (analysis, data treatment and interpretation); writing article.

F. Pierron: Conception and completion of the research project (sampling); writing article.

P. Couture: Conception and completion of the research project (sampling); writing article.

M. Baudrimont: Contribution to article writing.

L. Hare: Contribution to article writing.

P.G.C. Campbell: Conception of the research project, contribution to article writing, to data interpretation.

5- Rosabal, M., Hare, L., Campbell, P.G.C., Mounicou S., 2015b. Metal (Ag, Cd, Cu, Ni, Tl, Zn) binding to cytosolic ligands in field-collected larvae of the insect *Chaoborus* as determined by Size-Exclusion Chromatography coupled to Inductively Coupled Plasma-Mass Spectrometry. *Metallomics.*

M. Rosabal: Conception and completion of the research project (sampling, analysis, data treatment and interpretation); writing article.

L. Hare: Contribution to article writing.

P.G.C. Campbell: Contribution to article writing, to data interpretation.

S. Mounicou: Conception and completion of the research project (analysis, data interpretation); contribution to article writing.

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ABBREVIATIONS/ABRÉVIATIONS

BM: Biodynamic model

BLM: Biotic ligand model

ERE: Environmental risk assessment

HSP: Heat-stable proteins

HDP: Heat-denatured proteins

ICPMS: Inductively coupled plasma-mass spectrometer

MT: Metallothioneins

MTLP: Metallothionein-like proteins

SEC: Size exclusion chromatography

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1. Résumé récapitulatif

Introduction

Métaux traces dans l'environnement. Évaluation de risques environnementaux

Les activités humaines ont considérablement augmenté le flux de nombreux métaux (Nriagu & Pacyna, 1988). Ces contaminants peuvent atteindre des systèmes aquatiques avoisinants à partir d'émissions provenant des activités minières et métallurgiques (Helios Rybicka, 1996). Une fois dans les milieux aquatiques, ils peuvent être accumulés par des animaux via l'alimentation ou par l'eau; cette accumulation pouvant entraîner des effets toxiques (Luoma & Rainbow, 2008). Les approches, ayant comme objectif d'évaluer la probabilité d'effets délétères causés par l'exposition aux contaminants métalliques, ont généralement trois principales composantes : l'exposition, la bioaccumulation et la toxicité de métaux traces (Figure 1.1) (Campbell *et al.*, 2006, US EPA, 2007). Les approches pour l'évaluation des risques environnementaux (*ERE)* sont conçues pour protéger avec une certaine probabilité des communautés et des populations, à partir des informations prédictives sur le niveau de contamination exerçant des effets biologiques délétères. Le succès de l'*ERE* sera déterminé en grande partie par l'exactitude avec laquelle les modèles appliqués vont prédire ces trois composantes sous différentes conditions environnementales.

Modèles pour prédire la bioaccumulation et la toxicité de métaux

Au cours des 30 derniers ans, différents modèles ont été développés afin de relier la spéciation chimique des éléments traces à la bioaccumulation et la toxicité chez un grand nombre d'espèces aquatiques (Figure 1.1). Dans ce contexte, le modèle du ligand biotique (*BLM*) (Di Toro *et al.*, 2001), une manifestation plus récente du modèle de l'ion libre (Campbell, 1995), a été utilisé pour prédire l'accumulation et la toxicité des métaux traces tels que le Cd, le Cu, le Fe, le Mn, le Ni, le Pb et le Zn (Paquin *et al.*, 2002). Ces modèles stipulent que l'accumulation et la toxicité d'un métal sont proportionnelles à l'activité de l'ion libre du métal en question, à pH et dureté constants, dans le milieu aqueux externe (Morel, 1983).

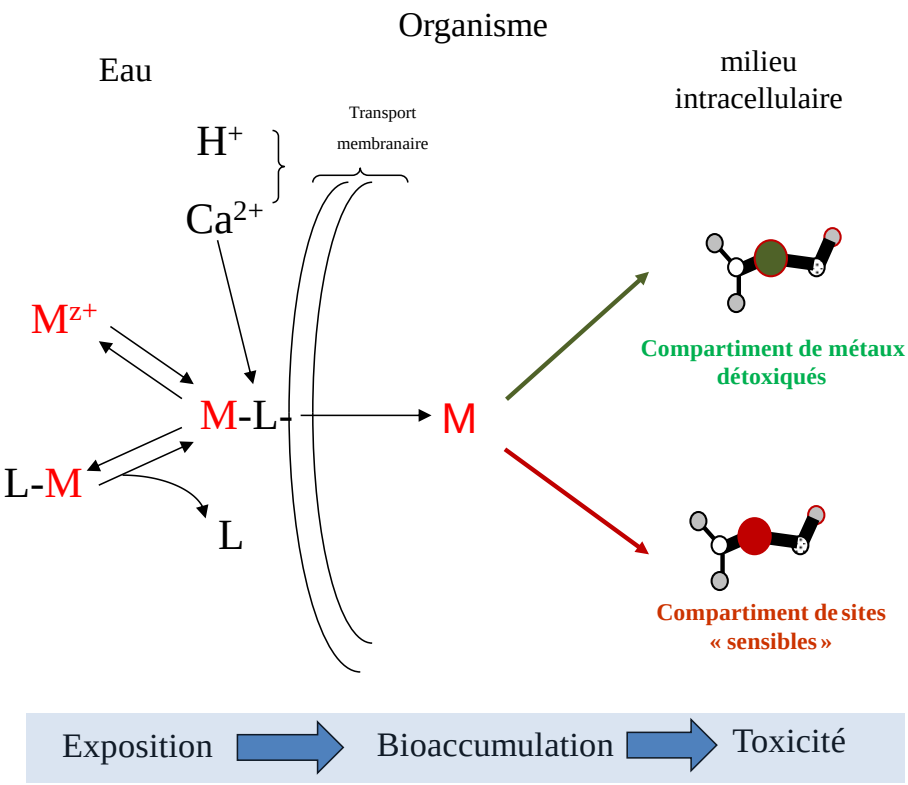

Figure 1.1: Représentation schématique des liaisons entre l'exposition aux métaux (spéciation dans l'eau, complexes de métaux), la bioaccumulation (transport membranaire, répartition subcellulaire de métaux) et la toxicité (compartiment de métaux sensibles) pour un organisme aquatique. Adaptée de Campbell *et al.* (2009).

Bien que le *BLM* ait été utilisé pour établir des critères de qualité d'eau et pour réaliser des analyses d'*ERE*, il doit être raffiné d'avantage afin de prédire l'accumulation et la toxicité des métaux traces sous, par exemple lorsque le pH et la dureté varient en fonction du temps et pour des expositions chroniques (Paquin *et al.*, 2002). Ces modèles demeurent encore inutilisables pour des animaux pour lesquels l'eau ne représente pas une importante voie d'accumulation de métaux traces ou pour les cas où le transport de métaux traces à travers la membrane cellulaire se produit autrement que par le transport facilité de cations (Campbell *et al.*, 2002). Dans ces deux cas, les prévisions de *BLM* ne semblent pas être adéquates pour protéger les communautés et les populations de métaux traces.

Ainsi, l'incorporation des voies alimentaires et aqueuses dans la modélisation de l'accumulation de métaux traces chez les organismes aquatiques est nécessaire pour mieux prédire la bioaccumulation de métaux traces. Le modèle biodynamique (*BM*)

(Luoma & Rainbow, 2005) s'avère un outil intéressant capable d'intégrer des variables importantes pour la prise en charge alimentaire (p. ex. : efficacité d'assimilation, concentration du métal en question dans la nourriture, taux d'ingestion), et de les séparer des variables décrivant la prise en charge aqueuse (p. ex. : concentration de l'ion libre du métal en question). Cependant, les paramètres nécessaires pour la modélisation biodynamique, tels que les constantes de vitesse pour la prise en charge à partir de l'eau, ou le taux d'ingestion de la nourriture contaminée, ne semblent pas être très exacts. Ils sont fréquemment estimés à partir de données obtenues par des expériences d'exposition de durée courte au laboratoire où les organismes sont souvent exposés à des conditions peu réalistes. De plus, ces mesures ont typiquement été effectuées sans tenir compte du fait que les organismes sont capables de s'acclimater aux conditions d'exposition, ce qui peut entraîner des réductions importantes dans leur taux d'accumulation de métaux traces (McGeer *et al.*, 2007). Luoma *et al.* (2005) ont réalisé une revue de la littérature portant sur des études appliquant le *BM*, et ils ont démontré que les prédictions de bioaccumulation de métaux étaient en accord avec les concentrations de métaux mesurées en laboratoire comme sur le terrain pour un nombre important d'organismes aquatiques. Néanmoins, le *BM* n'est pas capable de déterminer si le métal bioaccumulé est biologiquement actif et responsable d'effets néfastes (Wallace *et al.*, 2003a). Des mesures de fractionnement subcellulaire de métaux traces, entre les formes détoxiquées et les fractions contribuant aux effets toxiques, sont nécessaires pour mieux décrire la relation entre la bioaccumulation et la toxicité des métaux traces (Wallace *et al.*, 2003a, Wang, 2013b). Pour contourner ces problèmes, certaines études ont été effectuées en combinant les informations obtenues du *BM* ou du *BLM* avec des mesures subcellulaires de métaux dans le compartiment de métaux détoxiqués et celui de fractions dites sensibles (Campana *et al.*, 2015, Croteau & Luoma, 2009, Liao *et al.*, 2011, Tan & Wang, 2012). Cependant, nos connaissances sur la répartition subcellulaire de métaux traces et les stratégies intracellulaires de gestion des métaux demeurent encore limitées. Avec ces informations, nous devrions pouvoir mieux comprendre la toxicité des métaux au niveau cellulaire chez les organismes exposés dans la nature.

Exposition environnementale.

En ce qui concerne l'exposition des organismes aquatiques aux métaux traces, il est généralement reconnu que les expositions environnementales sont plus réalistes que celles effectuées en laboratoire où les conditions sont parfois peu naturelles (Luoma, 1995). Les résultats obtenus à partir d'expositions au laboratoire sont fréquemment douteux lorsque ces expositions sont de durée courte, impliquant l'exposition de l'organisme cible à un seul métal trace, à des concentrations irréalistes pour l'environnement. De plus, les réponses physiologiques déclenchées par les organismes en laboratoire peuvent différer de celles observées avec des organismes récoltés sur le terrain. Pour cette raison, les mesures subcellulaires nécessaires pour réaliser des analyses d'*ERE* de métaux traces seront plus pertinentes avec des organismes de différents groupes taxonomiques exposés dans le milieu naturel. Dans ce contexte, des organismes tels que les larves de l'insecte *Chaoborus punctipennis* et des anguilles américaines (*Anguilla rostrata*) et européennes (*Anguilla anguilla*) chroniquement exposés à ces contaminants sont de très bons candidats pour ces études.

Choix des organismes.

Les larves de *Chaoborus* (Insecta, Diptera, Chaoboridae) ont été proposées pour estimer des concentrations de Cd (Hare & Tessier, 1996), Ni (Ponton & Hare, 2010) et Se (Ponton & Hare, 2013) accumulées dans les chaînes alimentaires planctoniques des milieux lacustres. Ces organismes sont capables de tolérer des grandes gammes de concentrations en métaux traces ([Cd] : 0.1-20 nmol L^{-1}, [Cu] : 5-1 700 nmol L^{-1}, [Ni] : 10-2 110 nmol L^{-1}, [Zn] : 1-5 000 nmol L^{-1}), de dureté ([Ca] : 30-2 000 µmol L^{-1}) et des variations de la température (4-32 ^{o}C). Par ailleurs, la tolérance des larves de *Chaoborus* aux eaux très acides, où d'autres biomoniteurs (p. ex. : mollusques, crustacés et poissons) ne sont pas capables de vivre, fait de ces organismes des candidats très utiles pour étudier la relation entre la répartition subcellulaire et la résistance aux métaux traces. Les larves de *Chaoborus* sont abondantes et largement distribuées dans le milieu lacustre. Elles sont faciles à identifier au niveau de l'espèce, contrairement à la plupart des larves d'insectes aquatiques. Elles fournissent suffisamment de tissu pour réaliser des analyses de métaux traces même avec un seul individu. Les larves de *Chaoborus punctipennis*, comme espèce

migratoire, peuvent être recueillies pendant la journée (dans les sédiments où elles se cachent pour éviter la prédation des poissons) ou dans la nuit (dans la colonne d'eau où elles se nourrissent de plancton). Hare *et al.* (2008) ont rapporté que les larves de cet insecte peuvent intégrer les changements de concentrations de Cd dans leur environnement sur une période de temps relativement courte, variant de quelques semaines à un mois.

Les larves de *Chaoborus* passent par quatre stades larvaires, suivis d'un stade de pupe avant d'émerger comme adultes. Leur cycle de vie peut varier en durée de quelques semaines, dans les lacs tropicaux, à années dans les climats froids (Carter & Kwik, 1977, Goldspink & Scott, 1971, Hare & Carter, 1986). Les espèces *C. punctipennis*, *C. albatus*, *C. americanus, C. trivittatus* et *C. flavicans* se trouvent dans les lacs du Québec (Borkent, 1981, Garcia & Mittelbach, 2008). En ce qui concerne les proies de *Chaoborus*, elles vont varier en fonction de l'état larvaire, de la profondeur dans la colonne d'eau, de la migration verticale et de la grandeur de la bouche (« mouth gape »). Marianne V. Moore (1988) a rapporté que les petits rotifères (p. ex. : *Kellicottia*, *Gastropus*, *Polyarthra* et *Trichocerca*) sont plus souvent ingérés par les larves de premiers stades (I, II), tandis que les grands rotifères (p. ex. : *Keratella crassa* et *Asplanchna priodonta*) et certains crustacés tels que *Daphnia* sont principalement les proies pour les larves aux stades III et IV.

D'autre part, les anguilles (Teleostei, Anguilliformes, Anguillidae) américaines (*Anguilla rostrata*) et européennes (*Anguilla anguilla*) ont été incluses dans nos études comme modèles de vertébrés, et, particulièrement, comme poissons. Contrairement à la tolérance observée chez *Chaoborus*, les populations des deux espèces d'anguilles, historiquement largement répandues dans des systèmes d'eau douce, ont montré une diminution importante au cours des 30 dernières années (Haro *et al.*, 2000). Dans ce contexte, *A. anguilla* est considérée en situation de danger critique d'extinction par l'Union internationale pour la conservation de la nature (Dekker, 2003). La Communauté européenne a donc mis en place un plan de gestion régionale pour la protection d'*A. anguilla*, impliquant l'application de règlements pour la pêche, l'évaluation des impacts anthropiques et le dénombrement des stocks d'anguilles (CIEM, 2011). Du côté de l'anguille américaine, l'état de leurs stocks a conduit à leur désignation comme espèce

préoccupante (« *species of special concern* ») au Canada en 2006 et comme une espèce en voie de disparition dans la province de l'Ontario (COSEWIC, 2006).

Une caractéristique importante des anguilles est leur cycle biologique, qui comprend deux étapes de métamorphose, deux migrations à travers l'océan Atlantique et une étape de maturation somatique à long terme (de 8 à 20 ans). Les deux espèces d'anguilles fraient dans la mer des Sargasses, et les larves « leptocéphales» migrent vers des cours d'eau côtiers de l'Amérique du Nord (*A. rostrata*) et de l'Europe (*A. Anguilla*), portées principalement par le courant du *Gulf Stream*. À la fin du stade larvaire, ils se métamorphosent en civelles transparentes (ou cristallines). À mesure que les civelles transparentes s'approchent des côtes, elles deviennent des civelles pigmentées et elles changent leur nourriture, d'un comportement pélagique à se nourrir plutôt d'animaux benthiques. Elles se déplacent ensuite vers l'amont des rivières où elles changent vers la couleur jaune (anguilles jaunes), mais elles restent sexuellement immatures; elles vont inclure dans leur nourriture des invertébrés et de petits poissons. Ces anguilles adultes sexuellement immatures vont habiter des écosystèmes d'eau douce très divers, soit des lacs, des réservoirs comme de petits ruisseaux. Après 8 à 20 ans, quand elles deviennent sexuellement matures (connues sous le nom «anguilles argentées»), elles commencent leur migration de retour à la mer des Sargasses pour frayer. Pendant leur maturation somatique à long terme, les anguilles jaunes sont susceptibles d'être chroniquement exposées à des contaminants, incluant les métaux traces, et par conséquent elles constituent de très bonnes candidates pour explorer la relation entre l'accumulation de métaux traces non essentiels et la répartition subcellulaire de ces contaminants.

Objectifs et hypothèses

À la lumière des faits mentionnés ci-dessus, ce projet doctoral visait les objectifs suivants :

1- Déterminer la répartition subcellulaire des éléments non essentiels chez les larves de l'insecte *Chaoborus* (Cd et Ni; larve entière) et chez les anguilles jaunes américaines et européennes (As, Ag, Cd, Ni, Pb et Tl; foies).
   - hypothèses (*Chaoborus*)

- La fraction de protéines *HSP* jouera un rôle important dans la détoxication du Cd et du Ni chez les larves de ces insectes.

- Des concentrations élevées de ces métaux non essentiels seront aussi mesurées dans les fractions sensibles, suggérant une détoxication incomplète.

- hypothèses (*A. anguilla* et *A. rostrata*)
  - Les protéines du cytosol thermostables de forte affinité pour les métaux traces (par exemples, les *MT*) représenteront une importante voie de détoxication de métaux.

  - La formation de granules pourra jouer un rôle important dans la séquestration de métaux traces.

  - Des liaisons « inappropriées » de métaux traces avec des ligands physiologiquement importants confinés dans les mitochondries, le cytosol (autrement dit les protéines thermostables) et d'autres organites seront probablement observées.

2- Caractériser les ligands cytosoliques liés aux métaux traces (p. ex. : Ag, Cd, Cu, Zn et Tl) chez les larves de *Chaoborus* à l'aide d'une approche « métallomique » basée sur la chromatographie d'exclusion stérique couplée à la spectrométrie de masse élémentaire (*SEC-ICPMS*).

  - Le Cd serait majoritairement associé aux *MT*, tandis que le Ni serait séquestré par d'autres ligands thermostables ne contenant pas de groupements thiols.

  - Une co-élution du Cd serait observée avec d'autres métaux mous étudiés tels que l'Ag, Cu et le Zn.

D’autres études portant sur la gestion intracellulaire de métaux chez les larves de *Chaoborus* sont abordées dans cette thèse. Ces résultats sont présentés dans le Chapitre 2 (*Assessment of a subcellular metal partitioning protocol for aquatic invertebrates: preservation, homogenization, and subcellular fractionation)* et au Chapitre 3 (*Uptake and subcellular distributions of cadmium and selenium in transplanted aquatic insect larvae)* de la section II « Articles scientifiques ».

Méthodologie générale

Sites d’étude. Échantillonnage.

Les larves de *Chaoborus* en stade larvaire IV ont été récoltées en 2009 et en 2010 dans des lacs situés près des villes canadiennes de Rouyn-Noranda (Québec) et de Sudbury (Ontario). Ces lacs ont été exposés pendant longtemps à des métaux provenant de l'activité minière locale ainsi que des opérations de fonderies situées dans ces villes (Banic *et al.*, 2006, Dixit *et al.*, 2007). Ils sont donc différemment contaminés en métaux potentiellement toxiques tels que le Cd, le Ni, le Cu et le Zn. Des anguilles jaunes ont été récoltées en 2012 dans les systèmes fluviaux-estuariens de la Gironde (France) et du fleuve Saint-Laurent (Canada), ces systèmes ayant été historiquement contaminés par des composés organiques et inorganiques. Dans l’estuaire de la Gironde, des études géochimiques datant des années 80 ont démontré les effets d’activités minières et d’activités agricoles intensives sur la qualité de l’eau et des sédiments (Audry *et al.*, 2006, Coynel *et al.*, 2007, Latouche, 1988). Ainsi, le Cd a été alors particulièrement identifié comme un contaminant important (Blanc *et al.*, 1999, Claisse, 1989). D’autres métaux incluant l’Ag, l’Cu, le Pb et le Zn sont aussi trouvés à des concentrations importantes dans certains compartiments environnementaux de l’estuaire français (Deycard *et al.*, 2014, Lanceleur *et al.*, 2011). Durrieu *et al.* (2005) ont observé que les anguilles européennes accumulaient des concentrations élevées de Cd, Cu, Hg et Zn, classifiant l’espèce comme étant la deuxième plus contaminée en métaux parmi d’autres espèces de poissons incluant l'alose feinte *Alosa fallax,* le bar *Dicentrarchus labrax,* le maigre *Argyrosomus regius* et la sole *Solea vulgaris.* Quant au système fluvial-estuarien nord-américain, des effluents urbains et industriels et les retombées atmosphériques ont été identifiés comme des sources importantes de la contamination en métaux traces du

fleuve Saint-Laurent (Gobeil, 1999, Gobeil *et al.*, 1995, Quémerais *et al.*, 1999, Quémerais & Lum, 1997, Tremblay & Gobeil, 1990). Les études menées sur la contamination chez des anguilles américaines ont surtout visé la contamination organique, avec relativement peu de travaux sur la contamination par les métaux traces et leurs effets (à l'exception notable du Hg) (Carignan *et al.*, 1994, Hodson *et al.*, 1994) Malgré les efforts consacrés pour réduire la charge de ces contaminants dans les effluents industriels et urbains le long du fleuve Saint-Laurent, la contamination dans cette immense région, où l'urbanisation et l'industrialisation sont de plus en plus importantes, demeure un problème environnemental préoccupant. Pour déterminer le fractionnement subcellulaire de métaux traces chez les anguilles américaines et européennes, quatre sites d'échantillonnage ont été choisis dans chaque système en tenant compte des données déjà disponibles pour les contaminations métalliques dans l'eau, dans les sédiments et chez certains organismes, y compris les anguilles américaines et européennes (Carignan *et al.*, 1994, Durrieu *et al.*, 2005, Hodson *et al.*, 1994)

Les approches utilisées

Dans ce travail, nous avons utilisé deux approches pour déterminer la répartition subcellulaire des métaux traces : (i) un protocole faisant appel à la centrifugation différentielle (*Chaoborus* : larves entières; *Anguilla* : foies) et (ii) une séparation par chromatographie liquide (exclusion stérique) suivie d'une analyse « on line » de l'éluat par spectrométrie de masse élémentaire (*SEC-ICPMS*), cette technique couplée étant appliquée aux cytosols et aux fractions *HSP* de *Chaoborus*.

Répartition subcellulaire

Pour déterminer la répartition subcellulaire de métaux traces nous avons appliqué un protocole permettant d'obtenir de façon opérationnelle six fractions subcellulaires : débris cellulaire plus noyaux (1), granules (2), mitochondries (3), microsomes plus lysosomes (4), protéines cytosoliques thermostables (5, *HSP)* et protéines thermosensibles (*HDP*, 6). Le protocole comprend des étapes de centrifugation différentielle, une digestion avec l'hydroxyde de sodium et un traitement thermique; il a été adapté des protocoles déjà appliqués par Wallace *et al.* (2003a), Giguère *et al.* (2006) et Lapointe *et al.* (2009). Pour les deux tissus récoltés (foies et larves entières), nous avons utilisé des techniques

mécaniques pour le broyage cellulaire. L'efficacité de chaque protocole a été évaluée à l'aide de marqueurs enzymatiques localisés exclusivement dans certaines fractions subcellulaires (par exemple : cytosol) ou organites tels que les mitochondries. Ces résultats ont montré des niveaux acceptables de brisure cellulaire et d'intégrité des organites. Des calculs de bilan de masse ont été effectués pour vérifier la récupération globale des métaux lors de la séparation de l'homogénat en fractions subcellulaires. Ces résultats de rendements (%) ont été jugés acceptables (82-112%). Une fois les différentes fractions subcellulaires obtenues, le dosage des métaux dans chaque fraction a été réalisé par *ICPMS*. Nous avons exploré les relations entre les concentrations de métaux ou la contribution en métaux de chaque fraction subcellulaire avec la concentration totale du métal en question en appliquant différents tests statistiques.

Approche SEC-ICPM

Le cytosol et la fraction *HSP* de larves de *Chaoborus* ont été séparés par chromatographie d'exclusion stérique couplée à la spectrométrie de masse élémentaire (*SEC-ICPMS*) pour déterminer les profils de métaux traces séquestrés par les bioligands localisés dans les deux fractions (Figure 1.2).

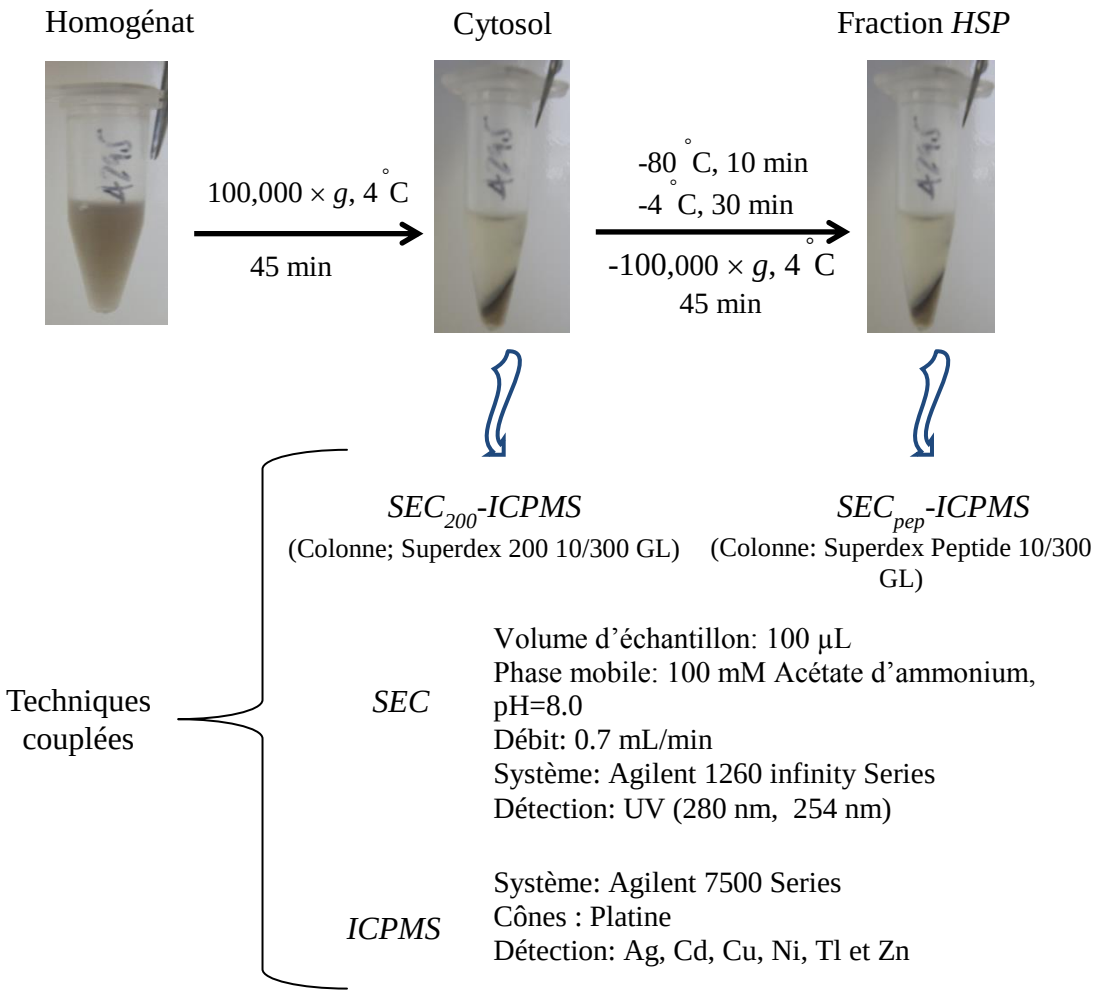

Figure 1.2: Approche métallomique appliquée au cytosol et aux fractions *HSP* des larves de *Chaoborus*.

Bilan des résultats et de discussion

Dans ce résumé, je vais comparer nos résultats pour le Cd chez *Chaoborus* et les anguilles avec ceux obtenus pour le bivalve *Pyganodon grandis* (glande digestive) (Bonneris *et al.*, 2005) et la perchaude *Perca flavescens* (foie) (Giguère *et al.*, 2006). Par rapport au Ni, la comparaison sera faite entre les résultats de fractionnement subcellulaire chez *Chaoborus*, les deux espèces d'anguilles et la perchaude. Nous allons également discuter des résultats les plus importants obtenus lors de l'application de l'approche métallomique au cytosol et aux fractions *HSP* chez *Chaoborus*.

L'approche de répartition subcellulaire de métaux traces a été appliquée à des organismes aquatiques chroniquement exposés, non seulement à un métal, mais plutôt à un mélange de métaux et probablement à d'autres stress environnementaux. Pour certains de ces métaux (Ag, As, Cd, Ni, Pb et Tl), nous avons déterminé leur répartition entre le compartiment détoxiqués (incluant la fraction *HSP* et les granules) et le compartiment dites sensibles (mitochondries, *HDP* et microsomes plus lysosomes). Les tissus utilisés pour ces études varient d'un organisme à l'autre. Malgré ces différences, l'analyse de la répartition subcellulaire de l'As, Ag, Cu, Ni, Pb et Tl, déterminée chez différents organismes récoltés sur le terrain, a révélé des similitudes remarquables dans le comportement du même métal chez différentes espèces. La technique *SEC-ICPMS* appliquée chez *Chaoborus* a également fourni des informations utiles et complémentaires à celles obtenues par l'approche de fractionnement subcellulaire.

La répartition intracellulaire des métaux traces étudiés semble suivre les prédictions de Nieboer et Richardson (1980) quant à la formation de complexes métal-ligand (et donc de la toxicité des métaux) basées sur les préférences de liaison de chaque métal. À cet égard, la répartition subcellulaire de l'Ag et du Cd entre le compartiment de métaux détoxiqués et le compartiment de sites sensibles était très similaire. Par exemple, des concentrations très élevées d'Ag et de Cd ont été trouvées dans le compartiment de métaux détoxiqués par rapport à celles retrouvées dans le compartiment sensible, et cela pour la plupart des organismes aquatiques étudiés. Chez les deux espèces d'anguilles et chez *C. punctipennis*, l'Ag accumulé dans le compartiment de métaux détoxiqués était surtout associé à la fraction *HSP*, ce qui suggère que les *MT* ou *MTLP* vont jouer un rôle important dans la complexation de ce métal.

En ce qui concerne le Cd, la majorité du Cd intracellulaire a été retrouvée dans le compartiment de métaux détoxiqués pour tous les organismes comparés (Figure 1.3). Cette similitude dans la gestion intracellulaire du Cd a été observée chez les deux espèces d'anguilles, même si elles avaient accumulé des concentrations en Cd très différentes (la concentration hépatique maximale du Cd chez *A. anguilla* était quatre fois plus élevée que celle d'*A. rostrata*). Par exemple, il n'y avait pas de différence significative ($P > 0,05$) entre les deux espèces par rapport à la pente de la régression linéaire entre la concentration du Cd dans le compartiment détoxiqué versus la concentration totale du Cd

dans les foies. Ces résultats suggèrent que les animaux étudiés utilisent les mêmes stratégies intracellulaires de gestion de Cd afin de minimiser les effets délétères causés par l'exposition à ce contaminant. Le rôle prédominant de la fraction *HSP* pour la détoxication du Cd a été observé chez tous les animaux étudiés. Les analyses métallomiques par *SEC-ICPMS* dans le cytosol et dans les fractions *HSP* des larves de *Chaoborus* ont confirmé que des *MTLP* (identification basée sur la similitude de leur poids moléculaire avec ceux connus pour les *MT* chez *Drosophila melanogaster*) sont directement impliqués dans la détoxication du Cd. Ces résultats chromatographiques ont aussi révélé que des isoformes de *MT* (ou de *MTLP*) semblent jouer des rôles différents dans ce processus de complexation/ détoxication du Cd. Ces analyses ont également montré que les réponses de détoxication chez *Chaoborus* deviennent plus importantes à des concentrations totales plus élevées, une observation qui est en accord avec notre étude de terrain avec des larves de *Chaoborus* exposées de façon chronique au Cd (Chapitre 1).

Signalons que des similitudes entre le Cd et l'Ag ont été observées lors des analyses par *SEC-ICPMS* chez *Chaoborus*; les deux métaux étaient complexés par les *MT* or *MTLP* détectés dans le cytosol et la fraction *HSP*. Ainsi, la spéciation subcellulaire de l'Ag et du Cd est probablement déterminée par la liaison préférentielle de ces métaux de classe B (Nieboer *et al.*, 1980) aux groupements sulfhydryles des *MTLP* impliquées dans leur détoxication.

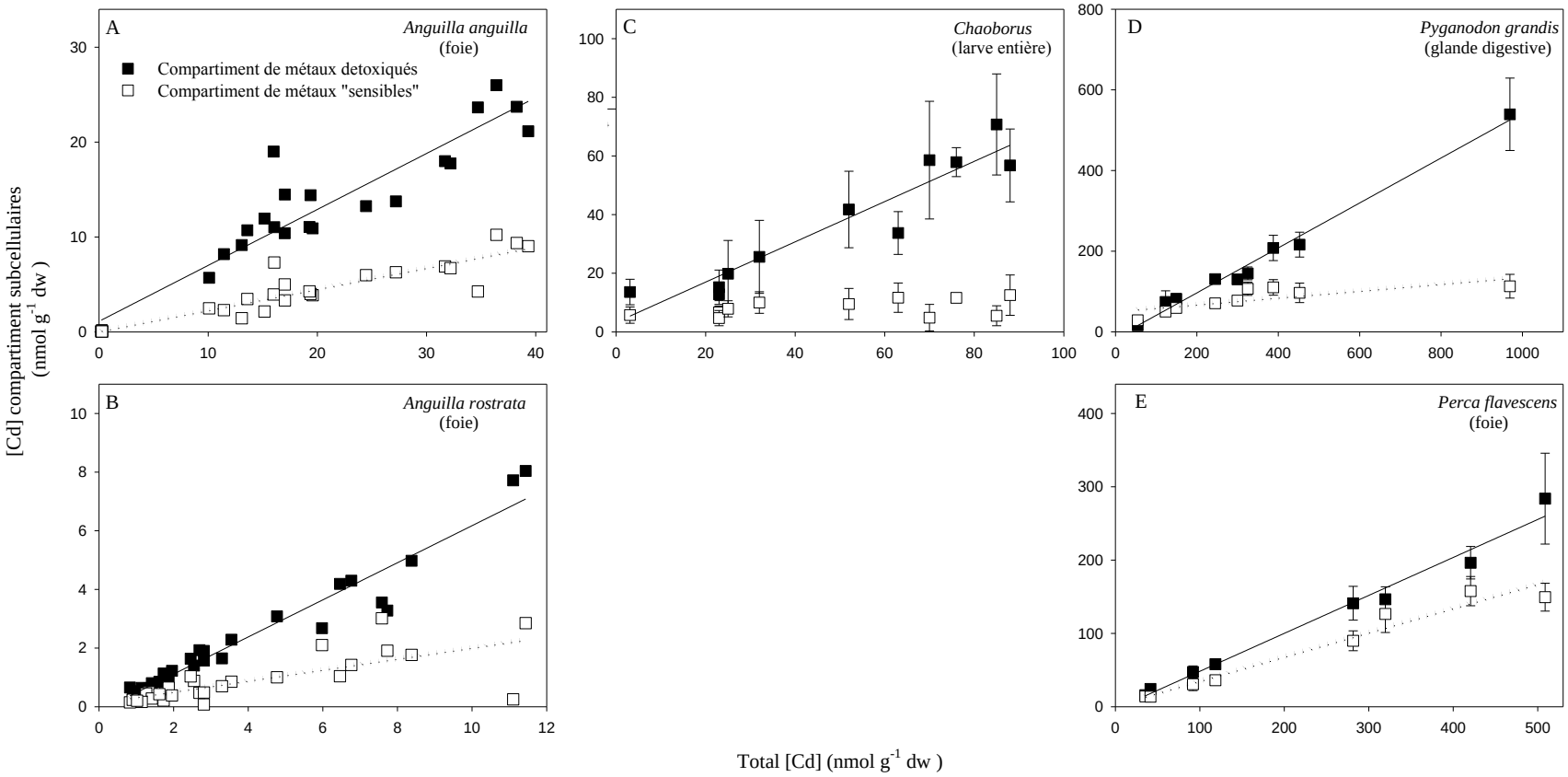

Figure 1.3: Relation entre la concentration totale de Cd dans l'organe (anguilles, perchaude, bivalve) ou dans l'animal entier (moucheron) et la concentration de Cd dans le compartiment de métaux détoxiqués (carrés noirs) et dans le compartiment de sites sensibles (carrés blancs) chez *Anguilla anguilla* (foie, A), *Anguilla rostrata* (foie, B), larves de *Chaoborus* (larve entière, C), *Pyganodon grandis* (glande digestive gland, D) et *Perca flavescens* (foie, D). Les régressions significatives y ont été représentées ($P < 0.05$).

D'autre part, le plomb (métal de comportement intermédiaire) et le thallium (métal de classe B) (Nieboer *et al.*, 1980), capables d'interagir avec de groupes sulfhydryles et aminés, a été aussi trouvé dans le compartiment de métaux détoxiqués chez les anguilles, mais à des proportions moins importantes que celles mesurées pour l'Ag et le Cd. Contrairement à ces deux derniers métaux fortement associés aux *MTLP*, le Pb et le Tl « détoxiqués » n'ont pas été limités à la fraction *HSP* où les *MTLP* se trouvent. Des accumulations importantes étaient aussi mesurées dans la fraction granules, où d'autres ligands portant de groupements HS^- ou N pourraient être impliqués dans la séquestration de ces contaminants potentiellement toxiques. Les résultats des analyses chromatographiques chez *Chaoborus* ont montré que l'accumulation du Tl dans la fraction *HSP* était associée à des biomolécules de faibles poids moléculaires (< 1.7 kDa), possiblement dans une forme méthylée ($(CH_3)_2Tl^+$) (Schedlbauer & Heumann, 2000).

Pour ce qui est du Ni, des concentrations importantes du Ni « détoxiqué » ont été observées pour les trois espèces de poissons qui ont été étudiées (*A. anguilla*, *A. rostrata* et *P. flavescens*) ainsi que pour les larves de *Chaoborus*. Néanmoins, des différences dans la stratégie de détoxication utilisée ont été constatées parmi les organismes comparés. Pour les larves de *Chaoborus* et la perchaude, le Ni a été séquestré majoritairement par des protéines cytosoliques thermostables dans la fraction *HSP*, tandis que chez les anguilles le Ni a été incorporé surtout dans des granules minéraux. Tenant compte de la faible affinité du Ni pour les groupements HS^- ou N: par rapport aux autres métaux (p. ex. : l'Ag et le Cd), la séquestration de ce métal dans la fraction de granules pourrait être déterminée par la présence de granules comportant le Fe ou le Ca/Mg comme élément principal, plutôt que par la présence de granules riches en soufre réduit. L'analyse des fractions *HSP* de larves de *Chaoborus* en utilisant l'approche chromatographique (*SEC-ICPMS*) a montré que les *MT* et les *MTLP* n'étaient pas impliquées dans la complexation du Ni; d'autres ligands encore inconnus sont responsables de la complexation du Ni dans cette fraction. Considérant que le Ni est un métal plus difficile à polariser que l'Ag et le Cd, d'après la classification dur-mou décrite par Nieboer *et al.* (1980), cet élément ne semble ni induire la biosynthèse de la *MT* ni interagir avec les groupements sulfhydryles des *MT*.

En comparant les concentrations du Ni dans le compartiment de métaux détoxiqués pour les quatre organismes étudiés, on peut constater de différences importantes entre *Chaoborus* et *P. flavescens*, deux organismes récoltés dans la même région affectée par des activités minières et avec des gradients de bioaccumulation similaires (Figure 1.4). Pour les larves de *Chaoborus*, le pourcentage du Ni total accumulé qui a été « détoxiqué » dépasse le 50 % pour l'ensemble des organismes récoltés. En contraste, les cellules hépatiques de la perchaude ont détoxiqué moins qu'un quart du Ni hépatique accumulé.

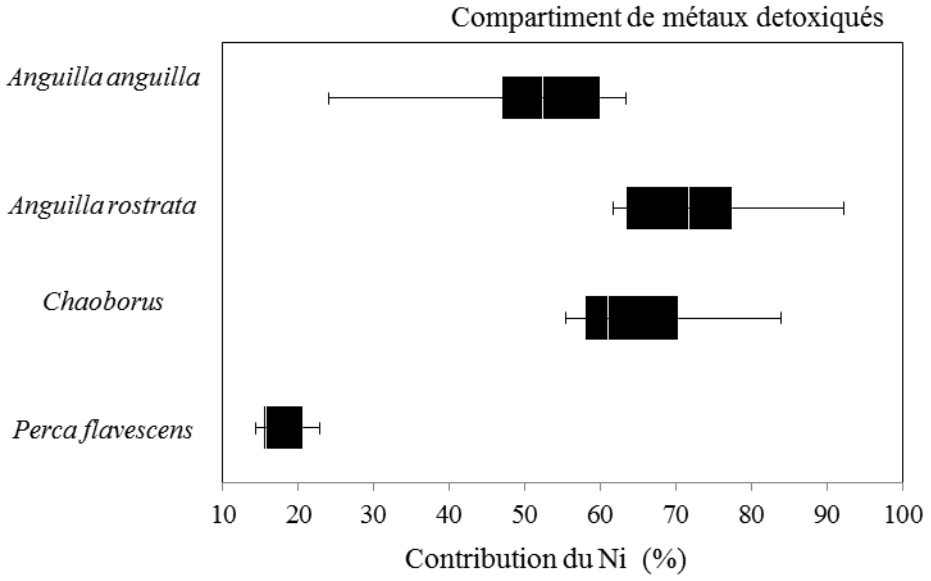

Figure 1.4: Distribution « boîte à moustaches » de la contribution en pourcentage (%) du compartiment de métaux détoxiqués à la concentration totale du Ni chez *Anguilla anguilla* (foie, n = 11), *Anguilla rostrata* (foie, n = 28), *Chaoborus* (larve entière, n = 12) et *Perca flavescens* (foie, n = 5). Dans chaque boîte, la ligne verticale en blanc représente la médiane, les deux extrémités indiquent les percentiles 25 et 75%, et les moustaches, l'étendue des valeurs.

En ce qui concerne l'As, Nieboer et Richardson (1980) ont classé l'As(III) comme intermédiaire, c'est-à-dire, sans affinité prédominante du type A ou B pour les ligands intracellulaires. Comme nous n'avons pas déterminé la distribution de l'As parmi les différentes espèces chimiques (As(III), As(V), formes organo-As; Thomas *et al.* (2001)) il est difficile d'établir un lien entre les préférences de liaison de l'As et sa répartition subcellulaire à partir de nos résultats.

Malgré les réponses de détoxication observées, des accumulations importantes de divers métaux ont été retrouvées dans le compartiment sensible, ce qui suggère que les mécanismes de détoxication n'ont pas été complètement efficaces. Des métaux non essentiels se trouvaient associés aux fractions de protéines thermosensibles du cytosol, dans les mitochondries ou dans d'autres organites. En fait, les concentrations de ces métaux dans le compartiment « sensible » a significativement augmenté en fonction de la concentration totale intracellulaire pour la plupart des organismes comparés. Même chez les organismes fortement contaminés, les concentrations mesurées dans ce compartiment ont dépassé celles obtenues dans le compartiment de fractions impliquées dans la détoxication (p. ex. : Tl chez *A. anguilla*, Ni chez *P. flavescens*, puis As et Ni chez *A. rostrata*). Ces résultats suggèrent que le modèle de « débordement cellulaire » (Jenkins & Mason, 1988, Mason & Jenkins, 1995) n'est pas adéquat pour décrire les stratégies de gestion de métaux chez les animaux exposés de façon chronique dans le milieu aquatique. Nous n'avons pas mesuré des effets toxiques chez les organismes étudiés, mais nos résultats suggèrent qu'il pourrait y avoir des effets néfastes sur les fonctions biologiques effectuées par les bioligands avec lesquels ces métaux sont liés (Mason *et al.*, 1995).

Parmi les fractions sensibles, les mitochondries, où des processus biochimiques clés pour l'organisme se produisent, se révèlent comme la fraction la plus importante, en renfermant des concentrations élevées de ces contaminants potentiellement toxiques (As, Cd, Pb et Tl) (Figure 1.5). À cet égard, la proportion de métaux trouvée dans le compartiment de fractions sensibles qui a été confinée dans les mitochondries a varié pour l'Ag (*A. anguilla*: 14 - 65%; *A. rostrata*: 2 - 62%), As (*A. anguilla*: 9 - 59%; *A. rostrata*: 1 - 72%), Cd (*A. anguilla*: 6 - 72%; *A. rostrata*: 1 - 71%), Ni (*A. anguilla*: 15 - 65%; *A. rostrata*: 12 - 57%), Pb (*A. anguilla*: 19 - 65%; *A. rostrata*: 11 - 60%) et Tl (*A. anguilla*: 9 - 62% ; *A. rostrata*: 8 - 73%).

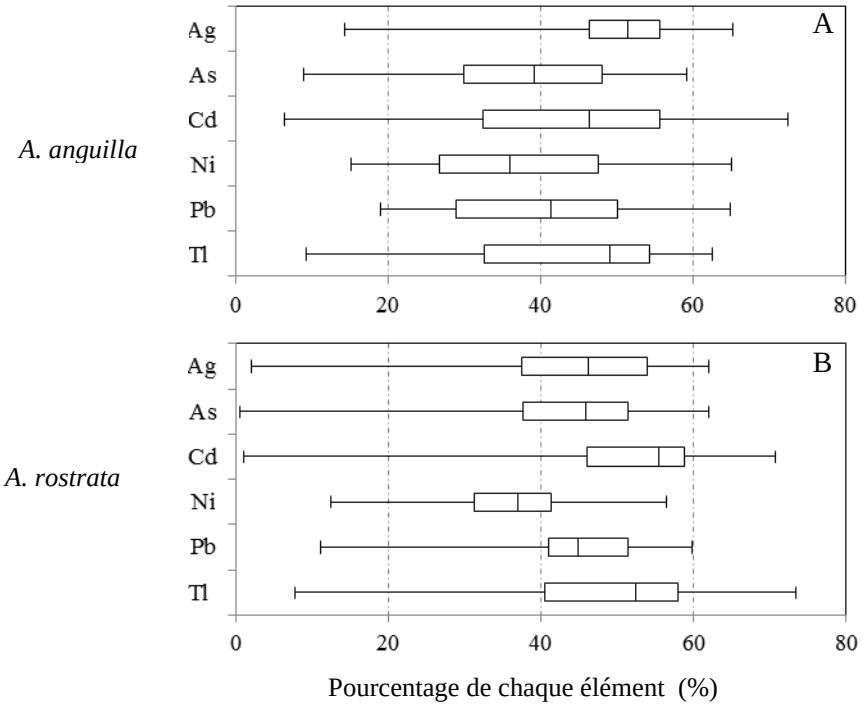

Figure 1.5: Distribution « boite à moustaches » de la contribution en pourcentage (%) de chaque élément étudié dans la fraction de mitochondries chez les foies de l'*Anguilla anguilla* (A) et *Anguilla rostrata* (B). Dans chaque boîte, la ligne verticale noire représente la médiane, les deux extrémités indiquent les percentiles 25 et 75%, et les moustaches, l'étendue des valeurs.

Notez que les pourcentages les plus élevés ont été atteints pour le Cd chez *A. Anguilla* (Figure 1.5A) et pour le Tl, le Cd et l'As chez *A. rostrata* (Figure 1.5B). La contribution importante observée pour les mitochondries par rapport aux autres fractions sensibles montre une localisation subcellulaire où ces contaminants potentiellement toxiques pourraient produire des effets délétères dans le milieu intracellulaire.

En général, les résultats de la répartition subcellulaire ont mis en évidence des différences parmi les métaux étudiés, entre les deux espèces d'anguilles jaunes pour certains métaux incluant l'As, le Ni et le Pb.

Perspectives

Les mesures subcellulaires de métaux traces effectuées pour distinguer l'accumulation de métaux dans le compartiment de fractions sensibles de celle du compartiment de métaux détoxiqués permettent aux chercheurs de déterminer les stratégies de gestion de métaux

utilisées par les organismes aquatiques pour faire face à de fortes concentrations de ces contaminants (Campbell *et al.*, 2005, Campbell *et al.*, 2009). En ce qui concerne les stratégies de détoxication de métaux, l'incorporation de métaux aux granules et la complexation de métaux par des protéines cytosoliques thermostables (par exemple, *MT* ou *MTLP*) ont été reconnues pour protéger les organismes contre la toxicité des métaux, mais la caractérisation détaillée des biomolécules impliquées dans ces réponses de détoxication demeure encore nécessaire pour mieux comprendre les rôles spécifiques de ces fractions dans la répartition intracellulaire de métaux traces.

Dans cette thèse, des analyses chromatographiques d'une série de métaux traces ont été déterminées chez *Chaoborus*, ce qui a permis de réaliser une caractérisation de complexes métal-ligand en fonction de leurs poids moléculaires. Cependant, plus d'études doivent être réalisées pour isoler, identifier et quantifier les complexes métalliques avec ces biomolécules intracellulaires. Par exemple, il serait intéressant de déterminer les caractéristiques biochimiques (poids moléculaire, composition et séquence en acides aminés et stœchiométrie de la liaison métal-ligand) de ces protéines et leurs fonctions spécifiques dans la détoxication de métaux. De même, davantage d'études devraient être faites pour élucider la séquestration de ces contaminants par des granules et plus particulièrement pour identifier les biomolécules directement impliquées dans la séquestration de métaux.

À ce sujet, les mesures analytiques de ces complexes métalliques peuvent être effectuées par l'application de techniques couplées (« *hyphenated* ») impliquant le couplage de techniques de séparation à haute résolution avec l'*ICPMS* (Lobinski *et al.*, 1998). Le couplage de la spectrométrie de masse élémentaire et moléculaire (MS) à la chromatographie liquide a déjà été utilisé pour caractériser certaines isoformes de Cd-*MT* (Mounicou *et al.*, 2010), mais cette approche, qui offre de nouvelles possibilités analytiques, devrait être appliquée à d'autres organismes aquatiques et à d'autres métaux. Des informations analytiques concernant des complexes métalliques avec des biomolécules intracellulaires sensibles seraient également utiles pour raffiner la relation entre la bioaccumulation d'un métal et son effet biologique.

Une autre question importante à considérer est que la nature des fractions subcellulaires n'est pas complètement connue. Par exemple, l'isolement de la fraction *HSP* du cytosol est basé sur les propriétés thermostables de protéines incluant les *MTLP*, mais il n'est pas évident que toutes les protéines présentes dans le surnageant après le traitement thermique (les thermostables) soient impliquées dans la détoxication de métaux traces. L'application de techniques couplées (par exemple, *HPLC-ICPMS*) combinées à des analyses protéomiques serait très utile pour révéler la nature de ces fractions subcellulaires.

Les études avec des organismes récoltés sur le terrain, et ayant été exposés de façon chronique aux métaux traces, ont montré des liaisons inappropriées dans les fractions sensibles aux métaux, même avec des organismes exposés à de faibles concentrations de métaux (Bonneris *et al.*, 2005, Giguère *et al.*, 2006). Des effets adverses ont été reportés pour certains des organismes étudiés (Campbell *et al.*, 2009), mais pour les larves de *Chaoborus* et les deux espèces d'anguilles il sera nécessaire de déterminer s'il y a des effets toxicologiques associés à l'apparition de métaux non essentiels dans les fractions dites sensibles.

2. Introduction

2.1 *Trace elements*

The term *trace metal* refers to elements found at "trace" levels, in "mass fractions" of 100 parts per million or less (Duffus, 2002). However, when Al, As, Mo or Se is included, the "less objectionable umbrella" term *trace elements* is preferred (Duffus, 2002, Hübner *et al.*, 2010). In biological samples the term *trace* would correspond to a concentration of around ppm, µg/g or mg/kg (dry weight), but Luoma *et al.* (2005) reported that some elements with ecotoxicological significance can exceed such concentrations. In the present thesis *trace metal* and *trace element* will be interchangeably used to refer, as proposed by Luoma *et al.* (2008), to either Lewis "soft" acids or elements having properties intermediate between Lewis "soft" and "hard" acids (Nieboer *et al.*, 1980).

2.2 *Incorporating trace elements in biological systems*

Elements naturally occur in the environment. The relative abundance of the elements on Earth, particularly in the crust, the aqueous zone and the atmosphere resulted from the Big Bang and other astronomic events dated from 5×10^9 years ago (R. J. P. Williams, 2002). Early forms of life emerged 3.5×10^9 years ago when vital biochemical processes required energy inputs and catalysts. In this context, the most abundant and available elements such as Mn, Fe, Co, Ni and Mo, which could function as catalysts or be involved in oxidation-reduction reactions related to energy inputs, were selectively assimilated to play essential roles. Other elements that were less abundant/available (e.g., As, Ag, Cd, Hg, Pb, Pd, Po) are not used for essential purposes in cells. As a result, living cells evolved mechanisms not only to acquire the required elements and to maintain them at physiological levels, but also to detoxify the non-essential elements to which early cells were exposed (Williams & Frausto da Silva, 1996).

In the Anthropocene, the environment has been altered due to increasing human activities that have increased the flux of elements from the earth's crust. Consequently, organisms now tend to be exposed to higher concentrations of non-essential trace elements than those that existed prior to this era. In response to these changes, some organisms have

developed tolerance to the higher concentrations of trace elements in their environments (Amiard *et al.*, 2006, Roesijadi *et al.*, 1982, Wallace *et al.*, 1998). Although trace metal essentiality differs somewhat among organisms, essential elements usually include Co, Cr, Cu, Fe, Mn, Se, Ti, V and Zn, whereas nonessential elements include As, Ag, Au, Cd, Hg, Ni, Pd, Pt and Tl.

2.3 *Prediction of metal accumulation and toxicity in aquatic organisms*

As mentioned above, human-related activities have increased the fluxes of many trace elements from the geosphere to the atmosphere and to the biosphere (J.O. Nriagu *et al.*, 1988). Trace elements can reach freshwater ecosystems by direct inputs from mines, metal smelters and other industries (Helios Rybicka, 1996, Jerome O. Nriagu *et al.*, 1998) or by indirect sources such as atmospheric transport and deposition, chemical erosion (weathering) and physical erosion (Dickson, 1986, Stevens *et al.*, 2009, Weber *et al.*, 2009). Once in aquatic systems, trace elements can be accumulated by freshwater animals from water and/or food (Luoma *et al.*, 2005), which can lead to toxic effects (Luoma *et al.*, 2008). As a result of the need to evaluate the effects of such contaminants on the environment, ecological risk assessments (ERA) for trace elements in freshwater ecosystems have been developed, which has led to the creation of regulatory criteria (Campbell *et al.*, 2006).

According to the US Environmental Protection Agency (US, 1992), ERA is a process that "evaluates the likelihood that adverse ecological effects are occurring or may occur as a result of exposure to one or more stressors". In this way, ERA aims to protect populations and communities based on predicted information about the level of contamination resulting in adverse biological effects (e.g., lethal concentration, LC_{50}; effect concentrations, EC_{50}). In addition, the ERA approach is used to rank risks posed by different stressful conditions (e.g., mixtures of trace elements), to establish site-specific guidelines and to assess the success of remediation actions (Campbell *et al.*, 2006). In general, the ERA frameworks used to assess the risk posed by trace metal contamination involve three main components: metal availability, metal bioaccumulation and metal toxicity. The success of ERA approaches will be thus determined by how well the models

used are able to estimate or predict these three components under various environmental conditions.

Over the last 30 years, significant progress has been made in relating the chemical speciation of trace elements to their bioaccumulation and toxicity for a variety of aquatic organisms (Figure 2.1). In this regard, the free-ion metal activity model (FIAM) (Campbell, 1995) and the biotic ligand model (BLM) (Di Toro *et al.*, 2001, Niyogi & Wood, 2004, Paquin *et al.*, 2002) have been developed and applied to many types of aquatic organisms (e.g., algae, invertebrates, fish) for a range of trace elements (e.g., Cd, Cu, Fe, Mn, Ni, Pb and Zn). Both models consider "the universal importance of free metal ion activities in determining the uptake and toxicity of trace elements" (Morel, 1983).

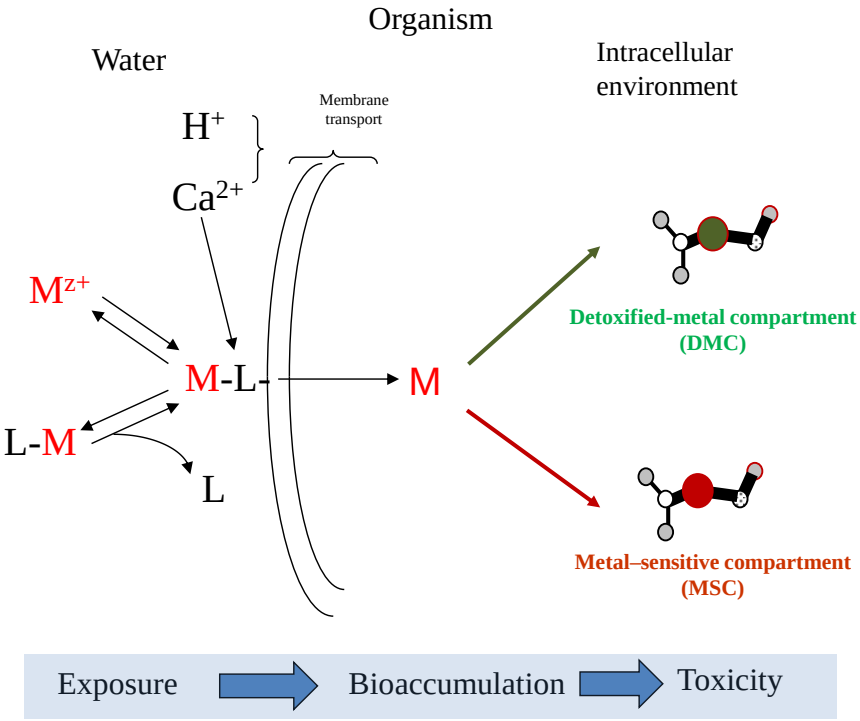

Figure 2.1: Schematic illustration showing the links between metal exposure (metal speciation in water, metal complexation), bioaccumulation (transmembrane transport, subcellular metal partitioning) and toxicity (metal-sensitive compartment) for a given aquatic organism. Adapted from Campbell and Hare (2009).

Although the BLM has been used to develop water quality criteria and perform aquatic risk assessments, this model is unable to predict the accumulation and toxicity of trace elements under certain conditions, for example, where the pH and/or water hardness vary over time, as well as under chronic exposure to low concentrations of metals (Paquin *et*

al., 2002). Other shortcomings of the BLM are evident when this model is applied to aquatic organisms for which diet is the major source of trace metals or where metal transport across cell membranes occurs by means other than cation-facilitated transport (Campbell *et al.*, 2002).

Biodynamic models have been widely used to model and predict metal concentrations in aquatic animals (Luoma *et al.*, 2005). They consider the uptake of trace elements into animals from both water and food, as well as trace element losses, either physiological or through growth. Luoma and Rainbow (2005) reviewed applications of biodynamic modelling to a wide range of animals and exposure conditions and concluded that there was reasonably good agreement between predictions of metal bioaccumulation and measurements in the laboratory or in nature. In spite of this, if the rate constants used in such models are obtained in short-term experiments with metal-naïve animals, they are likely to lack environmental realism. For example, acclimation by fish has been shown to reduce the accumulation of trace elements (McGeer *et al.*, 2007). Furthermore, because biodynamic modeling approaches are generally used to estimate metal accumulation in whole organisms, they do not tend to reflect the biologically active portion that is likely to be responsible for adverse effects in living cells (Adams *et al.*, 2011). In this context, information on the distribution of metal burdens between detoxified forms and biologically active pools that contribute to toxic responses is required to better describe the relationship between metal bioaccumulation and its biological effects (Campbell *et al.*, 2005, Wallace & Luoma, 2003b).

Several studies have attempted to predict metal toxicity by combining information from biodynamic models (Croteau *et al.*, 2009, Rainbow & Luoma, 2011a) or the BLM (Liao *et al.*, 2011) and measurements of subcellular metal detoxification (Campana *et al.*, 2015, Rainbow, 2007, Tan *et al.*, 2012). However, important knowledge gaps remain and accurate measurements of the subcellular distribution of trace elements as well as improved understanding of the metal-handling strategies of aquatic organisms under various environmental conditions are crucial to predict metal toxicity.

2.4 *Metals within cells.*

Once a metal crosses the cell membrane it faces an intracellular environment that is very different from that found outside of the cell. First, in contrast to the wide range of pHs that can be found in the external environment (e.g., lake water), the intracellular pH is generally neutral (around 7) with the exception of lysosomes (acidic pH is required for the activity of digestive enzymes) (Madshus, 1988, Roos & Boron, 1981). The intracellular environment can have a less-oxidized redox potential than does the extracellular environment (Auchinvole *et al.*, 2012, Menon & Goswami, 2007). In addition, the nature of most intracellular metal-binding ligands is different from those found outside of the cell. In the intracellular environment, a large number of ligands have been identified as important cellular "sinks" including metal-binding proteins, lysosomes and mineralized and organic-based concretions (Mason *et al.*, 1995). Such ligands, which can have long biological half-lives, contribute to the persistence of essential and nonessential metals in the intracellular environment. They are extremely variable in form and cellular location; however they appear to function in a similar way to control the internal availability of metals. The intracellular metal speciation must answer the same questions (*bound to what* and *in what species or form* metals are found) that are asked when speciation is considered in water or sediment and one is attempting to establish relationships between metal exposure and biological effects. Additionally, *where* the metal is located in the intracellular environment must be also considered.

With regards to the metal binding to intracellular ligands, Nieboer *et al.* (1980) proposed a classification of metals into three groups to predict the metal complex in the intracellular environment based on ligand-binding preferences for various organic ligands (Figure 2.2).

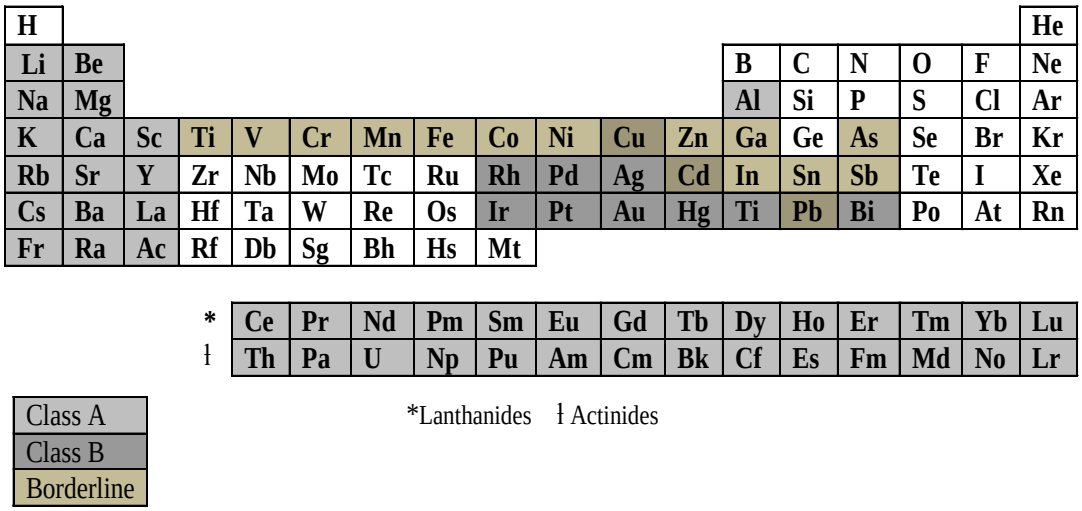

Figure 2.2: The classification of metals into Class A, Borderline and Class B categories based up Lewis acid properties. Nieboer and Richardson (1980) and Pearson (1969).

Metals of class A (e.g., K^{+1}, Li^{+1}, Na^{+1}, Ba^{+1}, Ca^{+2}, Mg^{+2} and Al^{+3}) are non-polarizable and highly stable and preferentially bind anions with oxygen as electron donors. Metals of class B (e.g., Ag^{+1}, Au^{+1}, Cd^{+2}, Hg^{+2}, Pb^{+2}, Cu^{+1}) exhibit a pronounced preference for sulphur and nitrogen donors located in cells. Borderline metals (e.g., Mn^{+2}, Fe^{+3}, Co^{+2}, Ni^{+2}, Cu^{+2}, Zn^{+2}) show ligand-binding characteristics that are intermediate between those for class A and B cations.

2.5 *Metal-detoxification strategies*

To prevent metal toxicity, a variety of complementary mechanisms have been evolved by organisms to control the uptake, sequestration and efflux of trace elements (Rainbow, 2002). Metal uptake can also be minimized by behavioural avoidance (Mason *et al.*, 1995).

2.5.1 Limiting metal uptake

Aquatic organisms have evolved a variety of strategies for limiting or preventing metal uptake (Banerjee, 2004, Mason *et al.*, 1995). Several organisms are known to modify the mode, rate and route of metal uptake by changing the speciation and bioavailability of elements in the surrounding environment through the secretion of chemical modifiers.

For example, the thermophylic green alga *Cyanidium caldarium* limits metal internalization by incorporating elements as sulphides into microcrystals associated with its external cell membrane (Wood & Wang, 1983). In addition, a decrease in the permeability of epithelial surfaces by introducing extracellular barriers has also been identified as a strategy for preventing toxicity for a number of different elements in such diverse organisms as algae, bacteria, polychaetes and fish (Mason *et al.*, 1995).

2.5.2 Favoring metal efflux

Metal detoxification based on increasing metal efflux using mechanisms such as ATPase pumps and chemiosmotic membrane transporters has been reported in prokaryotes and eukaryotes (Silver & Phung, 2005, Tsai *et al.*, 1997). Metal-efflux systems have been shown to be involved in limiting the concentrations of Ag, Co, Cu, Ni, and Zn (Silver & Phung, 1996, Silver *et al.*, 2005), albeit at very high ambient concentrations of these elements. Note that for efflux to take place, metals must first be taken up and complexed by intracellular ligands that can then be involved in the excretion of these elements.

2.5.3 Sequestering intracellular metal

Organisms can sequester elements as a detoxification strategy and thereby accumulate high quantities of trace elements without apparent detrimental effects. Tolerance to metal toxicity has therefore been related to high body burdens of trace elements that are bound to a variety of ligands (e.g., metal-binding proteins, lysosomes, mineralized and organic-based concretions).

The sequestration of intracellular elements by metal-binding ligands, mainly metallothioneins and metallothionein-like proteins, has been studied in animals as diverse as mammals, reptiles, amphibians, fish, molluscs, crustaceans, annelids and aquatic insects (Amiard *et al.*, 2006, Nordberg, 1998, Sigel *et al.*, 2009). They are involved in the cellular regulation and detoxification of certain trace metals (e.g., Ag, Cd, Cu, Hg and Zn). MT are cytosolic non-enzymatic proteins of low molecular weight that generally lack aromatic amino acids and are heat stable. One of their most pertinent characteristics is their high cysteine content (e.g., mammals: 33%; crustaceans: 31%; insects: 25-28%) (Sigel *et al.*, 2009). The presence of thiol groups (-SH) in cysteine provides MT with a high affinity for sequestering trace elements in the cytoplasm and/or controlling the

concentrations of essential elements (Templeton & Cherian, 1991). Repetitive amino acid patterns in MT sequences (Cys-Cys, Cys-X-Cys and Cys-X-Y-Cys, where X and Y are amino acids other than cysteine) allow MT to be grouped into three classes based on the occurrence and similarity of their sequence patterns. Class I includes MT with sequences similar to those found in mammalian cells and includes the MT of molluscs and crustaceans. Class II MT include those from bacteria, yeast, fungi and dipteran insects, which are not homologous with mammalian MT. The non-proteinaceous metal-binding ligands of plant cells known as phytochelatins are included in Class III. Binz and Kagi (1999) proposed an alternative classification that takes into account phylogenetic features as additional classification criteria. In this light, MT are classified into 15 families including those found in molluscs (family 2), crustaceans (family 3) and dipteran insects (family 5).

With regards to the determination of metal to protein stoichiometric ratios, Scheuhammer and Cherian (1986) reported that under certain conditions mammalian MT can bind approximately 17 to 18 atoms of Ag(I), indicating a possible metal : thiol ratio of 1:11. Such evidence is in agreement with recent work on MT isolated from organisms exposed to metal mixtures, which demonstrated that the metal complex Cu_nZn_{7-n}–MT as well as "supermetallated" MT forms with Me : MT stoichiometries greater than the accepted 7:1 or 12:1 ratios can exist (Duncan & Stillman, 2006). Some reports have suggested that the substitutive exchanges of metal in MT that can occur *in vivo* appear to follow the *in vitro* hierarchical sequences of metal binding defined in the order: Hg > Ag > Cu > Cd > Zn (Nordberg, 1998, Vašák, 1991) but that these ranks of affinity appear not to be completely respected by MT isolated from mussel digestive glands (Funk *et al.*, 1987).

Biochemical and physiological studies have provided important insights into the biological role of MT in the homeostasis, metabolism and detoxification of some trace elements (Amiard *et al.*, 2006, Nordberg, 1998, Sigel *et al.*, 2009). The widespread occurrence and the conservative character of MT in a range of taxonomic groups gives rise to the conclusion that MT perform a crucial role in such vital processes, however, their specific biological functions are still a subject of controversy (Amiard *et al.*, 2006). Mason *et al.* (1995) suggest that MT play two main roles: homeostatic control of essential elements (e.g., Cu and Zn) and detoxification of nonessential elements (e.g., Cd and Hg).

With regards to the first function, several studies have revealed the potential roles of MT in the physiological "buffering" of cellular Cu and Zn during normal cellular functioning (Dallinger, 1996, Roesijadi, 1996, Viarengo & Nott, 1993). Such proteins are thought to act as an essential metal reservoir available for fulfilling enzymatic and other metabolic demands, allowing the maintenance of crucial cellular processes. In addition to this role, MT can also prevent metal toxicity by avoiding the binding of excess essential elements or nonessential elements to physiologically important sites where they can cause deleterious effects (Hidalgo *et al.*, 2009, Roesijadi, 1992). In this respect, the induction of MT is thought to be associated with the enhancement of metal tolerance in various aquatic organisms (Dallinger *et al.*, 1997, Wallace & Lopez, 1997). Moreover, MT appear to be involved in cellular protection against ionizing radiation (Cai *et al.*, 1999) and in scavenging free radicals during oxidative damage (Viarengo *et al.*, 2000).

In human cells, the MT gene superfamily comprises seven functional genes (Karin *et al.*, 1984) that code for two major protein isoforms, termed MT1 and MT2 (Quaife *et al.*, 1994, Schmidt *et al.*, 1985). The expression of different isoforms is not limited to mammals, however; a number of distinct isoforms of such proteins have been identified in several types of organisms, some of which have been associated with different biological functions. For example, Dallinger *et al.* (1997) reported in terrestrial snails strong evidence for the existence of distinct MT isoforms, differing in their binding properties for trace metals, that can accomplish different functions. Comparative analysis of MT expression in several variants of helicoid snails (*Helix pomatia* and *Arianta arboustorum*), after exposure to a contaminated diet, revealed the occurrence of a Cu-MT isoform concerned with the regulation of Cu whereas a Cd-MT isoform involved in the detoxification of the metal was exclusively expressed in animals fed on a Cd-enriched diet. Similar results were also reported for the fruit fly *Drosophila melanogaster* (Lauverjat *et al.*, 1989) and for the crab *Callinectes sapidus* (Brouwer & Brouwer-Hoexum, 1991). In the latter study, evidence was obtained for the metal-specific induction of different MT isoforms involved in different physiological functions. Strong evidence for the expression of distinct MT isoforms playing different biological functions in cells has been confirmed using MT gene amplification or duplication (Amiard *et al.*, 2006). The occurrence of multiple forms of MT, and the fact that the response after

induction may be associated with an isoform type, makes it crucial to understand the different induction properties of MT isoforms and their specific physiological roles (Amiard *et al.*, 2006).

Given the high affinity of metals for thiol-containing molecules and the presence of thiols other than MT, it is not surprising that the intracellular fate of metals is also determined by other metal-binding ligands. Thus the sequestration of intracellular elements by other thiol-containing ligands, including glutathione (GSH) and other small biomolecules, has been identified in several aquatic organisms (Mason *et al.*, 1995).

Reduced GSH (l-γ-glutamyl-l-cysteinyl-glycine) is a low-molecular-weight, non-protein tripeptide thiol occurring at relatively high concentrations (mM) in cells (Kosower & Kosower, 1978, Valencia *et al.*, 2001). GSH is synthesized intracellularly from its constituent amino acids by the sequential action of gamma-glutamylcysteine synthetase (EC 6.3.2.2) and glutathione synthetase (GSS; EC 6.3.2.3) (Griffith, 1999, Zhang & Forman, 2012). This sulfhydryl-containing molecule can be present in different forms in cells but the most studied forms in aquatic organisms are reduced GSH (also referred as free GSH) and glutathione disulphide (GSSG), the oxidized form. The dominant form of GSH in the cell depends on the activity of glutathione peroxidase (GPX; EC 1.11.1.9) and glutathione reductase (GR; EC 1.8.1.7) (Asensi *et al.*, 1999, Kosower *et al.*, 1978). The former is responsible for removing H_2O_2 from cells through GSH oxidation thereby increasing the level of GSSG (Deneke & Fanburg, 1989), whereas GR plays an important role in reducing GSSG to a form of GSH (Ithayaraja, 2011). As a result of the activity of these enzymes, one GSH form prevails in cells over the other. In this way, depending on its form, GSH is involved in a variety of cellular reactions (synthesis, reduction, oxidation, conjugation, etc.) and physiological processes comprising cellular defense against the toxic action of xenobiotics, oxyradicals as well as metal cations (Biswas & Rahman, 2009, Chan & Cherian, 1992, Kosower *et al.*, 1978, Meister, 1995, Valencia *et al.*, 2001). GSH has been reported to provide cellular protection against metal toxicity by altering rates of metal uptake and elimination and/or by chelating cellular elements as soon as they enter the cell (Canesi *et al.*, 1999, Valencia *et al.*, 2001). From a toxicological perspective, studies with mammals have suggested that GSH acts as a first line of defense against elements by temporarily complexing elements that enter the

cytosol, before the induced synthesis of MT is able to establish effective MT levels (Chan *et al.*, 1992, Wimmer *et al.*, 2005).

Mason *et al.* (1995) identified other low molecular weight cytosolic metal-binding ligands such as homarine, taurine, thiol esters, amino acids, N-heterocyclic compounds and dipeptides (Gly2, Cys-Gly). They have been operationally identified as ligands with a molecular weight lower than 5,000 Daltons (Da). In contrast to MT and GSH, which are involved in metal detoxification, metal binding by these ligands has been associated with the onset of metal-induced stress (Jenkins *et al.*, 1988, Sanders & Jenkins, 1984). Several laboratory exposures using aquatic organisms have demonstrated that metal binding to these low molecular weight ligands exhibits a threshold response in which signs of metal-induced sublethal toxicity (reduced growth, decreased fecundity and impaired reproductive success) have been identified. However, recent field studies on chronically-exposed freshwater animals (Bonneris *et al.*, 2005, Campbell *et al.*, 2009, Giguère *et al.*, 2006) have provided strong evidence for the inadequacy of this threshold model of metal toxicity.

2.5.4 Metal-containing granules

Studies on aquatic organisms using electron and/or light microscopy have reported the sequestration of a wide range of elements in mineralized accumulations (Marigómez *et al.*, 2002, Mason *et al.*, 1995). Such metal-rich structures have a variety of names (inclusions, concretions, storage vesicles and bodies), but the term granule is widely accepted (Mason *et al.*, 1995). Among the metal-containing ligands that occur in the intracellular environment, granules have shown the greatest variability in terms of cellular location, chemical composition and occurrence, such that few generalisations can be made about them. Despite their variability, several attempts have been made to classify these metal concretions in aquatic and terrestrial organisms. Hopkin (1989) suggested that metal-containing granules should be classified into four groups (types A, B, C and D). Later, for aquatic organisms, Mason *et al.* (1995) proposed a classification based on the inorganic composition and the cationic profiles that can be used to categorize these metal-containing ligands into three groups: Cu-containing, Fe-containing and Ca/Mg containing granules.

Cu-containing granules are characterized by their heterogeneous structure, spherical form and variable electron density (Hopkin, 1989, Walker, 1977). X-ray microanalysis of these granules reveals that their composition is more consistent than their appearance; they always contain sulphur as the major complexing anion in association with Cu as well as trace quantities of a number of class B metals and borderline metals such as Ag, Cd, Fe, Hg, Ni and Zn (Barka, 2007, Marigómez *et al.*, 2002, Mason *et al.*, 1995, Nassiri *et al.*, 2000, Viarengo *et al.*, 1993, Yamuna *et al.*, 2009). Other elements (e.g., Ca, K, P, Cl, P) have also been reported to be sequestered in Cu-containing granules (Mason *et al.*, 1995). Hopkin (1989) pointed out that for some cell types, these metal granules may play an important role as a Cu reservoir for the metabolic requirements of the cell. On the other hand, evidence suggests that Cu-containing granules may also be involved in metal detoxification (Marigómez *et al.*, 2002, Nassiri *et al.*, 1997, Viarengo *et al.*, 1993) and thus metal tolerance for invertebrates such as Cd-resistant *Littorina littorea* (Marigomez *et al.*, 1990), a Cd-resistant strain of *Drosophila melanogaster (Lauverjat et al., 1989)* as well as *Macrobrachium malcolmsonii* (Yamuna *et al.*, 2009). Metal-MT complexes can be sequestered in lysosomes in which degradation reactions likely take place, then these metals-SH complexes could be precipitated in Cu-containing granules (Barka, 2007, Hopkin, 1989, Mason *et al.*, 1995). It is worth noting, however, that the suggested MT-lysosome-granules linkage requires further study to determine the mechanisms of metal incorporation, detoxification and granule formation.

Fe-rich granules have been reported in isopods, echinoderms and gastropods (Marigómez *et al.*, 2002, Mason *et al.*, 1995). They can have a similar appearance to some Cu-containing granules, but they can be distinguished, using X-ray microanalysis, by the presence of an important peak corresponding to Fe (Hopkin, 1989). They can occur as amorphous structures (deposits of flocculent material) or granules with a crystalline structure (e.g., polyhedral inclusions) (P. G. Moore, 1979, Viarengo *et al.*, 1993). Other elements have been identified in such granules including P (reaching in some cases a proportion of 1:1 with Fe) and, at trace levels, Ca and S (Buchanan *et al.*, 1980). Since the number of Fe-containing granules appears not to be related to Fe concentrations in the environment, they probably function as intracellular Fe-stores (Mason *et al.*, 1984).

Whereas Cu-containing granules sequester metals (class B and some borderline metals) with S donors (see above), Ca/Mg-containing granules sequester mainly class A metals using oxygen donors such as carbonate, oxalate, phosphate and sulphates (Marigómez *et al.*, 2002, Mason *et al.*, 1995). These inclusions are generally found in specific tissues that have an excretory function (Hopkin, 1989, Masala *et al.*, 2002). It was initially assumed that these granules were involved in the storage of Ca and P, facilitating their mobilization under certain circumstances, but evidence of their cellular formation, induction and elimination has led several authors (Marigómez *et al.*, 2002, Masala *et al.*, 2002, Simkiss *et al.*, 1982) to associate them with storage-excretion functions of class A and borderline metals (Ca, K, Mg, Mn, Sr, and Zn). Mason *et al.* (1995) concluded that carbonate-rich granules are involved in the process of ion regulation and pH balance of body fluids in *Littorina littorea*.

2.6 *Metal toxicity at the intracellular level*

Metal toxicity is assumed to occur when the intracellular concentration of a metal exceeds a threshold value, such that the metal exerts inappropriate nonspecific binding to physiologically important sites such as biomolecules (small peptides, enzymes, DNA or RNA) or organelles (mitochondria, endoplasmic reticulum and nuclei), causing impairment of biological function. In this respect, Mason *et al.* (1995) propose three ways in which nonspecific metal binding can ultimately lead to cellular toxicity. Metal binding to intracellular ligands blocking the essential biological functional groups of biomolecules is considered an example of such aberrant binding. Examples include the inactivation by Hg^{+2} of the functional center of enzymes that contain sulfhydryl groups such as alkaline phosphate (EC 3.1.3.1), lactate dehydrogenase (LDH, EC 1.1.1.27), and glucose-6 phosphate (EC 3.9.1.1) (Clarkson, 1993, Vallee & Ulmer, 1972). Similarly, As^{+3} is reported to cause functional disruptions to pyruvate dehydrogenase (PDH) by the formation of a very stable complex thereby inhibiting the enzyme (Schiller *et al.*, 1977). The inactivation of the functional center of PDH and perhaps of other dithiol-containing enzymes by arsenite is thought to occur by chelating or complexing the thiol groups (Adamson & Stevenson, 1981, Aposhian & Aposhian, 2006).

Some other examples of perturbed function caused by elements have been reported when a displacement/substitution of essential metal ions in biomolecules, mainly proteins, occurs. In this case, toxicity is linked to the disruption of a biological function (Mason *et al.*, 1995). An example of this action has been reported for zinc-dependent carbonic anhydrase (CA) isolated from the liver of the teleost fish *Dicentrarchus labrax* (European seabass) (Ceyhun *et al.*, 2011). CA enzymatic activity was inhibited in a competitive manner when Ag, Al, Cu, Co, Hg or Pb replaced Zn at the catalytic site. Whereas the displacement by Ag, Co, Hg and Pb showed weaker inhibitory actions, Al and Cu were the most potent inhibitors of CA. In contrast, *in vitro* studies with purified CA from rainbow trout (*Oncorhynchus mykiss*) kidney showed a different susceptibility rank order of elements, that is, Co > Cu > Cd > Ag (Söyüt & Beydemir, 2012). Similar inhibitory effects have been reported for lactate dehydrogenase, succinate dehydrogenase (EC 1.3.5.1) and Na^+/K^+-ATPase (sodium-potassium adenosine triphosphatase; EC 3.6.3.9) for Cd in rat livers (Karthikeyan & Bavani, 2009). Inhibitory displacement/substitution of Zn in pyruvate kinase (EC: 2.7.1.40) and δ-aminolevulinic acid dehydratase (E.C. 4.2.1.24) by Pb has been measured in human cells (Feksa *et al.*, 2012).

Mason *et al.* (1995) include modification of the active conformation of biomolecules as another way in which elements can produce deleterious effects. Reactions of Hg^{2+} with proteins through disulphide bonds, which play an important role in the folding and stability of biomolecules, are able to alter the functional conformation of proteins and thus their biological activity (David *et al.*, 1974). Studies on As binding to cysteinyl side chain proteins suggest that the capture of a pair of thiols by As may result in significant changes in local secondary structure in the protein targets (Cline *et al.*, 2003, Ramadan *et al.*, 2007). Depending on the placement of cysteine residues and the plasticity of the resulting conformation, metal binding can favor and/or disfavor the biological function of the As-binding protein. All these putative metal-sensitive targets (e.g., alkaline phosphate, lactate dehydrogenase, PDH or CA) are confined in mitochondria, cell nuclei, the endoplasmic reticulum (smooth and rough), Golgi apparatus and peroxisomes in animal cells. Other types of biomolecules such as DNA, RNA, carbohydrates and lipids should not be excluded as potential targets of non-essential trace elements.

2.7 *Animals used in this study*

To determine the metal-handing strategies developed by aquatic organisms to cope with high trace metal concentrations, two groups of freshwater animals were selected. First, an invertebrate, the phantom midge *Chaoborus* (Insecta, Diptera, Chaoboridae) was chosen because it has been proposed for use as a sentinel to estimate concentrations of Cd (Hare *et al.*, 1996), Ni (Ponton & Hare, 2009) and Se (Ponton *et al.*, 2013) in lake planktonic food webs. *Chaoborus* larvae occur in lakes that encompass a wide range of total dissolved metal concentrations ([Cd]: 0.1-20 nmol L^{-1}; [Cu]: 5-1700 nmol L^{-1}; [Zn]: 1-5000 nmol L^{-1}; [Ni]: 10-2110 nmol L^{-1}), hardness ([Ca]: 30-2000 µmol L^{-1}) and temperature (4-32 °C). In addition, the tolerance of *Chaoborus* to highly acidic waters in which other potential sentinels (molluscs, crustaceans, and fish) are absent makes them a useful animal model to investigate metal resistance or tolerance over a wide range of environmental conditions. *Chaoborus* larvae are abundant and widely distributed in lakes throughout the world. Migratory species of the genus can be collected both during the day (in sediments where they hide to avoid fish predation) and at night (in the water column where they feed on plankton). *Chaoborus* larvae are easy to identify to the species level (Saether, 1972), in contrast to most aquatic insect larvae, and provide sufficient tissue for contaminant analysis, even on individuals. They are reported to integrate changes in Cd concentrations in their surroundings over a relatively short period of time from several weeks to a month (Hare *et al.* (2008).

Larvae of *Chaoborus* develop through four instars followed by a pupal stage before emerging as an adult. Their life-cycle can vary in length from weeks, in tropical lakes, to years in cold climates (Carter *et al.*, 1977, Goldspink *et al.*, 1971, Hare *et al.*, 1986). Female adults do not feed and lay a single raft of eggs. The species *C. punctipennis, C. albatus*, *C. americanus*, *C. trivittatus* and *C. flavicans* are found in Quebec lakes (Borkent, 1981, Garcia *et al.*, 2008). Different prey preference patterns among larval instars have been related to prey availability as determined by their depth in the water column, vertical migration and mouth gape (Fedorenko, 1975, Hare & Carter, 1987, Marianne V. Moore, 1988). For example, for *Chaoborus punctipennis*, Marianne V. Moore (1988) reported that small rotifers (e.g., *Kellicottia*, *Gastropus*, *Polyarthra*, *Trichocerca*) are more frequently ingested by early instars (I, II) whereas large rotifers

(e.g., *Keratella crassa*, *Asplanchna priodonta*) and some crustaceans (e.g., *Daphnia*) are only ingested by instars III and IV. Information about Cd and Ni bioaccumulation in *Chaoborus* has been obtained from experiments designed to determine the relative importance of water and food as metal sources (Munger and Hare, 1997; Ponton and Hare, 2010, respectively). These experiments also yielded rate constants for metal uptake and loss as well as metal assimilation efficiencies for inclusion in biodynamic models (Croteau *et al.*, 2001, Munger & Hare, 1997, Ponton *et al.*, 2010).

We also used fish as model animals in our study; specifically, the eels *Anguilla anguilla* and *Anguilla rostrata* (Teleostei, Anguilliformes, Anguillidae). Populations of both eel species were historically widespread in freshwater systems but have declined over the last 30 years (Haro *et al.*, 2000, ICES, 2011). In this context, *A. anguilla* is considered to be critically endangered by the International Union for the Conservation of Nature (Dekker, 2003, ICES, 2011). The European Community has implemented a regional eel management plan, involving fishing regulations and the assessment of anthropogenic impacts and eel stocks in most regions (ICES, 2011), including the Gironde estuary in south-western France. Likewise, the poor stock status of American eels (*A. rostrata*) led to their designation as a "species of special concern" in Canada in 2006 and as an "endangered species" in the province of Ontario (COSEWIC, 2006). One important characteristic of this species is their unusual life cycle, which includes two metamorphosis steps, two Atlantic migrations and long-term somatic maturation ranging from 8 years to 20 years. Both eel species spawn in the Sargasso Sea, migrate as leptocephalus larvae to coastal streams of North American (*A. rostrata*) and of Europe (*A. anguilla*), carried on surface currents of the Gulf Stream (van Ginneken & Maes, 2005). At the end of the larval stage, they undergo metamorphosis into a transparent larval stage called a "glass" eel (Arai *et al.*, 2000). When they enter estuaries, they darken as they change from being pelagic feeders to feeding on benthic animals; at this stage they are known as elvers. They then move upstream in rivers where they change color to yellow (yellow eels), but remain sexually immature (van Ginneken *et al.*, 2005). As yellow eels, their diets include invertebrates and small fish from which they can accumulate high quantities of organic and inorganic contaminants (Castonguay *et al.*, 1989). These sexually immature adult eels inhabit diverse freshwater ecosystems from lakes, reservoirs

to small streams. After 8–20 years, when they become sexually mature (when they are known as "silver eels"), they begin their migration back to the Sargasso Sea to spawn (van Ginneken *et al.*, 2005). During their long-term somatic maturation, yellow eels exposed to high concentrations of trace metals can be used to explore the relationship between the accumulation of non-essential trace metals and metal-induced toxicity.

2.8 *Environmental metal exposure. Study areas*

The biological effects of trace elements on aquatic organisms are often assessed by exposure in the laboratory to aqueous metal without considering metal-contaminated foods (Wang, 2013a). However, the results obtained under such conditions are often questionable, especially when exposures are of short duration and involve a single trace element at concentrations that are not environmentally relevant. In addition, animal responses in the laboratory can differ from those in the field because of the sensitivity of the test organisms to uncontrolled stimuli in the laboratory environment (Luoma, 1995). Thus we took advantage of the existence of trace metal exposure gradients in the field, to explore metal-handling strategies in *Chaoborus* larvae and American and European yellow eels that had been chronically subjected to metal stress.

Insect larvae and fish were collected from freshwater systems impacted by human activities. *Chaoborus* larvae were collected from lakes in mining-impacted areas near the Canadian cities of Rouyn-Noranda (Quebec) and Sudbury (Ontario), whereas eels were collected from the Saint Lawrence River and the Gironde River system.

2.8.1 Mining regions of Rouyn-Noranda and Sudbury

Lakes located in the mining regions of Rouyn-Noranda and Sudbury have received emissions from metal smelters for over 70 years. In the city of Rouyn-Noranda, a large Cu smelter has been in operation since 1927 and, until the 1970s, its feed came entirely from local ores. In 1976, Noranda's Horne mine closed and the plant started to smelt ores and metal-containing materials coming from around the world (Dixit *et al.*, 2007). Since the early 1980s, the atmospheric emissions from the smelter have largely been controlled, but the nearby lakes remain contaminated with elements such as Cd, Cu and Zn due to historical atmospheric deposition (Banic *et al.*, 2006, Perceval *et al.*, 2006). Similarly, lakes in the Sudbury region have been affected by historical inputs from the two major

smelters, which have been in operation for over 75 years. Smelter emissions peaked during the 1960s when Sudbury-area smelters constituted one of the world's largest point sources of SO_2 and metal emissions. After the emission reductions that were implemented in the late 1970's, substantial changes in the water quality of lakes were noted but many are still metal-contaminated (Borgmann *et al.*, 1998).

In both regions, the relative metal contamination of the lakes is determined primarily by their proximity to the smelting operations and by wind direction. Thus Lakes Dufault and Osisko are located downwind from the Horne smelter and exhibit higher metal concentrations in lake water than do Lakes Opasatica and Dasserat that are upwind of the smelter (Alpay *et al.*, 2006, Bonham-Carter *et al.*, 2006). A similar situation exists near the Sudbury smelters (Campbell *et al.*, 2003, Keller *et al.*, 2007).

2.8.2 Saint Lawrence River system (Canada) and the Gironde region (France)

Intensive agricultural activity and historical metal pollution from former mining and smelting operations that started to develop in the late nineteenth century have strongly impacted the environmental quality of the Gironde River and its estuary (Audry *et al.*, 2006, Coynel *et al.*, 2007). Cadmium contamination, which is the result of coal mining and Zn-ore treatment in the upstream reaches of the Gironde catchment, has been reported as a major problem in this fluvial system (Blanc *et al.*, 1999, Claisse, 1989). At the end of the 1970s, high Cd concentrations were reported in Gironde oysters that were 10 times higher than those reported in oysters collected from other non-industrial sites in France (Latouche, 1988). In addition to Cd, contamination by Zn, Pb, Ag and Cu has been reported in the Gironde as a consequence of human activities (Deycard *et al.*, 2014, Lanceleur *et al.*, 2011). The deterioration of aquatic resources in the Gironde system has been linked to this metal contamination (Audry *et al.*, 2004, Blanc *et al.*, 1999). In this context, Durrieu *et al.* (2005) reported that European eels collected from this freshwater ecosystem were the fish species having the second highest concentration of cadmium (Cd) in gills, muscles, and kidneys among 7 other fish species (e.g., twaite shad, *Alosa fallax*; bass, *Dicentrarchus labrax*; meagre, *Argyrosomus regius*; sole, *Solea vulgaris*). Although some regulatory decisions were implemented to reduce the observed environmental impacts (Buzier *et al.*, 2006), these contaminants remain an important

issue of concern for which monitoring programs and ecological risk assessments must be performed.

Contamination of the Saint Lawrence River system is the result of urban, industrial, and agricultural development in its drainage basins (Carignan *et al.*, 1994). With regard to metals, industrial and urban effluents as well as atmospheric deposition have been identified as important contributors of trace elements such as Ag, As, Cd, Hg and Pb (Charles Gobeil, 1999, C. Gobeil *et al.*, 1995, Quémerais *et al.*, 1999, Quémerais *et al.*, 1997, Tremblay *et al.*, 1990). Significant efforts have been made to reduce contaminant discharges from industrial and water treatment plants located along the Saint Lawrence River, but given the great area covered by this ecosystem and the increasing urbanisation and industrialisation near the river system, metal contamination remains an important environmental issue.

3. Research questions. Objectives and hypothesis

3.1 *Research context.*

Ecological Risk Assessments for aquatic ecosystems aim to relate ambient metal concentrations in water or sediment to those in animals, from which the likelihood of toxicity can be determined (Campbell *et al.*, 2006). However, the link between total metal concentrations in animals and toxicity is often weak because not all accumulated metal is likely to cause toxic effects (Wang, 2013b). To better relate metal bioaccumulation to metal toxicity, a subcellular metal partitioning approach developed by Wallace *et al.* (2003a) has been very useful in providing diagnostic information about the potential toxicity of internal trace metal doses (Campbell *et al.*, 2009, Wang & Rainbow, 2006). Such an approach allows researchers to distinguish between metal accumulated in detoxified metal fractions (e.g., metallothionein-like peptides, heat-stable proteins, granule-like concretions) that may not represent a toxicological risk, from metal bound to physiologically-sensitive target molecules (e.g., cytosolic enzymes) and organelles (e.g., mitochondria) where the metal could induce deleterious effects. Thus, access to subcellular measurements could increase the reliability of ERAs (Buchwalter *et al.*, 2008, Kraemer *et al.*, 2005, Wang *et al.*, 2006). Such subcellular measurements are also useful for comparing the efficiency of various animal groups at limiting the binding of (non-essential) metals to physiologically important sites. Lastly, this approach allows researchers to evaluate the likelihood that a predator consuming animal cells will be able to assimilate the elements that they contain (Rainbow *et al.*, 2007, Wallace *et al.*, 2003b). This approach could be refined by determining the molecular weight, composition and stoichiometry of metal-binding ligands such as MT as well as the molecules present in metal-sensitive fractions. Such measurements could be obtained by combining subcellular metal-partitioning measurements along with so called "hyphenated"[1] analytical techniques (Mounicou *et al.*, 2010).

[1] In analytical chemistry, the term « hyphenated techniques" refers to the linking of a separation technique (for example, High Performance Liquid Chromatography) to a detection technique (for example, Inductively Coupled Plasma Mass Spectrometry); such a combination would be referred to as HPLC-ICPMS.

Given these facts, the research described in this thesis was designed to respond to the following questions:

a) What are the metal-handling strategies used by chronically metal-exposed *Chaoborus* larvae and American and European yellow eels to cope with high concentrations of non-essential trace elements?
b) How efficiently do these animals detoxify non-essential trace elements?
c) Are elements in these animals bound to inappropriate cellular fractions such as mitochondria, heat-denatured cytosolic proteins and other organelles? If this is the case, which of these "sensitive" fractions contributes most to the metal burden found in the metal-sensitive compartment?
d) Do detoxification responses vary among the animal species and elements studied? In this regard, can the predicted reactivity of trace elements with bio-ligands (Mason *et al.*, 1995) be used to explain the way in which these animals handle the various trace elements?
e) Are there differences between the two eel species in terms of how they store elements in their liver cells?
f) How are elements bound to cytosolic bio-ligands?

3.2 *Objectives and Hypotheses*

The **main** objectives and hypotheses of this thesis are as follows:

1. To determine the subcellular partitioning of non-essential elements in two groups of freshwater animals: Larvae of the insect *Chaoborus* (Cd and Ni; whole organism) and American (*A. rostrata*) and European (*A. anguilla*) yellow eels (As, Ag, Cd, Ni, Pb, and Tl; liver cells).

   Hypotheses related to *Chaoborus*:

   - The subcellular partitioning protocol applied to *Chaoborus* larvae yields a representative, high-yield, low-artifact separation of subcellular fractions.

   - The HSP play an important role in the detoxification of Cd and Ni.

- The subcellular Cd partitioning in transplanted larvae show that the HSP are involved in the detoxification of this metal.

- The concentrations of Cd and Ni increase in putative metal-sensitive fractions along the metal exposure gradients.

Hypotheses related to the eels *A. anguilla* and *A. rostrata*:

- Cytosolic, thermostable, ligands (e.g., metallothionein, metallothionein-like peptides) are the main metal-detoxifying agents.

- Mineral concretions provide a complementary metal-handling strategy.

- Inappropriate binding of elements to physiologically sensitive compartments such as mitochondria, heat-denatured cytosolic proteins and other organelles is likely.

2. Determine how elements are bound to cytosolic biomolecules in field-collected *Chaoborus* (Ag, Cd, Cu, Zn and Tl) by size-exclusion chromatography coupled to inductively coupled plasma-mass spectrometry.

Hypotheses

- Cd will be largely associated with MT and MTLP proteins, whereas Ni will be handled by ligands other than MT.

- Co-elution of Cd with other trace elements such as Ag, Cu and Zn will be observed.

Additional aspects of metal-binding in *Chaoborus* larvae are also included in this thesis. These results are presented in Chapter 10 (*Assessment of a subcellular metal partitioning protocol for aquatic invertebrates: preservation, homogenization, and subcellular fractionation*) and in Chapter 11 (*Uptake and subcellular distributions of cadmium and selenium in transplanted aquatic insect larvae*) in the section that includes published and unpublished papers resulting from this research program.

4. Methods

4.1 *Subcellular metal partitioning protocols*

Procedures designed to determine the subcellular partitioning of metals generally involve a homogenisation step, followed by a series of differential centrifugation steps. The fractionation procedure applied to *Chaoborus* (whole larva) and *A. anguilla* and *A. rostrata* (liver) was adapted from protocols described by Wallace *et al.* (2003a), Giguère *et al.* (2006) and Lapointe *et al.* (2009). This procedure allows one to separate animal tissues into six operationally-defined fractions including: nuclei and debris; granules (also referred as the NaOH-resistant fraction); mitochondria; microsomes and lysosomes; HDP including cytosolic enzymes; and heat-stable proteins and peptides (HSP) such as metallothionein (MT) and glutathione (GSH) (Figure 4.1). Despite the fact that there were differences in the homogenisation buffer and in the cell breakage technique used, the subcellular fractionation applied to *Chaoborus* larvae was basically similar to that used for eel liver (Figure 4.1).

The basic protocol involved removing a 100-µL aliquot from the homogenate for determining total trace metal concentrations and for mass-balance quality-control measurements. The remainder of the homogenate was centrifuged at 800 × *g* for 15 min at 4 °C. The supernatant (S1) was transferred to a pre-weighed and acid-washed 1.5 mL polypropylene micro-centrifuge tube for further separations. The pellet from this centrifugation step (P1) was suspended in 0.5 mL of ultrapure water, heated at 100 °C for 2 min, and digested with an additional 500 µL of 1 M NaOH (99.998%, Sigma-Aldrich, Oakville, ON, Canada) at 65 °C for 60 min. Centrifugation at 10,000 × *g* for 10 min at ambient temperature (~ 20 °C) was performed to separate the NaOH-resistant fraction (P2, referred to henceforth as "granules") from the nuclei + debris fraction (S2) that includes cell membranes, unbroken cells and nuclei.

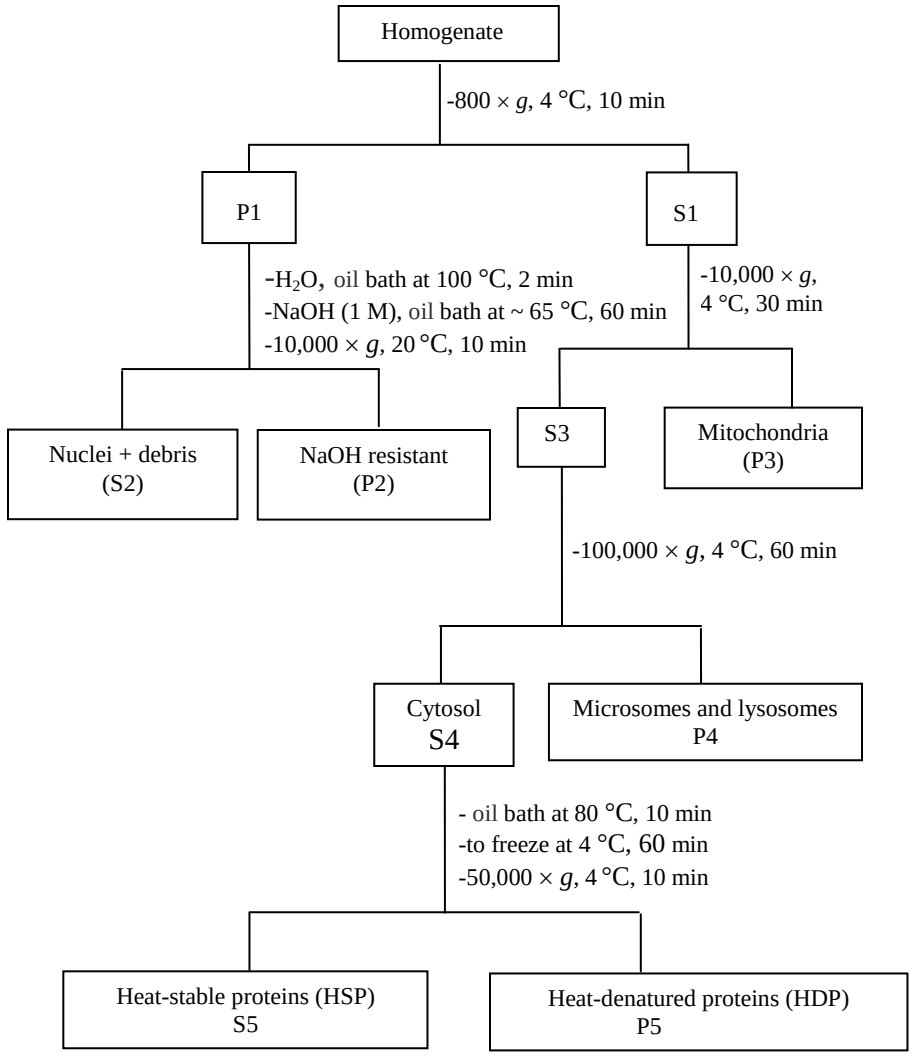

Figure 4.1: Partitioning procedure used to separate whole *Chaoborus* larvae and livers of *Anguilla anguilla* and *Anguilla rostrata* into various subcellular fractions.

The supernatant from the homogenate (S1) was centrifuged at 10,000 × *g* for 30 min at 4 °C to yield the mitochondrial fraction (P3). The resulting supernatant (S3) was subjected to an ultracentrifugation step at 100,000 × *g* for 60 min at 4 °C, giving a pellet containing organelles (microsomes and lysosomes) and the cytosolic fraction in the supernatant. To separate the heat-stable peptides and proteins (HSP) from the HSP, the cytosolic fraction (S4) was held at 80 °C for 10 min, left on ice for 1 hour and then centrifuged at 50,000 × *g* for 10 min at 4 °C. The resulting supernatant represents the HSP fraction, which includes MT and metallothionein-like proteins (MTLP).

High-speed centrifugations (≥ 50,000 × *g*) were performed using a WX ULTRA 100 centrifuge (Sorval, Ultra Thermo Scientific, Whitby, ON, Canada) equipped with a F50L-24 X1.5 rotor (Fisher Scientific, Whitby, ON, Canada) or using a Beckman TLA-100 centrifuge using a TLA-100.3 rotor (Beckman Counter, Mississauga, Ontario, Canada). For the other centrifugations, an IEC Micromax centrifuge (Thermo IEC, Arlington, MA, USA) was used. Each supernatant was acidified with nitric acid (final [HNO_3] 10%, v/v; Optima grade, Fisher Scientific, Whitby, ON, Canada) and kept at 4 °C until metal analyses were performed. Pellets were kept frozen at -80 °C until needed for analysis. They were then freeze-dried, digested with nitric acid (70%, v/v, Optima grade, Fisher Scientific, Whitby, ON, Canada) followed by hydrogen peroxide (30%, v/v; Optima grade, Fisher Scientific, Whitby, ON, Canada), and the final solutions were diluted to a fixed volume with ultrapure water. For the supernatants (HSP and nuclei/debris fraction), a similar digestion procedure was performed. For each metal, mass-balances were calculated to confirm acceptable metal recovery (Chapters 9 and 12).

To compare trends in the partitioning of a given metal between the detoxified-metal compartment (sum of metal in the HSP and in the granule fractions) and the metal-sensitive compartment (sum of metal in the mitochondria, HDP and microsomes + lysosomes fractions), metal concentrations in each compartment (calculated with respect to total dry weight; *Y*-axis) were plotted against total metal concentrations in a given animal or organ (*X*-axis). We compared the slopes of the relationships between metal in both compartments versus total metal concentrations to determine how efficiently these animals detoxify non-essential trace metals. To describe metal partitioning between these two compartments, the relative contribution (estimated as a percentage of the metal burden in each subcellular compartment divided by the total metal burden) was also examined (Appendix A). We also compared the contribution (%) of each individual fraction to its corresponding compartment (e.g., the contribution of the HSP fraction to the detoxified-metal compartment or of the mitochondrial fraction to the metal-sensitive compartment) along the bioaccumulation gradients (also shown in Appendix A). To compare subcellular metal partitioning among types of animals, we calculated the range in percentages (maximum % – minimum %) represented by each compartment or fraction

(e.g., mitochondria) for the metals of interest, as well as the median, the 25% and the 75% percentiles.

4.2 *Hyphenated techniques.*

We used so-called hyphenated techniques to separate the components of individual fractions. Specifically, chromatographic techniques were coupled on line to an ICPMS to separate and characterise metal complexes with various cytosolic biomolecules (Lobinski *et al.*, 1998). We performed chromatographic separations by applying SEC (Frelon *et al.*, 2013, Mounicou *et al.*, 2010) to the cytosol and to the HSP fractions of *Chaoborus* larvae (Figure 4.2); the eluate from the SEC step was analyzed on line by ICPMS (Figure A.1).

Separations of cytosol (100 µL) were carried out using a Superdex 200 (SEC_{200}) HR 10/30 size-exclusion column (linear mass separation range between 10 – 600 kDa), whereas the HSP fraction was fractionated on a Superdex peptide (SEC_{pep}) 10/30 size-exclusion column (linear mass separation range between 0.1 – 7 kDa). The Superdex 200 column was previously calibrated using molecular-weight standards such as thyroglobulin (670 kDa), ferritin (474 kDa), transferrin (80 kDa), Mn-SOD (40 kDa), myoglobulin (16 kDa), Cd-MT_2 (6.8 kDa) and vitamin B_{12} (1.3 kDa), whereas the Superdex peptide column was calibrated using carbonic anhydrase (10 kDa), Cd-MT_2 (6.8 kDa), vitamin B_{12} (1.3 kDa) and cysteine (0.12 kDa). For each column, fractionation was carried out using 100 mM of ammonium acetate (pH = 8.0) as the mobile phase at a flow rate of 0.7 mL min^{-1}. Prior to analyses, the column was flushed with 100 µL of the mobile phase containing 10 mM EDTA before conditioning with the mobile phase. Three injections of the EDTA solution (100 µL) were necessary to ensure acceptably low metal contamination from the stationary phase. The chromatographic effluent was monitored at 280 nm and at 254 nm and subsequently delivered to the ICPMS for metal measurements. All chromatographic equipment contained metal-free components, mainly PEEK (polyether ether ketone) in the sample flow-path, so as to ensure the integrity of bio-molecules and minimise unwanted surface interactions. For metal detection, platinum cones were used.

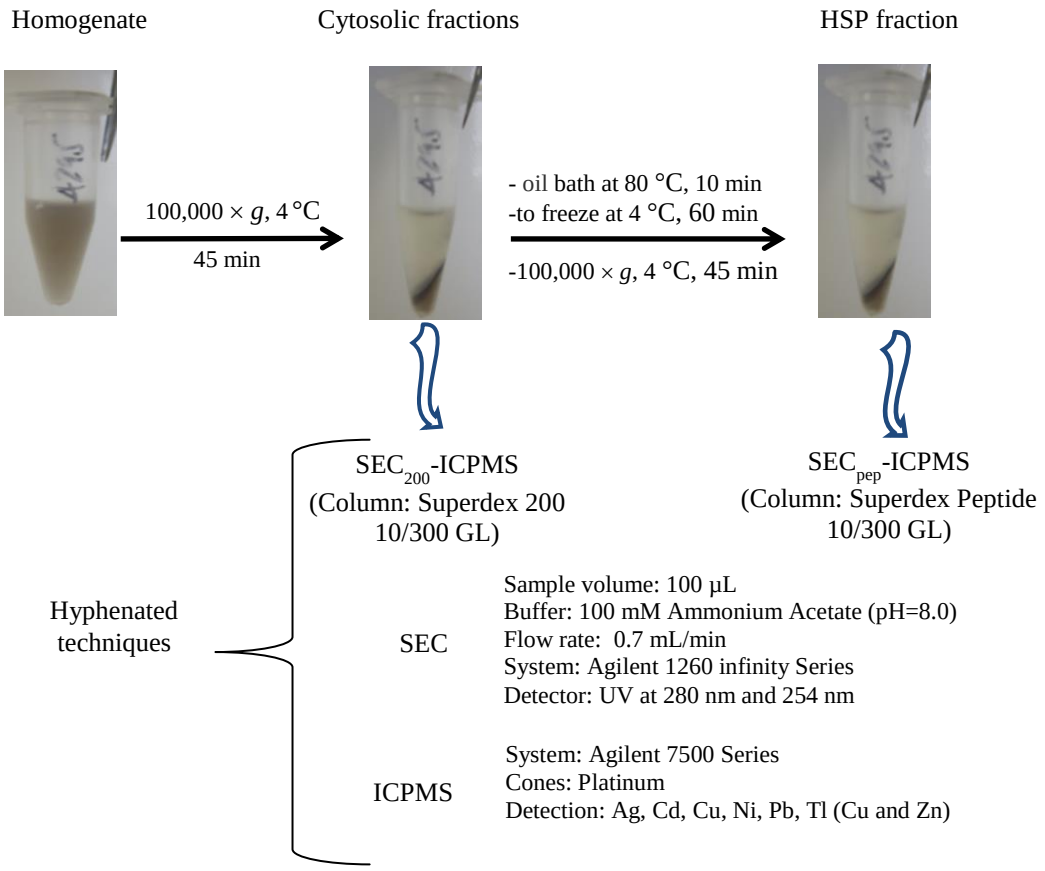

Figure 4.2: Schematic illustration of the hyphenated approach (SEC-ICPMS) applied to the cytosol and the HSP fraction of *Chaoborus* larvae.

For the cytosol, we determined the proportion (%) of total intensity detected for each metal studied among three MW pools that were operationally defined as being either high molecular weight (HMW, "2400" kDa – 40 kDa), medium molecular weight (MMW, 40 kDa – "0.7" kDa) or low molecular weight (LMW, < "0.7" kDa). For the HSP fractions, metal concentrations were normalized by the total dry weight of each SEC_{pep} fraction, which were operationally designated as fraction 1 ("31" kDa – 6.2 kDa), fraction 2 (6.2 kDa – < 1.7 kDa) and fraction 3 (< 1.7 kDa).

Note that the MW values in bold are outside of the recommended separation range for Superdex 200 (linear mass separation range: 10-600 kDa) and for Superdex peptide (linear mass separation range: 0.1 – 7 kDa) columns (for more details, see Chapter 13). These measurements in each SEC_{pep} fraction were plotted against total HSP metal

concentrations. For each metal studied, recoveries were calculated and they yielded acceptable recovery percentages for Ag, Cd, Ni, Pb and Tl (Chapter13).

5. Results and Discussion

Procedures designed to measure subcellular metal partitioning are inherently sensitive to potential artifacts that can complicate the interpretation of metal partitioning (see Chapter 10 for a detailed discussion of these problems). Thus the designation of some subcellular fractions as "granule-like" or "metallothionein-like" should be considered with caution. Furthermore, the grouping of fractions into a detoxified-metal compartment (granule-like and HSP) and a potentially metal-sensitive compartment (HDP, mitochondria, microsomes and lysosomes fractions), as in this thesis, is likely an oversimplification (Rainbow *et al.*, 2011b, Wallace *et al.*, 2003a). Metal accumulation in the microsomes and lysosomes fraction is particularly ambiguous since these organelles play different roles in handling non-essential metals (lysosomes: metal detoxification; microsomes: potential metal-sensitive targets), and the relative proportion of microsomes and lysosomes in this fraction is difficult to determine. Although we included microsomes and lysosomes in the detoxified-metal compartment, if this fraction is considered as neutral (that is, excluding the microsomes and lysosomes fraction from the metal-sensitive compartment) or as a part of the detoxified metal fractions, the partitioning results of metals between both compartments can be affected for As (in both eel species), Ni (in *A. rostrata* and in *P. flavescens*) and Pb (in *A. anguilla*) (see Chapter 12 and Table A.1 for more details).

The efficacy of the subcellular partitioning procedures was assessed by determining the distribution of marker enzymes located specifically in mitochondria, in the cytosol or in the microsomes and lysosomes (Chapter 10 for insect larvae, Chapter 12 for eel livers). These results suggest that a good balance between cell breakage and organelle integrity was achieved in the application of the subcellular fractionation procedure to whole insect larvae and to eel livers. Since the nuclei and debris fraction does not fit neatly into either the detoxified or sensitive categories, this fraction is generally ignored (cf., Cain *et al.* (2004); Cooper *et al.* (2010)). However, the relative contribution of the nuclei and debris fraction can be used as an indication of the efficacy of the homogenization procedure. In this context, we obtained a low (~ 35%) and constant percentage (standard deviation <

25%) contribution in this fraction, indicating that the homogenisation step was both effective and reproducible (Chapter 9, Chapter 12).

We discuss the subcellular partitioning results metal by metal in the following order: Ag, As, Cd, Ni, Pb and Tl. For Ag, As, Pb and Tl, we consider the results obtained for both eel species, while for Cd we compare the subcellular measurements for the animals studied in Chapter 9 and Chapter 12 with those obtained for the giant floater mollusc *Pyganodon grandis* (digestive gland; Bonneris *et al.* (2005)) and for liver of the yellow perch *Perca flavescens* (Giguère *et al.*, 2006) (Table 1). For Ni, we compare results for *Chaoborus*, eels and *Perca flavescens.* We also discuss the results obtained with the SEC-ICPMS approach as applied to the whole cytosol and the HSP fractions of *Chaoborus* larvae and both eel species.

Table 1. Summary information of the aquatic organisms compared for each element studied. The biological material used, the sampled regions indicating the sampling sites are also given.

Trace element	Organism (biological material)	Sampled region (sampling sites)	Reference
Ag	*Aguilla anguilla* (liver)	Gironde region, France (Dordogne, Garonne, Gironde estuary, Certes)	Rosabal *et al.* (2015)
	Aguilla rostrata (liver)	Saint Lawrence river region, Canada (Saint Jean River, Sud-Ouest River, Lake Saint Pierre, Lake Saint François)	
As	*Aguilla anguilla* (liver)	Gironde region, France (Dordogne, Garonne, Gironde estuary, Certes)	Rosabal *et al.* (2015)
	Aguilla rostrata (liver)	Saint Lawrence river region, Canada (Saint Jean River, Sud-Ouest River, Lake Saint Pierre, Lake Saint François)	
Cd	*Aguilla anguilla* (liver)	Gironde region, France (Dordogne, Garonne, Gironde estuary, Certes)	Rosabal *et al.* (2015)
	Aguilla rostrata (liver)	Saint Lawrence river region, Canada (Saint Jean River, Sud-Ouest River, Lake Saint Pierre, Lake Saint François)	
	Chaoborus punctipennis (whole insect)	-Rouyn Noranda region, Canada (Lake Daserrat-I, Lake Daserrat-II, Lake Dufault, Lake Marlon, Lake Opasatica, Lake Osisko) -Sudbury region, Canada (Lake Crooked, Lake Crowley, Lake Hannah, Lake Lohi, Lake Silver, Lake Swan)	Rosabal *et al.* (2012)
	Perca flavescens (liver)	-Rouyn Noranda region, Canada (Lake Opasatica, Lake Vaudray, Lake Osisko, Lake Dufault) -Sudbury region, Canada (Lake Laurentian, Lake Wavy, Lake Raft, Lake Hannah)	Giguère *et al.* (2006)
	Pyganodon grandis (digestive gland)	-Rouyn Noranda region, Canada (Lake Evain, Lake Opasatica, Lake Ollier, Lake Dufay, Lake Joannes, Lake Heva, Lake Caron) Lake Bousquet, Lake Vaudray)	Bonneris *et al.* (2005)

Trace element	Organism (biological material)	Sampled region (sampling sites)	Reference
Ni	*Aguilla anguilla* (liver)	Gironde region, France (Dordogne, Garonne, Gironde estuary, Certes)	Rosabal *et al.* (2015)
	Aguilla rostrata (liver)	Saint Lawrence river region, Canada (Saint Jean River, Sud-Ouest River, Lake Saint Pierre, Lake Saint François)	
	Chaoborus punctipennis (whole insect)	-Rouyn Noranda region, Canada (Lake Daserrat-I, Lake Daserrat-II, Lake Dufault, Lake Marlon, Lake Opasatica, Lake Osisko) -Sudbury region, Canada (Lake Crooked, Lake Crowley, Lake Hannah, Lake Lohi, Lake Silver, Lake Swan	Rosabal *et al.* (2012)
	Perca flavescens (liver)	-Rouyn Noranda region, Canada (Lake Opasatica, Lake Vaudray, Lake Osisko, Lake Dufault) -Sudbury region, Canada (Lake Laurentian, Lake Wavy, Lake Raft, Lake Hannah)	Giguère *et al.* (2006)
Pb	*Aguilla anguilla* (liver)	Gironde region, France (Dordogne, Garonne, Gironde estuary, Certes)	Rosabal *et al.* (2015)
	Aguilla rostrata (liver)	Saint Lawrence river region, Canada (Saint Jean River, Sud-Ouest River, Lake Saint Pierre, Lake Saint François)	
Tl	*Aguilla anguilla* (liver)	Gironde region, France (Dordogne, Garonne, Gironde estuary, Certes)	Rosabal *et al.* (2015)
	Aguilla rostrata (liver)	Saint Lawrence river region, Canada (Saint Jean River, Sud-Ouest River, Lake Saint Pierre, Lake Saint François)	

5.1 *Ag*

The range of total Ag concentrations in livers of *A. anguilla* (0.9 – 24 nmol g^{-1} dw) was similar to that of *A. rostrata* (1.7 – 16 nmol g^{-1} dw). For both eel species, the increase in Ag concentrations in the detoxified-metal compartment (slope$_{A.\ anguilla}$: 0.42 ± 0.07; slope$_{A.\ rostrata}$: 0.70 ± 0.05) was significantly higher ($P < 0.05$) than those observed for the metal-sensitive compartment (slope$_{A.\ anguilla}$: 0.28 ± 0.03; slope$_{A.\ rostrata}$: 0.12 ± 0.05) along the total hepatic Ag bioaccumulation gradient (Figure 5.1).

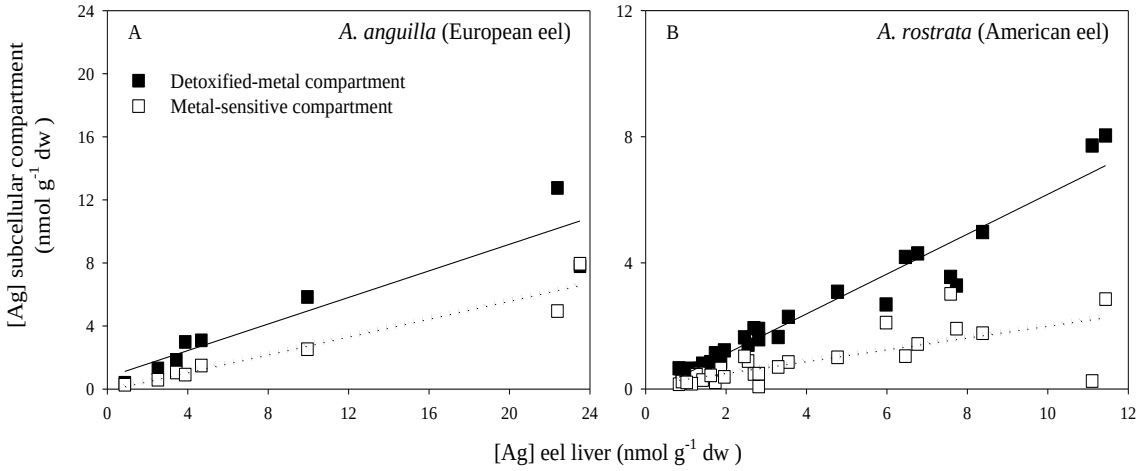

Figure 5.1: Relationships between total hepatic Ag concentrations (horizontal axis) and Ag concentrations in the detoxified-metal compartment (solid squares) and the metal-sensitive compartment (open squares) in *Anguilla anguilla* (panel A) and *Anguilla rostrata* (panel B). Each point represents an individual eel. Lines represent significant regressions ($P < 0.05$).

Silver in the detoxified-metal compartment was largely found in the HSP fraction in the American (contribution mean: 94% ± 6 %) and European eels (contribution mean: 95% ± 3%). These results suggest that both fish species detoxify this non-essential metal by binding it to MT or MTLP. The association of Ag with thermostable, cysteine-rich, low molecular weight proteins and peptides is consistent with the preference of this "soft" metal for the sulfhydryl groups of MT and MTLP (Nieboer *et al.*, 1980). However, some of the Ag in eels was likely bound to other S-rich ligands since we also measured significant quantities of Ag in the metal-sensitive compartments of both eel species (Figure 5.1), in which mitochondria were the major Ag-binding fraction (contribution mean $_{A.\ anguilla}$: 48% ± 16%, n = 8; contribution mean $_{A.\ rostrata}$: 51% ± 17, n = 20). These

results are consistent with those obtained using hyphenated analyses (e.g., SEC_{200} – ICPMS or SEC_{pep} – ICPMS) in *Chaoborus* larvae, where Ag binding to both MMW (where MT and MTLP are found) and HMW ligands was revealed (Chapter 13). Inappropriate Ag binding to metal-sensitive targets such as mitochondria and some cytosolic proteins could be associated with potential toxic effects (Mason *et al.*, 1995).

Similar proportions of hepatic Ag were found in the metal-sensitive compartment in the American (contribution mean: 25% ± 10%) and European (contribution mean: 24% ± 10%) eels (Figure A.2).

5.2 *As*

We measured higher maximum total hepatic As concentrations in European eels (5.2 – 320 nmol g^{-1} dw) compared to American eels (4.4 – 44 nmol g^{-1} dw). The slope of the As concentration in the detoxified-metal compartment compared to the total As concentration (slope: 0.45 ± 0.06) was similar ($P > 0.05$) to that observed for the metal-sensitive compartment (slope: 0.37 ± 0.04) in eels collected from the Gironde system (Figure 5.2A). In contrast to *A. anguilla*, the increase in As concentrations in the metal-sensitive compartment (slope: 0.55 ± 0.07) was stronger than that of the detoxified-metal compartment (slope: 0.15 ± 0.05) (Figure 5.2B) for the eels collected from the Saint Lawrence River system.

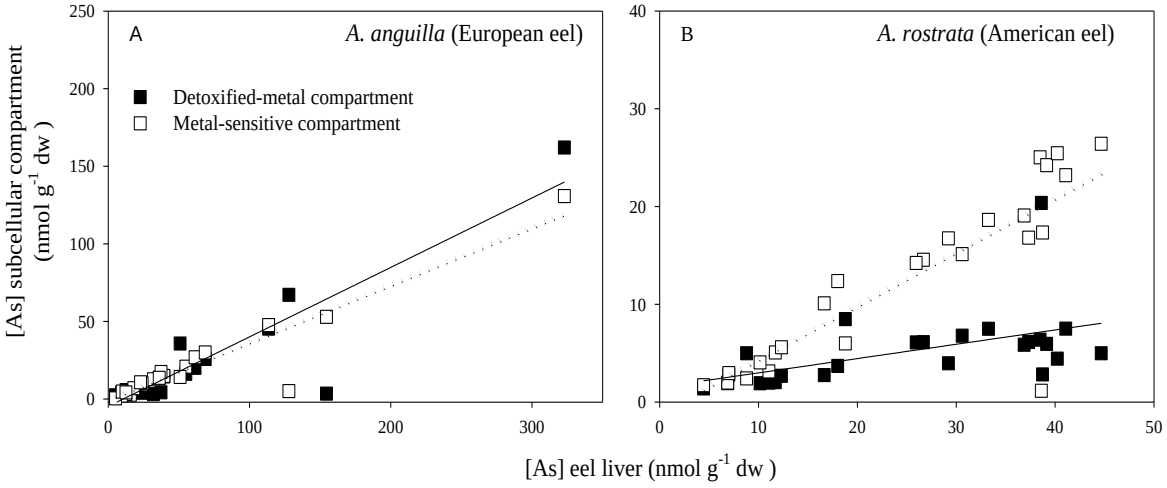

Figure 5.2: Relationship between total hepatic As concentrations (horizontal axis) and As concentrations in the detoxified-metal compartment (solid squares) and metal-sensitive compartment (open squares) in *Anguilla anguilla* (panel A) and *Anguilla rostrata* (panel B). Each point represents an individual eel. Lines represent significant regressions ($P < 0.05$).

The As concentrations measured in the metal-sensitive compartment in both eel species increased significantly to the total internalized As in the liver cells with a contribution mean of 41% ± 15% and 57% ± 18% for *A. anguilla* and *A. rostrata*, respectively. As was the case for Ag, the mitochondrial fraction contributed the most to the As accumulated in the metal-sensitive compartments of *A. anguilla* (contribution mean: 37% ± 14%) and *A. rostrata* (contribution mean: 45% ± 11%) (Figure. 3, Chapter 12). As suggested by Mason *et al.* (1995), As binding to these sensitive sites could provoke adverse effects.

5.3 *Cd*

Although the range in total Cd concentrations varied widely among *A. anguilla* (liver: 0.2 – 40 nmol g^{-1} dw), *A. rostrata* (liver: 0.8 – 11 nmol g^{-1} dw), whole *Chaoborus* (3 – 90 nmol g^{-1} dw), *Perca flavescens* (liver: 36 – 510 nmol g^{-1} dw) and *Pyganodon grandis* (digestive glands: 55 – 970 nmol g^{-1} dw), the majority of the total intracellular Cd was in all cases found in the detoxified-metal compartment (Figure A.3). Although the maximum hepatic Cd concentration in *A. anguilla* was 4-fold higher than that in *A. rostrata*, there was no significant difference ($P > 0.05$) between the two species with respect to the slope of the plots of Cd in the detoxified-metal compartment *vs.* total liver Cd (European eels, slope: 0.59 ± 0.05; American eels, slope: 0.63 ± 0.06) (Figure 5.3A, B). Overall, these results suggest that the animals studied use the same metal-handling

strategies to cope with incoming Cd. In this context, the predominant role of the HSP fraction in detoxifying Cd by MT or MTLP was confirmed in all the animals studied (Figure A.4).

Further analyses in the cytosol and in the HSP fractions of *Chaoborus* larvae (Chapter 13) consistently showed that MT (identification based on the similarity of their molecular weight with those known for MT for *Drosophila melanogaster*) are directly involved in Cd detoxification. The results obtained with *Chaoborus* larvae revealed that distinct MT isoforms (or MTLP) appeared to play different roles in Cd detoxification. With regards to Cd-handling, the hyphenated analysis also showed that Cd detoxification responses in *Chaoborus* are stronger at higher whole larval Cd concentrations (Chapter 13), an observation that is in accordance with our earlier field study on chronically metal-exposed *Chaoborus* (Chapter 9). In this field study, the measurements of subcellular Cd partitioning revealed that the Cd detoxification by the HSP fraction became more important when Cd concentrations exceeded an apparent threshold body concentration (Figure 7, Chapter 9). These observations are consistent with the results obtained with the analysis of the HSP fractions by SEC_{pep}-ICP MS, where at higher internalized Cd concentrations in the HSP fraction, more HSP ligands were involved in the complexation of this potentially toxic metal than at lower Cd bioaccumulation values (Figure 4E, Chapter 13).

Despite these Cd detoxification responses, Cd concentrations measured in the metal-sensitive compartment increased significantly with increasing total Cd concentrations in all animals except *Chaoborus* (Figure 5.3). Among the three metal-sensitive fractions, the HDP (*Chaoborus*) and the mitochondrial fractions (eel livers and digestive glands of *P. grandis*) were the major Cd-binding sites. Although the percentage of Cd in the HDP fraction in the metal-sensitive compartment decreased significantly along the Cd bioaccumulation gradient for four of the organisms studied (Figure A.5), the percentage of Cd in the mitochondrial fraction increased for all of the animals studied (Figure A.6).

Although Cd, a class-B cation (Pearson, 1969), appears to be readily detoxified by interacting with the sulfhydryl groups of MT, other S- and N-donating groups found in nucleic acids, enzymes and other biomolecules could be potential targets of this metal

(Mason *et al.*, 1995). In this context, up to a third of the internalized Cd (*A . anguilla*: 22% ± 7%, *A . rostrata*: 25% ± 10%, *Chaoborus*: 21% ± 9%, *Perca flavescens*: 34% ± 4%, *P. grandis*: 30% ± 12%) was found in the metal-sensitive compartment as biologically active pools that could contribute to toxic responses(Wallace *et al.*, 2003a). Potential effects could result from the interaction of Cd with metalloenzymes, leading to conformational changes or to the displacement of physiologically essential metals including Zn^{2+}, Cu^{2+}, Fe^{3+}, $Fe^{/2+}$, Mg^{2+} and Ca^{2+} (Moulis, 2010).

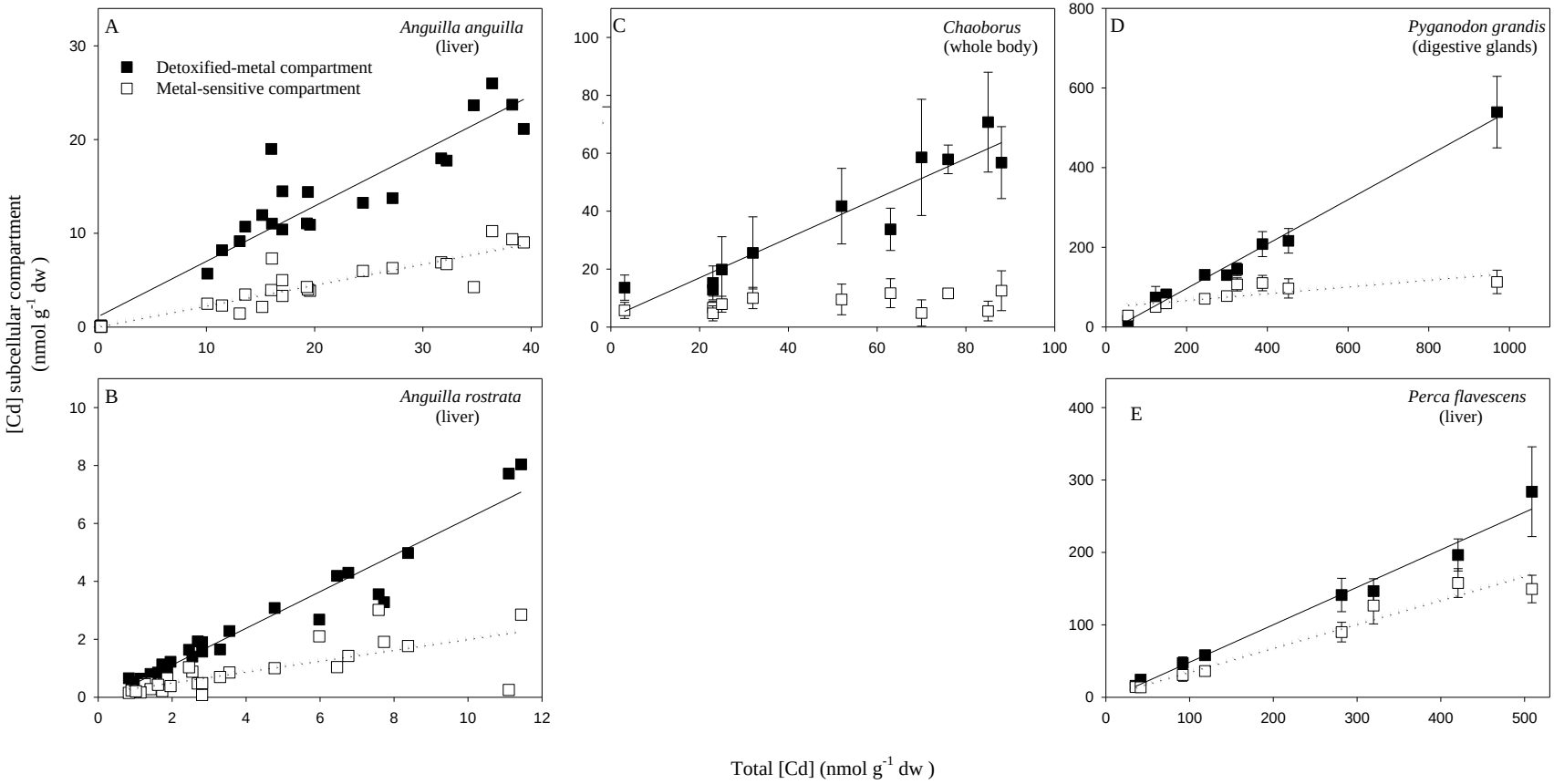

Figure 5.3: Relationship between total Cd concentrations (horizontal axis) and Cd concentrations in the detoxified-metal compartment (solid squares) and the metal-sensitive compartment (open squares) in *Anguilla anguilla* (liver, panel A), *Anguilla rostrata* (liver, panel B), *Chaoborus larvae* (whole body, panel C), *Pyganodon grandis* (digestive gland, panel D) and *Perca flavescens* (liver, panel E). Lines represent significant regressions ($P < 0.05$).

Our results suggest that the degree of Cd detoxification in *Chaoborus* larvae is similar to that in eel livers, but somewhat higher than that of liver cells of *P. flavescens* and digestive glands of *P. grandis* (Figure 5.4A).

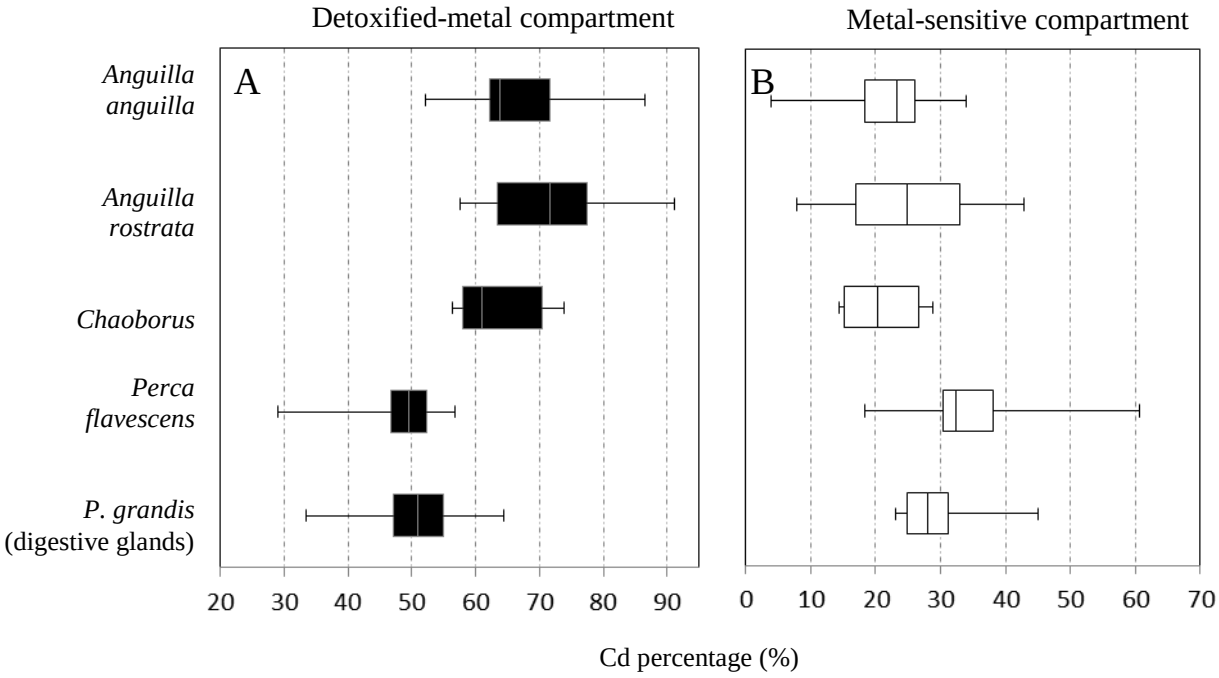

Figure 5.4: Box-Whisker plots of the Cd percentage contribution in the detoxified-metal compartment (panel A) and in the metal-sensitive compartment (panel B) for *Anguilla anguilla* (liver, n = 25), *Anguilla rostrata* (liver, n = 28), *Chaoborus* larvae (whole body, n = 12), *Perca flavescens* (liver, n = 7), *Pyganodon grandis* (digestive gland, n = 9). In each box plot, the central vertical line represents the median, the box gives the interval between the 25% and 75% percentiles, and the whisker indicates the range. "dg" represents digestive gland.

For these latter organisms, detoxified Cd did not exceed 60% of the total metal accumulated. With regards to the metal-sensitive compartment, the percentage of the total internalized Cd found in this compartment reached values as high as 54% for some digestive glands of *P. grandis* (Figure 5.4B). For the other aquatic organisms studied, the percentages of Cd in the metal-sensitive compartment were not greater than 41%. Note that for liver cells of juvenile yellow perch, more than the 30% of the total hepatic accumulated Cd (Figure 5.4B) was associated with physiologically-sensitive target molecules (e.g., cytosolic enzymes) and organelles (e.g., mitochondria) where the Cd could induce deleterious effects.

5.4 *Ni.*

For all the fish species studied, there was an increase in Ni concentrations in the detoxified-metal compartment along the total Ni bioaccumulation gradient (Figure 5.5). In *Chaoborus* larvae, major proportions of the total accumulated Ni were consistently found in the detoxified-metal compartment (Figure A.7).

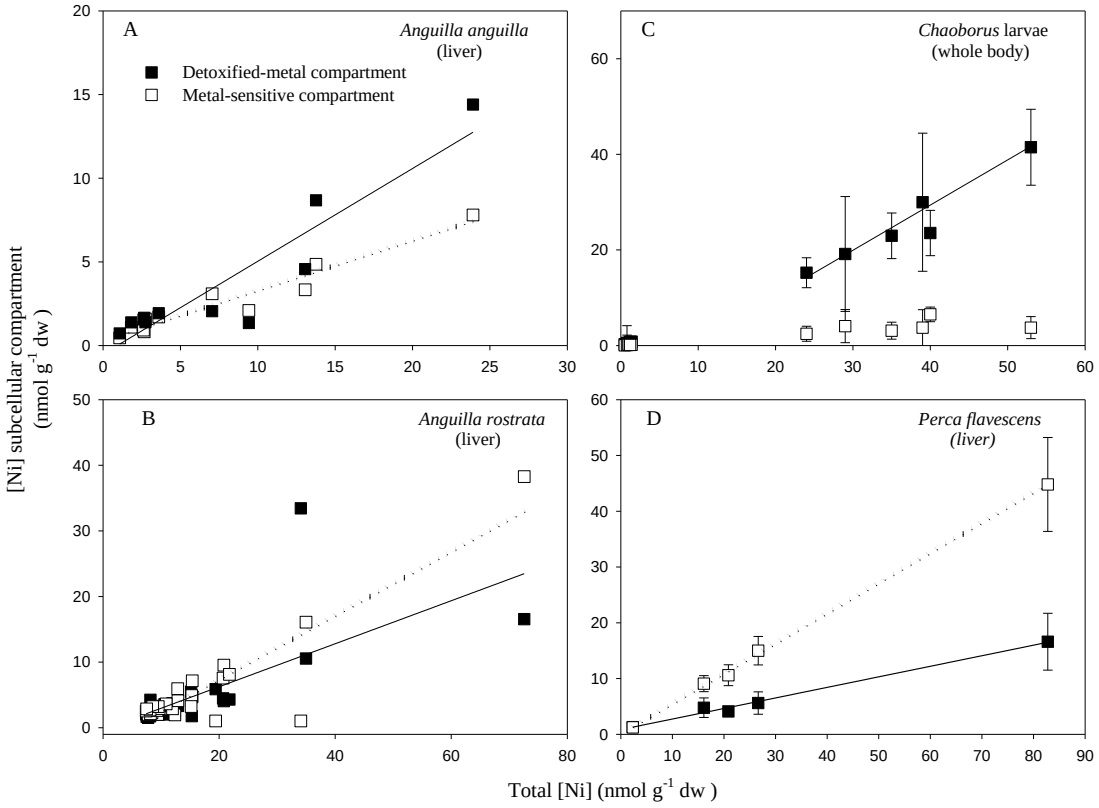

Figure 5.5: Relationship between total Ni concentrations (horizontal axis) and Ni concentrations in the detoxified-metal compartment (closed squares) and the metal-sensitive compartment (solid squares) in *Anguilla anguilla* (liver, panel A), *Anguilla rostrata* (liver, panel B), *Chaoborus* larvae (whole body, panel C) and *Perca flavescens* (liver, panel D). Lines represent significant regressions ($P < 0.05$).

In the case of highly Ni-contaminated larvae from the Sudbury lakes, the Ni concentration of the detoxified-metal compartment increased as a function of total larval Ni concentrations (Figure 5.5). However, the predominant strategy (HSP or granules) used to detoxify this non-essential metal differed among species. Whereas the HSP fraction in *Chaoborus* larvae (contribution mean: 62% ± 17%, Figure 4, Chapter 9) and in *Perca flavescens* (contribution mean: 67% ± 18%, (Giguère *et al.*, 2006)) markedly

contributed to the Ni measured in this compartment, Ni detoxification in the liver cells of the *A. rostrata* was predominantly in granules (contribution mean: 54% ± 22%, Figure 5, Chapter 12).

The sequestration of metals by mineralized structures containing Ca, Mg, Cu or Fe has been reported mostly in terrestrial (Hopkin, 1989) and aquatic invertebrates (Mason *et al.*, 1995), but it appears that metal-rich granules can also be involved in the detoxification of Ni in vertebrates (Lapointe *et al.*, 2009, Leonard *et al.*, 2014). Considering the weak preferential binding of this borderline metal for sulfhydryl-rich ligands as compared to other metals (Nieboer *et al.*, 1980), Ni sequestration in the granule fraction is likely to depend on the occurrence of Fe-containing or Ca/Mg-containing moieties. In contrast, the association of Ni with MT or MTLP, as well as its ability to induce the biosynthesis of such proteins in aquatic organisms, have not been demonstrated. In studies with *P. promelas* (Lapointe *et al.*, 2009), *O. mykiss* (Leonard *et al.*, 2014) and *P. flavescens* (Giguère *et al.*, 2006), Ni was mainly found in the fraction containing cytosolic thermostable proteins and peptides. However, analysis of the HSP fractions of *Chaoborus* larvae (Chapter 13) using hyphenated techniques (i.e., SEC-ICPMS) showed that MT and MTLP were not involved in Ni complexation in the HSP fraction. Since Ni is a "harder" metal than Cd or Ag in the hard-soft acid-base classification described by Nieboer *et al.* (1980), this element is not thought to be a potential inductor of MT biosynthesis nor to be able to interact with MT sulfhydryl groups.

In spite of the metal-detoxification response observed in these aquatic organisms, increases in Ni concentrations (and major Ni contributions) were observed in the metal-sensitive compartment along the bioaccumulation gradient (Figure 5.5, Figure A.7), suggesting that Ni detoxification was incomplete and that these animals could be experiencing deleterious Ni-induced effects. As was the case with Cd, the entities to which Ni is bound in the metal-sensitive compartment appear to be organism-specific. The HDP fraction in *Chaoborus* (contribution mean: 51% ± 11%) and *P. flavescens* (contribution mean: 47% ± 6%) was the major sensitive fraction to which Ni was bound, whereas for both eel species, among the metal-sensitive fractions, the mitochondria had the highest proportion of Ni. As mentioned above, the accumulation of a nonessential metal such as Ni in the metal-sensitive compartment where it can interact with

physiologically important biomolecules (e.g. O-, P-containing ligands) has the potential to produce toxic effects (Macomber & Hausinger, 2011). In this context, the results obtained with *Chaoborus* using hyphenated techniques showed that Ni binds to cytosolic HMW ligands (covering a MW range from 670 kDa to 200 kDa; considered to be metal-sensitive ligands, Figures 1 and 2, Chapter 13) that are not associated with other class B metals (e.g., Cd and Ag).

Comparing Ni concentrations in the metal-sensitive compartment and the detoxified-metal compartment, differences were observed between *Chaoborus* larvae and yellow perch collected from the same mining-impacted region and with similar Ni bioaccumulation gradients (Figure 5.6). For all *Chaoborus* larvae, the percentage of "detoxified" Ni exceeded 50% of the total Ni accumulated in the whole body (Figure 5.6A). However, as the Ni accumulated in the HSP fraction was not associated with detoxification ligands (e.g., MT or MTLP) in the HSP fractions (Chapter 13), these percentages are likely overestimates. In contrast to this Ni detoxification response, liver cells of *Perca flavescens* were not able to detoxify more than a quarter of the total hepatic Ni.

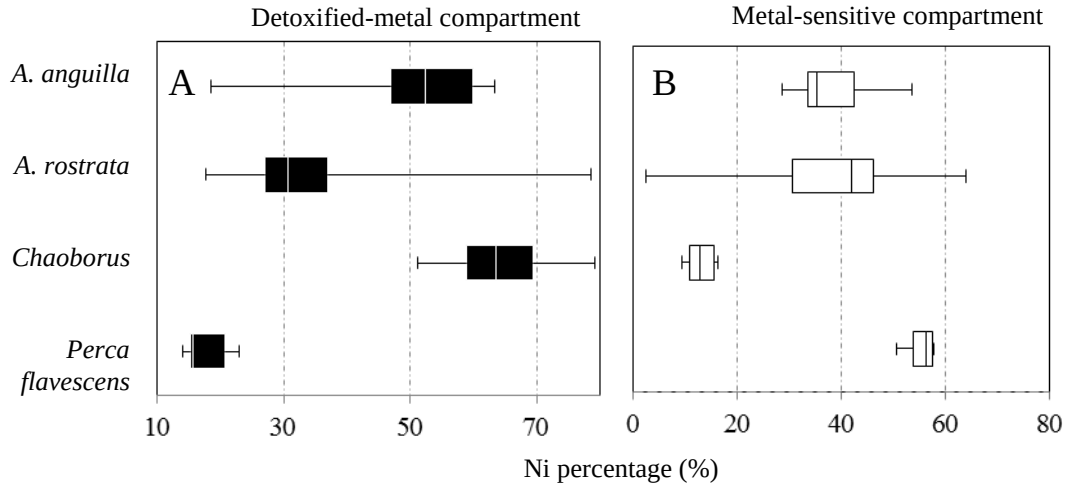

Figure 5.6: Box-Whisker plots of the Ni percentage contribution in the detoxified-metal compartment (panel A) and in metal-sensitive compartment (panel B) for *Anguilla anguilla* (liver, n = 11), *Anguilla rostrata* (liver, n = 28), *Chaoborus* larvae (whole body, n = 12) and *Perca flavescens* (liver, n = 5). In each box plot, the central vertical line represents the median, the box gives the interval between the 25% and 75% percentiles, and the whisker indicates the range.

Accordingly, Ni accumulation in the metal-sensitive compartment in *Perca flavescens* was much higher than that observed for *Chaoborus*, where less than the 20% of the total larval Ni concentration was found in this compartment (Figure 5.6B).

5.5 *Pb.*

The total hepatic Pb bioaccumulation range in *A. anguilla* (0.4 – 3.2 nmol g^{-1}) was lower than that in *A. rostrata* (0.6 – 13 nmol g^{-1}). Lead concentrations in both subcellular compartments increased significantly along the bioaccumulation gradient (Figure 5.7). The Pb detoxification response involved mainly Pb sequestration in insoluble granules in American (contribution mean: 56% ± 23%) and European (contribution mean: 54% ± 22%) eels. According to Nieboer *et al.* (1980), Pb is a borderline metal that has no clear preference for either O- or S- donating ligands. Lead detoxification in this fraction could involve any of the three granule types (Fe-containing, Ca/Mg-containing or Cu-containing granules). Both eel species also accumulated appreciable Pb in the HSP fraction. In this context, Van Campenhout *et al.* (2008) reported that in livers of field-collected European eels MT played only a limited role in Pb handling, but the SEC-ICPMS analysis showed association of this metal with MT or MTLP in *A. rostrata* (results not shown).

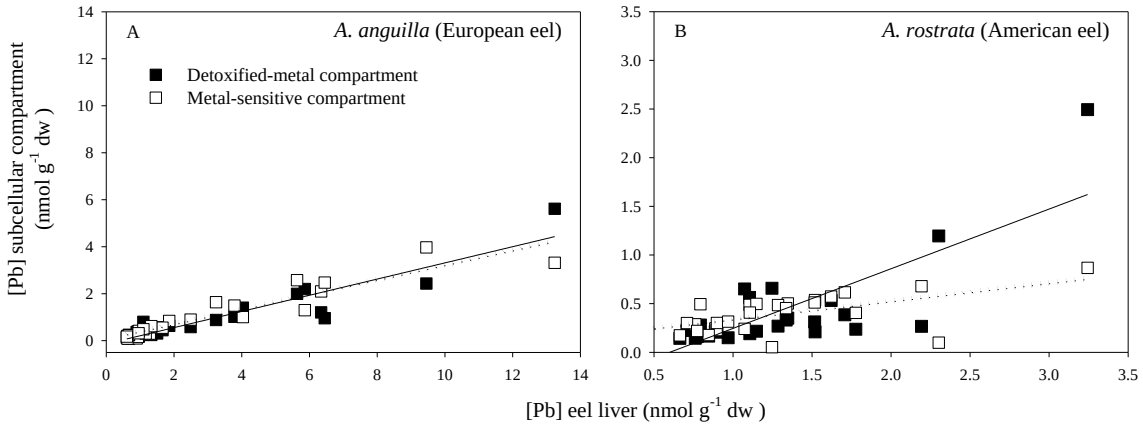

Figure 5.7: Relationship between total hepatic Pb concentrations (horizontal axis) and Pb concentrations in the detoxified-metal compartment (solid squares) and the metal-sensitive compartment (open squares) in *Anguilla anguilla* (panel A) and *Anguilla rostrata* (panel B). Each point represents an individual eel. Lines represent significant regressions ($P < 0.05$).

The above-mentioned metal-handling strategies were not completely successful in limiting Pb binding to metal-sensitive targets. The mitochondria were the major Pb-binding fraction in *A. anguilla* (contribution mean: 40% ± 12%) and *A. rostrata* (contribution mean: 42% ± 13%). Given the capacity of Pb to replace other metals involved in important biochemical processes, including Ca, Fe and Zn (Vallee *et al.*, 1972), its accumulation in metal-sensitive fractions could result in the disturbance of cellular functions. The proportion of the total accumulated Pb found in the metal-sensitive compartment represented 40% ± 13% and 37% ± 13% for the European and American eels, respectively.

5.6 *Tl.*

Hepatic Tl concentrations ranged from 0.018 to 0.44 nmol g^{-1} dw for both eel species. The subcellular partitioning of Tl between detoxified-metal and metal-sensitive compartments was not significantly different between the two eel species (Figure 5.8, Figure A.8). Thallium was mainly found in the HSP fraction in both *A. anguilla* (71% ±18%) and *A. rostrata* (73% ±16%), which initially suggested its potential association with MT and MTLP. Both eel species also detoxified Tl by incorporating it into mineralized inclusions in their liver cells. As is thought to be the case for other soft metals such as Ag and Cd, Tl (as Tl(I)) could be associated with S in Cu-containing granules (Mulkey & Oehme, 1993).

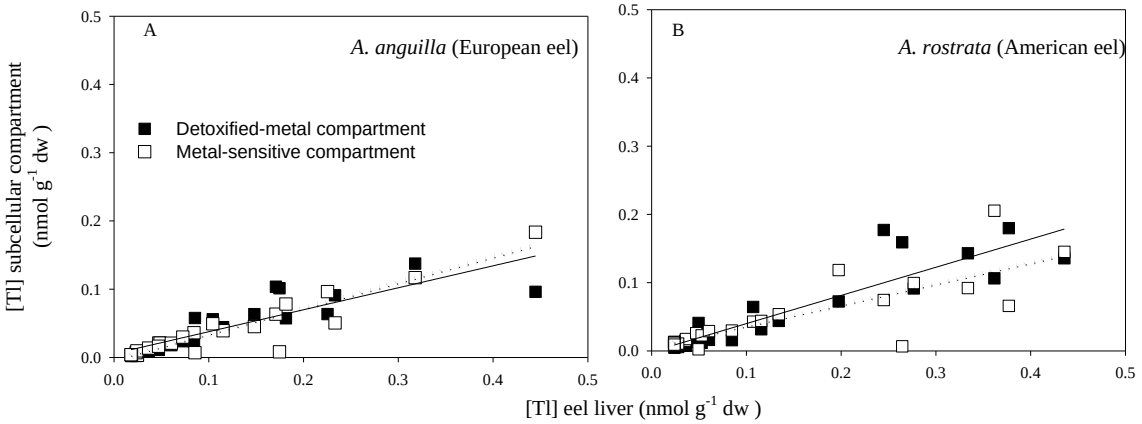

Figure 5.8 : Relationship between total hepatic Tl concentrations (horizontal axis) and Tl concentrations in the detoxified-metal compartment (solid squares) and the metal-sensitive compartment (open squares) in *Anguilla anguilla* (panel A) and *Anguilla rostrata* (panel B). Each point represents an individual eel. Lines represent significant regressions ($P < 0.05$).

However, in the cytosol and HSP fractions of *Chaoborus* larvae, SEC-ICPMS measurements showed that this metal was not associated with the MT pool (Chapter 13). Thallium was consistently found in the LMW fraction (HSP fraction: < 1.7 kDa), where it could be associated with sulfhydryl-containing groups in free amino acids such as cysteine or as methylated thallium, that is, $(CH_3)_2Tl^+$ (Schedlbauer *et al.*, 2000).

As was the case with the other non-essential metals, Tl in the metal-sensitive compartment could be a harbinger of toxic effects. Among metal-sensitive fractions, the mitochondrial fraction was again the major Tl-binding fraction. According to our results, about 40% of the accumulated Tl was found in the metal-sensitive compartment in the eel liver: *A. anguilla*, contribution mean 38% ± 13%; *A. rostrata*, contribution mean 42% ± 15%). As was the case for Ni, the contributions of this metal in the detoxified-metal compartment are probably overestimated.

6. Conclusions

The subcellular metal partitioning approach was applied to various aquatic organisms that had been chronically exposed not to a single metal, but rather to a mixture of metals and possibly to other environmental stressors; for some of the metals, we determined their subcellular partitioning between the detoxified-metal compartment and metal-sensitive compartment. The animal organs used for the analyses differed among species. For *Chaoborus* larvae we used the whole insect, whereas for fish (both eel species and yellow perch) the liver was the object of our analyses. For bivalves, the subcellular partitioning of trace metals in the digestive gland was included in our comparisons. Despite these differences, the subcellular partitioning of As, Ag, Cu, Ni, Pb and Tl determined in various chronically exposed field-collected aquatic invertebrates (*Chaoborus*, *P. grandis*) and vertebrates (*A. anguilla*, *A. rostrata*, *P. flavescens*) revealed remarkable similarities in the behaviour of the same metal in different species (Table 2).

Table 2. Summary information of the subcellular metal partitioning and SEC-ICPMS results. GRAN: granules; MITO: mitochondria; M+L: microsomes and lysosomes; HMW: high molecular weight, LMW: low molecular weight; nd: non detected, na: non analysed. For more detail, please see Chapters 9, 12, 13, Bonneris et al. (2005) and Giguère et al. (2006).

Trace element	Organism	Metal-detoxification strategies	Metal-sensitive fractions	SEC-ICPMS analysis (HSP fraction)
Ag	*Anguilla anguilla*	HSP	MITO > M+L, HDP	na
	Anguilla rostrata	HSP, GRAN	HDP	MT or MLP
	Chaoborus punctipennis	na	na	
As	*Anguilla anguilla*	HSP	MITO > M+L > HDP	na
	Anguilla rostrata			
Cd	*Anguilla anguilla*	HSP	MITO > M+L > HDP	na
	Anguilla rostrata			MT or MLP
	Chaoborus punctipennis		HDP > MITO > M+L	
	Pyganodon grandis		MITO > M+L ~ HDP	na
	Perca flavescens		MITO ~ M+L > HDP	
Ni	*Anguilla anguilla*	HSP, GRAN	HDP > MITO ~ M+L	na
	Anguilla rostrata	GRAN, HSP	HDP ~ MITO ~ M+L	LMW
	Chaoborus punctipennis	HSP, GRAN	HDP > MITO ~ M+L	
	Perca flavescens	HSP		na
Pb	*Anguilla anguilla*	GRAN, HSP	MITO > M+L > HDP	na
	Anguilla rostrata			MT or MLP
Tl	*Anguilla anguilla*	HSP, GRAN	MITO > M+L > HDP	na
	Anguilla rostrata			LMW
	Chaoborus punctipennis	na	na	

"Hyphenated" analysis (e.g., SEC-ICPMS) of the cytosol and the HSP fraction collected from *Chaoborus* also provided useful and complementary information to that provided by the subcellular measurements, in particular with respect to the nature of the ligands found in the "HSP" fraction. In this context, SEC-ICPMS analysis revealed that in the HSP fractions Cd and Ag are largely associated with MT or MTLP, whereas for Ni and Tl other bioligands are involved in their complexation.

As expected, the intracellular speciation of the metals studied was related to the classification described by Nieboer *et al.* (1980), in which metal-ligand complexes (and thus metal toxicity) were predicted on the basis of the ligand-binding preferences of various metals. In this regard, the analysis of the percentage contribution of the metal studied in the detoxified-metal compartment in both eel species (Figure 6.1A, B) showed a similarity between Ag and Cd. In both eel species, more than the 29% (*A. rostrata*, Figure 6.1B) and 47% (*A. anguilla*, Figure 6.1A) of the total Ag and Cd, respectively, in liver cells was associated with MT or MTLP . In addition, the proportion of both metals found in the metal-sensitive compartment did not exceed 44% (Ag) and 41% (Cd) of the total metal accumulated (Figure 6.1C, D). These results suggest a much higher degree of detoxification for both metals compared to the other elements studied, for which the contribution of the metal-sensitive compartment reached much higher percentages (Figure 6.1C, D). This similarity could be ascribed to the fact that both metals were consistently found in the HSP fraction where they were bound to cytosolic, thermostable MTLP, as revealed by measurements using SEC-ICPMS. Thus the subcellular speciation of Ag and Cd is likely determined by the preferential binding of these class B metals (Nieboer *et al.*, 1980) to the sulfhydryl groups of MT, which detoxifies them.

Lead (a borderline metal) and Tl (a class B metal) (Nieboer *et al.*, 1980), which interact with sulfhydryl groups (although less readily than other class B metals), tended to be present in lower proportions in the detoxified-metal compartment of eels than were Ag and Cd (Figure 6.1 A, B). As a corollary, Pb and Tl were present in higher proportions in the metal-sensitive compartment than were Ag and Cd (Figure 6.1 C, D). "Hyphenated" analysis of the HSP fraction showed that Tl in the HSP fraction of *Chaoborus* was present only in the LMW pool, perhaps as the methylated species $(CH_3)_2Tl^+$ (Schedlbauer *et al.*, 2000). In contrast to these metals, Ni (a borderline cation) was distributed about

equally between the detoxified metal and the metal-sensitive compartments in eel livers (Figure 6.1). Although high Ni concentrations were found in the HSP pool in *Chaoborus*, this metal was not associated with MT or MTLP, which suggests that it was bound to O- and P-containing bioligands.

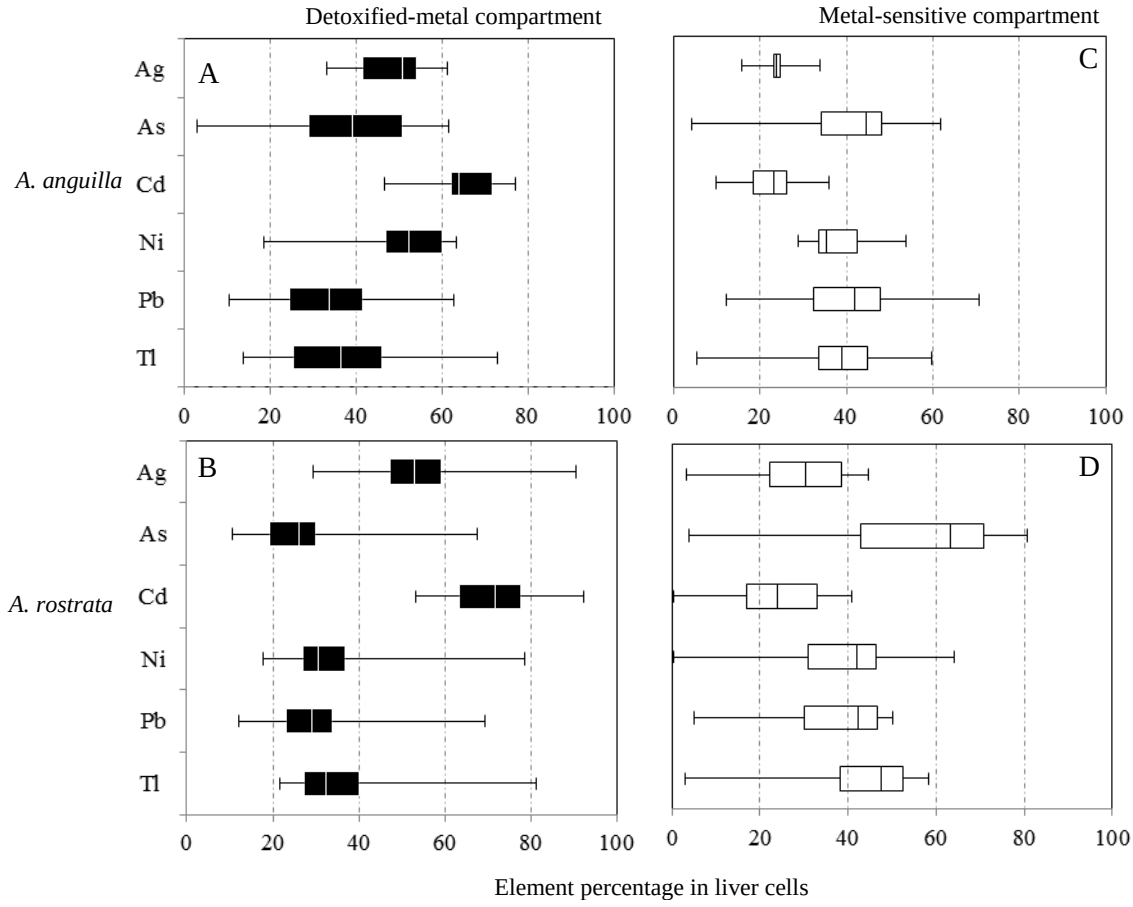

Figure 6.1: Box-Whisker plots of percentage contribution of Ag, As, Cd, Ni, Pb and Tl in the detoxified-metal compartment (panel A, C) and in metal-sensitive compartment (panel B, D) in *Anguilla anguilla* (upper panels) and *Anguilla rostrata* (lower panels). In each box plot, the central vertical line represents the median, the box gives the interval between the 25% and 75% percentiles, and the whisker indicates the range.

With regards to As, Nieboer *et al.* (1980) classified As(III) as a borderline metal, but as we did not determine As speciation (e.g., arsenite, arsenate, monomethylarsonic acid, dimethylarsinic acid (Thomas *et al.*, 2001)) and because this metal was not detected in the hyphenated analysis, we cannot speculate on the likelihood of As-induced deleterious effects.

The differences in subcellular metal partitioning that were noted among metals should be helpful in predicting the likelihood of metal toxicity. On the one hand, the binding preference of class B metals (e.g., Ag and Cd) for the S- and N-donating groups found in some amino acids (e.g., arginine, histidine, lysine, cysteine) could affect the functional integrity of proteins and enzymes (Bartlett *et al.*, 2002, Jimenez-Morales *et al.*, 2012). On the other hand, metals with pronounced class-A reactivity (e.g., Ni) would interact mainly with the O-donating ligands found in the side chain groups (-R groups) of amino acids (e.g., aspartic acid, glutamic acids) frequently located in the active sites of enzymes (Bartlett *et al.*, 2002, Jimenez-Morales *et al.*, 2012). According to Mason *et al.* (1995), non-essential metals bound to these sensitive sites can block functional groups, displace essential metals, or modify the active conformation of sensitive biomolecules, resulting in metal-induced toxicity.

From an ecotoxicological perspective, these results show that the trace element concentrations in the detoxified-metal compartment increased as a function of the total intracellular trace metal concentrations in all of the animals studied. Specifically, increased concentrations were found in the HSP fraction that includes MT, MTLP, GSH and free amino acids, with MT and MTLP generally considered to be major contributors to metal detoxification. Indeed, SEC-ICPMS analysis revealed the complexation of Cd and Ag by these thermostable bioligands. However, Ni, Pb and Tl were associated with granules, a result that points to an alternative strategy for limiting the binding of non-essential metals to physiologically important biomolecules.

Despite evident metal detoxification, it clearly was not completely effective in keeping non-essential metals away from metal-sensitive targets. Inappropriate binding of these contaminants to physiologically metal-sensitive compartments such as heat-denaturable cytosolic proteins, mitochondria and other organelles was consistently measured for all the studied metals. Accumulation in the putative metal-sensitive fractions increased along the metal bioaccumulation gradient and in the most-contaminated organisms the concentrations of some metals in the metal-sensitive compartment exceeded those observed in the detoxified-metal compartments (Tl in *A. anguilla*, Ni in *P. flavescens*, and As and Ni in *A. rostrata*). These results suggest that the "spillover" model (Jenkins *et al.*, 1988, Mason *et al.*, 1995) is not adequate to describe metal-handling strategies, at

least in chronically exposed animals in the field. Although we did not measure toxic effects in the present study, our results suggest that there could be impairment of the biological functions carried out by the bioligands with which these metals are associated (Mason *et al.*, 1995). The mitochondria, where key biochemical processes occur, appear to be a major binding fraction for As, Cd and Tl (Figure 6.2). For example, the proportion of the metal found in the metal-sensitive compartment that was confined to the mitochondria varied greatly for Ag (*A. anguilla*: 14% – 65%; *A. rostrata:* 2% – 62%), As (*A. anguilla*: 9% – 59%; *A. rostrata:* 1% – 72%), Cd (*A. anguilla*: 6% – 72%; *A. rostrata:* 1% – 71%), Ni (*A. anguilla*: 15% – 65%; *A. rostrata:* 12% – 57%), Pb (*A. anguilla*: 19% – 65%; *A. rostrata:* 11% – 60%) and Tl (*A. anguilla*: 9% – 62%; *A. rostrata:* 8% – 73%). Note that the highest percentages were reported for Cd in *A. anguilla* (Figure 5.2 A) and for Tl, Cd and As in *A. rostrata* (Figure 6.2 B).

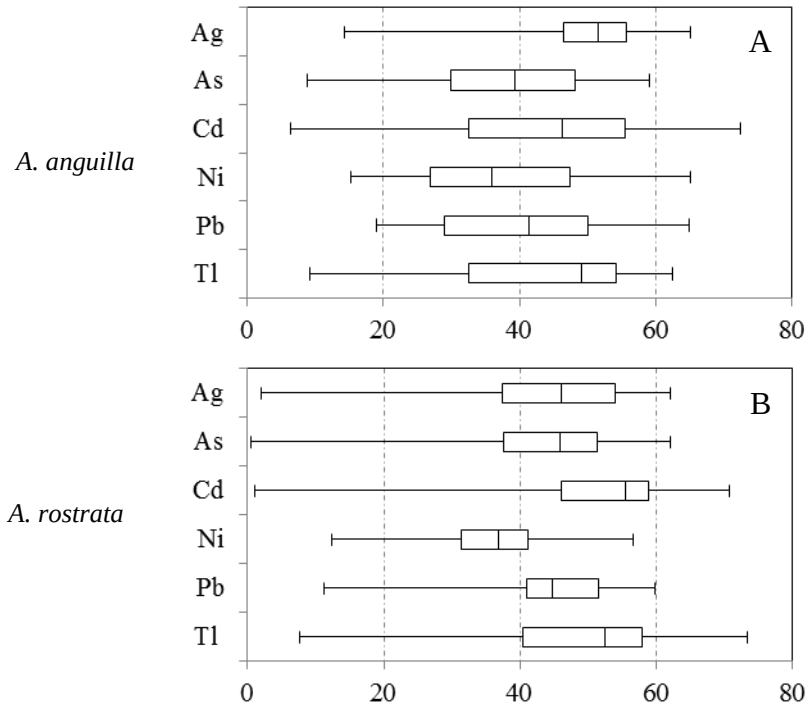

Figure 6.2: Box-Whisker plots of percentage contribution of Ag, As, Cd, Ni, Pb and Tl in the mitochondria to the metal-sensitive compartment in *Anguilla anguilla* (panel A) and *Anguilla rostrata* (panel B). In each box plot, the central vertical line represents the median, the box gives the interval between the 25% and 75% percentiles, and the whisker indicates the range.

The important contribution observed for the mitochondria, compared to the other metal-sensitive fractions, suggests that future work on metal-induced adverse effects should focus on these organelles.

Considering the preferential binding of these three metals (Nieboer *et al.*, 1980), we speculate that sulfhydryl-containing proteins involved in important biochemical pathways are the major metal-binding targets in this organelle. This observation is consistent with the *in vitro* results obtained for rat livers by Belyaeva and Korotkov (2003), who reported direct Cd effects on mitochondrial functions by the binding of this metal to both sulfhydryl-containing and metal-binding (e.g., Ca, Mg) enzymes.

Based on the metal-handling strategies used by the aquatic organisms studied, and considering the low proportions of Cd and Ni in the metal-sensitive compartment in *Chaoborus* larvae, we suggest that detoxification in *Chaoborus* was more efficient than in the other aquatic animals (cf., Cd: *P. flavescens* and the digestive glands of *P. grandis*). In addition, differences in terms of metal-handling strategies between both eel species (*A. anguilla* and *A. rostrata*) were noted for As, Ni, Pb.

7. Future directions

Subcellular metal measurements performed to discriminate between metals accumulated in the metal-sensitive compartment and in the detoxified-metal compartment allow researchers to determine the metal-handling strategies used by aquatic organisms to cope with high metal concentrations (Campbell *et al.*, 2005, Campbell *et al.*, 2009). Knowledge of subcellular metal partitioning in living cells is necessary for understanding and predicting metal toxicity. With regards to metal-detoxification mechanisms, metal incorporation into mineral inclusions and the binding of metals to thermostable cytosolic proteins (e.g., MT or MTLP) are known strategies to protect organisms against metal toxicity (Wallace *et al.*, 2003a), but detailed characterization of the entities involved in such detoxification responses is required to better understand the specific roles of these fractions in the intracellular trafficking of metals. In this thesis, the subcellular partitioning of several trace metals was determined in *Chaoborus* larvae and in two eel species and the characterisation of metal-ligand complexes in terms of molecular weights was performed by SEC-ICPMS analysis for the insect. However, such information is not sufficient to pinpoint the relationships between metal accumulation and metal toxicity as part of an ERA approach. Although several studies concerning the role of MT in metal detoxification and the formation of mineral granules have been published (Amiard *et al.*, 2006, Marigómez *et al.*, 2002, Nordberg, 1998), more effort should be addressed to isolate, identify and quantify metal complexes with such intracellular entities. For example, given the evidence of the expression of distinct MT isoforms with different physiological roles in metal sequestration (Dallinger *et al.*, 1997, Lauverjat *et al.*, 1989, Lemoine *et al.*, 2000), it would be interesting to investigate the biochemical characteristics (molecular weight; amino acid composition and sequences of the metal-binding moiety; stoichiometry of metal-ligand binding) of these proteins and their specific roles in intracellular metal-trafficking. Likewise, we need to elucidate the sequestration of these contaminants by metal-rich granules and more particularly to understand the formation of these structures. In this regard, analytical measurements of these metal complexes can be achieved by the application of "hyphenated" techniques, where high-resolution separation by chromatographic techniques or by electrophoresis is coupled on line to the ICPMS instrument (Lobinski *et al.*, 1998). Elemental molecular

mass spectrometric (MS) technology coupled with liquid chromatography has been already used to characterize Cd-MT isoforms (Mounicou *et al.*, 2010), but this approach has yet not been widely applied to other organisms.

It is also important to mention that these techniques should not be limited to metal-detoxified fractions. Analytical quantification of the metal complexes with sensitive biomolecules would be useful to refine the relationship between metal bioaccumulation and toxic effects. Such hyphenated analyses complemented with subcellular metal measurements should allow predictions of metal toxicity on the basis of metal accumulation in the metal-sensitive compartment (and more specifically the binding of metals to the bioligand targeted by these contaminants) rather than the total internalised metal concentrations (Rainbow *et al.*, 2015). Considering that non-essential metals can be bound to metal-sensitive ligands by distinct mechanisms (e.g., blocking functional groups, displacing essential metals or modifying the active conformation; Mason *et al.* (1995)), it would be interesting to investigate the nature of these biochemical interactions, which could reveal the functional impairments that these inappropriate metal bindings are causing. In addition to this biochemical information, these results could also help explain how certain metals consistently induce greater damage than others.

Another important issue concerning the use of the subcellular metal partitioning approach is the unknown nature of the subcellular fractions that have been operationally grouped in the detoxified-metal and metal-sensitive compartments (Wallace *et al.*, 2003a). For example, the isolation of the HSP fraction from the cytosol is based on the thermostable properties of cytosolic metal-binding proteins, including MT. However, the fact that the proteins and peptides found in the supernatant after heat treatment are "thermostable" does not mean that they are all involved in metal detoxification. The application of hyphenated systems (e.g., HPLC-ICPMS) combined with proteomic analysis would contribute to more rigorous interpretations of the subcellular metal distribution results. In addition, as the potential re-distribution of elements among intracellular ligands might occur as a consequence of the homogenisation and differential centrifugations steps, we recommend the use of microscopic techniques (e.g., histochemistry, autoradiography, electron microscopy, X-ray microanalysis and nanometer scale secondary ion mass

spectrometry) to validate the subcellular partitioning results and contribute to better interpretations.

Studies with field-collected organisms that have been chronically exposed to trace metals have reported inappropriate metal bindings in the metal-sensitive fractions, even at low metal exposure levels (Bonneris *et al.*, 2005, Giguère *et al.*, 2006). Although for some of these organisms evidence for metal-induced adverse effects has been reported (Campbell *et al.*, 2009), biological impairment as a consequence of metal exposure should be measured for *Chaoborus* larvae and both eel species and linked to metal bioaccumulation in the metal-sensitive compartment.

8. References

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PART-II : SCIENTIFIC ARTICLES

9. Subcellular metal partitioning in larvae of the insect *Chaoborus* collected along an environmental metal exposure gradient (Cd, Cu, Ni and Zn)

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Abstract

Larvae of the phantom midge *Chaoborus* are common and widespread in lakes contaminated by metals derived from mining and smelting activities. To explore how this insect is able to cope with potentially toxic metals, we determined total metal concentrations and subcellular metal partitioning in final-instar *Chaoborus punctipennis* larvae collected from 12 lakes situated along gradients in aqueous Cd, Cu, Ni and Zn concentrations. Concentrations of the non-essential metals Cd and Ni were more responsive to aqueous metal gradients than were larval concentrations of the essential metals Cu and Zn; these latter metals were better regulated and exhibited only 2–3-fold-fold increases between the least and the most contaminated lakes. Metal partitioning was determined by homogenization of larvae followed by differential centrifugation, NaOH digestion and heat denaturation steps so as to separate the metals into operationally defined metal-sensitive fractions (heat-denatured proteins (HDP), mitochondria, and lysosomes/microsomes) and metal-detoxified fractions (heat stable proteins (HSP) and NaOH-resistant or granule-like fractions). Of these five fractions, the HSP fraction was the dominant metal-binding compartment for Cd, Ni and Cu. The proportions and concentrations of these three metals in this fraction increased along the metal bioaccumulation gradient, which suggests that metallothionein-like proteins play an important role in metal tolerance of *Chaoborus* living in metal-contaminated environments. Likewise, a substantial proportion of larval Zn was in the HSP fraction, but its contribution did not increase progressively along the metals gradient. Despite the increases in Cd, Ni and Cu in the HSP fraction along the metal bioaccumulation gradient, some accumulation of non-essential metals (Cd and Ni) was observed in putative metal-sensitive fractions (e.g., HDP, mitochondria), suggesting that metal detoxification was incomplete. In the case of Cd, there appears to be a threshold body concentration of about 50 nmol Cd g^{-1} dry weight, above which Cd detoxification becomes more effective and below which *Chaoborus* does not "turn on" its detoxification machinery to the fullest extent.

Keywords: Subcellular partitioning; *Chaoborus*; Metals; Toxicity; Metallothioneins; Detoxification.

Résumé:

Les larves de l'insecte *Chaoborus* (couramment appelées «verre de cristal») sont très répandues aux lacs contaminés par des métaux traces provenant de l'activité minière locale ainsi que des opérations de fonderies. Pour comprendre la capacité de cet insecte à faire face à des métaux potentiellement toxiques, nous avons déterminé les concentrations de métaux et leur répartition subcellulaire chez les larves de *Chaoborus punctipennis* récoltées d'un ensemble de 12 lacs situés le long de gradients de concentrations aqueuses en Cd, Cu, Ni et Zn. Les concentrations de métaux non-essentiels étudiés (Cd et Ni) chez *C. punctipennis* ont varié plus en fonction des gradients métalliques aqueux que les concentrations larvaires pour les métaux essentiels (Cu et Zn); ces derniers métaux étaient mieux régulés et leurs concentrations totales chez les larves récoltées dans les lacs les plus contaminés n'étaient que de 2 à 3 fois plus élevées que leurs concentrations totales chez les larves provenant des lacs les moins contaminés. La répartition subcellulaire a été déterminée en appliquant une étape initiale d'homogénéisation suivie d'étapes de centrifugation différentielle, de digestion avec NaOH et de traitement thermique, afin de séparer opérationnellement les fractions sensibles aux métaux (*HDP*, mitochondries, microsomes et lysosomes) et les fractions contenant les métaux détoxiqués (*HSP*, granules). Parmi ces cinq fractions, la fraction *HSP* était le compartiment subcellulaire le plus important pour le Cd, le Ni et le Cu. Les contributions (%) et les concentrations de ces trois métaux dans cette fraction ont augmenté le long du gradient d'exposition des métaux, ce qui suggère que les protéines apparentées à la métallothionéine jouent un rôle important dans la tolérance de *Chaoborus*, leur permettant de tolérer des environnements contaminés par des métaux. De même, une proportion importante du Zn bioaccumulé se trouvait dans la fraction HSP, mais sa contribution n'a pas augmenté progressivement le long du gradient d'exposition aux métaux. Malgré l'augmentation des concentrations de Cd, de Ni et de Cu dans la fraction HSP le long des gradients de bioaccumulation des métaux, des accumulations importantes de métaux non essentiels (Cd et Ni) ont été observées dans les fractions sensibles (par exemple, *HDP*, les mitochondries), ce qui suggère que la détoxication de ces métaux était incomplète. Dans le cas de Cd, il semble y avoir une concentration seuil

d'environ 50 nmol Cd g^{-1} poids sec, au-dessus duquel la détoxication du Cd devient plus efficace et en dessous duquel *Chaoborus* ne "déclenche" pas ses réponses de détoxication à leur degré maximal.

Mots-clés : distribution subcellulaire, *Chaoborus*, métaux traces, toxicité, métallothionéines, détoxication.

Introduction

Essential trace metals are accumulated by living organisms and play key roles in numerous physiological and biochemical processes, acting as co-factors, as electron donors or acceptors in redox reactions, and as allosteric promoters (Finney and O'Halloran, 2003). However, these same metals can provoke toxicity when their concentrations exceed the metabolic requirements and storage capacity of living cells. Non-essential metals that enter the intracellular environment are subject to similar complexation reactions, but their involvement in these reactions is likely to lead to deleterious effects (Mason and Jenkins, 1995). Metal toxicity is assumed to occur when the intracellular concentration of a metal exceeds a threshold value, such that the metal binds to inappropriate, physiologically important sites such as biomolecules (small peptides, enzymes, DNA or RNA) or organelles (mitochondria, endoplasmic reticulum and nuclei), causing impairment of biological functions. Below such threshold values, the organism is assumed to be able to handle metals by controlling their rates of uptake or loss (Rainbow, 2002) or by sequestering them in intracellular compartments such as cytosolic metal-binding proteins, lysosomes and metal-rich granules (Marigómez et al., 2002; Mason and Jenkins, 1995; Wallace et al., 2003). In the cytosol, metallothioneins and metallothionein-like proteins are thought to be the main metal-binding molecules. These low molecular weight, heat-stable and cysteine-rich proteins bind essential and non-essential metals in most vertebrates and invertebrates (Amiard et al., 2006).

Metal resistance or tolerance has generally been attributed to the ability of an organism to preclude the binding of metals to inappropriate, physiologically important, metal-sensitive sites (Campbell et al., 2008; Mason and Jenkins, 1995). In principle, subcellular metal partitioning procedures could be used to reveal internal metal compartmentalization strategies used by organisms to cope with potentially toxic metals. One can distinguish between "metal-sensitive" fractions and "metal-detoxified" fractions in cells. To investigate how organisms can tolerate metal-contaminated environments in the field, one would need a sentinel organism that was abundant, easy to collect and identify, widespread, of adequate size, and tolerant of high ambient metal concentrations. Larvae of the phantom midge *Chaoborus* exhibit all of these properties and they have been used as sentinels for estimating biologically relevant concentrations of Cd and Ni in lakes

(Hare and Tessier, 1998; Ponton and Hare, 2009). The presence of metallothioneins or metallothionein-like proteins in *Chaoborus* larvae has been used to explain their persistence in metal-contaminated environments (Croteau et al., 2002), but little else is known about the subcellular compartmentalization of metals in the larvae.

We aimed to determine the relative distribution of Cd, Cu, Ni and Zn among various subcellular fractions in *Chaoborus* larvae living in lakes along an environmental metals gradient. To achieve this goal, we collected final-instar larvae of *Chaoborus punctipennis* from a dozen lakes located in the vicinity of metal smelters located in Rouyn-Noranda (Quebec) and Sudbury (Ontario), Canada. A subcellular partitioning procedure, using differential centrifugation, NaOH digestion and heat-denaturation steps, was then applied to separate larvae into metal-sensitive fractions (cytosolic enzymes, organelles) and detoxified metal fractions (metallothionein-like proteins, metal-rich granules). To determine how metal subcellular partitioning responded as a function of the metal bioaccumulation gradient, trace metal concentrations were measured in each subcellular fraction and in the whole insect. In addition, dissolved metal concentrations in lake water were measured to assess metal concentrations in its surroundings. Our results highlight the importance of the cytosolic heat-stable protein (HSP) fraction in metal detoxification in the intracellular environment of this insect, presumably allowing the larvae to cope with elevated concentrations of bioavailable trace metals in the environment.

Materials and methods

Study areas and sampling protocol

The 12 Canadian Precambrian Shield lakes we chose for this study were contaminated to various degrees by historical emissions from two metal smelters (Table 1), emissions that have been largely curtailed since the 1980s. Lakes near both smelters are contaminated with Cd, Cu and Zn (Banic et al., 2006; Perceval et al., 2006). Ni is also a metal of concern in the Sudbury area (Borgmann et al., 1998). From each of the 12 lakes we collected larvae of the insect *Chaoborus* (Diptera; Chaoboridae) in late May and early June of 2009 and 2010 (Table 1). Water samples were collected concurrently, with the exception of lakes Crowley and Silver for which we used aqueous metal concentrations measured in 2007 (Ponton and Hare, 2009). In each lake, larvae and water were collected

at a single station with the exception of Lake Dasserat in which two widely separated sampling sites were treated as individual "lakes". One station (DSII) was situated in a bay (Baie Arnoux) receiving acid mine drainage (Baie Arnoux) and the other (DSI) was located at the opposite (northwest) end of the lake (Goulet and Couillard, 2009).

Collection of water samples

In each lake, triplicate water samples were collected in the epilimnion, where *Chaoborus* larvae feed nocturnally, using *in situ* Plexiglas diffusion samplers (216 mm x 72 mm x 12 mm). These samplers contain eight 4-mL compartments separated from lake water by a 0.2 µm nominal pore-size polysulfone membrane (Gelman HT-200) (Croteau et al., 1998). Ultrapure water (Milli-Q system water; >18 Mohm cm) was used to fill the diffusion samplers and they were then sealed individually in clean plastic bags prior to their transport into the field. Water samplers were suspended about 1 m below the lake surface and left to equilibrate with the surrounding water for 3 days and then recovered. Details of the protocol used to collect water subsamples from the diffusion samplers are described in Ponton and Hare (2009). Subsamples of 4-mL, 1-mL, 3-mL and 4-mL were collected from the water samplers for the determination of dissolved organic carbon (DOC), dissolved inorganic carbon (DIC), pH and anions (Cl, NO_3 and SO_4), respectively. The remaining water was used for the analysis of metals (Cd, Cu, Ni and Zn) and for other cations (Al, Ca, Fe, K, Mg, Mn and Na). The collected samples were refrigerated until analysis (the samples for analysis of DOC, DIC, trace metals and other cations were all acidified before refrigeration).

Dissolved inorganic carbon was measured by gas chromatography (CombiPal injection and CP-Porabond U column, 3800 Varian, Mississauga, Ontario, Canada) and DOC was measured by combustion and transformation into CO_2 (TOC-VCPH, Shimadzu, Columbia, MD, US). The anions were determined by ion chromatography (AS-18 column, System ICS-2000, Dionex, Bannockburn, IL, US) and major cations were measured using an inductively coupled plasma-atomic emission spectrometer (ICP-AES, Vista AX CCD, Varian, Mississauga, Ontario, Canada). Total dissolved metal concentrations were measured by inductively coupled plasma-mass spectrometry (ICP-MS; Thermo Elemental X Series, Winsford, England, United Kingdom). For all water

quality measurements, blanks and appropriate standard reference materials were analyzed in accordance with INRS-ETE quality assurance/quality control protocols.

Collection of Chaoborus larvae

Final (fourth) instar larvae of *Chaoborus* were collected after sunset from the water column using a plankton net with a mesh-aperture of 164 µm. Larval samples were held in lake water at field temperatures and transported to the laboratory for sorting according to species (Saether, 1972). The widespread species *Chaoborus punctipennis* was collected from all lakes. Two species, *C. punctipennis* and *Chaoborus albatus*, were present in samples from Lake Hannah and were combined; since these species belong to the same subgenus (*Sayomyia)* (Saether, 1972), they are very similar in terms of their size and morphology as well as in their accumulation of trace metals (Croteau et al., 2001; Hare and Tessier, 1998; Ponton and Hare, 2009). Two series of larval samples were prepared, one for metal analyses and the other to determine metal subcellular partitioning. In the first series, three pooled samples of 10-30 individuals were placed separately on pieces of pre-weighed, acid-washed Teflon sheeting held in 1.5-mL polypropylene microcentrifuge tubes (Fisher Scientific, Whitby, Ontario, Canada) and then frozen at -20 °C until analysis. In the second series, five replicate samples of ~30 pooled larvae were prepared for each study lake. These samples were kept in acid-washed and pre-weighed 1.5-mL polypropylene microcentrifuge tubes until analysis.

Metal subcellular partitioning procedure

A homogenization and differential centrifugation procedure was used to separate each larval sample into six operationally-defined subcellular fractions (Fig. 1). We used a subcellular fractionation procedure adapted from protocols described by Wallace et al. (2003), Giguère et al. (2006) and Lapointe et al. (2009). In preliminary experiments, the fractionation procedure was optimized for *Chaoborus* larvae. Various parameters of the homogenization step (e.g., duration and intensity of the homogenization step; tissue to buffer ratios) were adjusted so as to minimize both the amount of cellular debris and damage to cell organelles (M. Rosabal, INRS-ETE, unpublished results).

Each composite larval sample was placed on ice until partially thawed and then homogenized in 25 mmol L^{-1} Tris-buffer (OmniPur, EM Science, affiliate of MERCK

KGaA, Darmstadt, Hesse, Germany) (pH = 7.4) at a ratio of 1:4 (insect weight : buffer volume) using a Pellet Pestle (Kontes, Vineland, NJ, USA) for 2 s at 30 s intervals for 5 min (Lapointe et al., 2009). The larval homogenates were subjected to centrifugation twice at 800 × g to reduce the presence of cellular debris (mainly unbroken cells, cell fragments and cell membranes) in the nuclei/debris fraction (S2). After the first centrifugation step, the supernatant was collected and the pellet was diluted again with the same Tris buffer (1:2 ratio), vigorously mixed (vortex, 40 s) and recentrifuged at 800 × g. Finally, the two supernatants were combined (S1) and transferred to a new pre-weighed and acid-washed 1.5-mL polypropylene microtube to be subjected to the differential centrifugation and heat-treatment procedure.

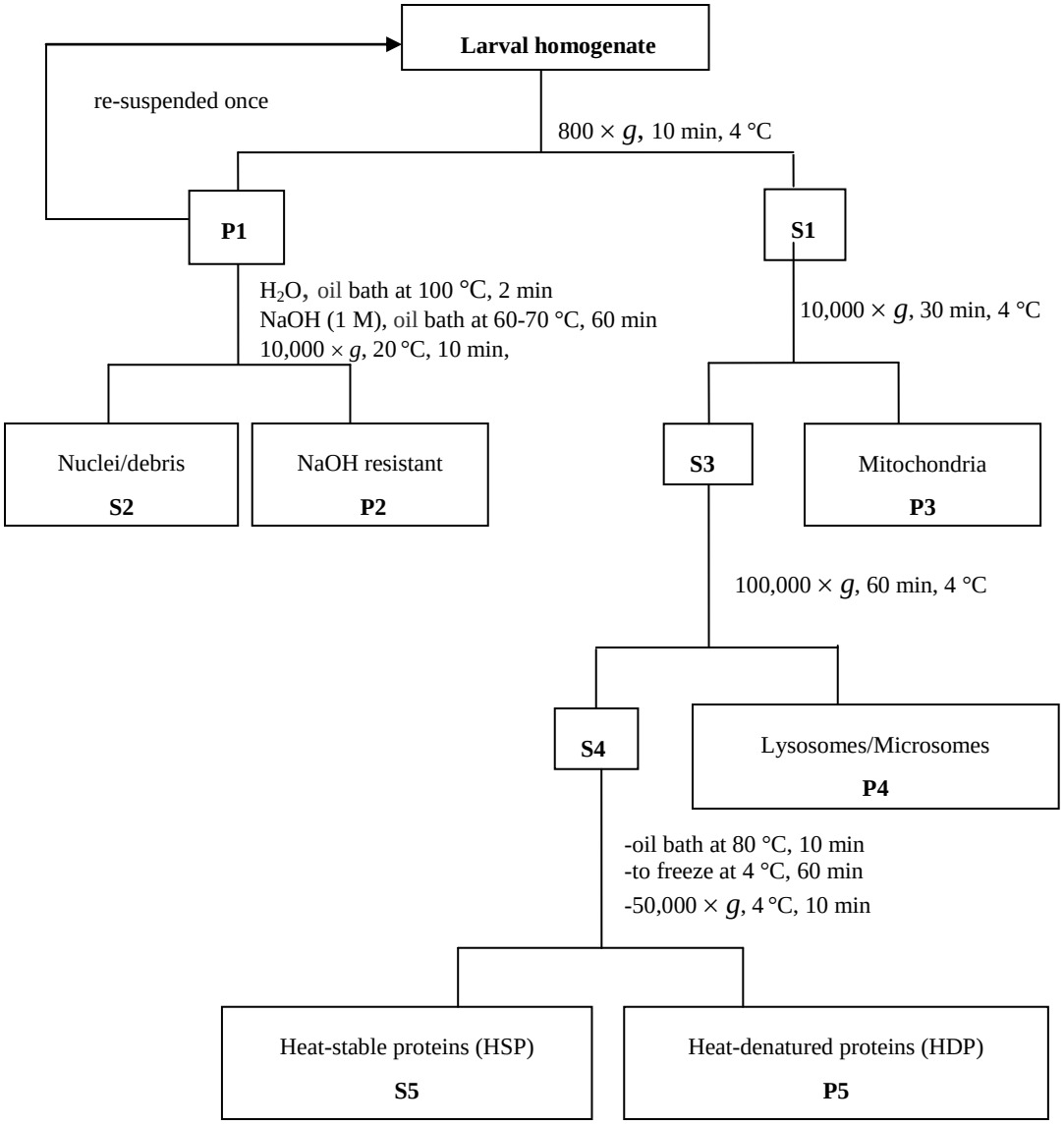

Fig. 1. Subcellular partitioning procedure used to separate larvae of *Chaoborus* into subcellular fractions.

Each final pellet (Fig. 1: P1) was first suspended in 500 µL of ultrapure water and heated for 2 min at 100 °C on a hot plate (Cole-Parmer, Ottawa, Ontario, Canada). Sodium hydroxide solution (500 µL, 1 N NaOH; 99.998%, Sigma-Aldrich, Oakville, Ontario, Canada) was added and the mixture was heated at ~65 °C for 60 min followed by centrifugation of 10,000 × g for 10 min at ambient temperature (~20 °C) to separate the NaOH resistant fraction (also referred to as granule-like; P2) from the supernatant comprising the nuclei/debris fraction (S2). In the fractionation protocol, the exoskeleton

would be expected to report first to pellet P1, which is then subjected to the NaOH digestion step at 65 °C. Given the high chitin content of the exoskeleton, and the known solubility of chitin in hot alkaline solution (Pillai et al., 2009), it is highly likely that the exoskeleton is dissolved in the NaOH digestion step and thus is found in the nuclei/debris fraction. Other workers have reached similar conclusions (Cain et al., 2006; Martin et al., 2007).

The collected supernatant (S1) was recentrifuged at 10,000 × g for 30 min at 4 °C to isolate the mitochondrial fraction (P3). The remaining supernatant (S3) was subjected to an ultracentrifugation step at 100,000 × g for 60 min at 4 °C in order to separate the lysosomes/microsomes (P4) from the cytosol (S4). To separate the HSP fraction (S5) from the HDP fraction (P5), the cytosol (S4) was heated at 80 °C for 10 min, cooled on ice for 1 h and subjected to a final centrifugation at 50,000 × g for 10 min at 4 °C. The heat-stable proteins, including MT and MTLP, remained in the final supernatant. The 50,000 × g and 100,000 × g centrifugations were carried out using a Beckman TLA-100 centrifuge using a TLA-100.3 rotor (Beckman Counter, Mississauga, Ontario, Canada), and for the other centrifugations, an IEC Micromax centrifuge (Thermo IEC, Arlington, MA, USA) was used. The volume of each sample of supernatant was estimated and then each supernatant was acidified with 2% nitric acid (Optima grade, Fisher Scientific, Whitby, Ontario, Canada) and kept at 4 °C until metal analysis. Pellets were frozen at -80 °C until lyophilisation and metal analysis.

Metal determinations and quality control.

To minimize accidental metal contamination, the polypropylene microcentrifuge tubes, the Teflon sheeting and all other lab-ware were soaked in 15% nitric acid and rinsed in ultrapure water prior to use. Samples for total larval metal analysis and the centrifugation pellets obtained in the subcellular fractionation procedure were freeze-dried (72 h; FTS Systems TMM, Kinetics Thermal Systems, Longueil, Quebec, Canada) and weighed (XS205 Dual Range Analytical Balance, Mettler Toledo, Mississauga, Ontario, Canada). All freeze-dried samples for whole insect analyses were placed in acid-washed HDPE bottles, whereas subcellular pellet samples were kept in their original microcentrifuge tubes to limit metal loss during transfer. The first digestion was carried out using

concentrated nitric acid (Optima grade, Fisher Scientific, Whitby, Ontario, Canada) at a ratio of 100 µL per mg of sample dry weight (dw) for 2 days at room temperature. Digestions of the subcellular solid samples were then transferred to acid-washed HDPE bottles and a hydrogen peroxide digestion (Optima grade, Fisher Scientific, Whitby, Canada) was performed at a ratio of 40 µL per mg dw for 1 day. The final digested volume was completed to 1 mL per mg of dw with ultrapure water. Each supernatant sample was digested by a similar procedure, with the approximation that each 1 mL of sample represented 1 mg tissue.

Total Cd, Cu, Ni and Zn concentrations in whole insects and in all subcellular fractions were measured using an inductively coupled plasma-mass spectrometer (ICP-MS; Thermo Elemental X Series, Winsford, England, United Kingdom). Concurrently, samples of similar weight of a certified reference material (lobster hepatopancreas, TORT-2, National Research Council of Canada, NRCC, Halifax, Nova Scotia, Canada) were subjected to the same digestion procedure and analyzed. The recovery of TORT-2 reference metals (n = 4) was within the certified range for Cd (101% ± S.D of 1%), Cu (92% ± S.D of 2%), Ni (91% ± S.D of 5%) and Zn (102% ± S.D of 2%). Metal concentrations from blanks were usually below the detection limit for Cd (0.003 µg L^{-1}), Cu (0.2 µg L^{-1}) and Ni (0.04 µg L^{-1}). The Zn concentration values for blanks were higher than the Zn detection limit (0.2 µg L^{-1}) but were negligible in comparison to the relatively high concentrations measured in the digested samples (96-163 µg L^{-1}). A 90-µL aliquot was collected from the first homogenization step in each sample and analyzed in order to verify the recovery of metals in the various subcellular fractions by a mass balance calculation. Mean values (± SD) of metal mass balance recoveries, considered as the ratio of sum of metal burdens in the six fractions divided by the metal burden estimated from the 90-µL aliquot and then multiplied by 100, were close to 100% for all studied metals: Cd, 101± 5%; Cu, 96 ± 6%; Ni, 121± 9%; and Zn 118 ± 10%.

Metal speciation calculations using WHAM

Metal free ion concentrations were estimated using the Windermere Humic Aqueous Model (WHAM, model 6.0.1) (Tipping, 2002). Input parameters included pH, total dissolved concentrations of major cations (Ca, Mg, Na and K), anions (CO_3^{2-}, SO_4^{2-}, NO_3^{2-} and Cl^-), and the metals of interest (Cd, Cu, Ni and Zn) as well as the

concentrations of metals (Al and Fe) that could be competitors for binding sites on dissolved organic matter (DOM). The concentrations of humic and fulvic acids required as input parameters to WHAM were estimated from our measurements of DOC. We assumed that the ratio of DOM:DOC was 2:1 (Buffle, 1988) and that, on average, 60% of DOM was composed of humic and fulvic acids in a ratio of 1:3 (Ritchie and Perdue, 2003). The original database of complexation constants provided with WHAM 6.0.1 for inorganic complexes was compared with a reliable source of thermodynamic data (Martell and Smith, 2004) and updated (if necessary).

Calculations and statistical analyses.

The relative contribution of each subcellular fraction to the total metal burden was estimated as a ratio defined by the metal burden in a given fraction divided by the sum of metal burdens in all fractions, multiplied by 100 to give results in terms of percentages (%). Metal concentrations in all subcellular fractions were expressed as total metal burden (nmol) divided by the total larval dry weight (g, dw). The determination of the wet weight : dry weight ratio was performed using five samples comprising ~30 larvae of *Chaoborus*.

All numerical data are represented by means ± standard deviations (SD). Relationships among environmental variables (i.e., aqueous metal concentrations, metal concentrations in the whole insect or in a given subcellular fraction) were initially examined in bivariate scatterplots and tested by simple correlation (Pearson r) after checking the assumption of normality (Shapiro-Wilk test). When normality was not respected, Box-Cox data transformation (Peltier et al., 1998) was applied with the exception of percentage data (relative contribution of each subcellular fraction to the total metal burden) which were arcsine transformed. If non-normality still persisted, a non-parametric correlation was reported (Spearman r). When bivariate plots indicated a possible linear relationship, simple regression models were tested using the ordinary least-squares equation when the necessary assumptions (normality and homoscedasticity of residuals) were satisfied. The Shapiro-Wilk test was used to verify the normality of distributions of the regression residuals and the Levene test was applied for assessing the equality of variances of the residuals. Extra sum-of-square F-tests were used to compare the slopes obtained from

linear regression analysis whenever the requirement of equality of residual variances was satisfied.

Differences in larval metal concentrations among lakes were assessed by one-way analysis of variance (ANOVA). Since the Levene test showed a significant heterogeneity of variance, a non-parametric Kruskal-Wallis test was applied, followed by a Tukey honestly significant difference (HSD) test, which was performed on ranks to discriminate among lakes. Statistical analyses were performed using STATISTICA version 6.1 software (StatSoft, Tulsa, Oklahoma, USA) and a probability of $P \leq 0.05$ was considered significant.

Results

Metal concentrations in lake water

As anticipated, water chemistry varied greatly among the lakes, indicating the existence of a strong spatial gradient (Table 1). Ratios of maximum to minimum values for trace metals ($[M]_{max}$ / $[M]_{min}$) were higher for Zn and Ni than for Cd and Cu; the highest total dissolved Zn and Ni concentrations were 110-fold and 123-fold higher than those obtained from the less-impacted lakes. Lakes Dufault, Crooked, Silver and Crowley were the most metal-contaminated lakes, whereas Lake Opasatica generally had the lowest metal concentrations. Aqueous Ni concentrations were much lower in lakes from the Rouyn-Noranda area than in those from the Sudbury region (Table 1), a result that reflects the proximity of the latter to two nickel smelters. Gradients in metal concentrations were even more pronounced when free-metal ion concentrations are considered (Table 1). The very high gradient calculated for Cu^{2+} concentrations is strongly influenced by the low Cu^{2+} values calculated for lakes Opasatica and Osisko.

Metal accumulation in Chaoborus

The gradients measured in aqueous metal concentrations were paralleled by marked differences among lakes in total metal concentrations in *Chaoborus* larvae (Fig. 2). Higher maximum to minimum values were calculated for Ni and Cd than for Cu and Zn. The highest larval Cu and Zn concentrations were only 2-3 times higher than the lowest concentrations of these essential metals. However, consistent with the potential use of this insect as a biomonitor (Hare and Tessier, 1998; Ponton and Hare, 2009), the

maximum Cd and Ni concentrations were 29-fold and 89-fold higher, respectively, than the minimum values for these metals. Total Ni concentrations in *Chaoborus* were markedly higher in the Sudbury lakes than in the Rouyn-Noranda lakes, as was the case for dissolved Ni concentrations. Larvae from lakes Marlon, Crooked, Crowley and Dasserat-II exhibited the highest values for total Cd, Cu, Ni and Zn concentrations, respectively, whereas metal concentrations in larvae from Lake Opasatica were consistently low, as was the case for the aqueous metal concentrations.

Table 1. Location, sampling date and chemical composition (pH, dissolved organic carbon (DOC) and dissolved metal concentrations and free-ion metal concentrations) of water collected in the epilimnion of our study lakes.

Lake (code)	Location	Sampling date	pH	[DOC] $mg.L^{-1}$	[Cd] (nM)	[Cu] (nM)	[Ni] (nM)	[Zn] (nM)	$[Cd^{2+}]$ (nM)	$[Cu^{2+}]$ (nM)	$[Ni^{2+}]$ (nM)	$[Zn^{2+}]$ (nM)
Rouyn-Noranda, Quebec												
Daserrat-I (DSI)	$48^0$14'N, $79^0$23'W	2010	7.6	5.1	1.8	86	15	400	0.50	0.034	7.6	93
Daserrat-II (DSII)	48^0 16'N, $79^0$23'W	2010	7.8	5.9	0.92	97	15	500	0.35	0.022	7.7	110
Dufault (DU)	48^0 20'N, $79^0$07'W	2010	7.7	3.8	3.3	160	14	714	1.6	1.1	8.7	240
Marlon (MA)	48^0 15'N, $79^0$03'W	2010	7.7	7.8	1.4	180	13	53	0.28	0.053	5.5	9.1
Opasatica (OP)	48^0 03'N, $79^0$16'W	2010	6.7	6.7	0.16	45	19	7.9	0.02	0.0015	6.4	0.7
Osisko (OS)	48^0 14'N, $79^0$00'W	2010	8.5	2.3	0.48	53	33	6.5	0.17	0.0016	16	10
Sudbury, Ontario												
Crooked (CK)	$46^0$25'N, $81^0$02'W	2010	6.7	4.2	1.1	350	1500	100	0.54	2.2	1000	46
Crowley (CW)	$46^0$23'N, $80^0$59'W	2007	6.3	3.2	0.91	130	870	120	0.41	1.3	540	29
Hannah (HA)	$46^0$26'N, $81^0$02'W	2010	7.9	3.5	0.47	200	1100	6.8	0.18	0.028	650	1.9
Lohi (LO)	$46^0$23'N, $81^0$02'W	2009	6.7	3.6	0.77	110	780	110	0.4	1.7	540	38
Silver (SL)	$46^0$22'N, $81^0$03'W	2007	5.9	2.7	2.2	160	1600	240	1.2	1.4	1100	130
Swan (SW)	$46^0$21'N, $81^0$03'W	2009	5.9	2.1	1.9	90	1100	110	0.95	6.6	800	51
Ratio Max/Min			400	3.7	21	7.8	123	110	80	4400	200	342

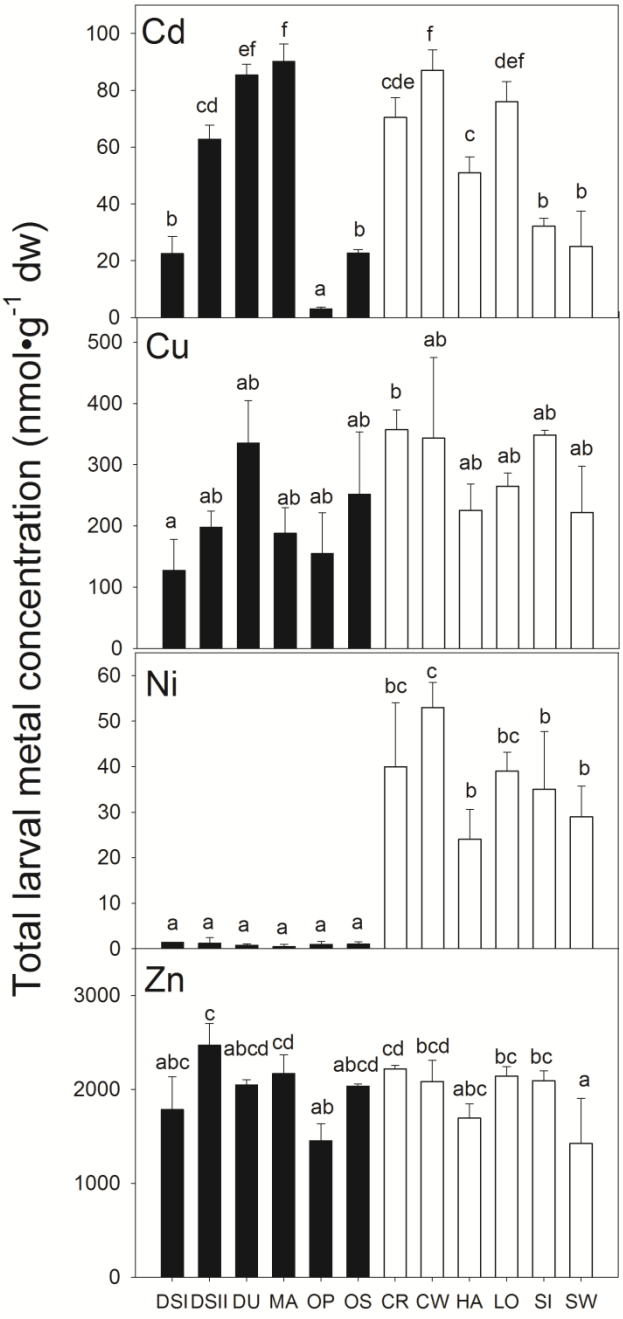

Fig. 2. Total Cd, Cu, Ni and Zn concentrations in larvae of *Chaoborus* collected from 12 lakes (means ± SD, n = 5). Bars with the same letter indicate no significant differences among lakes, whereas different letters indicate that the differences are significant (ANOVA followed by Tukey test, $P < 0.05$). Lakes from the Rouyn-Noranda area are represented by black bars, whereas those from the Sudbury area are represented by white bars. See Table 1 for the two-letter lake codes.

In only one of the 12 lakes (Lake Hannah) did we collect a *Chaoborus* species other than *C. punctipennis.* Our samples from this lake were a mixture of *C. punctipennis* and the

very closely related *C. albatus* (from the same subgenus). We chose to include Lake Hannah in our dataset because all of our past studies on *Chaoborus* indicate that *C. punctipennis* and *C. albatus* respond similarly to changes in ambient Cd concentrations. For example, Croteau et al. (2001) compiled data from four lakes where the two species co-existed and reported that their accumulated Cd concentrations were virtually identical. In the same paper, the biodynamic constants for Cd uptake and depuration were determined in the laboratory and shown to be very similar for the two species. To verify that the inclusion of the Lake Hannah data was not biasing our interpretations, we re-ran our statistical analyses with only 11 lakes (i.e., without Lake Hannah). The results were indistinguishable from those obtained using the original 12 lake data set.

Metal subcellular partitioning

To identify what proportion of the total cellular metal burden was found in each subcellular fraction, a ratio describing the relative contribution of each fraction (Y-axis) was plotted against total larval metal concentrations (X-axis) ("C" panels in Figs. 3-6). To describe how individual subcellular fractions tend to behave along the metal bioaccumulation gradients, metal concentrations in each fraction (calculated with reference to total larval dry weight) were plotted against metal concentrations in the entire insect ("A" panels for putative metal-sensitive fractions and "B" panels for metal-detoxified fractions; see Figs. 3-6) (Wallace et al., 2003). Since the nuclei/debris fraction is not readily categorized into one or the other of these two categories, we have not included it in panels "A" or "B". The relative contribution of this fraction for each metal is presented in the "C" panels, as an indication of the efficacy of the initial tissue homogenization procedure.

Cadmium

The majority of the larval Cd was found in the HSP fraction (average for all lakes of 59 ± 7%; Fig. 3C) and the percentage in this fraction was directly related to total larval Cd concentrations (r^2 = 0.60; $P < 0.001$). Along the metal bioaccumulation gradient, the contribution of the HDP fraction decreased significantly from 27% to 7% (r^2 = 0.81; $P < 0.001$), concurrent with the proportional increase in the HSP fraction.

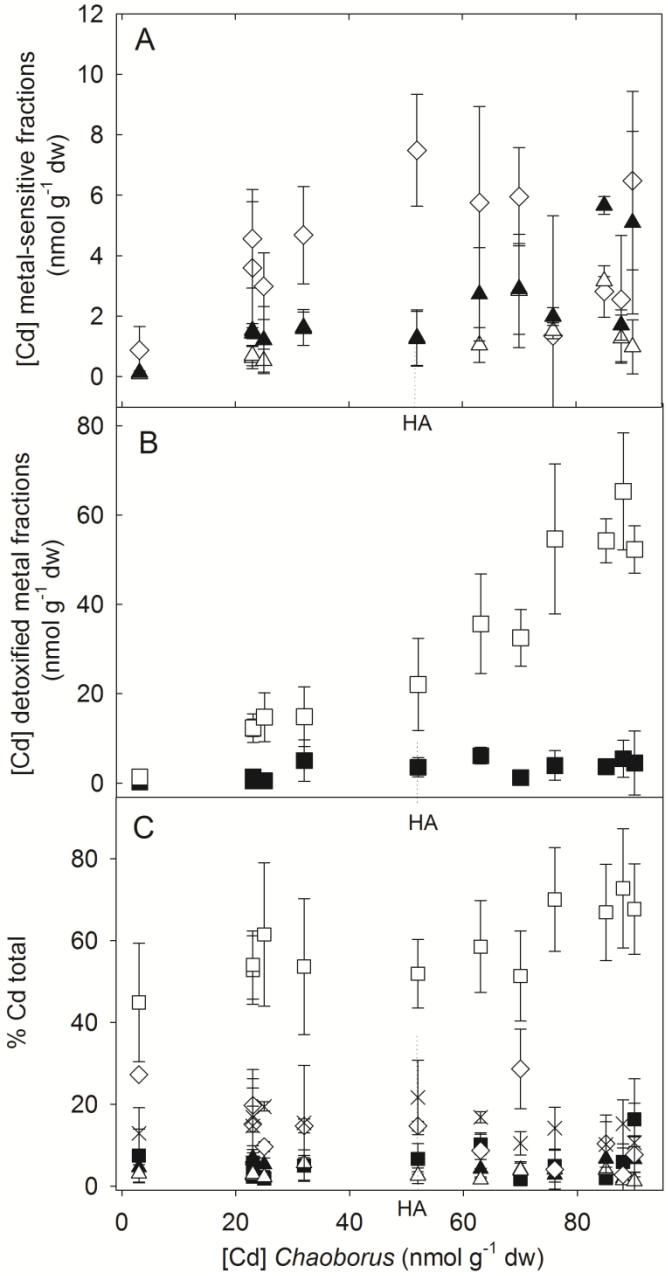

Fig. 3. Relationship between total larval Cd concentrations (horizontal axis) and Cd subcellular partitioning in larvae of *Chaoborus* for potentially metal-sensitive fractions (panel A) and for detoxified metal fractions (panel B). The relative contribution of each subcellular fraction (%) to the total Cd burden is shown in panel C. Each point represents data for a single lake (mean ± SD, n = 5). The Lake Hannah data are indicated by HA. Symbol definitions: □-HSP, ■-NaOH-resistant fraction, X-nuclei/debris, ◇-HDP, Δ-lysosomes/microsomes, ▲-mitochondria.

Cadmium concentrations in all of the subcellular fractions tended to increase as total Cd concentrations in the whole insect increased (Fig. 3A and B). With regards to the metal-

detoxified fractions (Fig. 3B), the response of the HSP fraction (slope = 0.66 ± 0.06; $P < 0.001$) was significantly stronger ($P < 0.001$) than that of the "granule-like" fraction (slope = 0.05 ± 0.01; $P < 0.001$). In the metal-sensitive fractions (Fig. 3A), significant increases were observed for the mitochondria (slope = 0.03 ± 0.01; $P < 0.005$) and microsomes/lysosomes fractions (slope = 0.02 ± 0.01; $P < 0.01$). However, highest concentrations were observed in the HDP fractions which tended to increase up to a total larval concentration of about 50 nmol g^{-1} and then remain reasonably constant (Fig. 3A).

Nickel

Total larval Ni concentrations were much lower in larvae collected in lakes from Rouyn-Noranda area than in those collected in the Sudbury lakes (Fig. 2). For larvae from the first group, the HSP (mean value = 29 ± 5%; n = 6) and the granule-like (mean value = 33 ± 7%; n = 6) fractions dominated the subcellular distribution of nickel (Fig. 4C). In larvae from the more contaminated Sudbury lakes, the HSP fraction increased with increasing larval Ni concentrations (slope = 0.9 ± 0.3; $P = 0.04$) but the opposite was the case for the granule-like fraction (slope = – 0.8 ± 0.2; $P < 0.01$) (Fig. 4C). In the metal-sensitive fractions (Fig. 4A), there was a step change between the Rouyn-Noranda and the Sudbury lakes, but no significant trend was observed along either regional bioaccumulation gradient, as was also the case for the granule-like fraction (Fig. 4B). In contrast, Ni concentrations in the HSP fraction were significantly related (r = 0.59; $P < 0.0001$) to total larval Ni concentrations (Fig. 4B).

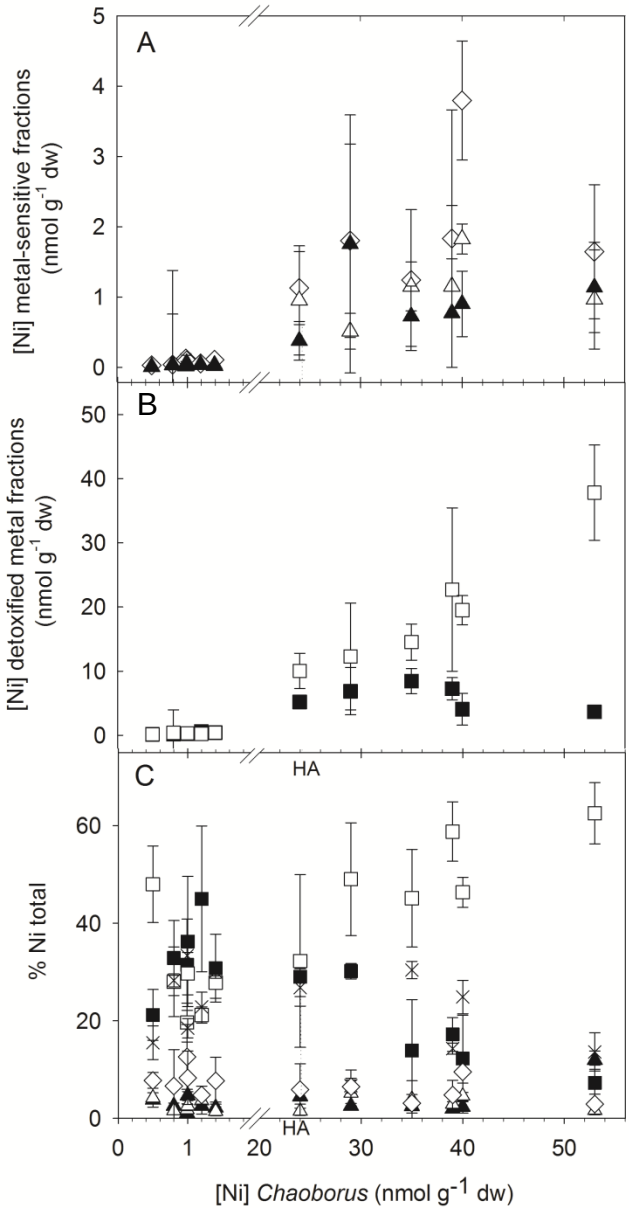

Fig. 4. Relationship between total larval Ni concentrations (horizontal axis) and Ni subcellular partitioning in larvae of *Chaoborus* for potentially metal-sensitive fractions (panel A) and for detoxified metal fractions (panel B). The relative contribution of each subcellular fraction (%) to the total Ni burden is shown in panel C. Each point represents data for a single lake (mean ± SD, n = 5). The Lake Hannah data are indicated by HA. Symbol definitions: □-HSP, ■-NaOH-resistant fraction, X-nuclei/debris, ◇-HDP, Δ-lysosomes/microsomes, ▲-mitochondria.

Copper

Unlike the case for previous two metals, the relative proportions of the different subcellular fractions remained more or less constant as Cu concentrations in whole larvae increased (Fig. 5C).

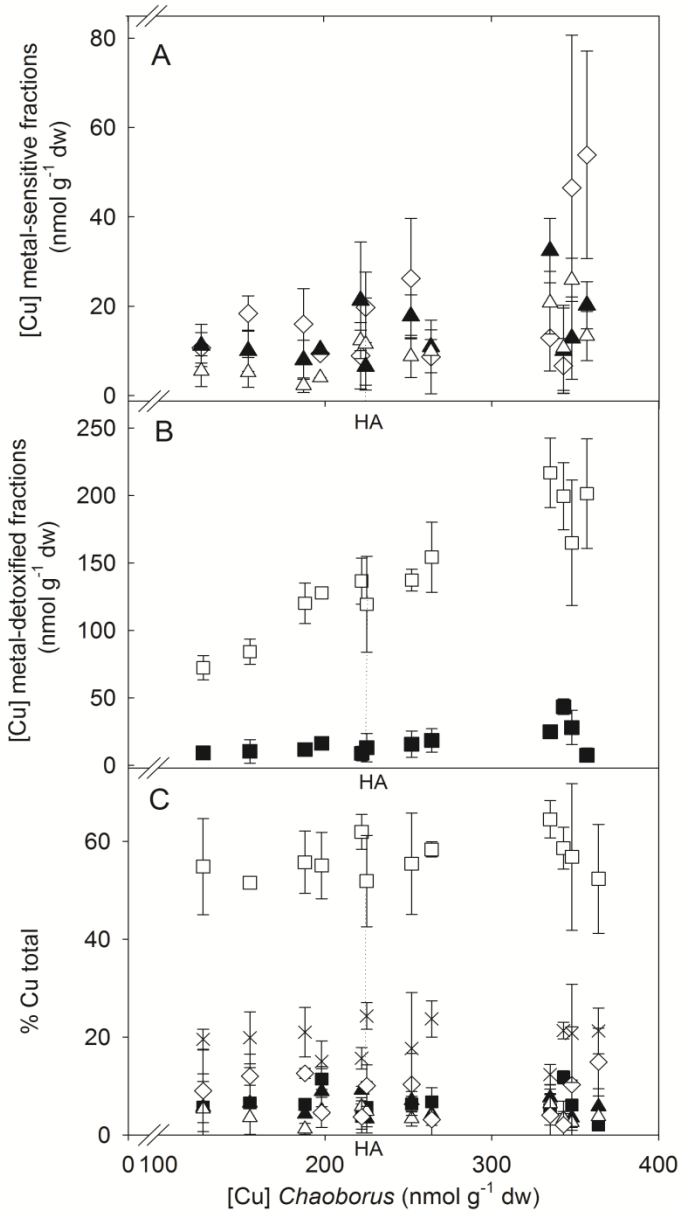

Fig. 5. Relationship between total larval Cu concentrations (horizontal axis) and Cu subcellular partitioning in larvae of *Chaoborus* for potentially metal-sensitive fractions (panel A) and for detoxified metal fractions (panel B). The relative contribution of each subcellular fraction (%) to the total Cu burden is shown in panel C. Each point represents data for a single lake (mean ± SD, n = 5). The Lake Hannah data are indicated by HA. Symbol definitions: □-HSP, ■-NaOH-resistant fraction, X-nuclei/debris, ◇-HDP, Δ-lysosomes/microsomes, ▲-mitochondria.

The HSP fraction again dominated among the subcellular fractions, with a mean contribution of 56 ± 3%. Copper concentrations in the various subcellular fractions showed the lowest ratio of maximum to minimum values among the four metals. The relative importance of the various fractions decreased in the order HSP > HDP ~ granule-like > lysosomes/microsomes ~ mitochondria. With regards to Cu concentrations,

significant increases were found in the lysosomes/microsomes (slope: 0.06 ± 0.02; $P < 0.004$) and in the HSP fractions (slope: 0.53 ± 0.06; $P < 0.0001$) (Fig. 5A-B) along the bioaccumulation gradient. In contrast to the observations for Cd and Ni, the metal-sensitive fractions showed no tendency to level off as total larval Cu concentrations increased (Fig. 5A).

Zinc

Total larval Zn concentrations varied little along the exposure gradient (by a factor of < 2). Not surprisingly, the relative contributions of the various subcellular fractions showed no trends along the limited Zn bioaccumulation gradient (Fig. 6C). However, the relative proportions were quite variable. For example, the contribution of the HSP fraction, which again was quantitatively dominant, varied from 22 to 50% (mean 34 ± 7%), a situation that contrasts markedly with that for Cu (Fig. 5C). The different behaviour of Zn is also seen in Fig. 6B, where the concentration of Zn in the HSP fraction did not increase along the bioaccumulation gradient, unlike the case for the other three metals. Among the metal-sensitive fractions, only in the mitochondria did the Zn concentration increase (slightly) along the gradient ($r^2 = 0.38$; $P = 0.03$) (Fig. 6A).

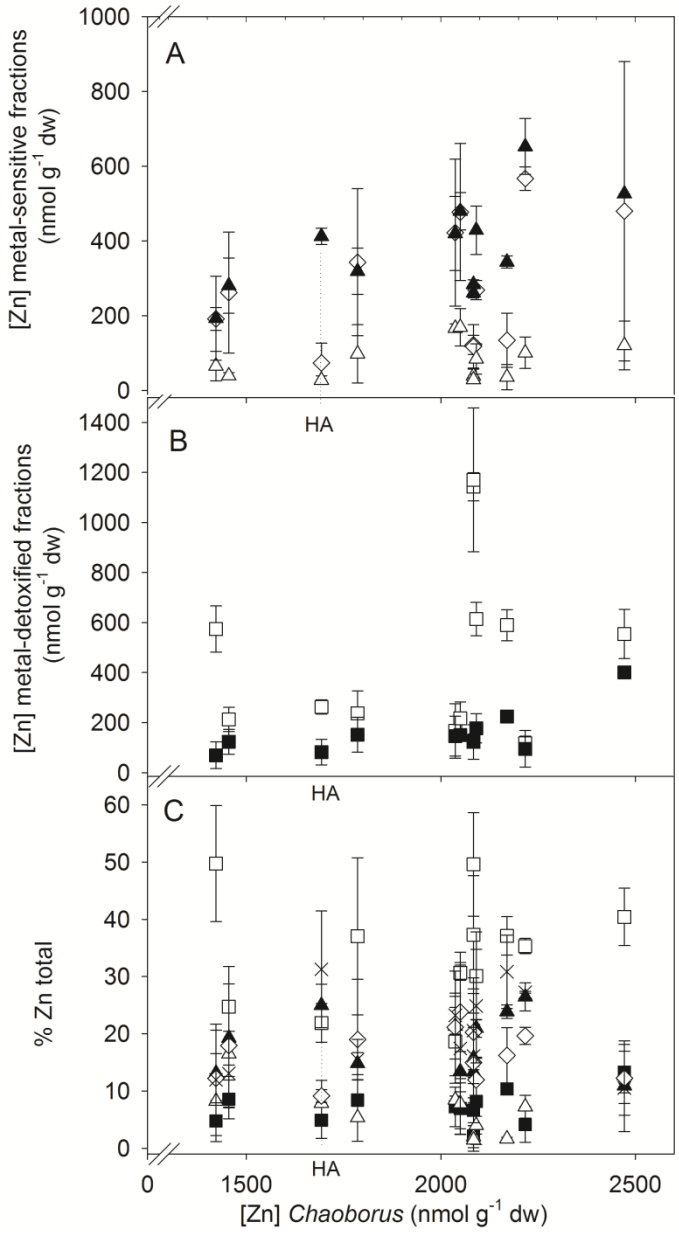

Fig. 6. Relationship between total larval Zn concentrations (horizontal axis) and Zn subcellular partitioning in larvae of *Chaoborus* for potentially metal-sensitive fractions (panel A) and for detoxified metal fractions (panel B). The relative contribution of each subcellular fraction (%) to the total Zn burden is shown in panel C. Each point represents data for a single lake (mean ± SD, n = 5). The Lake Hannah data are indicated by HA. Symbol definitions: □-HSP, ■-NaOH-resistant fraction, X-nuclei/debris, ◇-HDP, Δ-lysosomes/microsomes, ▲-mitochondria.

Discussion

Relevance of the observed dissolved metal exposure gradients

Studies concerning the relative importance of food and water as metal sources for *Chaoborus* larvae have revealed that Cd is taken up from food alone (Munger et al., 1999) and that food is likely the dominant uptake route for Ni (Ponton and Hare, 2010). Despite this fact, metal concentrations in this insect are consistently related to the free metal ion concentrations of these metals in lake water (Cd: Hare and Tessier (1998); Ni: Ponton and Hare (2009)), which suggests that these relationships are indirect, i.e. via the planktonic food chain on which this insect depends. Thus presenting trace metal concentrations in lake water is useful for comparison with those in *Chaoborus* larvae, as well as for demonstrating the extent of the environmental trace metal gradient to which the larvae of *Chaoborus* were chronically exposed.

If the importance of food as a vector for metal uptake applies not only to Cd and Ni, but also to the Cu and Zn, the apparent "regulation" of the concentrations of these essential metals may be occurring lower in the planktonic food chain, in the prey items ingested by *Chaoborus*, rather than in the predator itself.

When comparing metal-handling strategies in *Chaoborus* with those observed in its co-habitants in the Rouyn-Noranda lakes (see below), it is worthwhile noting that *Chaoborus* can respire anaerobically over long periods of time and can live in anoxic environments that are likely hostile in other chemical ways (e.g., high concentrations of H_2S). Having a relatively impermeable epidermis presumably confers certain advantages, compared to its co-habitants.

Subcellular metal partitioning

Subcellular partitioning protocols that employ differential centrifugation to separate various subcellular fractions, such as that shown in Fig.1, are inherently sensitive to potential artifacts such as the breakage or clumping of particles, the aggregation of organelles, the leakage of soluble constituents from organelles and overlap among subcellular fractions (De Duve, 1975). The presence of such artifacts in protocols based on tissue homogenization followed by differential centrifugation and NaOH-digestion steps can complicate the interpretation of the metal partitioning results. As other authors

have suggested (Giguère et al., 2006; Wallace et al., 2003), the separation and designation of each fraction as "NaOH-resistant", "mitochondria", "lysosomes/microsomes", etc., should be considered with caution. Similar to this operational classification, the grouping of fractions into "potentially metal-sensitive" (HDP, mitochondria, lysosomes/microsomes) and "detoxified-metal" (NaOH-resistant and HSP) categories (Wallace et al., 2003) is likely an oversimplification.

It should also be noted that the toxicological significance of metal accumulation in the "lysosomes/microsomes" fraction is inherently difficult to interpret, given the potential presence of organelles having different roles. If lysosomes dominate this fraction, then an increase in metal accumulation in this operationally defined fraction would suggest metal storage pending possible eventual elimination. On the other hand, if metals detected in this fraction were mostly in microsomes, then this accumulation could be indicative of metal binding to metal-sensitive sites (Giguère et al., 2006). As mentioned earlier, a similar ambiguity exists for the "nuclei/debris" fraction – some workers have grouped this fraction into the detoxified-metal compartment together with NaOH-resistant fraction, but others have chosen not to interpret the metal concentration in this fraction (e.g., Cain et al. (2004)) . Since its relative importance as a subcellular metal compartment will vary as a function of the efficacy of the homogenization step, the nuclei/debris fraction will not be considered in the following discussion.

Despite the caveats described above, protocols for determining subcellular metal partitioning do allow us to evaluate the relative contributions of a range of subcellular fractions to metal accumulation and to assess their toxicological significance.

Cd partitioning

The majority of Cd accumulated in larvae of *Chaoborus* was associated with the HSP fraction, which includes metallothioneins (MT) and metallothionein-like proteins. It is widely accepted that these cysteine-rich, low-molecular-weight proteins play an important role in metal sequestration and detoxification in aquatic and terrestrial invertebrates (Amiard et al., 2006; Dallinger, 1996; Mason and Jenkins, 1995). The significant increase in Cd concentrations in the HSP fraction as larval Cd concentrations increased, and the increase in the relative contribution of the $[Cd]_{HSP}$ fraction, suggest Cd

detoxification by MT and metallothionein-like proteins. Although we did not measure MT concentrations in the present study, this interpretation is consistent with the results reported by Croteau et al. (2002) in which metallothionein-like proteins, as measured by a Hg-saturation assay, were found to increase in several species of indigenous *Chaoborus* collected along environmental metal gradients. A strong relationship was also reported between the concentration of these MT-like proteins and the Cd concentration in the whole insect (Croteau et al., 2002), suggesting that metallothionein induction contributed to the presence of *Chaoborus* in highly metal-contaminated environments, as has been suggested for other organisms (Amiard et al., 2006).

In contrast to other aquatic organisms in which Cd tends to be mainly sequestrated in the granule-like fractions (Bonneris et al., 2005a; Bustamante et al., 2002; Goto and Wallace, 2010; Ng and Wang, 2005), larvae of *Chaoborus* preferentially reduce the availability of the non-essential metal Cd by binding it to metallothioneins or metallothionein-like proteins. The predominance of the HSP fraction in detoxifying Cd has been also observed in invertebrates exposed to Cd in the laboratory (Buchwalter et al., 2008; Cain et al., 2006; Dubois and Hare, 2009; Dumas and Hare, 2008; Xie and Buchwalter, 2011) and in field-collected organisms (Cain et al., 2004; Giguère et al., 2006; Martin et al., 2007; Michaud et al., 2005; Oyoo-Okoth et al., 2012).

Despite the increase in the concentration of the Cd-HSP fraction, $[Cd]_{HSP}$, increases in Cd concentrations were also observed in the metal-sensitive fractions (mitochondria and lysosomes/microsomes) along the Cd bioaccumulation gradient, without any threshold value. A similar Cd intracellular partitioning pattern was also noted in several indigenous invertebrates (Bonneris et al., 2005b; Cain et al., 2004; Voets et al., 2009) and vertebrates (Giguère et al., 2006; Goto and Wallace, 2010), suggesting that the "spillover" model is not adequate to represent Cd-handing in chronically exposed animals. The presence of Cd in these "inappropriate" fractions, as defined by Mason and Jenkins (1995), suggests that Cd detoxification is incomplete. Interestingly, the increases in Cd concentrations in two of these metal-sensitive fractions (lysosomes/microsomes and heat-denatured proteins) both seem to occur early in the bioaccumulation gradient, up to a whole-body larval concentration of about 50 nmol Cd g^{-1} dw (Lake DSII in Fig. 3A). In this same range of larval Cd concentrations, significant Cd accumulation in the HSP fraction was observed

(slope: 0.40 ± 0.06; $P < 0.003$; Fig. 3B) but this metal detoxification strategy seems not to be effective in avoiding the binding of Cd to such physiologically important sites. For larval Cd concentrations above this apparent threshold, Cd concentrations in these two fractions do not show any further tendency to increase ($P > 0.05$; Fig. 3A); above the threshold, the slope of the $[Cd]_{HSP}$ plot (slope = 1.0 ± 0.3; $P = 0.04$) is significantly higher ($P < 0.05$) than that observed for the lower range in Cd larval concentrations (< 50 nmol Cd g^{-1} dw; Fig. 3B). We speculate that these changes above the 50 nmol g^{-1} threshold reflect the induction of a more effective detoxification response. The plot of $[Cd]_{HDP}$ versus $[Cd]_{HSP}$ suggests a bell-shaped relationship (Fig. 7), in which a significant increase in $[Cd]_{HDP}$ was observed ($P < 0.01$) up to around 8 nmol Cd g^{-1} dw (Lake DSII in Fig.7) followed by a decrease as $[Cd]_{HSP}$ increased ($P = 0.04$). These results are consistent with the idea that the induced MT-like protein is better able to protect the HDP fraction under the high exposure scenario where $[Cd]_{HSP}$ is maximal.

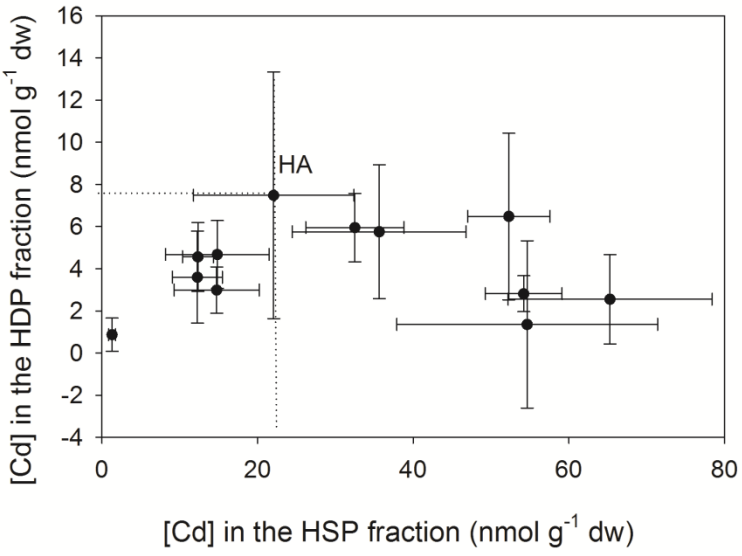

Fig. 7. Relationship between Cd concentrations in the HDP fraction and Cd concentrations in the HSP fraction in *Chaoborus* larvae. Each point represents data for a single lake (mean ± SD, n = 5). The Lake Hannah data point is indicated by HA.

In comparison to other indigenous freshwater animals, the giant floater mollusk (*Pyganodon grandis*) and the yellow perch (*Perca flavescens*), the degree of Cd

detoxification in *Chaoborus* seems to be reasonably comparable to that in the bivalve (Bonneris et al., 2005b) but somewhat more effective than in the fish (Giguère et al., 2006). To judge from the protection afforded by the HDP fraction and the Cd levels in the mitochondria fraction, our data suggest that Cd-handling strategies were more effective in the two invertebrates than in yellow perch. In both invertebrates, the HDP fractions did not exhibit a significant response as total bioaccumulated Cd increased, and only a modest increase in Cd concentrations was noticed in the mitochondria fraction. In contrast, both of these indicators, $[Cd]_{HDP}$ and $[Cd]_{mitochondria}$, increased markedly in livers of yellow perch collected along a Cd exposure gradient.

Ni partitioning

As was the case for Cd, the majority of the accumulated Ni was associated with the HSP fraction, and this was the only fraction that increased progressively along the Ni bioaccumulation gradient (Fig. 4). For all of the other fractions, there was a step-change increase in accumulated Ni concentrations between the animals collected in the Rouyn-Noranda lakes and those collected from the Sudbury lakes. However, within the Sudbury region there was no increase in $[Ni]_{HSP}$ despite the 2.3-fold increase in total larval Ni, suggesting that Ni detoxification was reasonably effective.

From a biochemical perspective, the association of Ni with the MT-like fraction is somewhat surprising, given the low affinity of Ni, a class A cation, for thiols and the apparent inability of this element to induce the biosynthesis of metallothioneins (Amiard et al., 2006). Since total larval Cd and Ni are correlated for the six Sudbury lakes (r^2 = 0.89; $P < 0.05$), we suspect that Cd induces the synthesis of MT-like proteins and that Ni then competes successfully for some of the MT binding sites. There is some precedent for the involvement of the MT-like fraction in binding subcellular Ni. Dumas and Hare (2008) exposed two aquatic invertebrates, *Tubifex tubifex* and *Chironomus riparius*, to Ni-contaminated sediments in the laboratory for two weeks and then determined the subcellular partitioning of Ni using a protocol similar to ours; most of the Ni accumulated in the HSP fraction. Although this determination of Ni partitioning was not performed on indigenous organisms, the animals were exposed to environmentally relevant Ni concentrations and under simulated natural environmental conditions. In the freshwater crustacean *Gammarus* exposed to Ni-contaminated water, the HSP fraction played an

important role in detoxifying Ni (Geffard et al., 2010). Likewise, in livers from yellow perch (*P. flavescens*) collected from lakes in the Sudbury region, there was also an increase in the concentration of Ni in the HSP fraction as the total hepatic Ni contamination increased (Giguère et al., 2006).

Nickel concentrations in the granule-like fraction were higher in *Chaoborus* larvae collected from the Sudbury lakes than in their counterparts from the Rouyn-Noranda lakes, but did not show a further increase along the Ni bioaccumulation gradient in the Sudbury lakes (i.e., the relative contribution of this fraction decreased along the bioaccumulation gradient). A much higher contribution from the NaOH-resistant fraction was reported by Vijver et al. (2006) for Ni in field-collected earthworms (*Aporrectodea caliginosa*), although it should be noted that the subcellular fractionation protocol used in this latter study differed slightly from that used here (the first centrifugation was performed at 10,000 × g rather than 800 × g).

The presence of Ni in the potentially metal-sensitive fractions (HDP; mitochondria; lysosomes/microsomes) suggests an incomplete detoxification of Ni. However, the trends along the Ni bioaccumulation gradient are far weaker than those reported for hepatic Ni in field-collected yellow perch (Giguère et al., 2006), indicating that *Chaoborus* larvae are better able to handle the incoming Ni than are yellow perch. A similar result was reported for blue oyster (*Crassostrea hongkongensis*) in which Ni accumulation in metal-sensitive fractions was negligible in comparison to the accumulation of this metal in metal-detoxified fractions (Wang et al., 2011).

Cu partitioning

Despite the wide range of aqueous Cu concentrations measured among our study lakes ($[Cu^{2+}]^{max}/[Cu^{2+}]^{min}$: 4400; $[Cu]^{max}/[Cu]^{min}$: 7.8), bioaccumulated Cu concentrations in *Chaoborus* larvae varied relatively little ($[Cu]^{max}/[Cu]^{min}$: 2.8). Along this modest Cu bioaccumulation gradient, all the individual subcellular fractions increased and their relative contributions remained unchanged (Fig. 5C). As was the case for the two preceding metals, Cd and Ni, the majority of the accumulated Cu was bound to the HSP fraction, as has been reported in fish (Giguère et al., 2006). Indeed, on a molar basis Cu was always present in this fraction at higher concentrations than the two non-essential

metals. Even at the low end of the Cu bioaccumulation gradient (e.g., in larvae from Lake Opasatica where $[Cd]_{HSP}$ was < 2 nmol g^{-1} and $[Ni]_{HSP}$ was < 0.2 nmol g^{-1}), the basal levels of $[Cu]_{HSP}$ were still > 70 nmol·g^{-1}. The persistence of $[Cu]_{HSP}$ in the uncontaminated lakes reflects the dual role of MT-like proteins, as a basal reservoir of bioavailable Cu(I) within the cytosol and as an inducible defense against the toxicity of non-essential metals such as Cd and Ni.

Croteau et al. (2002) reported a significant correlation between MT and larval Cu concentrations in indigenous *Chaoborus* collected along ambient metal gradients comparable to those studied in the present work. In contrast to Cd, however, there was no relationship between total MT concentrations and external ambient Cu, suggesting that this metal-binding protein was acting as a competitive ligand in the cytosol, sequestering part of the available Cu (Croteau et al., 2002). Other evidence for Cu sequestration by MT-like proteins in aquatic indigenous organisms has been reported for bivalves (Bonneris et al., 2005b; Couillard et al., 1995) and fish (Falfushynska and Stolyar, 2009; Giguère et al., 2006; Hogstrand et al., 1991).

Appreciable Cu bioaccumulation also occurred in the lysosomes/microsomes fraction. As mentioned in Section 4.1, the role of metals in this fraction is not easy to interpret. If Cu bound in this fraction corresponds to fragmented endoplasmic reticulum, then its presence could imply imperfect Cu regulation which could lead to effects on either protein synthesis and transport or on glycogen storage (Fowler et al., 1989). In contrast, if Cu in this fraction is mostly associated with lysosomes, then Cu storage and eventual excretion could be occurring. Considering the relationship observed between Cu accumulation in the HSP fraction and the lysosomes/ microsomes fraction ($r2 = 0.683$; $P < 0.05$), a turnover of MT in the lysosomes, as proposed by Viarengo (1989), could be suggested for Chaoborus.

Overall, we interpret these results as progressive Cu detoxification in *Chaoborus* larvae since accumulation was observed in metal-detoxified compartments. Progressive accumulation of Cu was also noted in potentially metal-sensitive fractions. Given the role of Cu as an essential element, its presence in these fractions is to be expected, but the observed increase in Cu concentrations along the metal bioaccumulation gradient does

suggest that control of internal Cu concentrations was not completely effective. Although this analysis is consistent with what has been suggested for other indigenous aquatic organisms (Bonneris et al., 2005a; Giguère et al., 2006; Goto and Wallace, 2010; Wang et al., 2011), the modest Cu bioaccumulation gradient should not be ignored when considering our Cu partitioning results.

Zinc partitioning

Although aqueous zinc concentrations varied markedly along the exposure gradient ($[Zn^{2+}]^{max}/[Zn^{2+}]^{min}$ = 342; $[Zn]^{max}/[Zn]^{min}$ = 110), Zn concentrations in *Chaoborus* increased only slightly ($[Zn]^{max}/[Zn]^{min}$ = 2). This result is not surprising since earlier field studies with several *Chaoborus* species (Croteau et al., 1998), as well as studies on other aquatic organisms collected from Rouyn-Noranda and Sudbury lakes (Bonneris et al., 2005b; Giguère et al., 2006), have consistently shown that internalized zinc concentrations are controlled within a very narrow range. One might have expected subcellular Zn fractions to be similarly "stable", but in fact several fractions proved quite variable along the bioaccumulation gradient (e.g., $[Zn]_{HSP}{}^{max}/[Zn]_{HSP}{}^{min}$ = 7; $[Zn]_{HDP}{}^{max}/[Zn]_{HDP}{}^{min}$ = 8). In half of the study lakes, Zn concentrations in the MT-like fraction were higher than in any other subcellular compartment, but unlike the other metals, $[Zn]_{HSP}$ did not increase progressively along the Zn bioaccumulation gradient (Fig. 6B). In other words, $[Zn]_{HSP}$ was quite variable along the overall metal contamination gradient, but this variability was not related to larval Zn concentrations. We speculate that the Zn in this fraction responds to the induction of MT-like proteins by Cd, but that $[Zn]_{HSP}$ is also affected by intracellular competition for the MT binding sites (both Cd and Cu are known to bind much more strongly to metallothionein than does Zn – e.g., Stillman (1995)). The only fractions that did show a progressive increase along the Zn bioaccumulation gradient were $Zn_{mitochondria}$ and, to a lesser extent, $Zn_{granule}$.

Overall, the very modest increase in total larval Zn concentrations along the Zn exposure gradient can be taken as evidence that *Chaoborus* copes rather well with the ~110-fold increase in aqueous Zn concentrations, not by sequestering the excess Zn but rather by down-regulating the uptake of Zn or by accelerating its ability to eliminate excess internalized Zn. The role of MT-like proteins in handling excess internalized Zn is not as obvious as it is for Cd, Ni and Cu, but nevertheless both $[Zn]_{mitochondria}$ and $[Zn]_{HDP}$ were

negatively correlated with $[Zn]_{HSP}$, these trends being consistent with a certain degree of protection being afforded by MT (Amiard et al., 2006). On the other hand, given the role of Zn as an essential element, its presence in these latter "metal-sensitive" fractions is hardly surprising. The high Zn concentrations in the HDP and in the mitochondria fractions in animals collected from reference sites as well as from contaminated lakes (Giguère et al., 2006; Hogstrand et al., 1991; Mason and Jenkins, 1995) suggest that the presence of Zn in these fractions is an indication of the essential biological role of the metal, rather than of the binding of Zn to "inappropriate" metal-sensitive sites. This latter interpretation is consistent with the degree of homeostatic control of larval Zn concentrations observed in *Chaoborus* as well as in other organisms.

Overall metal detoxification

In larvae of the insect *Chaoborus* collected in the field along a metal exposure gradient, trends in metal bioaccumulation differed between essential and non-essential metals. Larval Cd and Ni concentrations were more responsive to aqueous metal gradients than were larval concentrations of the essential metals, Cu and Zn; these latter metals were better regulated and exhibited only 2 – 3-fold increases between the least and most contaminated lakes.

Within the subcellular environment, the HSP fraction was the dominant metal-binding compartment for Cd, Ni and Cu, and for these metals it consistently was the fraction that increased the most along the metal bioaccumulation gradient. It was also an important contributor to the Zn subcellular burden, but as mentioned earlier, its contribution did not increase progressively along the metals gradient. A dominant role for the HSP fraction has been reported for Cd, Ni and Cu in other native aquatic organisms living along metal exposure gradients (bivalves Cd, Cu: Bonneris et al. (2005b); bivalves Cd: Voets et al. (2009); insect larvae Cd, Cu: Cain et al. (2004); fish Cd, Ni, Cu: Giguère et al. (2006), although the absolute M_{HSP} concentrations (expressed in $nmol \cdot g^{-1}$ dry weight) vary considerably among organisms.

From a biochemical perspective, the HSP fraction is normally considered to include metallothionein and MT-like peptides, GSH and free amino acids, with MT and MT-like peptides being the major contributors. Consistent with this interpretation, Croteau et al. (2002) collected *Chaoborus* species from lakes representing a metal exposure gradient

and reported that the total concentration of MT-like peptides, as determined by a Hg-saturation assay, increased along the gradient. Studies on yellow perch and the giant floater mussel have shown that the sum of the metal concentrations in the HSP fraction (calculated as metallothionein equivalents, using the accepted stoichiometry of M_7-MT for divalent metals such as Cd(II), Ni(II) and Zn(II), and M_{12}-MT for Cu(I)) was closely correlated to the total concentration of metallothionein, as determined independently by the same Hg-saturation assay (Campbell and Hare, 2009). In the present case we do not have an independent measure of the total MT concentration in *Chaoborus*, but we did calculate the sum of the metal concentrations in the HSP fraction for animals from each lake and were able to show that this estimate of total MT concentrations was positively correlated with the total larval Cd concentrations ($r = 0.60$, $P < 0.05$, $N = 12$), but not with other larval metal concentrations.

It should be emphasized that the sum of the metal concentrations in the HSP fraction is only a rough approximation of the total metallothionein concentration. Recent work on metallothioneins isolated from organisms exposed to metal mixtures, as is the case here, has demonstrated that mixed metal complexes may exist (e.g., $Cu_nZn_{(7-n)}$-MT) and in addition supermetallated forms of the peptide have been reported, where "supermetallation" describes metal:peptide stoichiometries greater than the accepted 7:1 or 12:1 ratios (Sutherland and Stillman, 2011).

Despite the increase in the HSP fraction that was observed along the metal bioaccumulation gradient, some accumulation of the non-essential metals (Cd and Ni) was observed in the putative metal-sensitive fractions (e.g., HDP, mitochondria), suggesting that metal detoxification was incomplete. Similar evidence of "spillover" of non-essential metals onto metal-sensitive sites has been reported for other wild aquatic organisms living along metal exposure gradients (Cain et al., 2004; Campbell and Hare, 2009; Goto and Wallace, 2010; Voets et al., 2009; Wang et al., 2011). In the specific case of Cd and C*haoborus*, there appears to be a threshold body concentration of about 50 nmol Cd g^{-1} dry weight, above which Cd detoxification becomes more effective (Figs. 3 and 7) and below which *Chaoborus* does not "turn on" its detoxification machinery to its fullest extent. With regards to the capacity of *Chaoborus* to handle these "inappropriate" metal bindings, we speculate that acclimation or adaptation of *Chaoborus* to these highly

metal-contaminated environments may have resulted in a capacity to tolerate some metal spillover without compromising essential biological functions such as growth and reproduction.

Finally, considering the importance of the HSP fraction and its apparent involvement in metal-detoxification strategies in larvae of *Chaoborus*, it would be interesting to investigate the biochemical nature of this fraction and to determine the relative contributions of cysteine, glutathione, and metallothionein-like peptides. For example, if the metal burden found in the HSP fraction were largely tied up not with MT but rather with glutathione, then the common assumption that the HSP fraction represents "detoxified metal" would be called into question (since metal binding to GSH might well compromise the cell's defenses against oxidative stress). To carry out such analyses, one could couple a chromatographic separation step (e.g., high performance liquid chromatography or capillary electrophoresis) with a sensitive metal-specific detector, as offered by "hyphenated" systems (Prange and Schaumloffel, 2002; Ryvolova et al., 2012). Given the convincing evidence for the existence of distinct metallothionein isoforms with different physiological roles in metal sequestration (Dallinger et al., 1997; Wallace and Lopez, 1997), this step might be coupled with an investigation of possible isoforms of these heat-stable proteins and their specific roles in metal-trafficking.

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10. Assessment of a subcellular metal partitioning protocol for aquatic invertebrates: Preservation, homogenization, and subcellular fractionation.

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Abstract

Measurements of the subcellular partitioning of trace metals have been used to predict assimilation efficiencies for metals between trophic levels and to evaluate metal detoxification mechanisms in aquatic organisms. Using field-collected larvae of the insect *Chaoborus*, we assessed the performance of two preservation protocols, three homogenization treatments and a cell fractionation protocol, to determine the best combination of techniques for assessing the subcellular partitioning of Cd, Cu, Ni and Zn. Preservation of larval samples for either two years at -80 °C or one year at -80 °C or -196 °C had little influence on the subcellular partitioning of these metals. Using several marker enzymes, i.e., cytochrome C oxidase and citrate synthase for mitochondria, β-hexosaminidase for lysosomes, and lactate dehydrogenase for the cytosol, we show that optimal and high-yield homogenization is achieved using a combination of mechanical disruption using a micro-pestle followed by vortexing and that the subsequent fractionation procedure employing differential centrifugation/NaOH digestion was effective. We conclude that the preservation and homogenization protocols as well as the fractionation procedure that we tested using *Chaoborus* larvae can serve as a model approach for improving current protocols and for designing protocols for previously untested biological samples.

Résumé

Les mesures de répartition subcellulaire de métaux traces ont été utilisées pour prédire l'efficacité d'assimilation des métaux entre les niveaux trophiques et pour évaluer les mécanismes de détoxication des métaux chez les organismes aquatiques. En utilisant des larves de l'insecte *Chaoborus* récoltées dans l'environnement, nous avons évalué la performance des deux protocoles de préservation d'échantillons, de trois stratégies d'homogénéisation et d'un protocole de fractionnement cellulaire, pour déterminer la meilleure combinaison de techniques permettant d'évaluer la répartition subcellulaire de Cd, de Cu, de Ni et de Zn. La conservation des échantillons larvaires soit pour deux ans, à -80° C ou d'un an à -80 °C ou 196 °C avait peu d'influence sur la répartition subcellulaire de ces métaux. L'utilisation de plusieurs marqueurs enzymatiques (la cytochrome C oxydase et la citrate synthèse pour les mitochondries, la β-hexosaminidase de lysosomes, et la lactate déshydrogénase pour le cytosol) ont montré que la stratégie d'homogénéisation optimale et avec haut rendement était celle qui impliquait une combinaison de rupture mécanique à l'aide d'un micropilon, suivie d'une agitation en utilisant un vortex, et que la procédure de fractionnement subcellulaire subséquent utilisant la centrifugation différentielle et une digestion avec NaOH était efficace. Nous concluons que les protocoles de conservation et d'homogénéisation ainsi que la procédure de fractionnement que nous avons testés en utilisant des larves de *Chaoborus* peuvent servir comme un modèle pour améliorer les protocoles de répartition subcellulaire et pour la conception de protocoles pour les échantillons biologiques non testés précédemment.

Introduction

The subcellular distribution of trace metals can be used to probe different questions relating to the internal trafficking / detoxification of metals and their trophic transfer from prey to predator (Wallace et al. 2003; Wang and Rainbow 2006). For example, the concentration of nonessential metals in heat-stable proteins or granule-like structures is thought to represent metal detoxification, whereas the accumulation of such metals in organelles and some cytosolic enzymes can be a harbinger of metal-induced toxicity (Campbell and Hare 2009; Wallace et al. 2003). The subcellular distribution of trace metals in prey items has been shown to influence the facility with which metals are transferred along some aquatic food chains (Rainbow et al. 2007; Wallace and Luoma 2003).

Protocols for determining subcellular metal partitioning typically involve the initial preservation of biological samples, followed at some later date by a homogenization step and then a series of differential centrifugation, heat denaturation and digestion steps designed to separate whole animals, organs or tissues into subcellular fractions (Table A1). Sample preservation is usually achieved by freezing in either liquid nitrogen or in a low-temperature freezer; once frozen, samples may be held for months or years prior to analysis. Reports dealing with the efficacy of such preservation methods tend to focus more on the viability of cells rather than on the integrity of cell structures and their contents. Some studies examining preservation conditions have reported minor changes in cellular properties when biological samples were stored at -80 °C (Katayama et al. 1997; Sputtek et al. 2005), whereas several other reports found little evidence of cellular disruption (Riesgo et al. 2012; Wang et al. 2006). To our knowledge, no published studies have examined the effects of long-term cryopreservation on metal partitioning within biological tissues.

Analysis of cryopreserved samples begins by thawing and sample homogenization, the goal of which is to achieve the highest possible degree of cell breakage while using a minimum of force, so that there will be no damage to subcellular components such as organelles (Graham 1997). Techniques used to rupture cells can be either physical, e.g., mechanical contact, sonication, hypo-osmotic shock, freeze-thaw cycles (Chaiyarit and

Thongboonkerd (2009); Lavoie et al. (2009); Wallace et al. (2003)), or chemical, e.g., surfactants or hydrolytic enzymes (Klein et al. (1983)). However, information about the homogenization efficiency achieved using these different methods is rarely reported. Since cell homogenization could be either too gentle, leaving cells intact, or too aggressive, thereby damaging organelles and releasing their contents, it is important to determine the efficacy of the homogenization step as well as its impact on the character of each subcellular fraction.

In addition to selecting a method to disrupt the cells, one must also choose a homogenization buffer. If the buffer includes thiol-containing compounds such as dithiothreitol or β-mercaptomethanol (as antioxidants), or phenylmethanesulfonyl fluoride (as a protease inhibitor), they can introduce artifacts either by adding metals as trace contaminants or by binding metals; both actions can perturb metal distributions and concentrations in the various subcellular fractions (Lobel 1989; Roesijadi and Drum 1982).

Once obtained, the homogenate is usually separated into fractions using differential centrifugation (De Duve 1975), either directly or using density gradients (Hinton and Mullock 1997). Note that the agents used to create density gradients (e.g., sucrose, Percoll, Iodixanol) can contaminate the homogenate with additional metals. Alternative cell fractionation techniques include immunoisolation (Salamero et al. 1990) and electrophoresis (Tulp et al. 1996). Among these methods, differential centrifugation is by far the most widely applied method in subcellular metal partitioning studies (Table A1). This approach is effective in separating the majority of subcellular components, without requiring pre-concentration procedures or the addition of reagents. However, the differential centrifugation approach can produce artifacts because of either the clumping of organelles or their breakage with the subsequent leakage of their soluble constituents (De Duve 1975; Graham and Rickwood 1997). Marker enzymes, known to be specific to a particular organelle or subcellular fraction, have been used to quantify organelle integrity following subcellular fractionation and to test for such artifacts (Hinton and Mullock 1997).

Finally, in addition to the possible redistribution of metals from (damaged) organelles and their release into the cytosol (Lavoie et al., 2009), there is also potential for metal redistribution within the cytosol itself, when heat treatment is used to distinguish between heat-stable and heat-denatured proteins. The proteins contributing to the heat-denatured fraction (HDP) may lose their metals on denaturation, leading to an underestimate of the metal originally bound to these ligands.

Given the potential for artifacts, it is important to assess the effectiveness of the preservation, homogenization and separation methods used, especially when working with animals for which fractionation protocols have not yet been evaluated. We chose the phantom midge *Chaoborus punctipennis* to conduct such tests, and have focused on the subcellular partitioning of cadmium (Cd), copper (Cu), nickel (Ni) and zinc (Zn). The predatory larvae of this insect have been used as sentinels to estimate bioavailable concentrations of Cd (Hare and Tessier 1996), Ni (Ponton and Hare 2009) and Se (Ponton and Hare 2013) in lake waters. They have the advantage of being very widespread, and occur even in highly-acidic, metal-contaminated, lakes where other potential sentinels (mollusks, crustaceans and fish) are likely to be absent.

To assess subcellular metal partitioning protocols for *Chaoborus* larvae, we first compared the performance of three homogenization strategies to rupture its cells without breaking cell organelles. We then assessed the performance of a differential centrifugation protocol used to separate the homogenate into subcellular fractions. A set of marker enzymes specific to organelles (mitochondria: cytochrome C oxidase (CCO) and citrate synthase (CS); lysosomes: β-hexosaminidase, HEX) or to putative subcellular fractions (cytosol: lactate dehydrogenase, LDH) was chosen for this purpose. Since the metal partitioning patterns obtained with such protocols may also be affected by the conditions used to preserve the original biological samples, we evaluated the integrity of larval samples preserved at -80 ^{o}C for two years and compared the influence of two preservation methods (-80 ^{o}C in a freezer or -196 ^{o}C under liquid nitrogen, for 1 year).

Materials and Procedures

Collection of Chaoborus larvae

Larvae of *Chaoborus* used in the present work were collected in 2010 from Crooked Lake (46°25'N, 81°02'W) and Lake Opasatica (48°03'N, 79°16'W) and in 2011 from Pine Lake (46°22'N, 81°01'W). These lakes are located on the Canadian Shield in the mining areas of Sudbury, Ontario (lakes Crooked and Pine) or Rouyn-Noranda, Quebec (Lake Opasatica); metal concentrations in Lake Opasatica are reasonably low and the lake can be considered as a reference site, whereas the other two lakes are metal-contaminated (Cd, Ni, Cu, Zn; Ponton and Hare (2009); Rosabal et al. (2012)). All larvae used were in their fourth (final) instar and they were collected after sunset by hauling a plankton net with a mesh aperture of 164-µm horizontally in the water column, then transported in lake water to the laboratory for sorting to species (Saether 1972). We used *Chaoborus punctipennis* (from lakes Crooked and Opasatica) for testing the homogenization and centrifugation protocols; final instar *C. punctipennis* (from Crooked Lake) and its sister-species *Chaoborus albatus* (from Pine Lake) were used to assess the conservation time and preservation methods, respectively.

Samples of ~30 similar-sized larvae were pooled, placed on acid-washed, pre-weighed pieces of Teflon sheeting in acid-washed 1.5-mL polypropylene microcentrifuge tubes and then immediately frozen in liquid nitrogen. In the laboratory they were subsequently held at either -80 °C in a freezer or -196 °C in liquid nitrogen. Larval samples from Crooked Lake were used for most measurements, whereas those from Lake Opasatica were used only to determine the metal contributed by each subcellular fraction to the total metal burden in the whole insect. *Chaoborus punctipennis* larvae from Lake Opasatica had Cd and Ni concentrations of 3.1 and 1.0 nmol g^{-1} dry weight (dw), respectively. The Cd, Ni, Cu and Zn concentrations in larval samples collected from Crooked Lake were 70, 44, 360 and 2200 nmol g^{-1} dw and the corresponding values for larvae from Pine Lake were 13, 12, 125 and 1030 nmol g^{-1} dw, respectively.

Homogenization treatments

The performance of three homogenization strategies was compared using enzymatic biomarkers for the cytosol, mitochondria and lysosome compartments (see below). Each

larval sample was placed on ice until partially thawed and then suspended in 25 mM Tris-buffer (OmniPur, EM Science, affiliate of MERCK KGaA, Darmstadt, Hesse, Germany) (pH 7.4) at a ratio of 1:2 (insect wet weight[g] : buffer volume[mL]). Each homogenization protocol was performed in triplicate. The first homogenization strategy (A) involved simple cell breakage, whereas the others (B and C) consisted of two combined homogenization steps, with the details as follows:

A: Larval cells were disrupted using a Pellet Pestle (Kontes, Vineland, New Jersey, USA) for 2 s at 30 s intervals for 5 min (Lapointe et al. 2009). The resulting homogenate was centrifuged at 800 × g (Fig. 1) and the supernatant was collected for the enzymatic analyses described below and for protein concentration measurements.

B: Following treatment A, the resulting pellet was resuspended in Tris buffer (1:2 ratio) and vigorously mixed using a vortex (Analog Vortex Mixer, Fisher Scientific, Whitby, Ontario, Canada) for 40 seconds and then centrifuged at 800 × *g*. The vortex treatment has been shown in earlier work to improve the purity of the organelle and cytosolic fractions (Lapointe et al. 2009; Ng et al. 2011). The two supernatants were combined to determine enzymatic activities and protein concentrations.

C: Following treatment A, the pellet was resuspended in Tris buffer (1:2 ratio) and re-homogenized using the Pellet Pestle for 3 min (2 s at 30 s intervals). The resulting homogenate was centrifuged at 800 × g and the supernatant combined with that from the first homogenization step for enzymatic assays and protein concentration measurements.

Assessment of the subcellular fractionation procedure

Triplicate composite samples (3 × 180 mg fresh weight) of *C. punctipennis* were subjected to a subcellular partitioning procedure (Fig. 1) adapted from protocols described by Wallace et al. (2003), Giguère et al. (2006) and Lapointe et al. (2009) that have been applied to a variety of aquatic organisms (Supporting information Table).

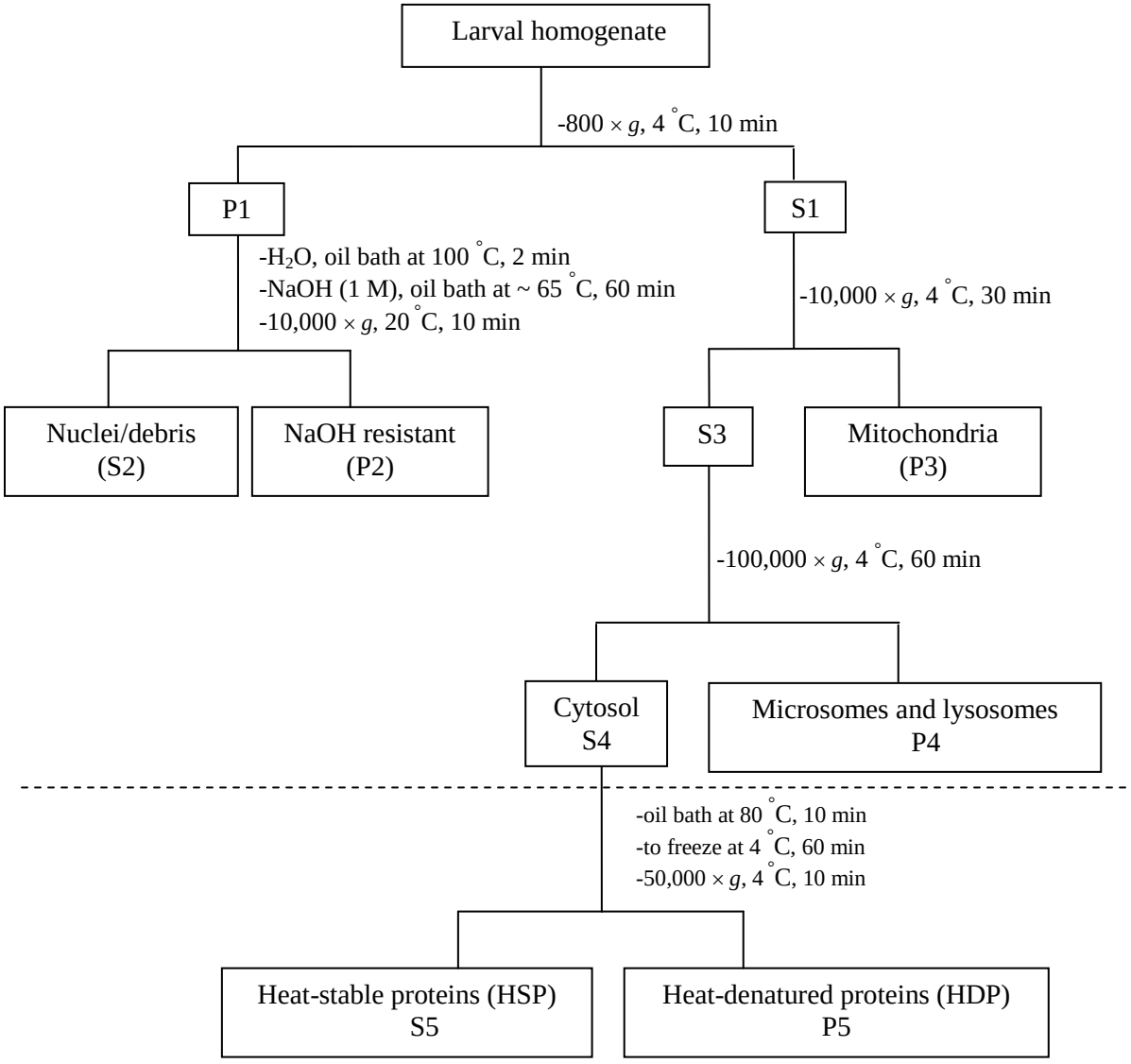

Fig. 1. Schematic illustration of the subcellular fractionation procedure used to separate larvae of *Chaoborus* into operationally-defined fractions. The separations above the dashed horizontal line were tested with marker enzymes.

Each larval sample was homogenized using procedure B, as described above. The final pellet resulting from the first centrifugation step (800 × g) was suspended in 500 µL of ultrapure water, heated for 2 min at 100 °C, mixed with 500 µL of NaOH (1 M; 99.998%, Sigma–Aldrich, Oakville, Ontario, Canada), and then heated at 65 °C for 60 min. To separate the nuclei/debris fraction (S2) from the NaOH-resistant granule-like (P2) fraction, the sample was centrifuged at 10,000 × g for 10 min at ambient temperature (20 °C). The combined supernatants resulting from homogenization protocol B were centrifuged at 10,000 × g for 30 min at 4 °C to isolate the mitochondrial fraction (P3). The remaining supernatant (S3) was subjected to an ultracentrifugation step at 100,000 × g for 60 min at 4 °C to separate the lysosomes/microsomes (P4) from the cytosol (S4). This final step was carried out using a Beckman TLA-100 centrifuge with a TLA-100.3 rotor (Beckman Counter, Mississauga, Ontario, Canada). An IEC Micromax centrifuge (Thermo IEC, Arlington, Maine, USA) was used for the other centrifugation steps. This entire sequence of steps was carried out twice, on separate replicate samples, once for analysis of the marker enzymes (n = 3) and once for determination of the subcellular distribution of Cd and Ni (n = 5) in *C. punctipennis* from Crooked Lake and Lake Opasatica.

Enzyme assays

A set of enzymatic biomarkers specific to particular organelles or subcellular fractions was used to assess the homogenization treatments and the nature of each subcellular fraction. All enzyme reactions were performed in 96-well microplates (Microlon 200, Greiner Bio-One, Sigma Aldrich, Oakville, Ontario, Canada) and the spectrophotometric measurements were carried out using a Cary 50 MPR microplate reader (Varian, Mississauga, Ontario, Canada). The specific activity of each enzyme is expressed as unit (U) of enzyme per mg of total protein (U or mU mg^{-1}). One U is defined as the amount of the enzyme that catalyzes the conversion of 1 µmol of substrate or the production of 1 µmol of product per min under defined conditions of temperature and pH. Blanks were run through the same procedure for each enzymatic assay, which was performed in triplicate. The determination of total protein was measured using the Bio-rad Protein Assay (Bio-rad, Mississauga, Ontario, Canada), which is based on the method of Bradford (1976), using bovine serum albumin as the standard. Prior to enzyme-activity

measurements, each pellet (NaOH-resistant, mitochondria and lysosomes/microsomes; P2, P3 and P4) was suspended with 200 µL of Triton X-100 (1%; Molecular Grade, Fisher Scientific, Whitby, Ontario, Canada) in Tris buffer (25 mM, pH 7.4) to solubilize membranes, and was then mixed lightly by vortex agitation.

Lactate dehydrogenase assay: Lactate dehydrogenase (LDH; EC 1.1.1.27), a specific marker of cellular cytosol, was assayed by measuring the reduction of pyruvate (99%, Sigma Aldrich, Oakville, Ontario, Canada) to lactate with the concomitant transformation of NADH to NAD (nicotinamide adenine dinucleotide; 98%, Sigma Aldrich, Oakville, Ontario, Canada) and H^+ (Worthington Enzyme Manual 1998). To measure LDH activity, 10 µL of each sample was added to each well containing 170 µL of a reaction mixture composed of NADH (0.16 mM) in a phosphate buffer (0.1 M, pH 7). Reaction components were incubated for 2 min in the spectrophotometer before making a 5-min absorbance measurement at 340 nm to set the baseline. The reaction was started by adding 20 µL of pyruvate substrate (5 mM) to each well. Enzyme activity was determined by monitoring the decrease in absorbance at 340 nm for 5 min, a result of the oxidation of NADH. An extinction coefficient of 6.22 mM^{-1} cm^{-1} was used. LDH activity is expressed as the oxidation of 1 µmol of NADH per minute per mg of protein at 25 °C and pH 7.

Cytochrome c oxidase assay: Cytochrome c oxidase (CCO; EC 1.9.3.1), a large enzymatic complex located on the inner mitochondrial membrane, was measured according to Storrie and Madden (1990) with a few modifications. The reaction mixture was composed of a phosphate buffer (0.1 M, pH 7) with the substrate cytochrome C (>95%, Sigma Aldrich, Oakville, Ontario, Canada) that was previously reduced by the addition of sodium dithionite (>85%, Sigma Aldrich, Oakville, Ontario, Canada). $K_3Fe(CN)_6$ (>99%, Sigma Aldrich, Oakville, Ontario, Canada) was added to the reaction mixture for the reference samples. To assess CCO activity, 10 µL of each sample was added to 190 µL of the reaction mixture in each sample well, whereas 200 µL of the mixture containing $K_3Fe(CN)_6$ was added to reference samples (n = 4 in each run). The determination of CCO activity was based on the decrease in absorbance at 550 nm of ferrocytochrome C, a result of its oxidation by CCO over 5 min. An extinction coefficient

of 19.1 mM^{-1} cm^{-1} was used for the calculation of CCO activity, which is reported as the oxidation of 1 µmol of ferrocytochrome C min^{-1} mg^{-1} at 25 °C and pH 7.

Citrate synthase assay: Citrate synthase (CS; EC 2.3.3.1), a specific marker of the mitochondrial matrix, was measured according to the method of Srere (1969). The reaction mixture was composed of phosphate buffer (1 mM, pH 8), Tris (100 M; OmniPur, EM Science, affiliate of MERCK KGaA, Darmstadt, Hesse, Germany), acetyl coenzyme A (0.1 M; CoA; >93%, Sigma Aldrich, Oakville, Ontario, Canada) and 5,5'-dithio-bis (2-nitro-benzoic acid) (0.1 M; DTNB; Sigma Aldrich, Oakville, Ontario, Canada). To assay CS activity, 10 µL of each sample was added to 170 µL of reaction mixture in each well. The samples were allowed to stabilize for 2 min before making 5-min absorbance measurements at 412 nm to set the baseline. The reaction was started by adding 20 µL of the substrate oxaloacetate (98%, Sigma Aldrich, Oakville, Ontario, Canada). The CS activity was measured as the increase in absorbance of DTNB-SH over 5 min at 412 nm, a result of the sulfhydryl group (-SH) transfer reaction from CoA to DTNB. Enzyme activity was calculated on the basis of an extinction coefficient of 13.6 mM^{-1} cm^{-1} and expressed as the formation of 1 µmol of DTNB-SH min^{-1} mg^{-1} at 25 °C and pH 8.

β-Hexosaminidase assay: β-hexosaminidase (HEX, EC 3.2.1.52), a lysosomal enzyme, was assayed by detecting spectrophotometrically the absorbance of 4-nitrophenol at 405 nm according to the procedure optimized by Marciniak et al. (2006). The reaction mixture included one solution of a citrate-phosphate buffer (100 mM, pH 4.7) and another solution of the artificial substrate 4-nitrophenyl-N-acetyl-β-D-Glucosaminide (7.5 mM; >99%, Sigma Aldrich, Oakville, Ontario, Canada). To measure HEX activity, 10 µL of each sample was added to 30 µL of reaction substrate and 40 µL of citrate phosphate buffer in each well. The plate containing the samples was incubated at 37 °C for 60 min with constant mixing, after which time the reaction was stopped by the addition of 200 µL of borate buffer (200 mM, pH 9.8). The concentration of 4-nitrophenol generated was determined from a calibration curve based on appropriate amounts of 4-nitrophenol (>99%, Sigma Aldrich, Oakville, Ontario, Canada) in 100 mM citrate-phosphate buffer (pH 4.7). HEX activity is reported as the formation of 1 µmol of 4-nitrophenol min^{-1} mg^{-1} at 25 °C and pH 4.7.

Efficacy of preservation conditions

Five replicate samples of *C. punctipennis* from Crooked Lake were submitted to a subcellular partitioning procedure after either two months or two years in storage in a refrigerator at -80 °C. In a separate study, three replicate samples of *C. albatus* from Pine Lake were stored for one year at either -80 °C in a refrigerator or in liquid nitrogen (-196 °C). All of these preserved samples were processed using the same subcellular partitioning protocol (Fig. 1). In both treatments, the cytosol (S4) was separated by heating at 80 °C for 10 min, cooling on ice for one hour and centrifuging at 50,000 × *g* for 10 min at 4 °C. Heat-stable proteins (HSP) are found in the supernatant whereas heat-denatured proteins (HDP) are located in the final pellet.

Metal determinations and quality control

To minimize accidental metal contamination, the polypropylene microcentrifuge tubes, the Teflon sheeting and all other lab-ware were soaked in 15% nitric acid for 7 days and rinsed in ultrapure water prior to use. Samples for total larval metal analysis and the centrifugation pellets obtained in the subcellular fractionation procedure were freeze-dried (72 h; FTS Systems TMM, Kinetics Thermal Systems, Longueil, Quebec, Canada) and weighed (XS205 Dual Range Analytical Balance, Mettler Toledo, Mississauga, Ontario, Canada). All freeze-dried samples for whole insect analyses were placed in acid-washed HDPE (High-Density Polyethylene) bottles, whereas subcellular pellet samples were kept in their original microcentrifuge tubes to limit metal loss during transfer. The first digestion was carried out using concentrated nitric acid (Optima grade, Fisher Scientific, Whitby, Ontario, Canada) at a ratio of 100 µL per mg of sample dw for 2 days at room temperature (Croteau et al. 2001). Digestions of the subcellular solid samples were then transferred to acid-washed HDPE bottles and a hydrogen peroxide digestion (Optima grade, Fisher Scientific, Whitby, Ontario, Canada) was performed at a ratio of 40 µL per mg dw for one day. The final digested volume was completed to 1 mL per mg dw with ultrapure water. Each supernatant sample was digested by a similar procedure, with the approximation that each 1 mL of sample represented 1 mg tissue. In order to avoid analytical problems related to the high salt content and elevated Na concentration in the NaOH extract, all debris fractions were diluted before metal measurements.

Total Cd, Cu, Ni and Zn concentrations in whole insects and in all subcellular fractions were measured using an inductively coupled plasma-mass spectrometer (ICP-MS; Thermo Elemental X Series, Winsford, England, United Kingdom). Concurrently, samples of similar weight (range of 0.5-1.0 mg dw) of a certified reference material (lobster hepatopancreas, TORT-2, National Research Council of Canada, NRCC, Halifax, Nova Scotia, Canada) were subjected to the same digestion procedure and analyzed. The percentage recovery for each metal was satisfactory (Cd: 103 ± 3%; Cu: 98 ± 4%; Ni: 105 ± 6%; Zn: 106 ± 12%; n = 10). The relative contribution of each subcellular fraction to the total metal burden was estimated as a ratio defined by the metal burden in a given fraction divided by the sum of metal burdens in all fractions, multiplied by 100 to give the results in terms of percentages (%). A 90-µL aliquot was collected from the first homogenization step for each sample and analyzed in order to verify the recovery of metals in the various subcellular fractions by a mass balance calculation. The recovery (means ± standard deviations) of metal in the subcellular fractions was calculated as the ratio of the sum of metal burdens in the five fractions divided by the total metal burden estimated from an aliquot collected from the larval homogenate. Good recoveries were obtained (n = 26), that is, 95% ± 2% for Cd, 107% ± 12% for Cu, 120% ± 6% for Ni and 105% ± 10% for Zn.

Calculations and statistical analyses

All numerical data are presented as means ± standard deviations (SD). The percentage of enzymatic activity was estimated as a ratio defined by the enzymatic activity of a biomarker in a given subcellular fraction divided by the sum of the enzymatic activities for the same marker in all fractions, multiplied by 100. Similarly, the percentage of metal in a given subcellular fraction (also referred to as 'relative contribution') was calculated considering the metal burden in the fraction divided by the sum of metal burdens in all fractions, multiplied by 100 to give results in terms of percentages (%). Metal concentrations in each subcellular fraction were expressed as the metal burden in the fraction (nmol) divided by the total larval dw (g).

Differences in enzymatic activities among the three homogenization treatments, as well as in relative enzymatic activity (expressed as the percentage of total enzymatic activity in the whole insect) among the subcellular fractions, were assessed by one-way

analysis of variance (ANOVA). When a significant difference in mean values was detected, Tukey's multiple comparison tests was used to identify which fractions were different. For the study of preservation methods, a t-test for independent samples was used to identify significant differences in relative metal burdens (also expressed as % of the total metal burden) among subcellular fractions. Preliminary conditions for parametric statistical tests (normality and homogeneity of variance) were verified. When any of these conditions were not respected, a Box-Cox data transformation was applied, except for the percentage data (relative contribution of enzymatic activity and relative metal burden in each subcellular fraction), which were arcsine transformed. Statistical analyses were performed using STATISTICA version 6.1 software (StatSoft, Tulsa, Oklahoma, USA). A *P* value of 0.05 was used as a threshold for the statistical tests.

Assessment

Homogenization treatments

The principal aim of any homogenization technique is to achieve the highest possible degree of cell breakage while using a minimum of force so that there will be little or no damage to subcellular components such as organelles (Graham 1997). Most of the homogenization procedures used in studies of metal subcellular partitioning involve a one-step approach (see Table A1), but some authors have included an additional step (Lapointe et al. 2009; Ng et al. 2011) to improve the degree of cell disruption. Accordingly, we included in our homogenization assessment both the simple one-step approach and two alternatives involving additional treatments. To assess the degree of cell breakage, we measured the activity of lactase dehydrogenase (LDH) in the supernatant obtained after homogenization and gentle centrifugation to remove cell debris (800 × *g*, 10 min). Since this enzyme is found exclusively in the cytoplasm (Xia et al. 2011), it has been used as an indicator of cell breakage (Haslam et al. 2000; Seib et al. 2006).

Compared to the one-step homogenization treatment (A), increased effectiveness of cell disruption was provided by treatments B and C (Fig. 2). The more aggressive treatment C was only slightly more effective than was treatment B (Fig. 2). This finding is corroborated by the significant increase observed in the CCO activity in the supernatants

collected after the application of homogenization treatments B and C in comparison with that for homogenization treatment A; the higher CCO activity reflects the greater numbers of mitochondria released following cell breakage.

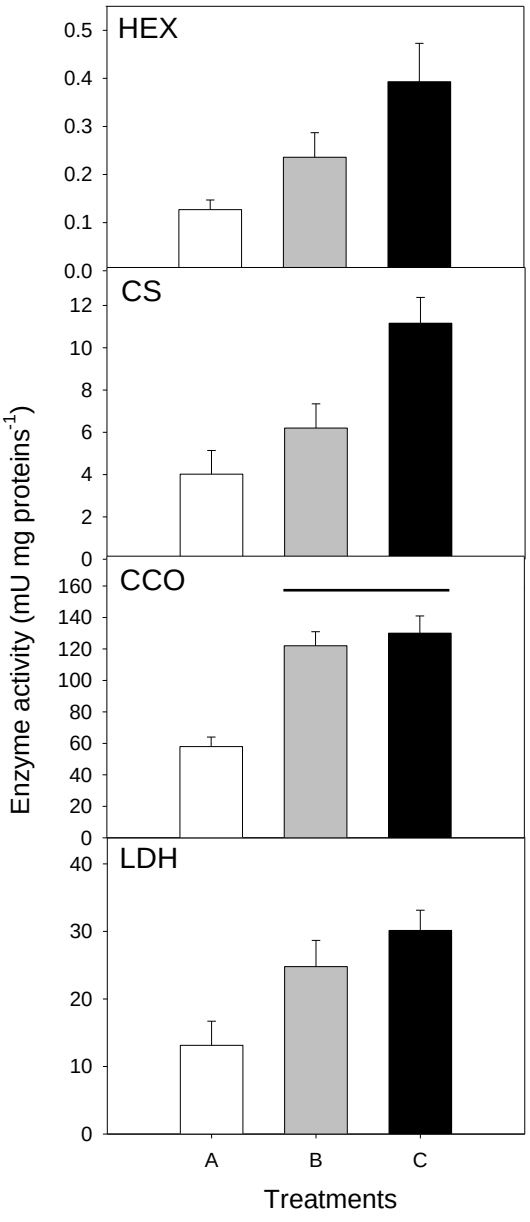

Fig. 2. Specific enzyme activity of lactose dehydrogenase (LDH), cytochrome C oxidase (CCO), citrate synthase (CS) and β-hexosaminidase (HEX) in cytosolic fractions of *Chaoborus punctipennis* larvae that were subjected to three different homogenization treatments (A, B or C). Error bars are standard deviations (n=3). All differences among treatments were significant, except for CCO, where the horizontal line above treatments B and C indicates a non-significant difference (ANOVA followed by Tukey test, $P > 0.05$).

Although treatments B and C were more effective in breaking cells, they led to greater damage to subcellular components. Thus the cytosolic activities of marker enzymes used to assess organelle integrity (HEX and CS) were significantly higher ($P < 0.05$) in treatments B and C than in treatment A (Fig. 2).

These results suggest increasing disruption of mitochondria (CS) and lysosomes (HEX) in the treatment order A < B < C. Damage to physiologically important organelles by mechanical methods has also been reported by Klein et al. (1983), who advocated the use of more gentle methods. However, since these gentler methods involve the use of metal-containing reagents (e.g., enzymes) or metal-binding agents (e.g., EDTA) (Klein et al. 1983), they can bias the results of studies designed to determine the distribution of metals within cells. We conclude that homogenization treatment B (Pellet Pestle + vortex mixer) is the best option of the three since it is effective in breaking cells (unlike A) and yet does not cause excessive damage to organelles (compared to C).

Evaluation of the subcellular fractionation protocol

Subcellular partitioning procedures employing differential centrifugation are inherently sensitive to artifacts such as the clumping of organelles, the overlap of fractions having similar centrifugation properties (e.g., lysosomes/microsomes), the breakage of organelles with subsequent leakage of their soluble constituents and the potential repartitioning of metals among subcellular fractions (De Duve 1975; Graham and Rickwood 1997). It follows that the designation of certain subcellular fractions as "granules", "mitochondria" or "lysosomes and microsomes" remains operational in character. The identity of the "debris" fraction is particularly problematic, and accordingly its contribution to metal partitioning is often ignored, but qualitatively this fraction does provide information about the efficacy of the homogenization treatment applied.

To evaluate the efficacy of the subcellular fractionation protocol most commonly used in trace-metal studies, we homogenized fresh samples of *C. punctipennis* larvae using treatment B (the most efficient of the 3 procedures tested), and applied the fractionation protocol illustrated in Fig. 1. We used enzyme assays to assess the efficacy of cell

disruption, and to determine the integrity of organelles and the distribution of intracellular components among the operationally-defined subcellular fractions.

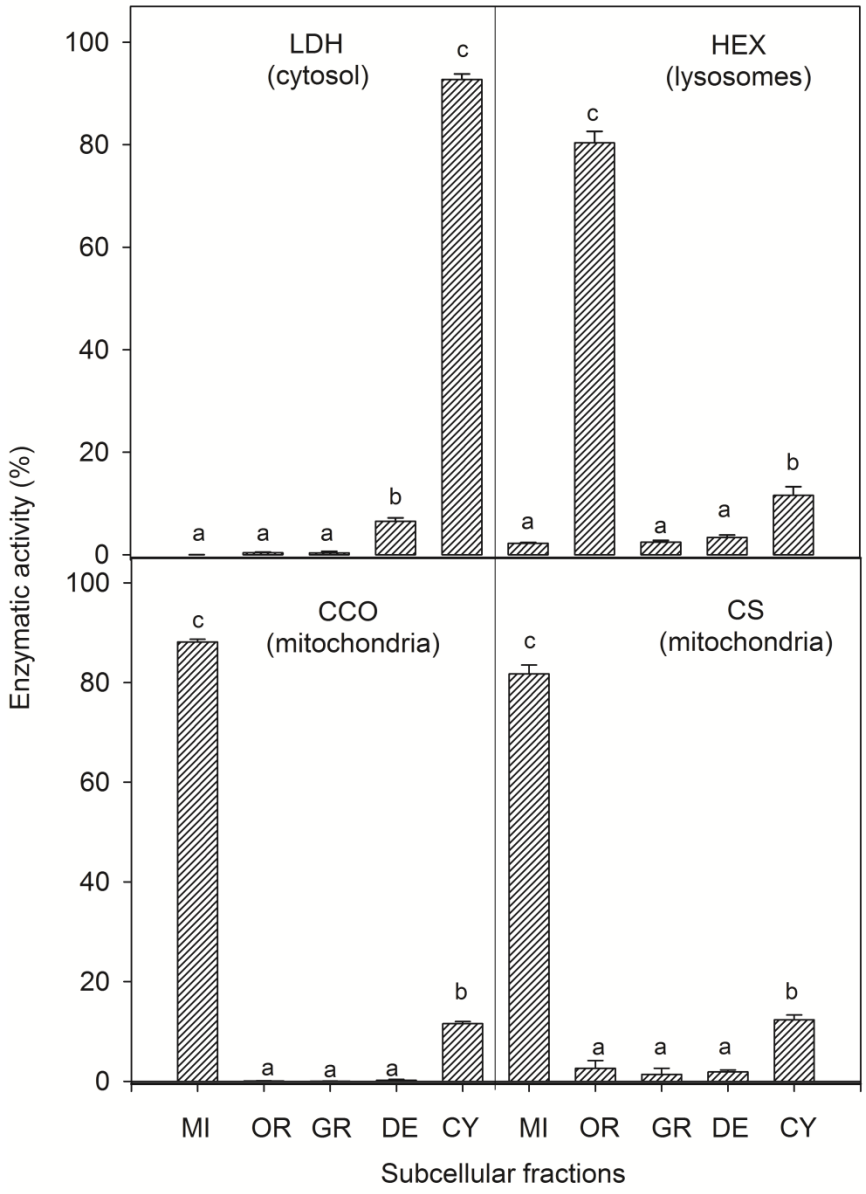

Fig. 3. Percentage (%) of the LDH, CCO, CS and HEX enzymatic activity in each subcellular fraction of *Chaoborus punctipennis* larvae. Error bars are standard deviations (n = 3).The same lowercase letter indicates a non-significant difference, whereas different letters indicate that differences are significant (ANOVA followed by Tukey test, $P < 0.05$). MI: mitochondria; OR: lysosomes/microsomes; GR: NaOH-resistant fraction; DE: nuclei/debris; CY: cytosol.

The results of the LDH assay (cytosolic marker) revealed that ~93% of the total activity of this enzyme was found in the cytosol fraction of the homogenate (Fig. 3), which

confirms that cell breakage was effective (Graham 1997). Low LDH activity (~7%) was found in the nuclei/debris fraction (after surfactant-induced lysis of any intact cells present), which suggests that only a small proportion of larval cells remained intact following homogenization using treatment B.

Lysosome pelleting into the lysosomes/microsomes fraction was efficient since ~80% of the total HEX activity (lysosome marker) was found in this fraction (Fig. 3), but ~12% HEX activity was measured in the cytosol fraction (Fig. 3), suggesting that some lysosomes were damaged by the disruptive forces used during the homogenization step. However, if the cytosolic isoform hexosaminidase C were present in *C. punctipennis*, it might transform the artificial substrate used in the enzymatic assay (Gao et al. 2001). This potential artifact could contribute in part to the HEX activity measured in the cytosol.

Mitochondrial pelleting was also efficient, with more than 88% of the total CCO activity (mitochondrial marker) reporting to the mitochondrial fraction (Fig. 3). In addition, mitochondrial integrity appears to have been largely maintained since more than 80% of the total CS activity (also a mitochondrial marker) was found in the mitochondrial fraction (Fig. 3). However, the presence of some CS activity in the cytosol (~12%; Fig. 3) suggests that there was a small contribution of internal components from the mitochondria to the cytosol fraction.

Redistribution of solutes from organelles to the cytosol as a result of homogenization has been reported by Preller and Wilson (1992), who detected the type III isoenzyme of hexokinase (EC 2.7.1.1) in the soluble fraction of human tissue homogenates rather than in the nuclear periphery fraction where this enzyme is known to be located. Similar redistribution of DNA polymerase from the nucleus to the cytosol has also been reported (Graham 1997). In the present context, such redistributions could lead to artifacts if they occurred with metal-containing organelles. Because both mitochondria and lysosomes provide physiologically important metal-sensitive sites to which non-essential metals (e.g., Cd and Ni) could bind, their rupture could bias subcellular metal distributions. Accordingly, we carried out a quantitative assessment of the possible contribution of metals from mitochondria and lysosomes to the cytosol fraction using *C. punctipennis*

larvae that had either very high (Crooked Lake) or very low (Lake Opasatica) concentrations of Cd and Ni; total larval Cd and Ni concentrations were respectively 44× and 23× higher in the contaminated lake than in the reference lake. The contribution of the cytosol to the total Ni and Cd burdens in larval samples from the low and high metal lakes ranged from 32% to 65% (Ni) and from 70% to 75% (Cd), respectively (Fig. 4).

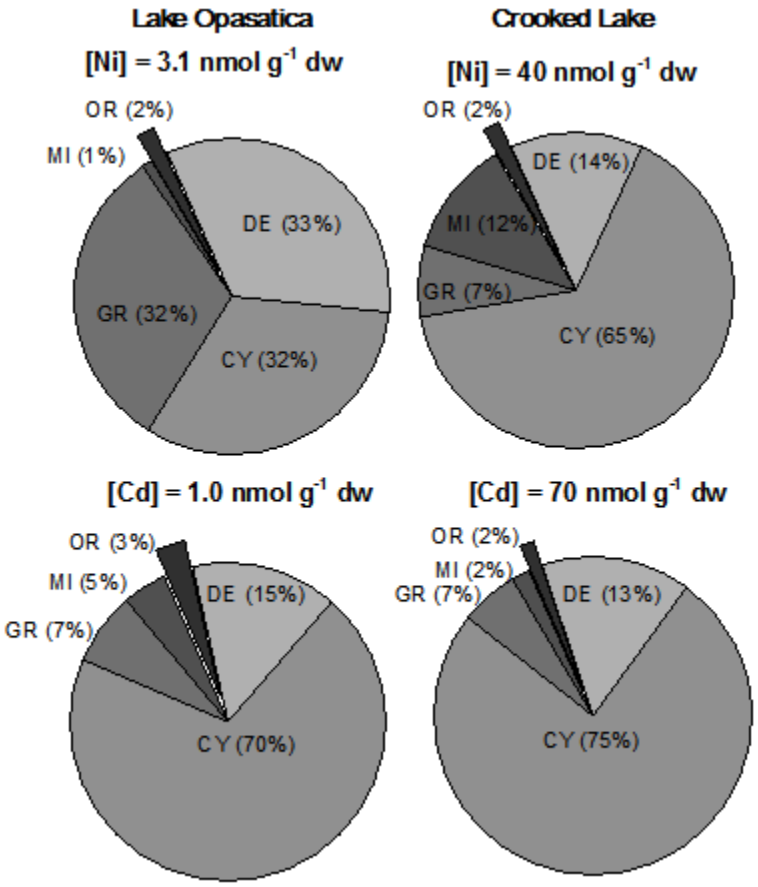

Fig. 4. Distribution (%) of Ni (upper pie-charts) and Cd (lower pie-charts) among subcellular fractions in *Chaoborus punctipennis* larvae (n = 5) collected from Lake Opasatica (left) and Crooked Lake (right). Total metal concentrations in the whole insect (above each pie chart in bold) are expressed in terms of larval dry weight. See Fig. 3 for fraction abbreviations.

In contrast, the contribution of the lysosomes/microsomes fraction (OR in Fig. 4) did not exceed 3% of the total Cd or Ni burden in the whole insect. Likewise, the contribution of the mitochondrial fraction (MI in Fig. 4) did not exceed 12% of the total metal burden.

Thus if any redistribution of Cd or Ni occurred from the lysosome/microsomes or mitochondrial fractions to the cytosol, it would have had little effect on subcellular metal distributions, since the majority of the Cd and Ni burdens was already present in the cytosolic fraction. We conclude that the subcellular partitioning protocol applied to *Chaoborus* larvae yields a representative, high-yield, low-artifact separation of subcellular fractions.

Efficacy of preservation conditions

To assess the integrity of *C. punctipennis* preserved at -80 °C for two years, we compared the percentage distribution of Cd, Ni, Cu and Zn in the subcellular fractions of these larvae to the distribution determined in larval samples collected at the same time but processed only two months after collection.

Other than the slight differences observed in some subcellular fractions (Fig. 5), preservation of the larval samples for two years at -80 °C had little influence on the subcellular partitioning of these metals. The subcellular fractions for which we noted statistically significant temporal differences (Fig. 5: Ni in mitochondria; Cd in the NaOH-resistant fraction; Zn in lysosomes/microsomes; Cu in the NaOH-resistant and lysosomes/microsomes fractions) were those that had the lowest percentage metal content and thus were nearest to the analytical detection limits for the various metals. In a comparative study of storage methods, Gorokhova (2005) showed that freezing microcrustaceans at -80 °C did not result in significant changes in terms of the quantity or quality of their mRNA.

We also compared the effect of storage for one year at either -80 °C in a refrigerator or -196 °C in liquid nitrogen on the subcellular partitioning of Cd, Cu, Zn and Ni in larvae of *C. albatus* (Fig. 6). For three of these metals, only one of the fractions showed a statistically significant effect (Ni, lysosomes/microsomes; Cd and Zn, nuclei/debris), whereas for Cu two fractions (HDP and NaOH-resistant) were slightly affected by the choice of preservation temperature. These findings are consistent with those reported for blood stem cells in which there were no significant differences in cell viability parameters between protocols that included storage in liquid nitrogen or freezing at -80 °C (Mccullough et al. 2010). Overall, although some changes undoubtedly occur during cell

storage (Massie et al. 2013), our results indicate that storage of *Chaoborus* larvae for one year at -80 °C is an effective means of preserving these insects without substantially altering the subcellular distributions of the metals.

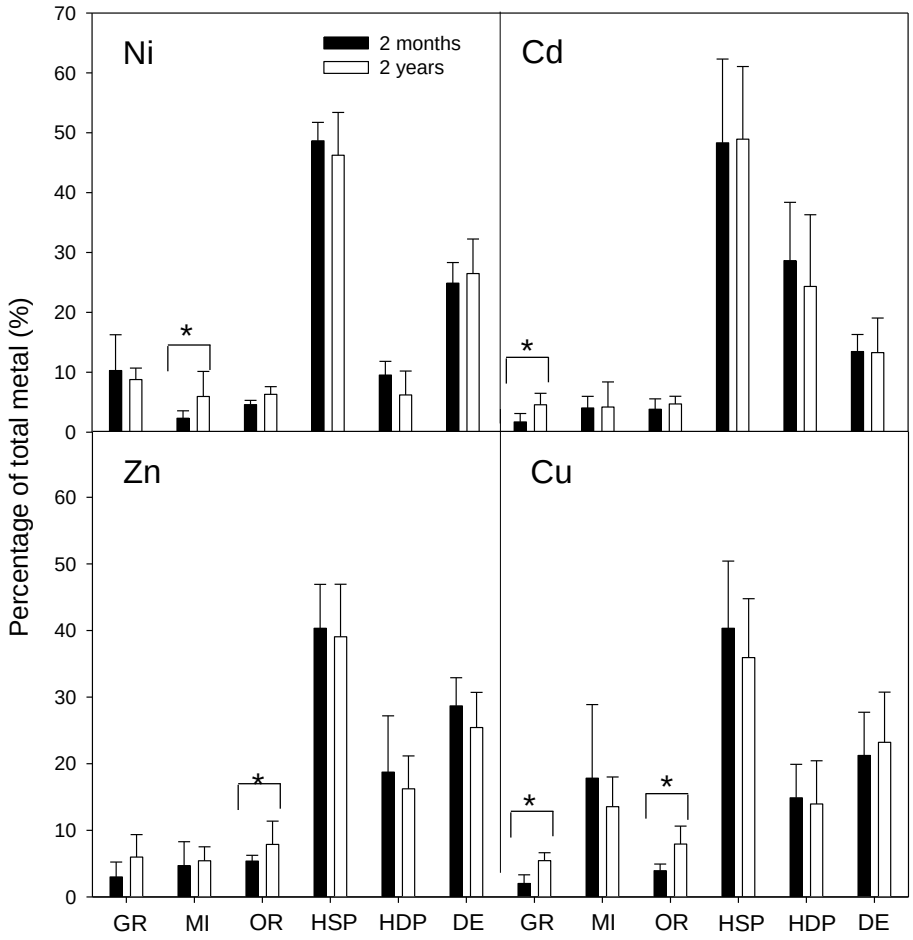

Fig. 5. Percentage distribution (%) of Ni, Cd, Zn and Cu in subcellular fractions of larvae of *Chaoborus punctipennis* following either 2 month storage (black bars) or 2 year storage (white bars) at -80 °C. Error bars are standard deviations (n=5). Significant differences ($P < 0.05$) for a given subcellular fraction are denoted by *. HSP: heat-stable proteins, HDP: heat-denatured proteins. See Fig. 3 for other fraction abbreviations.

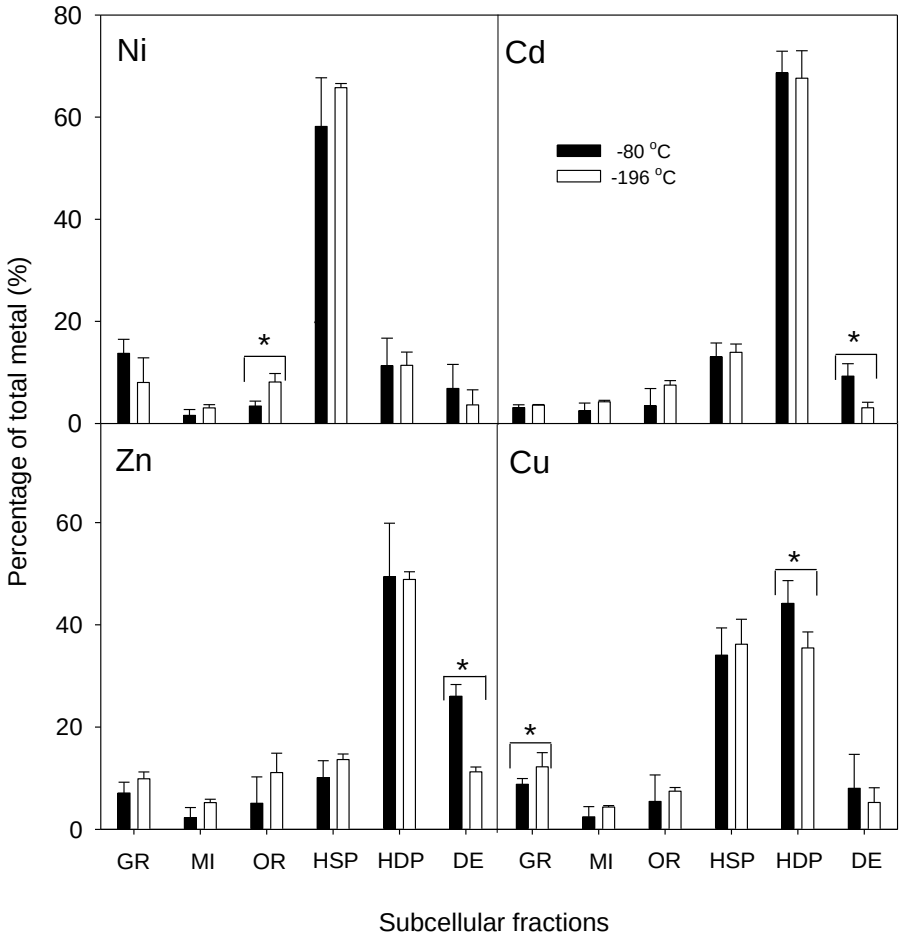

Fig. 6. Percentage distribution (%) of Ni, Cd, Zn and Cu in subcellular fractions of larvae of *Chaoborus albatus* obtained after one year of storage at -80 °C (black bars) or in liquid nitrogen (white bars). Error bars are standard deviations (n=3). Significant differences ($P < 0.05$) for a given subcellular fraction are denoted by *. See Figs. 3 and 4 for fraction abbreviations.

Discussion

Procedures designed to reveal how nonessential trace metals are distributed within animal cells allow researchers to evaluate whether these contaminants are stored in a way that would mitigate or exacerbate toxic effects as well as the likelihood that a predator

consuming prey cells will be able to assimilate the metal that they contain (Web Appendix, Table 1). Such procedures involve three basic steps, that is, sample preservation, homogenization and fractionation. We evaluated the efficacy of procedures currently used to achieve these ends. For this purpose we collected large numbers of *Chaoborus* larvae from both highly metal-contaminated lakes and a relatively pristine lake.

Biological samples destined for subcellular fractionation procedures are usually frozen for some time prior to analysis. In spite of this, the impact of freezing on subcellular metal partitioning has not been previously studied. Our data suggest that subcellular metal distributions in *Chaoborus* larvae stored at -80 ^{o}C or at -196 ^{o}C are very similar. Similarly, metal partitioning among subcellular fractions in samples stored -80 ^{o}C for two years was virtually identical to that in samples that had been stored at the same temperature for two months. Overall, our results suggest that cryopreservation of samples prior to homogenization and fractionation does not alter subcellular metal partitioning.

Following storage by freezing, cells need to be homogenized and then fractionated to determine the partitioning of metals among subcellular fractions of various types. We show that measurements of the activity of marker enzymes specific to particular organelles or subcellular fractions offer a simple and effective approach for assessing the effectiveness of both homogenization and cell fractionation. For our study, we used a set of enzymes localized in either the cytosol (LDH), lysosomes (HEX), or mitochondria (CCO and CS) to assess the efficacy of a subcellular metal partitioning protocol applied to *Chaoborus* larvae. Enzyme markers have also been used for the assessment of subcellular fractionation protocols for algae (CCO and CS) (Lavoie et al. 2009), bivalves (CCO) (Taylor and Maher 2012) and mammalian cells (LDH) (Seib et al. 2006).

With regard to sample homogenization methods, previous reports suggest that mechanical cell breakage is more effective than are chemical and physical methods since it efficiently disrupts cells with minimal damage to intracellular organelles (Simon et al. 2005). Given this observation, we tested the efficacy of mechanical homogenization of *Chaoborus* larvae using either a Pestle alone or in combination with Vortex mixing (Lapointe et al. 2009; Ng et al. 2011). The combined action of these two cell-disruption methods was

very effective, resulting in 93% cell breakage. Enzyme assays showed that these disruption methods resulted in little damage to mitochondria and lysosomes, and metal release from damaged organelles to the cytosol during homogenization would have had a negligible influence on metal concentrations in the cytosol of *Chaoborus* larvae. It would however be important to assess and if necessary correct for this potential artifact for other metals and other metal-exposed organisms.

In addition to this redistribution of metals, there is also a potential for metal redistribution when heat treatment is used to distinguish between heat-stable and heat-denatured proteins in the cytosol. Although this issue was examined in earlier work on yellow perch (Giguère et al. 2006), which suggested that this potential "leakage" was not quantitatively important, similar work might well be considered when a heat treatment step is considered for other organisms.

Since such optimization and verification steps are essential for all subcellular partitioning protocols using all types of animals, we believe that the assessment procedure described here can serve as a model approach for improving current protocols and for designing protocols for previously untested biological samples.

Comments and recommendations

Although there have been many articles reporting subcellular metal distributions in animals, the protocols used are rarely assessed (see Table A1). Since different organs, or the same organ from different animals, can differ markedly in chemical composition and structure, we would recommend that subcellular metal partitioning protocols be tested and optimized before their application to the samples of interest. Of particular importance here are the tissue homogenization conditions and the choice of the appropriate centrifugation parameters (centrifugal force and time). The validation approach used in the present study can serve as a template for evaluating the efficacy of protocols designed to measure the subcellular distribution of trace metals in previously untested biological samples.

Overall, the protocol tested provided a reliable estimate of metal subcellular distributions in *Chaoborus*. One caveat is that the exoskeleton of dipteran insects such as *Chaoborus* is quite thin, as opposed say to that of a mayfly or a caddisfly, and is not underlain by

calcium carbonate, as is that of many crustaceans. Whereas the thin chitinous exoskeleton of *Chaoborus* larvae is likely to be soluble in hot alkaline solution (Pillai et al. 2009), and thus found in the nuclei/debris fraction, those of other groups of invertebrates having a thicker or more heterogeneous exoskeleton are likely to be found in the NaOH-resistant fraction. We suggest including this point in the assessment of any subcellular partitioning protocol to be applied to organisms having a well-developed exoskeleton.

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Supporting information for:

Assessment of a subcellular metal partitioning protocol for aquatic invertebrates: Preservation, homogenization, and subcellular fractionation.

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One supplementary table

In Table A1 we have compiled descriptions of selected studies on aquatic invertebrates in which the subcellular distribution of one or more metals has been reported. The table is arranged taxonomically (taxonomic reference: http://eol.org/). Where more than one study was found for a given species, we have chosen to present the study in which the experimental procedures were described in most detail. In the column headed "Subcellular protocol," we give details of the experimental protocols used in the various studies. The first entry lists homogenization conditions (e.g., composition of the buffer, the pH), where * indicates that the sample had been preserved by freezing at ~ –80°C or under liquid nitrogen, and † indicates that the thawed sample was homogenized mechanically—in other words, these steps were carried out in the same manner as in our present assessment. An ING on the first line in this column indicates that this type of information was not given in the publication. The second entry details the subcellular fractions that were separated and analyzed, where ‡ indicates that the researchers provided a metal mass balance by which they compared the total amount of metal in the original homogenate with the sum of the metal quantities found in the various fractions. In the narrow column to the right of the central column, the presence of a check mark (√) indicates that the researchers adjusted their subcellular fractionation protocol either as a function of the biological tissue studied or as a function of the metals of interest, and that they report this information in their paper.

The following abbreviations are used in Table A1:

β-M: β-mercaptoethanol
HDP: heat-denatured proteins
PMSF: phenylmethanesulfonylfluoride
HSP: heat-stable proteins
DTT: dithiothreitol
MTLP: metallothionein-like protein
TRIS: 2-amino-2-hydroxymethyl-propane-1,3-diol
TAM: trophically available metal
HEPES: 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

Table A1. Selected subcellular metal partitioning studies on aquatic Annelida, Arthropoda, and Mollusca.

Classification		Genus & species	Subcellular protocol	√	Metal(s)	Reference
Phylum	**Class, Order**					
Annelida	Oligochaeta, Haplotaxida	*Tubifex tubifex*	25 mM Tris, pH 7.4*† Cellular debris, NaOH-resistant, organelles, HDP, HSP‡		Ni and Tl	Dumas and Hare (2008)
	Oligochaeta, Lumbriculida	*Lumbriculus variegatus*	25 mM Tris, 2 mM β-M, 0.2 mM PMSF, pH 7.2* Cellular debris, NaOH-resistant, organelles, HDP, HSP‡	√	Cu	Ng et al. (2012)
	Polychaeta, Aciculata	*Diopatra neapolitana*	† Insoluble fraction (cellular debris, NaOH-resistant) and soluble fraction (organelles, HDP, HSP)		Al, As, Cd, Cr, Cu, Hg, Ni, Pb, and Zn	Freitas et al. (2012)
		Neanthes virens	20 mM Tris, pH 7.6*† TAM (HDP, HSP, organelles), non-TAM (cellular debris, NaOH-resistant)‡		Cd and Hg (Me-Hg)	Goto and Wallace (2009)
	Polychaeta, Capitellida	*Arenicola marina*	20 mM Tris, pH 7.6* Cellular debris, NaOH-resistant, organelles, HDP, HSP‡		As	Casado-Martinez et al. (2012)
		Capitella capitata	Tris solution, pH 7.6*† Cellular debris, NaOH-resistant, organelles, HDP, HSP‡		Cd and Zn	Goto and Wallace (2007)
	Polychaeta, Phyllodocida	*Nereis diversicolor*	20 mM Tris, 5 mM β-M and 0.1 mM PMSF, pH 7.6* Cellular debris, NaOH-resistant, organelles, HDP, HSP		Ag, As, Cd, Cr, Cu, Fe, Pb, and Zn	Dang et al. (2012)
Arthropoda	Branchiopoda, Anostraca	*Artemia franciscana*	20 mM Tris, pH 7.6* Nuclei/cellular debris, NaOH-resistant, organelles, HDP, HSP‡		Cd and Zn	Seebaugh and Wallace (2004)
	Branchiopoda, Cladocera	*Daphnia magna*	25 mM Tris, pH 7.4† Nuclei/cellular debris, NaOH-resistant, organelles, HDP, HSP‡		Ni and Tl	Lapointe et al. (2009)

	Malacostraca, Amphipoda	*Gammarus lawrencianus*	* Cellular debris, NaOH-resistant, organelles, HDP, HSP		Cd	Seebaugh et al. (2006)
		Gammarus fossarum	10 mM Tris-HCl, 5 mM β-M, 0.1 mM PMSF, pH 7.4*† Cellular debris, NaOH-resistant, organelles, cytosol (MTLP and non-MTLP; HWM, MTL-MW, and LWM)‡	√	Cd, Ni and Pb	Geffard et al. (2010)
		Gammarus mucronatus, *Leptocheirus plumulosus*	20 mM Tris, pH 7.6*† TAM (HDP, HSP, organelles), non-TAM (cellular debris, NaOH-resistant)‡		Cd and Hg (Me-Hg)	Goto and Wallace (2009)
		Gammarus pulex	10 mM Tris-HCl, pH 7.6*† Nuclei/cellular debris, NaOH-resistant, organelles, HDP, HSP		Cu and Zn	Khan et al. (2011)
	Malacostraca, Decapoda	*Palaemonetes pugio*	Tris solution, pH 7.6*† Cellular debris, NaOH-resistant, organelles, HDP, HSP		Cd and Hg	Seebaugh and Wallace (2009)
		Palaemonetes varians	* Cellular debris, NaOH-resistant, organelles, HDP, HSP		Cu	Rainbow and Smith (2013)
		Procambarus clarkii	250 mM Sucrose, 25 mM HEPES, pH 7.4* Nuclei/cellular debris, NaOH-resistant, mitochondria, lysosomes/microsomes, cytosol (HDP, HSP)‡		U	Frelon et al. (2013)
		Uca pugnax	20 mM Tris, pH 7.6*† Insoluble fraction (cellular debris, NaOH-resistant), organelles, HDP, HSP‡		Ag, As, Cd, Co, Cr, Cu, Mn, Mo, Ni, Se, V, and Zn	Khoury et al. (2009)
	Maxillopoda, Cirripedia	*Balanus amphitrite*	Cellular debris, NaOH-resistant, organelles, HDP, HSP		Ag, Cd, and Zn	Cheung and Wang (2005)
	Insecta, Diptera	*Chaoborus* spp.	25 mM Tris, pH 7.4*† Nuclei/cellular debris, NaOH-resistant, mitochondria, lysosomes/microsomes, HDP, HSP‡	√	Cd, Cu, Ni, and Zn	Rosabal et al. (2012)
		Chironomus dilutus	20 mM Tris, pH 7.6*† TAM (HDP, HSP, organelles), non-TAM (cellular debris, NaOH-resistant)‡		Cd and Hg (Me-Hg)	Goto and Wallace (2009)

		Chironomus riparius	25 mM Tris, pH 7.4*† Nuclei/cellular debris, NaOH-resistant, organelles, HDP, HSP‡		Ni and Tl	Dumas and Hare (2008)
	Insecta, Ephemeroptera	*Baetis* spp., *Epeorus albertae*, *Serratella tibialis*	50 mM Tris-HCl, pH 7.4*† Debris (nuclei/cellular debris, mitochondria), microsomes, NaOH-resistant, HDP, HSP‡		Cd, Cu, and Zn	Cain et al. (2004)
		Maccaffertium modestum, *Isonychia* sp.	Debris (cellular debris, NaOH-resistant), organelles, microsomes, HDP, HSP‡		Hg	Xie et al. (2009)
		Maccaffertium ithaca, *Rhithrogena morrisoni*, *Isonychia tusculanensis*, *Isonychia* sp. 2, *Ephemerella subvaria*, *Ephemerella excrucians*, *Drunella grandis*	50 mM Tris-HCl, pH 7.4Debris (cellular debris, NaOH-resistant), organelles, microsomes, HDP, HSP		Cd	Buchwalter et al. (2008)
	Insecta, Plecoptera	*Claassinea sabulosa*, *Paragnetina* sp., *Calineuria californica*, *Acroneuria abnormis*, *Hesperoperla pacifica*, *Isogenoides hansonni*, *Baumanella alameda*	* Debris (nuclei/cellular debris, mitochondria), microsomes, NaOH-resistant, HDP, HSP		Cd	Martin et al. (2007)
		Doroneuria baumanni, *Skwala* sp., *Pteronarcys dorsata*	50 mM Tris-HCl, pH 7.4 Debris (cellular debris, NaOH-resistant), organelles, microsomes, HDP, HSP		Cd	Buchwalter et al. (2008)
	Insecta, Trichoptera	*Cheumatopsyche* sp. *Rhyacophila* sp.	50 mM Tris-HCl, pH 7.4 Cellular debris/NaOH-resistant, mitochondria, lysosomes/microsomes, HDP, HSP		Cd	Buchwalter et al. (2008)
		Chimarra sp., *Hydropsyche betteni*	Debris (cellular debris, NaOH-resistant), organelles, microsomes, HDP, HSP‡		Hg	Xie et al. (2009)

		Hydropsyche sp., *Arctopsyche grandis*	50 mM Tris-HCl, pH 7.4*† Debris (nuclei/cellular debris, mitochondria), microsomes, NaOH-resistant, HDP, HSP‡		Cd, Cu, and Zn	Cain et al. (2004)
Mollusca	Bivalvia, Arcoida	*Anadara trapezia*	Ca2+/Mg2+ free saline buffer*† Nuclei/cellular debris, NaOH-resistant, mitochondria, lysosomes/microsomes, HDP, HSP‡	√	Pb	Taylor and Maher (2012)
	Bivalvia, Myoida	*Potamocorbula amurensis*	20 mM Tris, pH 7.6*† Cellular debris, NaOH-resistant, organelles, HDP, HSP‡		Cd and Zn	Wallace et al. (2003)
	Bivalvia, Mytiloida	*Crenomytilus grayanus*	50 mM Tris-HCl, 250 mM sucrose, 500 mM NaCl, 10 mM $MgCl_2$, pH 7.5 Nuclei/cellular debris, NaOH-resistant, mitochondria/lysosomes, microsomes, HDP, HSP		Cd	Podgurskaya and Kavun (2006)
		Mytilus edulis	Cellular debris, NaOH-resistant, organelles, HDP, HSP		Ag, Cd, and Zn	Rainbow and Smith (2010)
		Mytilus galloprovincialis	* Cellular debris, NaOH-resistant, organelles, HDP, HSP		Ag, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Se, and Zn	He and Wang (2013)
		Perna viridis	30 mM Tris, pH 8.0* Cellular debris, NaOH-resistant, organelles, HDP, HSP		Ag, Cd, and Zn	Rainbow et al. (2007)
		Septifer virgatus	Cellular debris, NaOH-resistant, organelles, HDP, HSP		Ag, Cd, and Zn	Cheung and Wang (2005)
	Bivalvia, Ostreoida	*Crassostrea gigas, Aequipecten opercularis*	Cellular debris, NaOH-resistant, organelles, HDP, HSP		Ag, Cd, and Zn	Rainbow and Smith (2010)
		Crassostrea hongkongensis Crassostrea angulata	30 mM Tris-NaCl, 150 mM NaCl, 5 mM β-M, and 0.1 mM PMSF, pH 8.0* Cellular debris, NaOH-resistant, organelles, HDP, HSP‡		Ag, As, Cd, Cr, Cu, Ni, Pb, and Zn	Wang et al. (2011)

		Crassostrea virginica	Cellular debris, NaOH-resistant, organelles, HDP, HSP		Cu	Blanchard et al. (2009)
		Ostrea edulis	20 mM Tris, 150 mM NaCl, pH 8.6† Insoluble fraction (cellular debris, NaOH-resistant), and soluble fraction (organelles, HDP, HSP)		Ag, Cd, Cu, and Zn	Bragigand et al. (2004)
		Saccostrea cucullata, Chlamys nobilis	30 mM Tris-NaCl, pH 8.0* Cellular debris, NaOH-resistant, organelles, HDP, HSP		Ag, Cd, and Zn	Rainbow et al. (2007)
		Saccostrea glomerata	Cellular debris, NaOH-resistant, organelles, HDP, HSP		Ag, Cd, and Zn	Cheung and Wang (2005)
		Patinopecten yessoensis	* Cellular debris, NaOH-resistant, organelles, HDP, HSP		Ag, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Se, and Zn	He and Wang (2013)
	Bivalvia, Pholadomyoida	*Laternula elliptica*	20 mM Tris-HCl, 150 mM NaCl, 1.4 mM β-M, 0.5 mM PMSF, pH 7.6* Insoluble fraction (cellular debris, NaOH-resistant, organelles) and soluble fraction (HDP, HSP)		Cu	Choi et al. (2003)
	Bivalvia, Pterioida	*Isognomon isognomon*	20 mM Tris, 250 mM sucrose, 1 mM PMSF in DTT, pH 8.6† Insoluble fraction (cellular debris, NaOH-resistant, organelles) and soluble fraction (HDP, HSP)		As, Cd, Co, Cr, Mn, and Zn	Hedouin et al. (2010)
	Bivalvia, Unionoida	*Pyganodon grandis*	25 mM Tris, pH 7.2*†Nuclei/cellular debris, NaOH-resistant, mitochondria, lysosomes/microsomes, HDP, HSP‡		Cd, Cu, and Zn	Bonneris et al. (2005)
	Bivalvia, Veneroida	*Cerastoderma edule*	† Insoluble fraction (cellular debris, NaOH-resistant) and soluble fraction (organelles, HDP, HSP)		Al, As, Cd, Cr, Cu, Hg, Ni, Pb, and Zn	Freitas et al. (2012)
		Dosinia exoleta	Ultrapure water, pH 7† Insoluble fraction (cellular debris, NaOH-resistant) and soluble fraction (organelles,		Cd, Cu, Pb, and Zn	Darriba and Sanchez-Marin (2013)

			HDP, HSP)			
		Dreissena polymorpha	20 mM Tris, 5 mM β-M, 0.1 mM PMSF, pH 7.4*† Cellular debris, NaOH-resistant, organelles, HDP, HSP		Cd, Cu, and Zn	Voets et al. (2009)
		Gafrarium tumidum	20 mM Tris, 250 mM sucrose, 1 mM PMSF in DTT, pH 8.6† Insoluble fraction (cellular debris, NaOH-resistant) and soluble fraction (organelles, HDP, HSP)		As, Cd, Co, Cr, Mn, and Zn	Hedouin et al. (2010)
		Macoma balthica	20 mM Tris, pH 7.6*† Cellular debris, NaOH-resistant, organelles, HDP, HSP‡		Cd and Zn	Wallace et al. (2003)
		Mactra veneriformis	Tris solution Cellular debris, NaOH-resistant, organelles, HDP, HSP‡		Cd and Zn	Shi and Wang (2004)
		Marcia hiantina	30 mM Tris-NaCl, pH 8.0* Cellular debris, NaOH-resistant, organelles, HDP, HSP		Ag, Cd, and Zn	Rainbow et al. (2007)
		Meretrix meretrix	* Cellular debris, NaOH-resistant, organelles, HDP, HSP		Ag, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Se, and Zn	He and Wang (2013)
		Ruditapes decussatus	*† Insoluble fraction (cellular debris, NaOH-resistant, organelles) and soluble fraction (HDP, HSP)		As, Cd, Cr, Cu, Hg, Ni, Pb, and Zn	Freitas et al. (2012)
		Ruditapes philippinarum	30 mM Tris-NaCl, pH 8.0* Cellular debris, NaOH-resistant, organelles, HDP, HSP		Ag, Cd, and Zn	Rainbow et al. (2007)
		Scrobicularia plana	20 mM Tris, 5 mM β-M, 0.1 mM PMSF, pH 7.6* Cellular debris, NaOH-resistant, organelles, HDP, HSP		Ag, As, Cd, Cr, Cu, Fe, Pb, and Zn	Dang et al. (2012)

	Cephalopoda, Octopoda	*Octopus vulgaris*	10 mM Tris, 150 mM NaCl, 1 mM PMSF, pH 7.4* Nuclei/cellular debris/NaOH-resistant, mitochondria, lysosomes, microsomes		Cu, Cd, Pb, and Zn	Raimundo et al. (2008)
	Gastropoda, Archaeogastropoda	*Haliotis discus hannai*	250 mM Sucrose, 5 mM β-M, 0.1 mM PMSF, pH 7.5† Cellular debris, NaOH-resistant, organelles, HDP, HSP		Cu and Zn	Guo et al. (2013)
	Gastropoda, Hygrophila	*Haliotis diversicolor*	20 mM Tris-HCl, 2 μM PMSF, pH 8.0*† Cellular debris, NaOH-resistant, organelles, HDP, HSP		Ag, Cd, and Hg	Huang et al. (2010)
		Lymnaea stagnalis	20 mM Tris, 2 mM β-M, 0.2 mM PMSF, pH 8.6* Cellular debris, NaOH-resistant, organelles, HDP, HSP‡		Cu	Ng et al. (2011)
	Gastropoda, Littorinimorpha	*Crepidula onyx*	 Cellular debris, NaOH-resistant, organelles, HDP, HSP		Ag, Cd, and Zn	Cheung and Wang (2005)
	Gastropoda, Neogastropoda	*Babylonia areolata*	* Cellular debris, NaOH-resistant, organelles, HDP, HSP		Ag, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Se, and Zn	He and Wang (2013)
		Hinia reticulata	 Cellular debris, NaOH-resistant, organelles, HDP, HSP		Ag, Cd, and Zn	Rainbow and Smith (2010)
		Nassarius festivus	30 mM Tris-NaCl, pH 8.0* Cellular debris, NaOH-resistant, organelles, HDP, HSP		Ag, Cd, and Zn	Rainbow et al. (2007)
		Thais clavigera	† Cellular debris, NaOH-resistant, organelles, HDP, HSP		Ag, Cd, and Zn	Cheung and Wang (2005)
	Gastropoda, Prosobranchia	*Monodonta labio*	 Cellular debris, NaOH-resistant, organelles, HDP, HSP		Ag, Cd, and Zn	Cheung and Wang (2005)

*Samples preserved at –70°C/–80°C or in liquid nitrogen; †Homogenized using mechanical techniques; ‡Mass-balance results report.

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11. Uptake and subcellular distributions of cadmium and selenium in transplanted aquatic insect larvae.

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Abstract: We transplanted larvae of the phantom midge *Chaoborus punctipennis* from a lake having lower concentrations of Cd and Se (Lake Dasserat) to a more contaminated lake (Lake Dufault) located near a metal smelter in Rouyn-Noranda, Quebec. Transplanted individuals were held in mesh mesocosms for up to 16 days where they were fed with indigenous contaminated zooplankton. Larval Cd and Se burdens increased over time, and came to equal those measured in indigenous *C. punctipennis* from contaminated Lake Dufault. Larval Se burdens increased steadily, whereas those of Cd showed an initial lag phase that we explain by a change in the efficiency with which this insect assimilated Cd from its prey. We measured Cd and Se in subcellular fractions and found that larvae sequestered the majority (60%) of the incoming Cd in a detoxified fraction containing metal-binding proteins, whereas a minority of this non-essential metal was in sensitive fractions (20%). In contrast, a much higher proportion of the essential element Se (40%) was apportioned to metabolically-active sensitive fractions. Larvae took up equimolar quantities of these elements over the course of the experiment. Likewise, Cd and Se concentrations in wild larvae were equimolar.

Keywords: Transplantation, Subcellular partitioning, Cadmium, Selenium, *Chaoborus*, Metallothionein.

Résumé

Nous avons transplanté des larves de *Chaoborus punctipennis* d'un lac ayant de faibles concentrations de Cd et Se (lac Dasserat) à un lac plus contaminé (lac Dufault) situé près d'une fonderie à Rouyn-Noranda, Québec. Les larves transplantées ont été mises dans des mésocosmes de maille définie pour un maximum de 16 jours où elles ont été nourries avec du zooplancton récolté du lac contaminé. Les teneurs en Cd et en Se chez les larves transplantées ont augmenté durant l'expérience de transplantation et ces mesures sont devenues égales à celles des larves de *C. punctipennis* indigènes vivant dans le lac contaminé. La teneur en Se chez les larves transplantées a augmenté progressivement, tandis que celle en Cd a montré une phase de latence initiale que nous expliquons par un changement dans l'efficacité avec laquelle cet insecte a assimilé le Cd à partir de ses proies. Nous avons mesuré ces deux éléments dans les fractions subcellulaires et nous avons trouvé que les larves séquestraient la majorité (60 %) du Cd incorporé dans la fraction impliquée dans la détoxication de métaux, contenant des protéines avec une forte affinité pour des métaux, tandis qu'une minorité de ce métal non essentiel était dans les fractions sensibles (20 %). En revanche, une proportion beaucoup plus élevée de l'élément essentiel Se (40 %) a été répartie dans les fractions sensibles et métaboliquement actives. Les larves incorporaient des quantités équimolaires de ces éléments au cours de l'expérience; les concentrations de Cd et de Se chez les larves sauvages étaient également équimolaires.

Mots-clés : Transplantation, distribution subcellulaire, Cadmium, Sélénium, *Chaoborus*, Métallothionéines.

INTRODUCTION

Lakes near metal-smelting operations are usually contaminated by several metals.[1] A case in point is the region of Rouyn-Noranda (Quebec, Canada), where the concentrations of copper (Cu), zinc (Zn), cadmium (Cd) and selenium (Se) are elevated in the water, sediments and animals of many nearby lakes.[2-4] Of these elements, Cd is thought to be the major contributor to toxic effects in invertebrates[5] and fish[6] in these lakes, whereas Se has been reported to influence the uptake of Cd by aquatic invertebrates.[7]. Because larvae of the phantom midge *Chaoborus* are tolerant to trace elements, they have been used to monitor bioavailable Cd[8] and Se[3] in lakewater from this region. Although Se is an essential element, *Chaoborus* larvae do not regulate its concentrations (as they do those of Cu and Zn)[9] such that their Se concentrations are related to those in water and in their planktonic prey.[3] At high concentrations, both Cd[5-6] and Se[10] can be toxic to aquatic animals; Se can also protect animals from oxidative stress[11] caused by high concentrations of metals such as Cd in their cells.[12-14] These interactions are not well understood in aquatic animals such as *Chaoborus*, especially under conditions of changing Cd and Se exposure. To address this knowledge gap, we measured and compared Cd and Se concentrations in indigenous *Chaoborus punctipennis* larvae from 12 lakes situated near metal-smelters in Rouyn-Noranda and Sudbury (Ontario). From these lakes, we chose a low-metals lake and a high-metals lake and transferred *C. punctipennis* larvae from the former to the latter. Larvae were held in mesh mesocosms that allowed the free passage of contaminated lakewater and algae, but restrained the contaminated zooplankton prey that we added to the mesocosms.

We measured Cd and Se uptake over 16 days, both in whole larvae and in various subcellular fractions, to determine: (i) at what rates this insect takes up these elements; (ii) if the Cd and Se contents of transplanted larvae attain those found in indigenous larvae within the duration of the experiment; and (iii) to which subcellular fractions the incoming Cd and Se are bound. Measurements of subcellular partitioning allowed us to assess the likelihood that these elements would either cause toxic effects or be safely detoxified.[15]

METHODS

Study Sites. In June 2011, we transplanted larvae of the phantom midge *Chaoborus punctipennis* from Lake Dasserat, situated upwind from the Horne metal smelter in Rouyn-Noranda, Quebec, to Lake Dufault located < 1 km from the smelter and adjacent to several tailings ponds (Supporting Information, Table S1).[16] Although aerial emissions from the smelter were substantially reduced in the late 1980s,[17] trace-element concentrations in the latter lake have remained high in both water and plankton (Supporting Information, Table S1) due in part to continuous inputs from the drainage basin.[16] To compare Cd and Se concentrations in free-living larvae, we also measured these elements in *C. punctipennis* larvae, collected in 2007, from 12 other lakes situated near either Rouyn-Noranda (Marlon, Opasatica) or the metal-smelting center of Sudbury, Ontario (Clearwater, Crooked, Crowley, Hannah, McFarlane, McCharles, Raft, Ramsey, Silver, Tilton).[18] In addition, we collected bulk zooplankton (53-125 µm) from 7 Sudbury-area lakes (Crooked, Crowley, Hannah, Laurentian, McFarlane, McCharles, Ramsey). Lakewater collection (diffusion samplers) and analyses (pH, DOC, trace elements; Supporting Information, Table S1) were performed using previously published methods.[3, 19]

Invertebrate Collection and Experimental Design. *Chaoborus* larvae and zooplankton crustaceans were collected at night by hauling a 64 µm mesh-aperture plankton net horizontally in the epilimnion (1-3 m depth). Samples were transported in clean plastic bags filled with lakewater to the laboratory, where fourth-instar *Chaoborus* larvae were removed and identified to species.[20]

Chaoborus punctipennis from Lake Dasserat were grouped into nine lots of ~300 larvae each and held in plastic containers at 4 °C. The following day, one lot of larvae was added to each of nine mesocosms anchored at a water depth of 4 m in Lake Dufault. The cylindrical (1.2 m height by 0.5 m diameter) mesocosms were constructed of 64 µm mesh-aperture netting that allowed the free passage of lake water and phytoplankton but not the larger planktonic crustaceans on which late-instar *Chaoborus* larvae feed.[21] Lake Dufault sediments (without *Chaoborus* larvae) were placed in the bottom of each mesocosm as a refuge for transplanted *C. punctipennis* larvae because they feed in the

water column at night then burrow in the sediment during the day to avoid predators.[22] Since *Chaoborus* larvae do not feed when in sediments[23] and do not take up Cd from water,[21] they must take up Cd[22] and Se[24] from the prey they consume in the water column at night.

To feed *C. punctipennis* larvae in mesocosms, we collected crustacean prey by hauling a 64 µm mesh-aperture plankton net horizontally in the epilimnion of Lake Dufault at night. The collected zooplankton were sieved using a 500 µm mesh-aperture sieve to eliminate the large invertebrates on which *C. punctipennis* cannot feed.[25, 26] In the laboratory, prey density in plankton subsamples was estimated under a microscope. Based on these estimates, sufficient prey were added to each microcosm to attain densities of ~660 ± 15 (n = 5) prey per *C. punctipennis* larva. This procedure was repeated every 2-3 days. By this means, we maintained prey numbers well in excess of measured larval consumption.[21]

Larvae were collected from mesocosms at night, when they were in the water column, using a 100 µm mesh-aperture plastic sieve placed on the end of a wooden pole. Three replicate samples of 2-12 pooled larvae were collected from Lake Dasserat (day 0) and from three randomly-selected mesocosms on days 3, 6, 9, 11, 13, and 15 for total Cd and Se analyses. Subcellular trace element partitioning was measured in larvae collected both from Lake Dasserat (day 0) and from the mesocosms on days 6, 12 and 16 (30 larvae per sample; n = 3 -5 samples). Lastly, larvae were collected from Lake Dufault by hauling a 64 µm mesh-aperture plankton net horizontally in the epilimnion (1-3 m depth) on day 9 of the experiment. We assumed that trace element concentrations in day 9 larvae were representative of those in Lake Dufault larvae throughout the duration of our experiment. Larvae for total Cd and Se analyses were placed on acid washed (15% HNO_3; v/v) pieces of pre-weighed Teflon sheeting held in acid-washed 1.5-mL polypropylene micro-centrifuge tubes (Fisher Scientific) and then frozen at -20 °C. Larvae for the measurement of subcellular trace-element distributions were held in acid washed, pre-weighed, 1.5-mL micro-centrifuge tubes, frozen in liquid nitrogen and then held at −80 ^{o}C.

Subcellular Trace-Element Partitioning. We used a subcellular trace-element partitioning procedure, optimized for *Chaoborus*,[19, 27] that separated larvae into six operationally-defined fractions: (1) "debris" (nuclei and cellular debris); (2) "granules" (NaOH-resistant fraction); (3) mitochondria; (4) "organelles" (lysosomes and microsomes); (5) heat-denatured proteins (HDP) including enzymes; and (6) heat-stable proteins and peptides (HSP) including metallothionein and glutathione. The subcellular partitioning procedure (Supporting Information, Methods) is based on a series of differential centrifugations as well as an NaOH digestion and a heat treatment steps.

Cadmium and Selenium Analyses. All micro-centrifuge tubes, *Teflon* sheeting and other labware were soaked in 15% nitric acid (v/v; Omnitrace grade, Fisher Scientific), rinsed seven times with ultrapure water (18 MΩ cm) and dried under a laminar-flow hood to prevent inadvertent trace element contamination. Samples for total Cd and Se analyses in whole larvae and in centrifugation pellets were freeze-dried (72 h; FTS Systems TMM, Kinetics Thermal Systems), weighed (XS205 Dual Range Analytical Balance, Mettler Toledo) and digested in 100 µL of nitric acid (PlasmaPure Plus, SCP Science) per mg dry weight for two days at room temperature and then heated at 65 ºC for 6 h. After cooling, 40 µL per mg dry weight of hydrogen peroxide (H_2O_2; *Optima grade*, Fisher Scientific) was added and samples were held for 1 d in this solution after which time the digestate volume was completed to 1 mL per mg dry weight with ultrapure water.

Total Cd and Se concentrations were measured using an inductively-coupled plasma - mass spectrometer (ICP-MS; Thermo Elemental X Series). Samples of a certified reference material (lobster hepatopancreas, TORT-2, National Research Council of Canada) were concurrently subjected to the same digestion procedure and analyzed. Mean element recovery (n = 7) in samples of TORT-2 was 96 ± 3% and 90 ± 9% (± SD) for Cd and Se, respectively. For the subcellular partitioning study, a mass balance calculation was carried out by comparing element burdens estimated from the 100-µL aliquots removed from the first larval homogenate to the sum of Cd and Se burdens measured in the various subcellular fractions. Mean (± standard deviation) recoveries were close to 100% (Cd, 93 ± 13 %; Se, 96 ± 19 %).

Statistical Analyses. Relationships between temporal changes in Cd and Se concentrations in whole insects were initially examined in bivariate scatterplots. When these indicated a relationship, regression models were tested when the necessary assumptions (normality with the Shapiro-Will test and homoscedasticity using the Levene test) were satisfied. A t-test for independent samples was used to identify significant differences in dry weight, total trace element concentration, or total trace element quantity between indigenous larvae collected from Lake Dufault on day 9 and transplanted larvae collected on day 15 of the experiment. At the subcellular level, student t-tests were applied to compare trace element quantities in detoxified and metal-sensitive trace-element compartments on each sampling day. Temporal differences in the quantities of trace-elements in each subcellular fraction were assessed using the non-parametric Kruskal-Wallis test, followed by the Tukey-Kramer HSD test on ranks. Percentage data were arcsine transformed prior to applying parametric tests (ANOVA followed by HSD test). Statistical analyses were performed using STATISTICA version 6.1 (StatSoft, Tulsa) and JUMP 9.0.0 (SAS Institute Inc.). A probability level of 0.05 was used as the threshold for statistical significance.

RESULTS AND DISCUSSION

Cadmium and Selenium Concentrations in Wild *Chaoborus* Larvae and their Prey. Cadmium and Se concentrations in *C. punctipennis* larvae from Lake Dufault (96 and 62 nmol g^{-1}, respectively) were higher than those in larvae from Lake Dasserat (27 and 14 nmol g^{-1}, respectively; Supporting Information, Table S1). These values fell close to the regression line for Cd and Se in this insect species from 12 other eastern Canadian lakes (Figure 1), which suggests that our two study lakes are representative of those in this region. In transferring larvae between these two lakes, our goal was to monitor larval Cd and Se as their values increased from those in Lake Dasserat to those in Lake Dufault. We note that the relationship between Cd and Se concentrations in larvae is approximately linear (Figure 1). Furthermore, these insects are likely to take up most of their Cd[21] and Se[28] from their zooplankton prey, and there was also a linear relationship between these elements in zooplankton crustaceans from a subset of these lakes (Supporting Information, Figure S1).

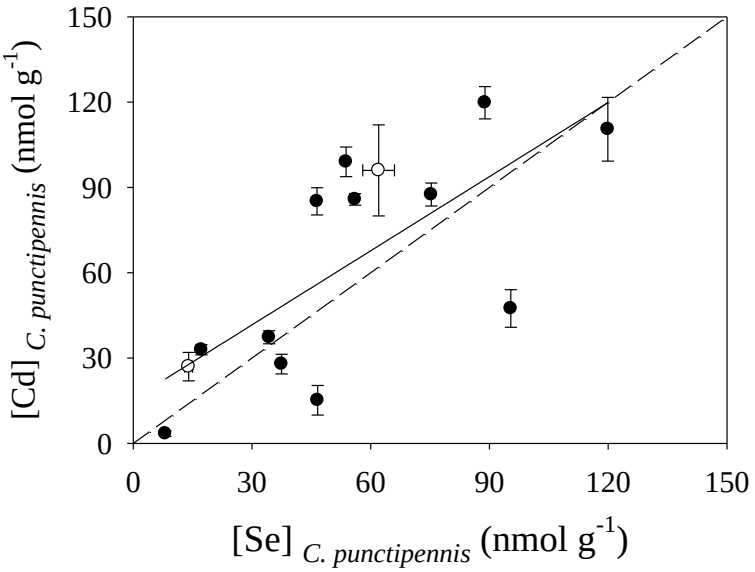

Fig. 1**.** Linear relationship (solid line; r^2 = 0.52, P = 0.003) between mean (± SD, n = 4-6) Cd and Se concentrations (nmol g^{-1}) in *Chaoborus punctipennis* (closed symbols) collected from 12 eastern Canadian lakes and comparative values for Lakes Dasserat and Dufault (open symbols). The broken line is the 1 to 1 line.

Temporal Changes in Larval Mass and Trace Elements. The mean dry weight (dw) of *C. punctipennis* larvae in mesocosms (Supporting Information, Figure S2) increased significantly from day 3 to day 15 (P = 0.02, r^2 = 0.79), which corresponds to a larval growth rate of 0.015 ± 0.004 (± standard error (SE)) mg $larva^{-1}$ day^{-1}. This value is very close to that reported previously for this species (0.013 ± 0.01 mg $larva^{-1}$ day).[22] Since transplanted larvae gained weight in the mesocosms, the zooplankton densities offered were clearly sufficient for larval growth. Note that the weight of larvae from Lake Dasserat (day 0) was higher than that of larvae in mesocosms on the first sampling day (3). However, this day 0 value is likely an artifact because, whereas all larvae collected from mesocosms were used to measure larval weight, those on day 0 were selected from a large number of larvae from which we inadvertently selected the largest larvae. Furthermore, *Chaoborus* larvae held in the laboratory show no measurable loss of weight over 3 days (data not shown).

We estimated the growth rate constant (k_g) for transplanted *C. punctipennis* larvae using the equation

$$W = W^0 e^{k_g t} \qquad (1)$$

where W^0 is the initial weight (mg dw larva^{-1} at day 3, that is, ignoring the day 0 value). Resolving this equation by least square analysis yields a growth rate constant of 0.03 ± 0.01 d^{-1} (n = 18; P = 0.01), which is close to the value of 0.02 ± 0.01 d^{-1} previously reported for *C. punctipennis*[21] larvae held in mesocosms at a prey to predator ratio (675) similar to that used in our experiment (660).

The Se content of *C. punctipennis* larvae transplanted to Lake Dufault increased linearly over time (r^2 = 0.79; P = 0.0001) such that by the end of the experiment it equaled that of indigenous Lake Dufault larvae (Figure 2A). The Cd content of larvae transplanted to Lake Dufault remained stable until day 6 and thereafter increased over time until it too reached that of indigenous Lake Dufault larvae by the end of the experiment (Figure 2C). Note that the time needed to equal the Cd and Se contents of indigenous larvae would likely have been longer had prey ingestion rates been lower (as in the study of Munger et al.).[21]

There was no change over time in the concentrations of Cd and Se in transplanted larvae because increases in larval Cd and Se contents (Figure 2A,C) were matched by increases in larval weight (Supporting Information, Figure S2). For this reason, we consider temporal changes in trace element quantities rather than concentrations. Because indigenous Lake Dufault larvae were smaller (Supporting Information, Figure S2), but contained as much Cd and Se as did transplanted larvae at the end of the experiment (Figure 3A,B), their concentrations of these elements were higher than those in transplanted larvae (Figure 2B,D). Larvae from contaminated Lake Dufault weighed less than those from Lake Dasserat (Supporting Information, Figure S2), likely because there was less zooplankton food available in this lake due to the effects of metals on these planktonic crustaceans.[29, 30]

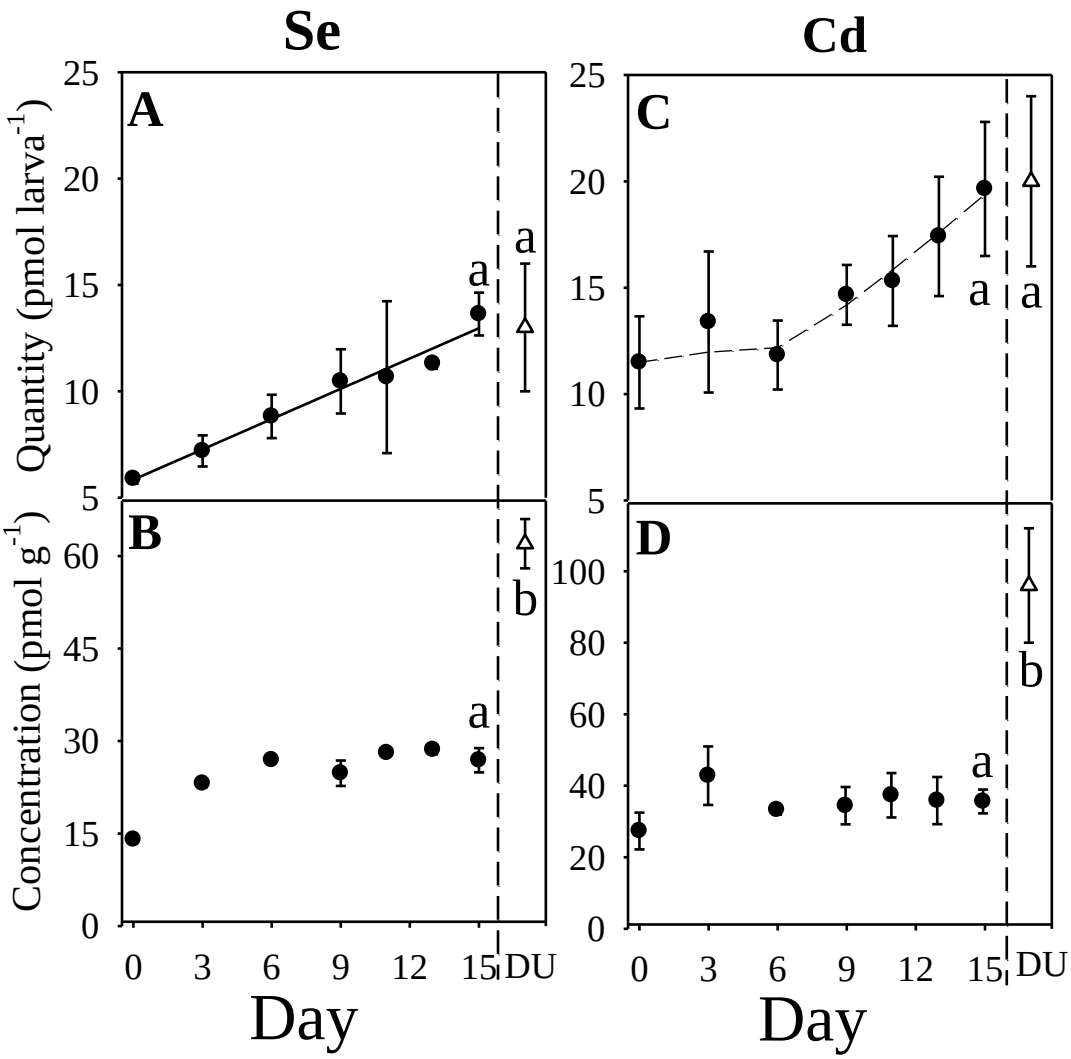

Figure 2. Temporal changes in the mean (± SD, n = 3-5) quantities (upper panels) and concentrations (lower panels) of Se (left panels) and Cd (right panels) in transplanted *Chaoborus punctipennis* larvae (closed circles) and in indigenous *C. punctipennis* larvae from Lake Dufault (DU, open triangles). The modeled dashed lines in panel C were generated using equation 3 (see text). Different lower-case letters indicate significant differences (Student's t-test, $P \leq 0.05$).

The fact that larval Cd content showed no increase during the first week of the experiment (Figure 2C), contrasts with the observation that the Cd content of *C. punctipennis* larvae increased steadily during the first week of a similar transplantation experiment.[21] To explore the reasons for this difference, we used a biodynamic model[31] to describe temporal changes in *Chaoborus* Cd concentrations ($[Cd]_{Chaoborus}$) as follows:

$$\frac{d[\mathrm{Cd}]_{Chaoborus}}{dt} = AE \times IR \times [\mathrm{Cd}]_{\mathrm{prey}} - k_e[\mathrm{Cd}]_{Chaoborus} - k_g[\mathrm{Cd}]_{Chaoborus} \qquad (2)$$

where the first term represents Cd uptake from prey, the second term represents physiological Cd loss and the third term represents Cd dilution by larval growth. Equation 2 ignores Cd uptake from water because *Chaoborus* larvae have been shown to take up all their Cd from food.[21, 32] Cadmium uptake from prey is represented as the product of the assimilation efficiency (*AE*; unitless), which is the proportion of the ingested Cd that crosses the gut membrane, the rate at which prey are ingested (*IR*; g of prey g^{-1} larval weight d^{-1}), and the Cd concentration in prey from Lake Dufault ($[Cd]_{prey}$; Table 1). The term k_e (d^{-1}) represents the rate constant for physiological Cd loss.

Since we maintained prey concentrations in the mesocosms in excess of larval needs, we assumed that the rate at which *C. punctipennis* larvae ingested prey (*IR*) was constant during the experiment. This supposition is supported by the fact that larval growth increased linearly throughout the experiment (Supporting Information, Figure S2) and by the absence of a lag in the accumulation of Se (Figure 2A). We conclude that the initial lag in Cd accumulation cannot be explained by an initially low ingestion rate. However, some studies have shown that increases in the concentration of metal-binding proteins in invertebrates and fish can increase the efficiency with which these animals assimilate Cd.[33, 34] With this possibility in mind, we hypothesize that a low initial (days 0-6) concentration of metal-binding proteins in the transplanted larvae led to a low Cd assimilation efficiency and Cd content, but that the concentration of metal-binding proteins, and thus AE, increased after day 6 leading to an increase in Cd content after this time (Figure 2C).

To test the plausibility of this hypothesis, we estimated Cd assimilation efficiencies separately for days 0-6, when larval Cd content did not vary, and days 6-15, when it increased, using the integrated form of equation 2, that is,

$$Q_{Cd-Chaoborus} = \frac{W_0 \cdot AE \cdot IR \cdot [Cd]_{prey}}{k_g + k_e}(e^{k_g t} - e^{-k_e t}) + Q^0_{Cd-Chaoborus} e^{-(k_g + k_e)t} \qquad (3)$$

where the quantity of Cd in *C. punctipennis* larvae is given by $Q_{Cd\text{-}Chaoborus}$. For this purpose we used an efflux rate constant (k_e) of 0.018 ± 0.005 d^{-1} and an ingestion rate (*IR*) of 0.9, as reported for a field experiment with similar prey densities.[21] This k_e value is comparable to values estimated for some mayflies,[35] but low compared to those reported for other aquatic insects.[35] We used our estimate of the growth rate constant (0.03 ± 0.01 d^{-1}) obtained from the data in SI (Figure S-1). The concentration of Cd in prey (zooplankton) was measured in Lake Dufault (66 ± 3 nmol g^{-1}). Using these values in equation 3 yielded an estimated Cd AE of 2.3% for days 0-6 and 4.6% for days 6-15. Model lines based on these estimates fit closely the measured data (Figure 2C; $r^2 = 0.95$; $P < 0.0001$), which suggests that changes in Cd assimilation efficiency during the experiment could explain the measured changes in larval Cd content. Although these AE estimates are low in comparison to those reported some predatory invertebrates,[36] they are close to that reported previously for *C. punctipennis* offered similar prey densities in a transplant experiment (2%)[21]. At lower prey densities, AE values for this species can reach 18%.[21, 22] Note that some other *Chaoborus* species can assimilate up to 58% of the Cd that they ingest.[22]A low Cd AE could help to explain the persistence of *C. punctipennis* in metal-contaminated lakes.

The reason that the Se content of *C. punctipennis* larvae did not follow the same time-course as Cd can be explained by the fact that this essential element is a constituent of many types of invertebrate proteins and thus its uptake would be less influenced by the concentrations of metal-binding proteins. Indeed, the fact that Se tends to be more efficiently assimilated than does Cd is consistent with the variety of metabolic processes in which it is involved. For example, larvae of the alderfly *Sialis velata* (Megaloptera) are reported to assimilate the majority of the Se that they ingest (55-97%),[37] as do crustaceans from several groups (mysids, copepods and amphipods: 45-80%).[38] These high values for Se contrast markedly with the low Cd assimilation efficiencies reported for *C. punctipennis* in this and previous studies (2-18%).[22] We note that Se is reported to reduce oxidative stress,[11] which can result in greater metal binding to metallothionein[39] and consequently a higher efficiency of Cd assimilation.[40] This potential interaction between the two elements suggests that it would be useful to measure ambient Se concentrations when attempting to predict the bioaccumulation and effects of Cd.

Subcellular Partitioning of Cadmium and Selenium. To determine how the Cd and Se accumulated by transplanted *C. punctipennis* larvae were stored in their cells, and if the subcellular Cd distributions in transplanted larvae were similar to those in chronically exposed animals, we measured these elements in various operationally-defined subcellular fractions.[19] Considering internal trace element "speciation" can be more effective for demonstrating potential effects than are measurements of total concentrations of contaminants. [15]

The highest quantities of larval Cd and Se were found at all times in the HSP (heat-stable protein) fraction (Figure 3A,C; $P < 0.05$), that is, the fraction containing metal-binding proteins and peptides. In proportional terms, 30-40% of the Se and 52-59% of the Cd (excluding cell "debris") were found in this fraction. The Se in this fraction is likely present as either seleno-cysteine in metal-binding proteins or covalently bound to sulfur in cysteine residues.[41] The high proportion of Cd in the HSP fraction is typical for *C. punctipennis* in nature.[19] Cadmium in this fraction is likely bound to metallothionein,[42] which could contribute to the ability of this species to maintain viable populations in Cd-contaminated waters.[8, 19]

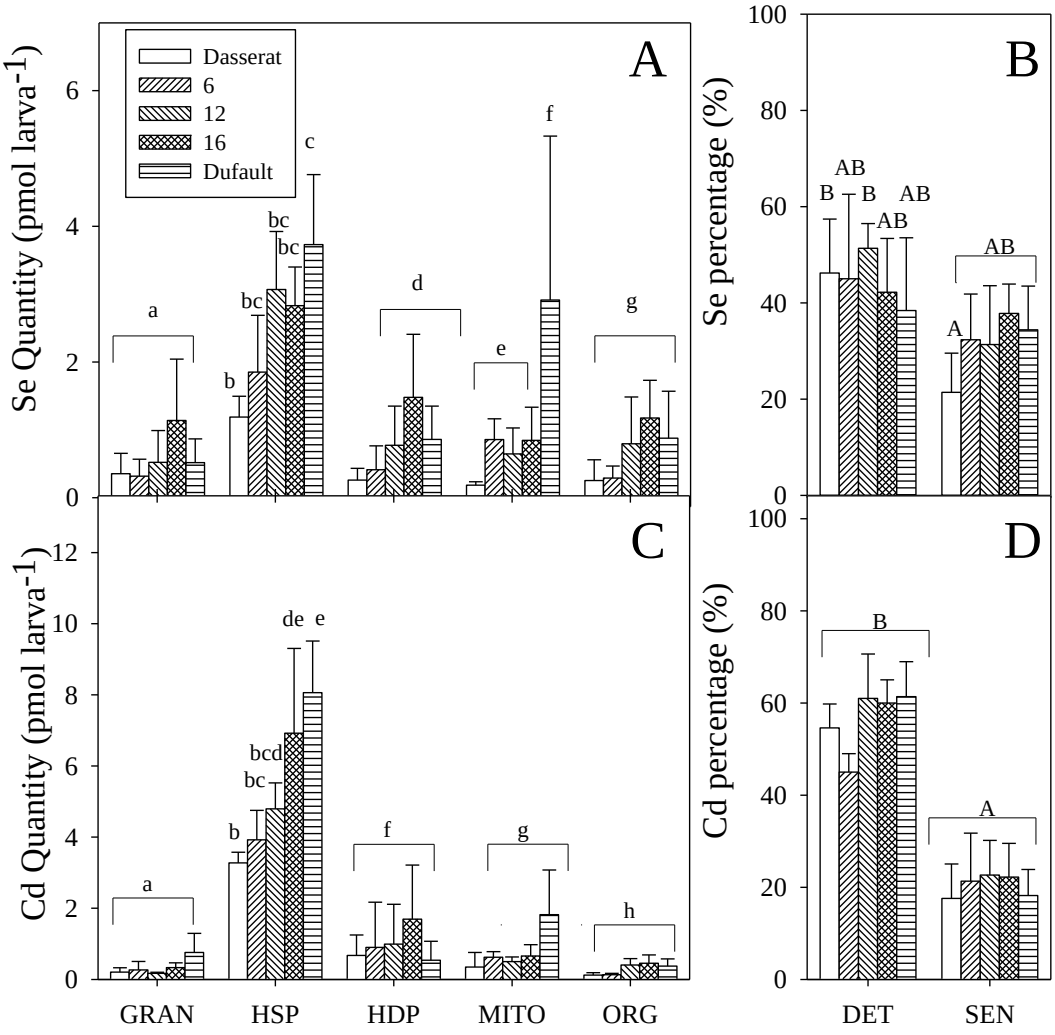

Fig. 3. Temporal changes in the mean (± SD, n = 3-5) quantities (A, C) and percentages (B, D) of Se (upper panels) and Cd (lower panels) in subcellular fractions and compartments of *Chaoborus punctipennis* larvae collected either directly from Lake Dasserat (day 0), or transplanted from Lake Dasserat and held in mesocosms in Lake Dufault for 6, 12 or 16 days, or collected from Lake Dufault. Fractions (A, B) include: GRAN = "granules", HSP = heat-stable proteins, HDP = heat-denatured proteins, MITO = mitochondria and ORG = "organelles", but exclude debris. Fractions (excluding debris) have been grouped into two compartments (C, D) depending on whether the trace elements they contain have been detoxified (DET = HSP + GRAN) or could cause toxicity (sensitive; SEN = HDP + MITO + ORG). Different letters indicate a significant difference (Kruskal-Wallis test followed by Tukey-Kramer HSD test, $P \leq 0.05$) between times for a given fraction (A and C) or between times and compartments (B and D).

Larvae from the more contaminated lake (Dufault) had significantly more Cd (P = 0.004; Figure 3C) and Se (P = 0.007; Figure 3A) in the HSP fraction than did indigenous larvae from the less contaminated lake (Dasserat). The mean quantity of Se (Figure 3A) and Cd (Figure 3C) in the HSP fraction of transplanted larvae tended to increase over

time (P = 0.0007), although these differences were non-significant for Se (Figure 3A; P = 0.07). The quantity of Cd in the HSP fraction did not show a significant increase until day 16. This time lag is consistent with the fact that in chronically metal-exposed *Chaoborus* there appears to be a threshold body concentration below which larvae do not activate their detoxification machinery and above which larvae induce metal-binding ligands to prevent toxicity.[19] After 16 days in the mesocosms, the quantity of Cd in the HSP fraction of transplanted *Chaoborus* larvae reached that of indigenous individuals from Lake Dufault (Figure 3C).

The quantities of Cd and Se in the other subcellular fractions showed no significant change over time (Figure 3A,C). In mitochondria, the quantity of Se was significantly higher (P = 0.008) in larvae from the contaminated lake (Figure 3A), which is consistent with the fact that mitochondria can generate high concentrations of reactive oxygen species[43] and Se is known to protect Cd-exposed animal cells from oxidative stress.[44, 45]

We grouped the various subcellular fractions into two compartments according to the likelihood that Cd, or an excess of Se, in these compartments would exert a toxic effect (sensitive compartment) or not (detoxified compartment).[15] The proportion of Se in a given compartment was constant over time (Figure 3B) and for a given time there was no significant difference (P > 0.05) in the proportion of Se in the detoxified and sensitive compartments, with the exception of larvae from Lake Dasserat, which had a significantly (P = 0.04) smaller proportion of their Se in the sensitive compartment than in the detoxified compartment (Figure 3B). Some aquatic insects[37] and crustaceans[46] maintain an even higher proportion of their Se in the sensitive compartment, which is consistent with the essential role that this element plays in animal metabolism. However, its presence in sensitive cell fractions can result in toxic effects if its concentration exceeds the narrow range between what is required by an animal and what is toxic.[28] In absolute terms, the quantity of Se in the potentially-sensitive compartment (mitochondria + HDP + "organelles") tended to increase throughout the experiment (data not shown) such that by day 16 it was significantly higher (P = 0.01) than that in larvae from Lake Dasserat (day 0) and equal to that in larvae from contaminated Lake Dufault (P = 0.70). A similar trend was observed for the quantity of Se in the detoxified compartment (HSP

+ "granules"), although the difference between the day 16 and Lake Dasserat values was not significant ($P = 0.06$).

In contrast to Se, the proportion of Cd in the sensitive compartment was significantly lower than that in the detoxified compartment at all times and in both study lakes (Figure 3D). Maintaining Cd at low concentrations in the sensitive compartment is clearly a priority for animals since this element has no essential role to play in their cells and thus can cause toxicity if it exceeds the cells' capacity to bind or eliminate this metal.[47] There was no significant temporal change in the proportion of Cd in either compartment, with that in the sensitive compartment remaining at approximately 20% of the total (Figure 3D). The Cd content of the detoxified compartment (data not shown) increased throughout the experiment such that the value at day 16 was about 2-times higher than that at day 0 ($P = 0.008$) and not significantly different ($P = 0.48$) from that of indigenous Lake Dufault larvae.

Overall, our subcellular partitioning results suggest that larvae exposed to low ambient concentrations of Cd and Se are able to manage higher concentrations of these elements as well as insects that have been chronically exposed to high concentrations of Cd and Se.

Relationships Between Cadmium and Selenium in *Chaoborus* Larvae. Relationships between Cd and Se in the metal-sensitive compartment (Figure 4B, open symbols; $r^2 = 0.97$, $P = 0.002$), as well as in its component fractions ($P < 0.004$ and $r^2 =$ 0.96-0.99 for the mitochondria and "organelles" fractions), were approximately linear, whereas the comparable relationship for the detoxified compartment was curvilinear (Figure 4B, solid symbols; $r^2 = 0.99$; $P = 0.01$). Consequently, the relationship for whole transplanted larvae was slightly curvilinear ($r^2 = 0.89$, $P = 0.01$, Figure 4A).

The total quantities of Cd and Se taken up by transplanted larvae (Figure 4A) were approximately equal (slope of 1.0 (± 0.2 (SE), $r^2 = 0.84$, $P = 0.004$) for linear relationship). Likewise, approximately equimolar relationships were measured between the concentrations of Cd and Se in wild *C. punctipennis* larvae (0.9 (± 0.2 (SE); Figure 1) and in their prey (1.0 (± 0.2 (SE); Supporting Information, Figure S1).

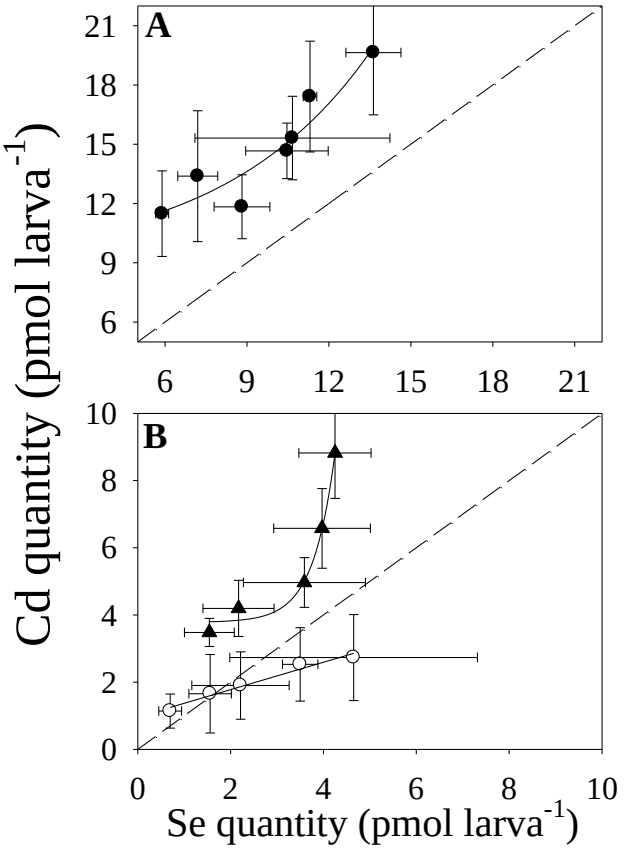

Fig. 4. Relationships between the quantities (pmol larva^{-1}) of Cd and Se in A: whole larvae, and B: detoxified (HSP + NaOH-resistant fractions; solid symbols) and potentially-sensitive (HDP + MITO + ORG; open symbols) compartments of indigenous and transplanted *Chaoborus punctipennis* larvae. Complete fraction names are given in the caption of Figure 3. The broken line is the 1 to 1 line.

These relationships suggest either that the distribution of these elements is linked at the subcellular level or that *C. punctipennis* larvae are exposed to similar concentrations of Cd and Se in our study lakes. The former possibility seems unlikely because the subcellular distributions of the two elements differed somewhat (Figures 3 and 4); measurements of Cd and Se in cytosolic fractions of various molecular weights would be useful for exploring this possibility. The latter possibility is supported by the fact that Cd and Se concentrations in wild *Chaoborus* larvae from our study area are related to those of bioavailable Cd[8] and Se[3] respectively, in lake water. Consequently, the ratio of Cd to Se in *Chaoborus* larvae from regions in which the bioavailable concentrations of Cd and Se differ widely is likely to differ from 1.

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Supporting information for:

Uptake and subcellular distributions of cadmium and selenium in transplanted aquatic insect larvae.

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One protocol, one table and two figures

Method Used for the Subcellular Partitioning of *Chaoborus* Larvae.

Larval samples were thawed on ice in Tris buffer adjusted to pH 7.4 with HCl (25 mmol L^{-1}, OmniPur, EM Science, affiliate of MERCK KGaA, Darmstadt, Hesse, Germany) at a ratio 1:2 (insect wet weight: buffer volume; mg : μL) and each sample was homogenized using a Pellet Pestle (Kontes, Vineland, NJ, USA) for 2 s at 30 s intervals for a total of 5 min. A 100 μL aliquot was removed from the homogenate for mass-balance quality-control measurements and the remainder was centrifuged at 800 × *g* for 15 min at 4 ºC. The resulting supernatant was removed and the pellet was re-suspended in homogenization buffer to a final ratio of 1:4. The sample was then homogenized a second time for 40 s using an Analog vortex mixer (Fischer Scientific) to rupture remaining whole cells. This second homogenate was also centrifuged at 800 × *g*. The two supernatants were combined and placed on ice until further separation.

The final pellet was suspended in 500 µL of ultrapure water, heated for 2 min at 100 ºC and digested with an additional 500 µL of 1N NaOH (99.998%, Sigma-Aldrich) at 65 ºC for 1 hour. Centrifugation of the digestate at 10,000 × *g* for 10 min at ambient temperature was performed to separate the NaOH-resistant fraction (granule-like fraction) from the solubilized fraction containing cellular debris and nuclei.

The combined supernatants from both homogenization steps were centrifuged at 10,000 × *g* for 30 min at 4 ºC to yield the mitochondrial fraction. The remaining supernatant was centrifuged at 100,000 × *g* for 60 min at 4 ºC, giving a pellet containing other organelles (lysosomes and microsomes). The resulting supernatant was held at 80 ºC for 10 min, left on ice for 1 hour and then centrifuged at 50,000 × *g* for 10 min at 4 ºC to obtain a pellet containing heat-denatured proteins (HDP) and a supernatant containing heat-stable proteins (HSP). High-speed centrifugations (≥ 50,000 × *g*) were performed using a Beckman TLA-100 centrifuge equipped with a TLA-100.3 rotor (Beckman Counter), whereas lower-speed centrifugations were performed using an IEC Micromax centrifuge (Thermo IEC, Arlington, MA, USA). Each supernatant was acidified with nitric acid (final [HNO_3] 10%; v/v; Optima grade, Fisher Scientific) and kept at 4 ºC until Cd and Se analyses. Pellets were frozen at −80 ºC until drying and trace element analysis as described in the text.

Table S1: Dissolved organic carbon ([DOC]), free cadmium ion ([Cd^{2+}]; calculated with the chemical equilibrium model WHAM 6*), selenium ([Se]) and calcium ([Ca]) concentrations and pH in water from Lakes Dasserat and Dufault. Also given are Cd and Se concentrations in larvae of *Chaoborus punctipennis* and in their prey (zooplankton) as well as the quantities of these trace elements in individual larvae and their dry weight. Ratios of values for the two lakes are also shown. Values (except pH) are means ± standard deviations (n = 2-7).

Variables	Lake Dasserat (DA) (48°16'N, 79°23'W)	Lake Dufault (DU) (48°18'N, 79°00'W)	DU/DA
Lakewater			
pH	7.6	7.7	
[DOC] (mg L^{-1})	5.1 ± 0.2	3.8 ± 0.1	1.3
[Cd^{2+}] (nmol L^{-1})	1.4 ± 0.2	5.1 ± 0.2	3.6
[Se] (nmol L^{-1})	0.9 ± 0.3	9.5 ± 5.1	10.6
[Ca] (mmol L^{-1})	0.21 ± 0.01	0.36 ± 0.01	1.7
Invertebrates			
[Cd] zooplankton (nmol g^{-1})	---	66 ± 3.1	---
[Se] zooplankton (nmol g^{-1})	63 ± 5.0	93 ± 2.4	1.5
[Cd] *Chaoborus* (nmol g^{-1})	27 ± 5.0	96 ±16	3.6
[Se] *Chaoborus* (nmol g^{-1})	14 ± 1.0	62 ±4.0	4.4
Cd *Chaoborus* (pmol $larva^{-1}$)	11 ± 2.0	20 ± 4.0	1.8
Se *Chaoborus* (pmol $larva^{-1}$)	5.9 ± 0.2	13 ± 3.0	2.2
Chaoborus weight (mg $larva^{-1}$)	0.4 ± 0.1	0.2 ± 0.1	0.5

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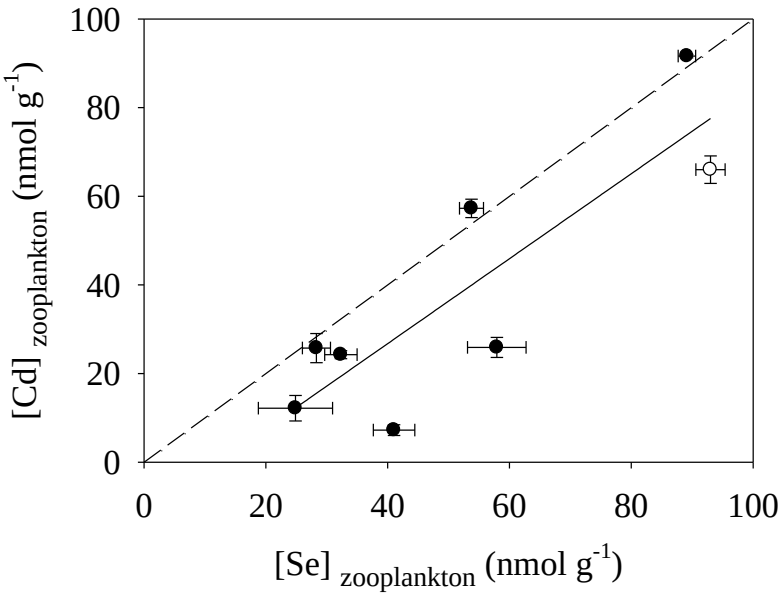

Figure S1: Linear relationship (solid line; $r^2 = 0.74$, $P = 0.007$) between mean (± SD, $n = 3$) Cd and Se concentrations (nmol g^{-1}) in zooplankton prey (53-125 µm) collected from 7 eastern Canadian lakes (solid symbols). The open circle is the value for Lake Dufault. The broken line is the 1 to 1 line.

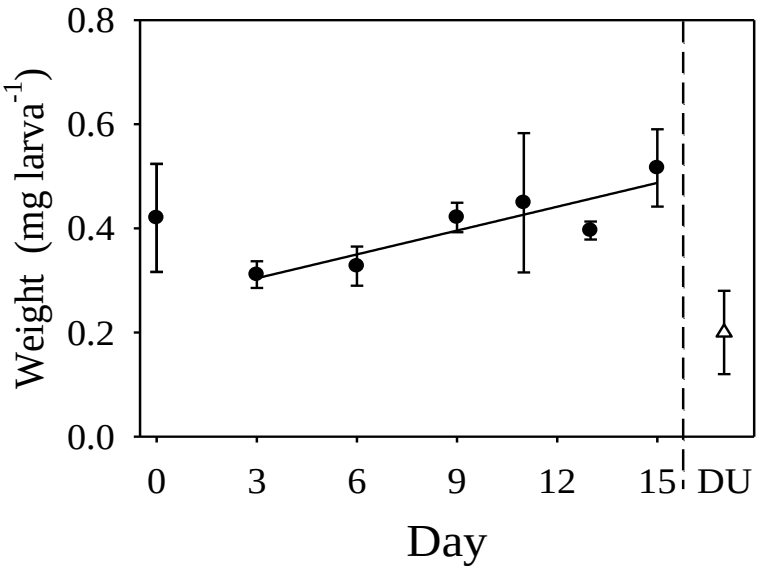

Figure S2. Temporal changes in mean individual dry weights (± SD, $n = 3$-5) of *Chaoborus punctipennis* larvae transplanted from Lake Dasserat to mesocosms in Lake Dufault (closed circles). The comparable value for indigenous Lake Dufault larvae (DU, open triangle) collected on day 9 is given on the right.

12. Subcellular partitioning of non-essential trace metals (Ag, As, Cd, Ni, Pb, and Tl) in livers of American (*Anguilla rostrata*) and European (*Anguilla anguilla*) yellow eels

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Abstract

We determined the intracellular compartmentalization of the trace metals Ag, As, Cd, Ni, Pb and Tl in the livers of yellow eels collected from the Saint Lawrence River system in Canada (*Anguilla rostrata*) and in the area of the Gironde estuary in France (*Anguilla anguilla*). Differential centrifugation, NaOH digestion and thermal shock were used to separate eel livers into putative "sensitive" fractions (heat-denatured proteins, mitochondria and microsomes + lysosomes) and detoxified metal fractions (heat-stable peptides/proteins and granules). The cytosolic heat-stable fraction (HSP) was consistently involved in the detoxification of all trace metals. In addition, granule-like structures played a complementary role in the detoxification of Ni, Pb and Tl in both eel species. However, these detoxification mechanisms were not completely effective because increasing trace metal concentrations in whole livers were accompanied by significant increases in the concentrations of most trace metals in "sensitive" subcellular fractions, that is, mitochondria, heat-denatured cytosolic proteins and microsomes + lysosomes. Among these "sensitive" fractions, mitochondria were the major binding sites for As, Cd, Pb and Tl. This accumulation of non-essential metals in "sensitive" fractions likely represents a health risk for eels inhabiting the Saint Lawrence and Gironde environments.

Keywords: Subcellular partitioning, *Anguilla*, eels, metal toxicity, detoxification

Highlights

- Handling of hepatic metals consistently involved cytosolic, thermostable ligands.
- Granule-like fractions are also involved in the detoxification of Ni, Pb and Tl.
- Despite these sequestration mechanisms, metal detoxification is incomplete.
- Along the metal gradient, concentrations increase in metal-sensitive fractions.
- This increase could represent a toxicological risk for the yellow eels.

Résumé

Nous avons déterminé la compartimentation intracellulaire des métaux traces Ag, As, Cd, Ni, Pb et Tl dans le foie des anguilles jaunes récoltées dans le système du fleuve Saint-Laurent au Canada (*Anguilla rostrata*) et dans la zone de l'estuaire de la Gironde en France (*Anguilla anguilla*). Des étapes de centrifugation différentielle, de digestion avec NaOH et de choc thermique ont été utilisées pour séparer les foies d'anguilles dans les fractions "sensibles" (protéines dénaturées à la chaleur ou *HDP*; mitochondries; microsomes + lysosomes) et les fractions de métaux détoxiqués (protéines thermostables ou *HSP*; granules). La fraction *HSP* était systématiquement impliquée dans la détoxication des métaux étudiés. En outre, les structures granulaires ont joué un rôle complémentaire dans la détoxication de Ni, de Pb et de Tl dans les deux espèces d'anguilles. Cependant, ces mécanismes de détoxication n'ont pas été totalement efficaces parce que les augmentations des concentrations de métaux traces dans les foies des anguilles ont été accompagnées par des augmentations significatives des concentrations de la plupart des métaux traces étudiés dans les fractions subcellulaires "sensibles", incluant les mitochondries, la fraction *HDP* et les microsomes + lysosomes. Parmi ces fractions "sensibles", les mitochondries ont été identifiées comme les principaux sites de liaison pour l'As, le Cd, le Pb et le Tl. Cette accumulation de métaux non essentiels dans les fractions "sensibles" représente probablement un risque pour la santé des anguilles qui peuplent les environnements Saint-Laurent et de la Gironde.

Mots-clés : partitionnement subcellulaire, *Anguilla*, anguilles, toxicité des métaux, détoxication.

Introduction

North Atlantic eel populations (*Anguilla* spp.) have declined drastically over the last 30 years (Haro et al., 2000; ICES, 2011). For *A. anguilla*, which are commercially exploited in European fresh, estuarine and coastal waters, a substantial reduction in their abundance has been reported (Dekker, 2003; ICES, 2011). Recent data for *A. anguilla* have shown that the recruitment of young yellow eels has dropped to about 4000 t/year, which represents less than 10% of the recruitment reported in the 1980s (ICES, 2011). Thus *A. anguilla* are considered to be critically endangered by the International Union for the Conservation of Nature (Dekker, 2003; ICES, 2011). The European Community has implemented a regional eel management plan, involving fishing regulations and the assessment of anthropogenic impacts and eel stocks in most regions (ICES, 2011), including the Gironde estuary.

North American eels have experienced a similar decline in their stocks in recent decades (COSEWIC, 2006; Haro et al., 2000). For example, juvenile eel recruitment in Lake Ontario, Lake Champlain, and the upper Saint Lawrence River has dramatically decreased by as much as 80% to 90% since the 1980s (Haro et al., 2000). In addition, the number of *A. rostrata* eels migrating up the Saint Lawrence River to Lake Ontario decreased 1000-fold between the early 1980s and the mid-1990s (Castonguay et al., 1994). A growing concern for the poor stock status of American eels led to their designation as a "species of special concern" in Canada in 2006 and as an "endangered species" in the province of Ontario (COSEWIC, 2006).

A variety of factors, including habitat loss, migration barriers, overfishing, introduced parasites, and changes in climate and oceanic sea currents, have been suggested as possible causes of the decline in recruitment of both eel species (Castonguay et al., 1994; COSEWIC, 2006; Dekker, 2003). Contaminants have also been included in the list of potential factors leading to these downward trends (Geeraerts and Belpaire, 2010; Maes et al., 2005) since eels are reported to have high concentrations of lipophilic organic contaminants and non-essential trace metals (Bordajandi et al., 2003; Durrieu et al., 2005; Pérez Cid et al., 2001; Pointet and Milliet, 2000; Usero et al., 2004). For example, indigenous *A. rostrata* are reported to be the fish with the highest

concentrations of polychlorinated biphenyls and mercury (Hg) in certain parts of the Saint Lawrence River (Abdelouahab et al., 2008; Hodson et al., 1994). In the Gironde ecosystem, European eels were ranked as the fish species having the second highest concentration of cadmium (Cd) in gills, muscles and kidneys among 7 other fish species (e.g., *Alosa fallax*, *Dicentrarchus labrax*, *Argyrosomus regius*, *Solea vulgaris*) (Durrieu et al., 2005). Since non-essential trace metals can affect physiological functions (Mason and Jenkins, 1995), the accumulation of such contaminants in *Anguilla* spp. might well contribute to their population decline (Pierron et al., 2008a).

Despite reports of contamination of both eel species by trace metals (Geeraerts and Belpaire, 2010; Hodson et al., 1994), the role that these contaminants play in the observed population declines has been little studied. In this context, the determination of the subcellular partitioning of these metals could in principle provide useful diagnostic information about their potential metabolic effects (Campbell and Hare, 2009; Wallace et al., 2003). Such approaches allow one to distinguish between metal accumulation in detoxified metal fractions (e.g., heat-stable proteins and granule-like structures), which do not represent a toxicological risk, from metal binding to physiologically sensitive target molecules (e.g., cytosolic enzymes) and organelles (e.g., mitochondria), where the inappropriate binding of non-essential metals can induce deleterious effects that could compromise the fitness and performance of both eel species.

With this in mind, we determined the subcellular partitioning of silver (Ag), arsenic (As), cadmium (Cd), nickel (Ni), lead (Pb) and thallium (Tl) in the livers of American and European yellow eels collected from the Saint Lawrence and Gironde environments, respectively. A subcellular partitioning procedure using differential centrifugation, NaOH digestion and thermal shock steps was applied to separate the liver samples into putative metal-sensitive fractions (heat-denatured proteins, mitochondria and microsomes + lysosomes) and detoxified-metal fractions (heat-stable proteins and NaOH-resistant granules). Trace metals were then measured in each subcellular fraction and changes in the partitioning between sensitive and detoxified compartment along the metal bioaccumulation gradient were used to assess the extent to which American and European eels are able to detoxify metals effectively.

Material and Methods

Site sampling and fish collection

A. anguilla and *A. rostrata* immature yellow eels were captured in late May and early June 2012 from the Saint Lawrence and Gironde region (Table 1). American eels were collected in the Saint Jean River (48º 51' 40" N; 64º 28' 47" W), the Sud-Ouest River (48º 22' 27" N; 68º 43' 02" W), Lake Saint Pierre (45º 09' 18" N; 74º 23' 04" W) and Lake Saint François (46º 19' 50" N; 72º 32' 06" W), whereas the European eels were collected from three sites in southwest France along the Gironde system (Dordogne: 44° 54' 30" N, 0° 15' 01" W; Garonne: 45° 12' 07" N, 0° 43' 35" W; Gironde estuary: 45° 12' 07" N, 0° 43' 35" W) and from Arcachon Bay, which is considered to be a pristine environment (Certes salt marshes: 44° 41' 18" N; 1° 1' 39" W). The sampling sites were selected on the basis of information on metal concentrations in water, sediments and biota previously reported for the Saint Lawrence River system (Carignan et al., 1994; Desrosiers et al., 2008; Saulnier and Gagnon, 2006) and the Gironde region (Durrieu et al., 2005; Pierron et al., 2008b). Some of the collection sites are highly metal-contaminated (e.g., Lake Saint Pierre, Lake Saint François and Garonne) compared to others that we consider to be reference sites (e.g., Saint Jean, Sud-Ouest and Certes).

Yellow eels from each sampling site were collected either by electrofishing or with fyke nets. After collection, fish were kept alive in well-oxygenated freshwater. Efforts were made to minimize stress on the eels during their capture and handling. Once the total length (± 1 mm) and weight (± 0.1 g) had been determined, the eels were decapitated. The liver was chosen as the target organ because of its importance in the detoxification of toxic substances, and because in previous eel studies the liver was shown to have high concentrations of trace metals (Durrieu et al., 2005; Pierron et al., 2008b). Livers were removed and held in 2-mL cryo-vials (Corning, Sigma-Aldrich, Oakville, ON, Canada), frozen in liquid nitrogen in the field and then kept at -80 ^{o}C in a laboratory freezer until analysis. The American eel capture and sampling protocols were approved by the INRS animal-care committee.

Subcellular partitioning procedure

We separated liver samples into six operationally-defined subcellular fractions (Fig. 1), that is, nuclei + debris; granule-like; mitochondria; microsomes + lysosomes; heat-denatured proteins (HDP) including cytosolic enzymes; and heat-stable proteins and peptides (HSP) such as metallothionein (MT) and glutathione (GSH). This fractionation procedure was adapted from protocols described by Giguère et al. (2006), Wallace et al. (2003) and Lapointe et al. (2009). The efficacy of this protocol was assessed by determining the distribution of marker enzymes located specifically in mitochondria (cytochrome C oxidase) or in the cytosol (lactate dehydrogenase) (Rosabal et al., 2014); these enzymes were measured in the subcellular fractions obtained from liver samples (n = 3) of *A. rostrata* (see Supplementary data, Fig. SI-7).

From each individual, 200 mg of hepatic tissue was chopped manually using a surgical steel razor blade and then homogenized in 1.5 mL of a solution of Tris (10 mM; OmniPur, EM Science, Darmstadt, Hesse, Germany; pH = 7.4) and sucrose (250 mM; Fisher Scientific, Whitby, ON, Canada) using five low-speed passages on a motorized Potter-Elvehjem homogenizer (Lapointe et al., 2009) equipped with a Teflon pestle (Fisher Scientific, Whitby, ON, Canada). A 100-µL aliquot was removed from the liver homogenate for determining total trace metal concentrations in the liver and for mass-balance quality-control measurements. The remainder of the liver homogenate was centrifuged at 800 × *g* for 15 min at 4 ºC. The supernatant (S1) was transferred to a pre-weighed and acid-washed 1.5 mL polypropylene microcentrifuge tube for further separations. The pellet from this centrifugation step (P1) was suspended in 0.5 mL of ultrapure water, heated at 100 ºC for 2 min, digested with an additional 500 µL of 1 M NaOH (99.998%, Sigma-Aldrich, Oakville, ON, Canada) at 65 ºC for 60 min. Centrifugation at 10,000 × *g* for 10 min at ambient temperature (~ 20 ºC) was performed to separate the NaOH-resistant fraction (P2, referred to henceforth as "granule-like") from the nuclei + debris fraction (S2) that includes cell membranes, unbroken cells and nuclei.

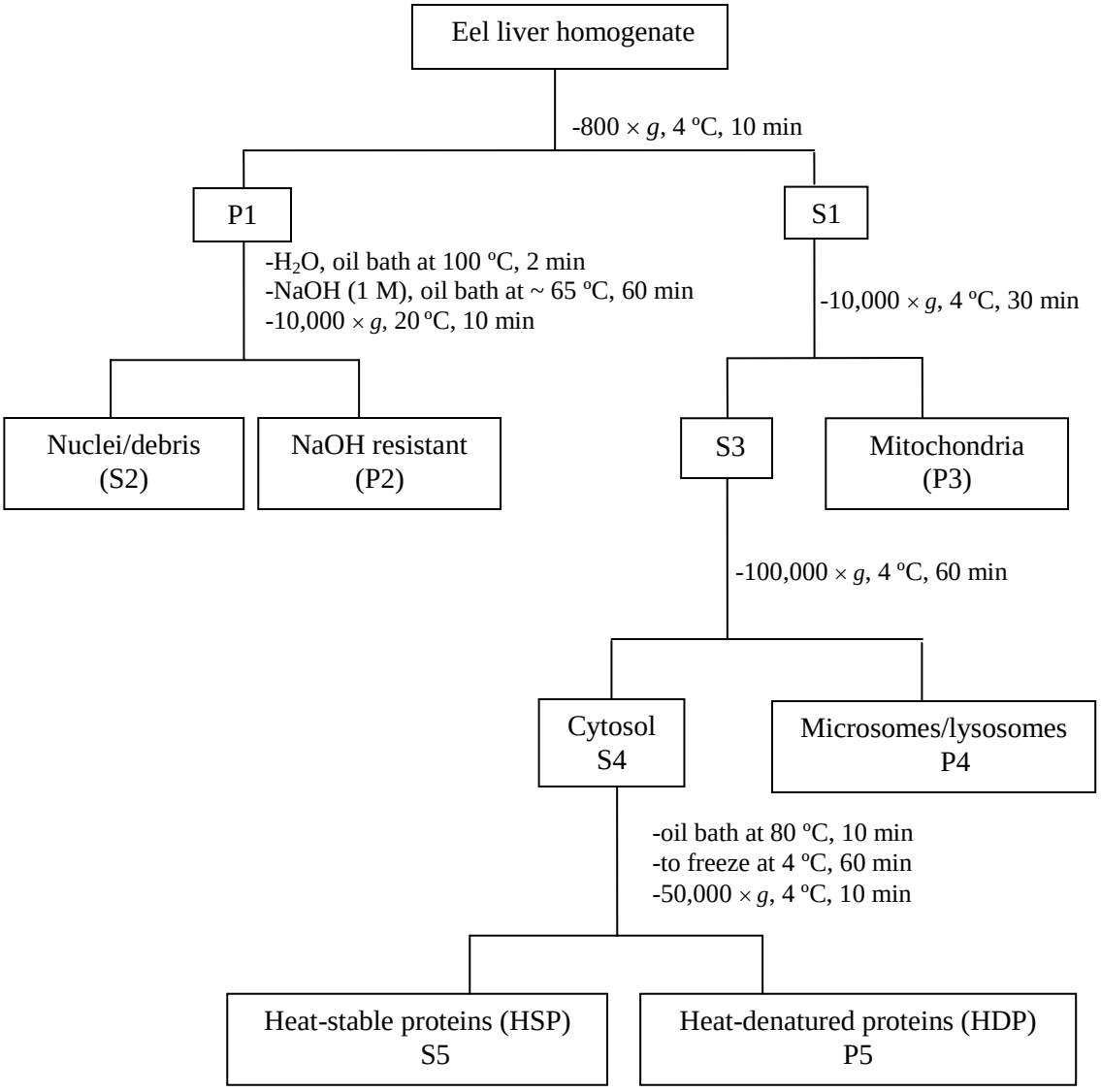

Fig. 1. Partitioning procedure used to separate the livers of *Anguilla anguilla* and *Anguilla rostrata* into subcellular fractions.

The supernatant from the original homogenisation step (S1) was centrifuged at 10,000 × *g* for 30 min at 4°C to yield the mitochondrial fraction (P3). The resulting supernatant (S3) was subjected to an ultracentrifugation step at 100,000 × *g* for 60 min at 4 ºC, giving a pellet containing other organelles (microsomes and lysosomes) and the cytosolic fraction in the supernatant. To separate the heat-stable peptides and proteins

(HSP) from the heat-denatured proteins (HDP), the cytosolic fraction (S4) was held at 80°C for 10 min, left on ice for 1 hour and then centrifuged at 50,000 × *g* for 10 min at 4 ºC. The HSP fraction, which includes MT and metallothionein-like proteins (MTLP) was collected from the supernatant.

High-speed centrifugations (≥ 50,000 × *g*) were performed using a WX ULTRA 100 centrifuge (Sorval, Ultra Thermo Scientific, Whitby, ON, Canada) equipped with a F50L-24 X1.5 rotor (Fisher Scientific, Whitby, ON, Canada), whereas lower-speed centrifugations were performed using an IEC Micromax centrifuge (Thermo IEC, Arlington, MA, USA). Each supernatant was acidified with nitric acid (final [HNO_3] 10%, v/v; Optima grade, Fisher Scientific, Whitby, ON, Canada) and kept at 4 ºC until the metal analyses were performed. Pellets were frozen at -80 ºC until the freeze-drying and trace metal analysis steps.

Trace metal measurements and quality control

All lab-ware was soaked in 15% nitric acid solution and rinsed seven times in ultrapure water before use, to minimize accidental metal contamination. Samples for total metal analysis (aliquots from the liver homogenates) and centrifuged pellets obtained in the subcellular partitioning procedure were freeze-dried (FTS Systems TMM, Kinetics Thermal Systems, Longueil, QC, Canada) for 72 h and weighed (XS205 DualRange Analytical Balance, Mettler Toledo, Mississauga, ON, Canada) to determine the dry weight (dw). The freeze-dried samples were digested at room temperature in 100 µL of nitric acid (70%, v/v, Optima grade, Fisher Scientific, Whitby, ON, Canada) per mg of sample (dw) for 3 days at room temperature, heated at 65 ºC for 6 h and then cooled before addition of hydrogen peroxide (30%, v/v; Optima grade, Fisher Scientific, Whitby, ON, Canada) at a ratio of 40 µL per mg of sample (dw). The final digestion volume was completed to 1 mL per mg of sample (dw) with ultrapure water. For the supernatants (HSP and nuclei/debris fraction), a similar digestion procedure was performed, with the approximation that 1 mL of each liquid sample represented 1 mg of tissue (dw).

Concentrations of Ag, As, Cd, Ni, Pb and Tl in liver homogenates and in all subcellular fractions were determined with an inductively coupled plasma-mass spectrometer (ICP-MS; Thermo Elemental X Series, Winsford, England, UK). Note that

although we refer to these elements as "metals", arsenic is, strictly speaking, a metalloid. Concurrently, samples of similar weight of two certified standard reference materials, TORT-2 (lobster hepatopancreas, National Research Council of Canada, NRCC, Halifax, NS, Canada) and DORT-4 (Dogfish liver, National Research Council of Canada, NRCC, Halifax, NS, Canada) were subjected to the same digestion procedure and then analyzed. Mean recoveries (± SD) of TORT-2 reference samples (n = 4) were within the certified ranges for As (107% ± 3%), Cd (101% ± 3%), Ni (95 % ± 3%) and Pb (88 % ± 2 %). For the DORT-4 reference samples, mean recoveries (± SD; n = 3-4) were also within the certified ranges for Ag (94% ± 2%), As (105% ± 2%), Cd (91% ±2%), Ni (101% ± 2%) and Pb (86% ± 2%). Note that we found no suitable certified reference material for which Tl concentrations are given.

The 100-µL aliquots collected from the first homogenization step were analyzed to verify metal recovery following subcellular partitioning. Recovery was calculated based on the ratio of the sum of the burdens of a given metal in the six fractions divided by the total metal burden in the 100-µL aliquot, multiplied by 100. Given the wide range in recovery values among individual fish, we included only individuals for which this value was between 60% and 150% for all metals studied (see footnote b in Table 1). Mean (± SD) recovery values (after rejecting values outside the 60-150% range) for each metal were close to 100% for both *A. anguilla*: Ag, 82% ± 15%, n = 8; As, 110 ± 15%, n = 22; Cd, 96% ± 14%, n = 25; Ni, 96% ± 21%, n = 11; Pb, 111% ± 15%, n = 25; Tl, 111% ± 19%, n = 24; and *A. rostrata*: Ag, 97% ± 18%, n = 20; As, 114 ± 13%, n = 25; Cd, 99% ± 15%, n = 28; Ni, 116% ± 16%, n = 28; Pb, 108% ± 23%, n = 27; Tl, 100% ± 15%, n = 26.

Calculations and statistical analysis.

Trace metal concentrations in all subcellular fractions were expressed as the total trace metal burden (nmol) divided by the total liver dry weight (g, dw). The relative contribution of each subcellular fraction to the total metal burden was estimated as a ratio defined by the metal burden in each fraction divided by the sum of the metal burdens in

all fractions, multiplied by 100 to give results in terms of percentages (%). Ratios of wet weight : dry weight were calculated for each sample.

All numerical data are represented by means ± standard deviations (SD). Relationships between total liver metal concentrations and metal concentrations or relative metal contributions (%) in a given subcellular fraction were initially examined in bivariate scatterplots and tested by simple correlation (Pearson r) after verifying the assumption of normality (Shapiro-Wilk test). For percentages (relative contribution of each subcellular fraction to the total metal burden), the data were arcsine transformed before parametric statistical analysis. Note that our statistical analyses were performed considering all individual fish for each species and not the sampling sites where they were collected. When bivariate plots indicated a possible relationship, regression models (e.g.; linear, exponential) were tested when the necessary assumptions were satisfied. Extra sum-of-square F-tests were used to compare the slopes obtained from linear regression analysis whenever the requirement of equality of residual variances was satisfied. When the total metal concentration ranges differed notably between *A. anguilla* and *A. rostrata* (e.g., for As, Ni and Pb), we re-ran our statistical analyses over similar ranges of metal concentrations (i.e., after truncating the wider concentration range so that it corresponded to the range covered by the less contaminated species). Similarly, when the distribution of the analytical data suggested that one or more points might be exerting an undue influence on the regression coefficient, we checked that the regressions remained significant after elimination of the "leverage points".

Principal component analysis (PCA) was used to infer similarity among the partitioning of metals in the detoxified and sensitive compartments. Data were grouped for each analysis into a two-component model, which accounted for 48-59% of the variance. Statistical analyses were performed using STATISTICA version 6.1 software (StatSoft, Tulsa, OK, USA) and the significance threshold used was $P < 0.05$.

Results

Metal accumulation in the eel liver

Liver metal concentrations did not vary significantly with the age, length or weight of the eel species studied. The whole livers of European *A. anguilla* had higher concentrations of most metals (Ag, As, Cd and Pb) than did those of North American *A. rostrata*, with the exception of Ni for which eels collected from the Saint Lawrence River system had higher concentrations than those collected in France (Table 1). Furthermore, the ratios of maximum to minimum metal concentrations ($[M]_{max}$ / $[M]_{min}$) were higher for European eels than for their American counterparts for all of the metals studied (Table 1). Ratios for *A. anguilla* decreased in the order Cd > As > Ag > Tl > Ni = Pb, whereas those for *A. rostrata* decreased in the order: Cd > Tl > As > Ni > Ag > Pb.

Table 1. Range (minimum – maximum) trace metal concentrations (nmol g^{-1} dw) in the liver of yellow eels collected in Europe (*Anguilla anguilla*) and North America (*Anguilla rostrata*). Also given are ranges in eel age, length and weight[a].

Variable	Trace metals					
	Ag	As	Cd	Ni	Pb	Tl
Anguilla anguilla (France)						
number of samples (n)[b]	8	22	25	11	25	24
[M] range (nmol g^{-1} dw)	0.9 - 24	5.2 - 320	0.2 - 40	1.1 - 24	0.6 - 13	0.018 - 0.44
$[M]_{max} / [M]_{min}$	27	63	200	22	22	24
age range (years)	5.0 - 15	3.0 - 15	3.0 - 18	3.0 - 15	3.0 - 18	3.0 - 18
length range (mm)	390 - 500	390 - 600	350 - 690	390 - 500	350 - 690	350 - 690
weight range (g)	78 - 230	73 - 400	73 - 480	97 - 240	78 - 480	74 - 480
Anguilla rostrata (Canada)						
number of samples (n)	20	25	28	28	27	26
[M] range (nmol g^{-1} dw)	1.7 - 16	4.4 - 44	0.8 - 11	7.4 - 73	0.4 - 3.2	0.024 - 0.44
$[M]_{max} / [M]_{min}$	9.4	10	103	9.8	8.0	18
age range (years)	6.0 - 16	6.0 - 16	6.0 - 16	6.0 - 16	6.0 - 16	6.0 - 16
length range (mm)	310 - 830	310 - 830	310 - 910	310 - 910	310 - 910	310 - 910
weight range (g)	80 - 1000	50 - 1100	50 - 1400	50 - 1400	50 - 1400	50 - 1400

[a]Note that in comparisons among metals for a given eel species, the number of livers varied for different metals, but the ranges of total length, body weight and age were the same for all metals. Maximum total length and body weight were however higher for the *A. rostrata* specimens than for those of *A. anguilla*.

[b]n represents the number of individual fish (from the 30 individual fish were initially analysed) for which the mass balance recovery was between 60% and 150% for each metal studied.

Metal subcellular partitioning

To describe how the metal concentrations in each subcellular fraction varied along the bioaccumulation gradients, metal concentrations (calculated with reference to total liver dry weight) in each detoxified metal fraction (HSP and granule-like fractions; panels A and C) and in each potentially metal-sensitive fraction (mitochondria, HDP and microsomes + lysosomes; panels B and D) were plotted against the total metal concentration (X-axis) in the liver homogenate (Figs. 2-7). Below, we discuss these results metal by metal. We also examined relationships between the relative contributions of each subcellular fraction (%) and total liver metal concentrations, as presented in the Supplementary data (Figures SI-1 to SI-6). To explore the behaviour of metals in the detoxified compartment ("Detoxified": sum of metal accumulated in the HSP and granule-like fractions, DMF) and the metal-sensitive compartment ("Sensitive": sum of metal accumulated in the mitochondria, HDP and microsomes + lysosomes fractions, MSF), and to determine the strongest response along the metal bioaccumulation gradients, we also compare the slopes of the relationships Detoxified *vs.* $[M]_{liver}$ and Sensitive *vs.* $[M]_{liver}$, and the slopes for the individual fractions.

Since the nuclei + debris fraction does not fit neatly into either the detoxified or sensitive categories, this fraction is generally ignored (cf., Cain et al. (2004); Cooper et al. (2010)). However, the relative contribution of the nuclei + debris fraction can be used as an indication of the efficacy of the homogenization procedure; a low and constant percentage contribution indicates that the homogenization step was both effective and reproducible. If the relative contribution of this fraction varied along the bioaccumulation gradient for a given metal (but not for the others) and where a correlation was observed between the metal concentration in this fraction and metal levels found in the putative metal-sensitive fractions, we assumed that this trend may be an indication that the given metal is associated with the nuclei rather than with general cellular debris. Only when this was the case we did consider the contribution of the nuclei + debris fraction.

Ag (silver)

The range in liver Ag concentrations was similar for the two species of eels (Fig. 2, Table 1).

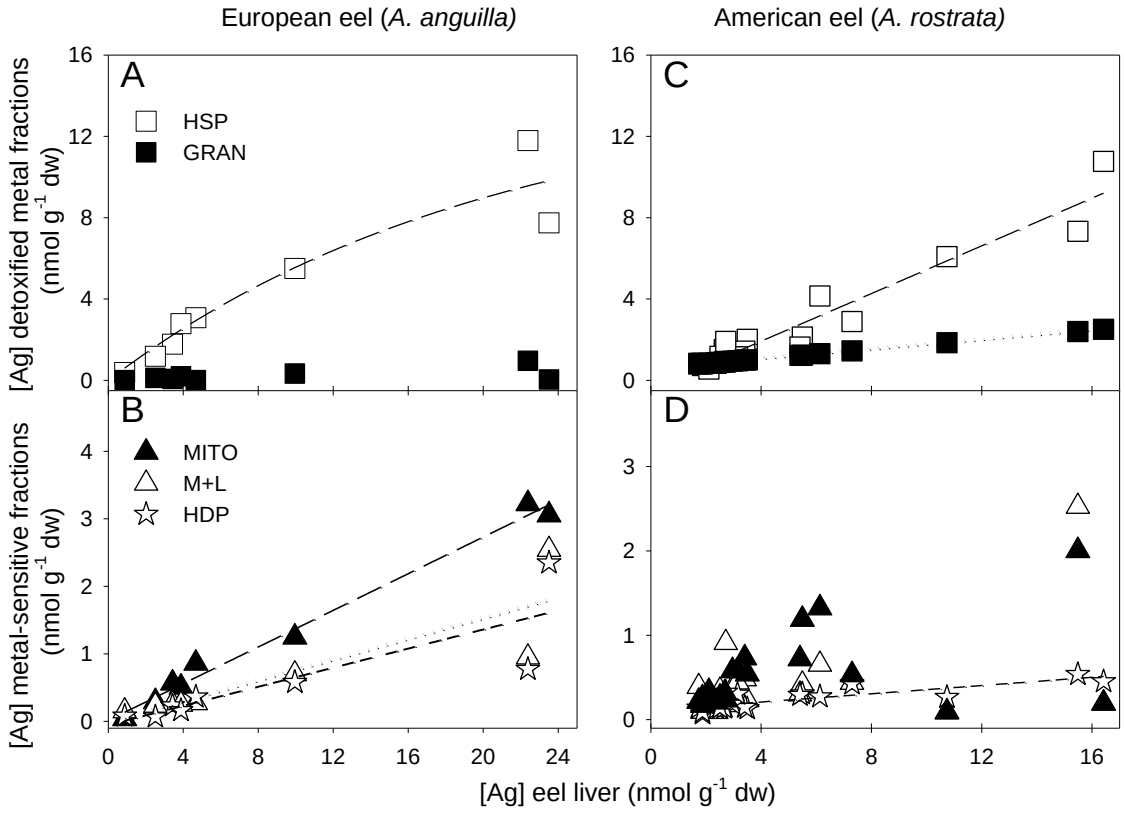

Fig. 2. Relationships between total hepatic Ag concentrations (horizontal axis) and Ag concentrations in various subcellular fractions (vertical axis) in *Anguilla anguilla* (left panels) and *Anguilla rostrata* (right panels) for detoxified metal fractions (panels A and C) and for metal-sensitive fractions (panels B and D). Each point represents an individual eel. Fraction abbreviations: HSP, heat-stable proteins; GRAN, NaOH-resistant granules; MITO, mitochondria; M+L, microsomes + lysosomes; HDP, heat-denatured proteins. Lines represent significant regressions ($P < 0.05$).

Silver concentrations in all subcellular fractions, except the granule-like fraction, increased significantly ($P < 0.003$) with increasing Ag concentrations in whole livers of *A. anguilla* (Figs. 2 A, B). The relationship between Ag concentrations in the HSP fraction and total liver Ag (Fig. 2 A) was best described by an exponential curve ($r^2 = 0.91$) rather than by a straight line (slope: 0.40 ± 0.06; $r^2 = 0.71$). The relative

contribution (%) of the HSP fraction ranged from 32% to 57% (mean 45 ± 9%) of the total. Among metal-sensitive fractions, significant increases in Ag concentrations were observed for the mitochondria (slope: 0.136 ± 0.006), microsomes + lysosomes (slope: 0.07 ± 0.02) and the HDP (slope: 0.07 ± 0.02) fractions (Fig. 2 B). Silver concentrations in the nuclei + debris fraction also increased slightly with increasing total hepatic Ag concentrations (slope: 0.04 ± 0.01). The increase in Ag concentrations in the detoxified metal compartment (slope: 0.42 ± 0.07) was significantly stronger ($P < 0.05$) than that of the metal-sensitive compartment (slope: 0.28 ± 0.03).

In *A. rostrata*, Ag concentrations in both the cytosol and the granule-like fraction increased ($P < 0.03$) as the total hepatic Ag concentrations increased (Figs. 2C, D). The response of the HSP fraction (slope: 0.59 ± 0.04) was significantly higher ($P < 0.05$) than that of the granule-like (slope: 0.11 ± 0.02) and HDP fractions (slope: 0.025 ± 0.004). The relative contribution of the HSP fraction increased significantly from 32% to 72% along the Ag bioaccumulation gradient ($r^2 = 0.33$; $P = 0.008$), whereas the contribution of the HDP fraction decreased from 9.6% to 3.0% ($r^2 = 0.28$; $P = 0.02$; Supporting data, Fig. SI-1). The increase in Ag concentrations in the metal-detoxified compartment (slope: 0.70 ± 0.05) was greater ($P < 0.05$) than that observed for the metal-sensitive compartment (slope: 0.12 ± 0.05).

As (arsenic)

The bioaccumulation gradient for As was considerably higher for *A. anguilla* (5.2 – 320 nmol g^{-1}) than for *A. rostrata* (4.4 – 44 nmol g^{-1}). For both eel species, the As concentrations in all subcellular fractions increased significantly ($P < 0.003$) as the total hepatic As concentrations increased, with the exception of the granule-like fraction in *A. rostrata* (Fig. 3C). Along the As bioaccumulation gradient, the increase in As concentrations in the metal-sensitive fractions followed the same increasing order for both eel species: HDP fraction (*A. anguilla*, slope: 0.08 ± 0.01; *A. rostrata*, slope: 0.12 ± 0.03) < microsomes + lysosomes (*A. anguilla*, slope: 0.12 ± 0.03; *A. rostrata*, slope: 0.20 ± 0.03) < mitochondria (*A. anguilla*, slope: 0.17 ± 0.02; *A. rostrata*, slope: 0.29 ± 0.03) (Figs. 3B, D). The increase in As concentrations in the HSP fraction (slope: 0.42 ± 0.05) of European eels was significantly ($P < 0.05$) greater than the increases observed

for the three putative metal-sensitive fractions and for the granule-like fraction (slope: 0.03 ± 0.01) (Fig. 3 A, B). In contrast, in American eels the increase along the bioaccumulation gradient observed for the HSP fraction (slope: 0.09 ± 0.03) was significantly lower ($P < 0.05$) than those reported for the individual metal-sensitive fractions (Figs. 3C, D).

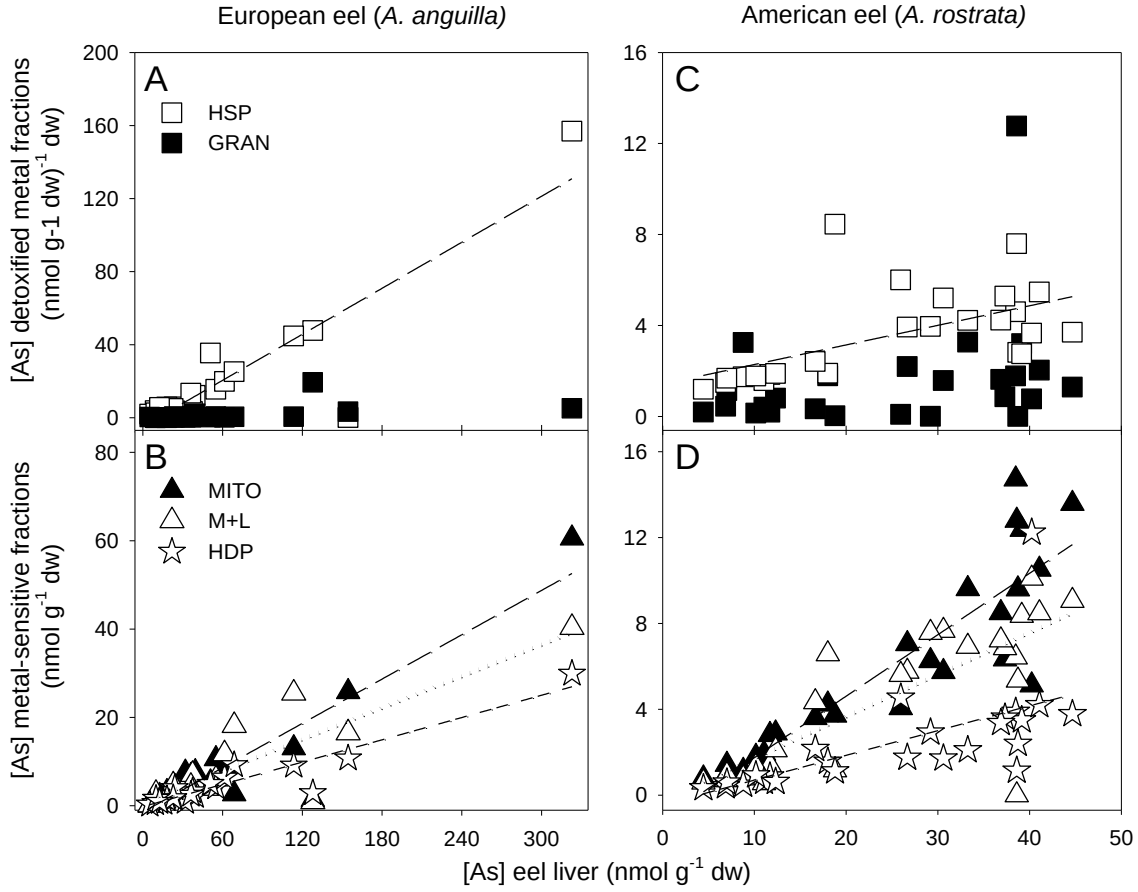

Fig. 3. Relationships between total hepatic As concentrations (horizontal axis) and As concentrations in various subcellular fractions (vertical axis) in *Anguilla anguilla* (left panels) and *Anguilla rostrata* (right panels) for detoxified metal fractions (panels A and C) and for metal-sensitive fractions (panels B and D). Each point represents an individual eel. Fraction abbreviations: HSP, heat-stable proteins; GRAN, NaOH-resistant granules; MITO, mitochondria; M+L, microsomes + lysosomes; HDP, heat-denatured proteins. Lines represent significant regressions ($P < 0.05$).

For eels collected in France, the increase in As concentrations in the detoxified-metal compartment (slope: 0.45 ± 0.06) was similar to that for the metal-sensitive compartment (slope: 0.37 ± 0.04). In contrast, the increase in As concentrations observed

for the metal-sensitive compartment (slope: 0.55 ± 0.07) for eels from the Saint Lawrence system was significantly higher ($P < 0.05$) than that found for the detoxified-metal compartment (slope: 0.15 ± 0.05). In *A. rostrata*, the relative contribution (%) of the HSP fraction to the total As burden significantly decreased along the As bioaccumulation gradient (slope: -0.003 ± 0.001), whereas the contribution of the metal-sensitive compartment significantly increased (slope: 0.007 ± 0.003).

Cd (cadmium)

The bioaccumulation gradient for Cd was higher for the European eels (0.2 – 40 nmol g^{-1}) than for their American counterparts (0.8 – 11 nmol g^{-1}). With the exception of the granule-like fraction, Cd concentrations in all subcellular fractions were significantly ($P < 0.001$) related to the total hepatic Cd concentration for both species (Fig. 4). The response of the HSP fraction was significantly stronger ($P < 0.05$) than those observed for the other subcellular fractions in both *A. anguilla* (slope: 0.57 ± 0.04; Figs. 4A, B) and *A. rostrata* (slope: 0.59 ± 0.03, Figs. 4C, D). The relative contribution of the HSP fraction to the total hepatic Cd burden varied from 43% to 76% in the European eels and from 44% to 76% in the American eels (Supporting information).

The increase in Cd concentrations in the mitochondrial fraction was higher ($P < 0.05$) than that observed for the microsomes + lysosomes and HDP fractions in *A. anguilla* (slope: 0.13 ± 0.02) and *A. rostrata* (slope: 0.09 ± 0.02). For both eel species (Figs. 4 B, D), no significant difference ($P > 0.05$) was found between the increase in Cd concentrations in the microsomes + lysosomes fraction (*A. anguilla,* slope: 0.05 ± 0.01; *A. rostrata,* slope: 0.05 ± 0.02) and in the HDP fraction (*A. anguilla,* slope: 0.04 ± 0.01; *A. rostrata,* slope: 0.04 ± 0.01). A similar increase in Cd concentrations was reported in the detoxified-metal compartment (*A. anguilla,* slope: 0.59 ± 0.04; *A. rostrata,* slope: 0.63 ± 0.03) and in the metal-sensitive compartment (*A. anguilla,* slope: 0.22 ± 0.02; *A. rostrata,* slope: 0.21 ± 0.03), for both eel species. In both cases, Cd accumulation in the subcellular compartment involved in metal detoxification was significantly higher ($P < 0.05$) than the increase observed for the putative metal-sensitive compartment.

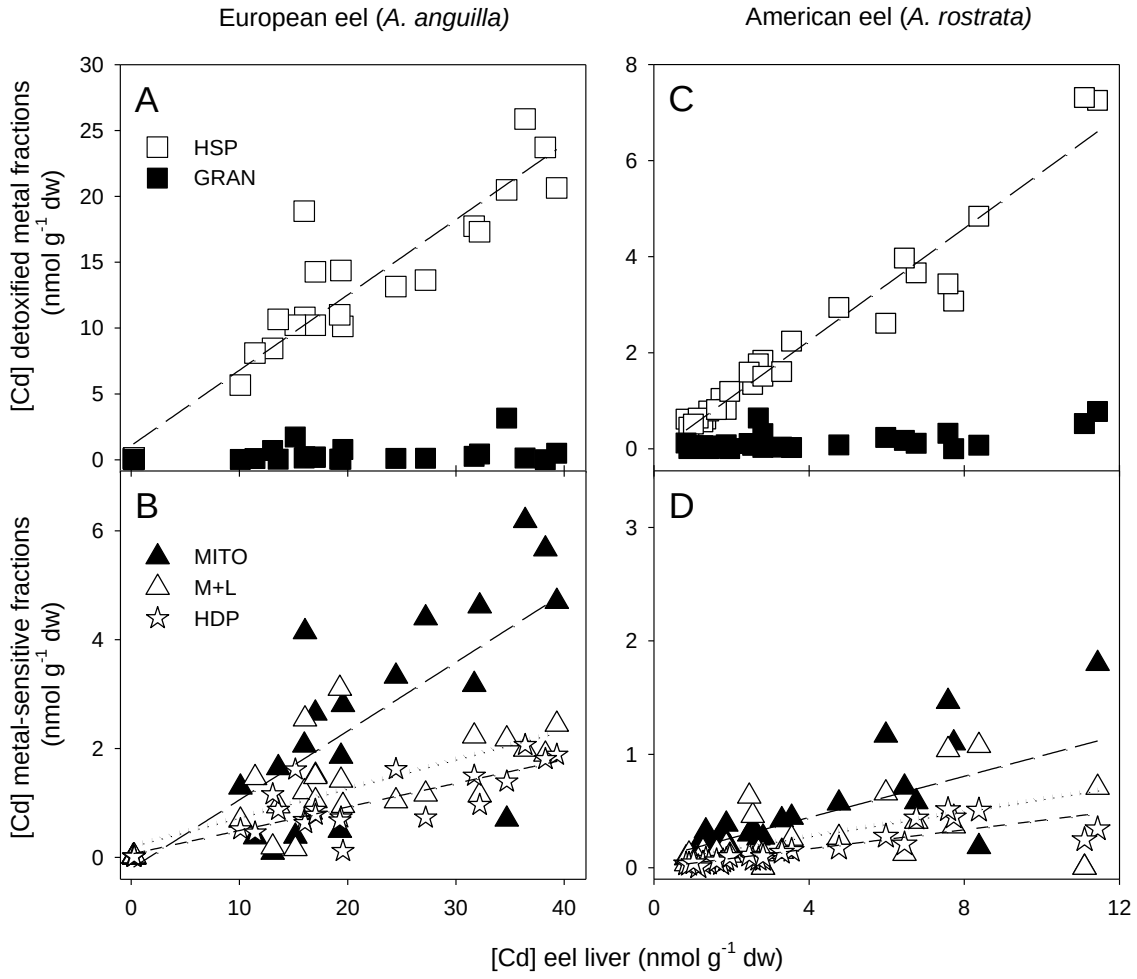

Fig. 4. Relationships between total hepatic Cd concentrations (horizontal axis) and Cd concentrations in various subcellular fractions (vertical axis) in *Anguilla anguilla* (left panels) and *Anguilla rostrata* (right panels) for detoxified metal fractions (panels A and C) and for metal-sensitive fractions (panels B and D). Each point represents an individual eel. Fraction abbreviations: HSP, heat-stable proteins; GRAN, NaOH-resistant granules; MITO, mitochondria; M+L, microsomes + lysosomes; HDP, heat-denatured proteins. Lines represent significant regressions ($P < 0.05$).

Ni (nickel)

In contrast to the other elements considered in this study, the bioaccumulation gradient for Ni was lower for the European eels (1.1 – 24 nmol g^{-1}) than for American eels collected in the Saint Lawrence River system (7.4 – 73 nmol g^{-1}). Nickel concentrations in all subcellular fractions increased ($P < 0.004$) with increasing total hepatic Ni accumulation in both eel species (Fig. 5). Within the detoxified-metal fractions

of *A. anguilla* (Fig. 5A), the increase in Ni concentrations in the granule-like fraction (slope: 0.45 ± 0.01) was higher ($P < 0.05$) than that in the HSP fraction (slope: 0.10 ± 0.02). However, in the interval from 0 to 25 nmol Ni g^{-1} dw, the increase observed for the granule-like fraction was not significant. In contrast, for *A. rostrata* yellow eels (Fig. 5C), there was no difference in Ni concentrations ($P > 0.05$) between the HSP fraction (slope: 0.17 ± 0.01) and the granule-like fraction (slope: 0.16 ± 0.08).

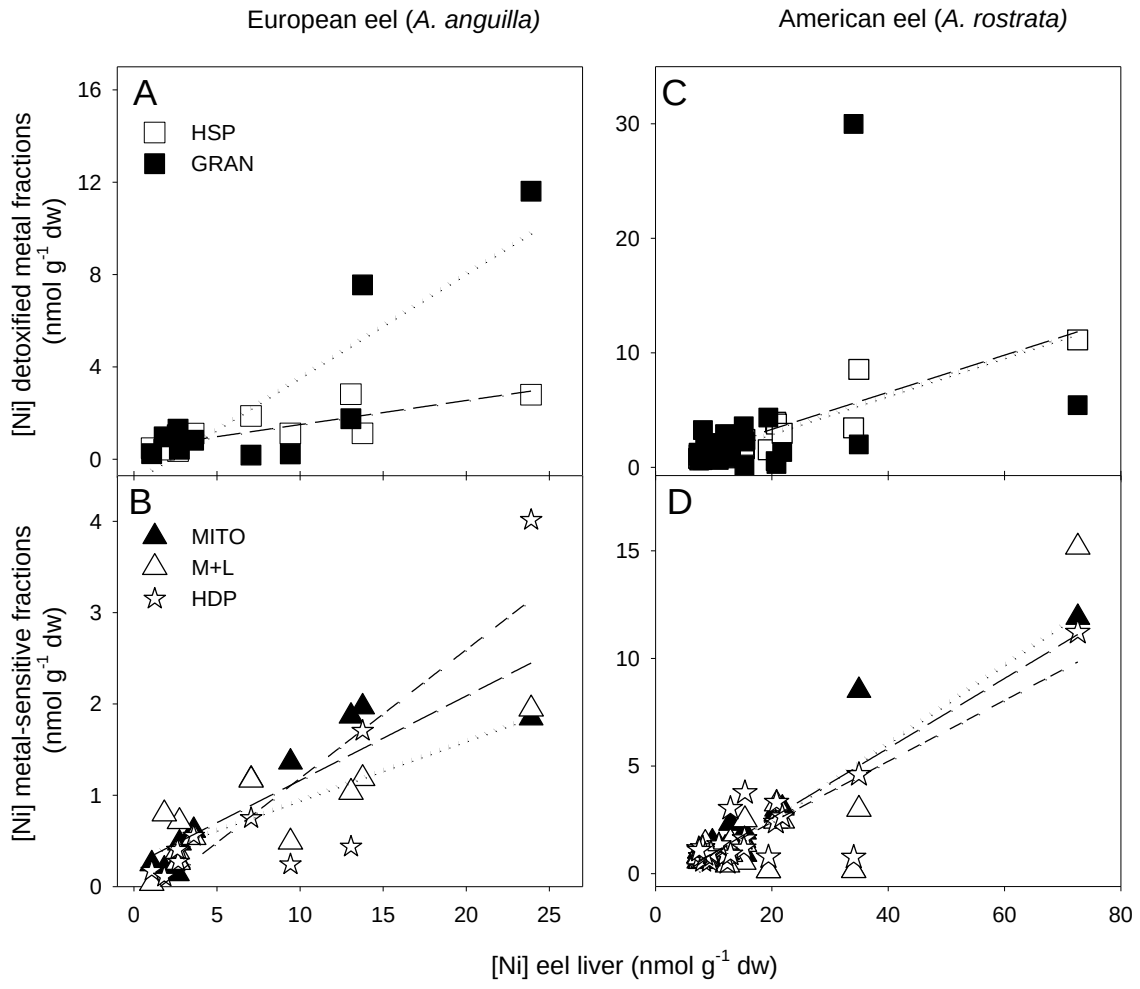

Fig. 5. Relationships between total hepatic Ni concentrations (horizontal axis) and Ni concentrations in various subcellular fractions (vertical axis) in *Anguilla anguilla* (left panels) and *Anguilla rostrata* (right panels) for detoxified metal fractions (panels A and C) and for metal-sensitive fractions (panels B and D). Each point represents an individual eel. Fraction abbreviations: HSP, heat-stable proteins; GRAN, NaOH-resistant granules; MITO, mitochondria; M+L, microsomes + lysosomes; HDP, heat-denatured proteins. Lines represent significant regressions ($P < 0.05$).

Among the metal-sensitive fractions, the Ni concentrations in yellow eels collected from the French sampling sites increased in the following order: microsomes + lysosomes (slope: 0.07 ± 0.01) ~ mitochondria (slope: 0.09 ± 0.01) < HDP (slope: 0.14 ± 0.03) (Fig. 5B). In contrast, no significant differences ($P > 0.05$) were observed among the metal-sensitive fractions in the American yellow eels: HDP (slope: 0.14 ± 0.01) ~ mitochondria (slope: 0.16 ± 0.02) ~ microsomes + lysosomes (slope: 0.18 ± 0.02) (Fig. 5D).

Total Ni accumulation in the metal-sensitive compartment (slope: 0.49 ± 0.05) was higher than the increase in Ni concentrations observed for the detoxified-metal compartments (slope: 0.33 ± 0.07) in eels from the Saint Lawrence River. For the European eels, however, the response of the metal-sensitive compartment (slope: 0.30 ± 0.03) was lower than that of the compartment involved in Ni detoxification (slope: 0.55 ± 0.08).

Pb (lead)

The bioaccumulation gradient for Pb was higher for *A. anguilla* (0.6 – 13 nmol g^{-1}) than for *A. rostrata* (0.4 – 3.2 nmol g^{-1}). The increase in Pb concentrations in the detoxified-metal compartment (*A. anguilla*, slope: 0.31 ± 0.03; *A. rostrata*, slope: 0.6 ± 0.1) was significantly higher than the increase in Pb concentrations in the metal-sensitive compartment (*A. anguilla*, slope: 0.34 ± 0.03; *A. rostrata*, slope: 0.19 ± 0.05) in yellow eels from both species (Fig. 6). The granule-like fraction (*A. anguilla*, slope: 0.25 ± 0.04; *A. rostrata*, slope: 0.6 ± 0.1) showed the highest increase in Pb concentrations among all the subcellular fractions (Figs. 6A, C). For both eel species, the Pb concentrations in the other subcellular fractions increased ($P < 0.01$) along the Pb bioaccumulation gradient in the increasing order: HDP (*A. anguilla*, slope: 0.07 ± 0.01; *A. rostrata*, slope: 0.020 ± 0.001) < microsomes + lysosomes (*A. anguilla*, slope: 0.08 ± 0.01; *A. rostrata*, slope: 0.05 ± 0.02) < HSP (*A. anguilla*, slope: 0.10 ± 0.01; *A. rostrata*, slope: 0.52 ± 0.01) < mitochondria (*A. anguilla*, slope: 0.17 ± 0.01; *A. rostrata*, slope: 0.11 ± 0.03) (Fig. 6). Note that with the notable exception of the HSP fraction, these slopes were always higher for the European eels than for the American eels.

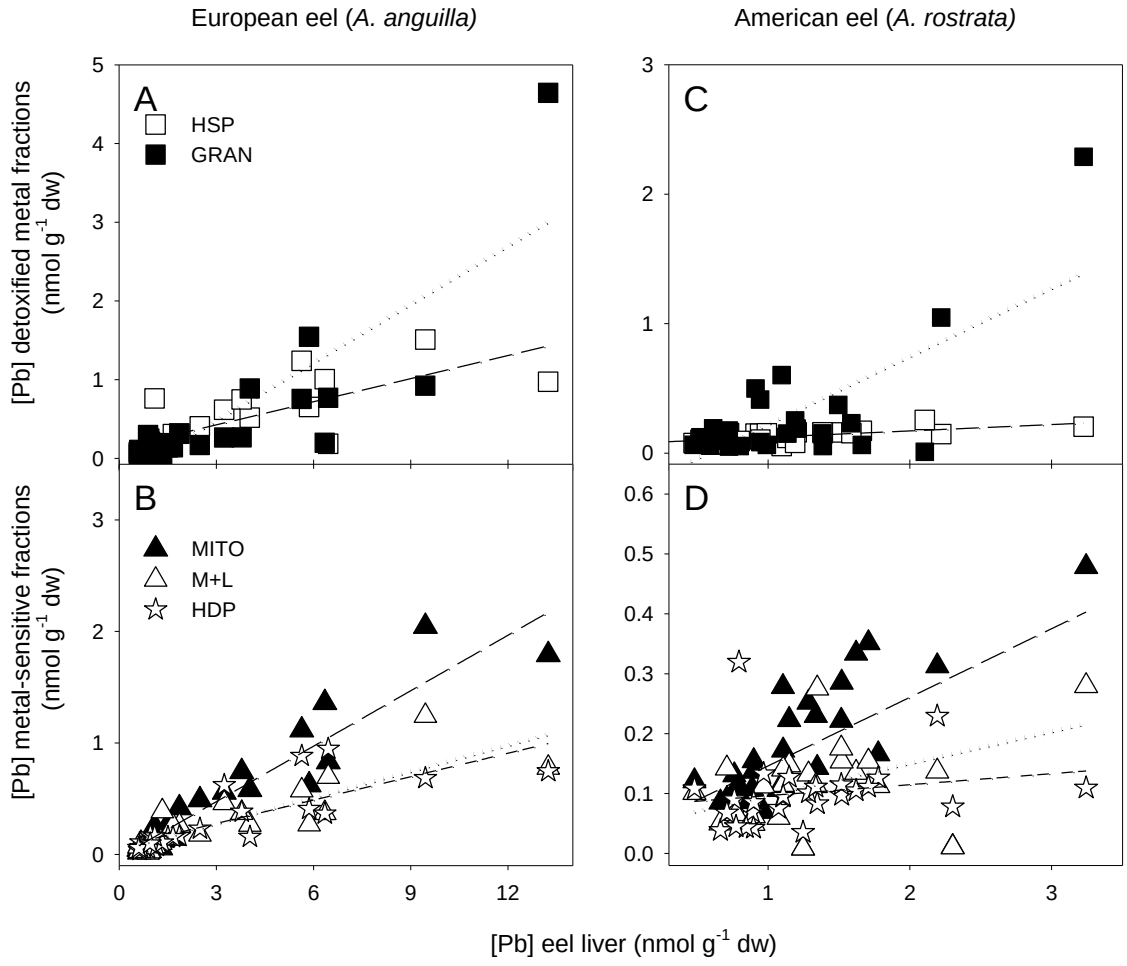

Fig. 6. Relationships between total hepatic Pb concentrations (horizontal axis) and Pb concentrations in various subcellular fractions (vertical axis) in *Anguilla anguilla* (left panels) and *Anguilla rostrata* (right panels) for detoxified metal fractions (panels A and C) and for metal-sensitive fractions (panels B and D). Each point represents an individual eel. Fraction abbreviations: HSP, heat-stable proteins; GRAN, NaOH-resistant granules; MITO, mitochondria; M+L, microsomes + lysosomes; HDP, heat-denatured proteins. Lines represent significant regressions ($P < 0.05$).

Tl (thallium)

The bioaccumulation gradient for thallium was virtually identical for the two study areas: *A. anguilla* (0.018 – 0.44 nmol g^{-1}) and *A. rostrata* (0.024 – 0.44 nmol g^{-1}). In both eel species, the Tl concentrations in each subcellular fraction were significantly ($P < 0.0001$) and linearly related to the total hepatic Tl concentration (Fig. 7), with the exception of the granule-like fraction (Figs. 7A, C), for which a sigmoidal relationship was observed along the Tl bioaccumulation gradient for both eel species (*A. anguilla*, $r^2 = 0.33$, $P < 0.0001$; *A. rostrata*, $r^2 = 0.66$, $P < 0.0001$). Thallium concentrations in the

HSP fraction (*A. anguilla*, slope: 0.30 ± 0.02; *A. rostrata*, slope: 0.29 ± 0.02) responded more strongly to the bioaccumulation gradient than the other subcellular fractions (Fig. 7). Among the metal-sensitive fractions, the increase in Tl concentrations was in the order: HDP (*A. anguilla*, slope: 0.040 ± 0.003; *A. rostrata*, slope: 0.030 ± 0.004) < microsomes + lysosomes (*A. anguilla*, slope: 0.12 ± 0.01; *A. rostrata*, slope: 0.11 ± 0.03) < mitochondria (*A. anguilla*, slope: 0.21 ± 0.03; *A. rostrata*, slope: 0.16 ± 0.02) (Figs. 7B, D).

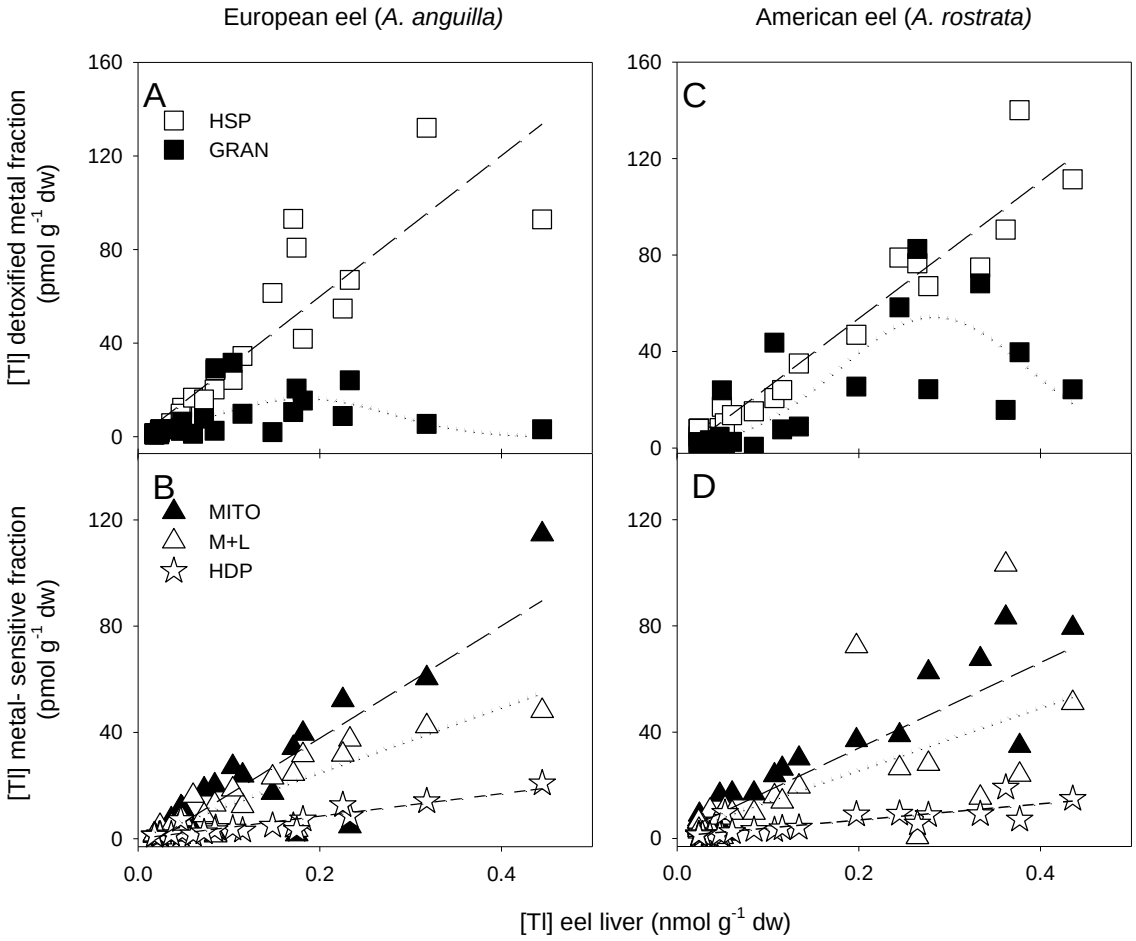

Fig. 7. Relationships between total hepatic Pb concentrations (horizontal axis) and Pb concentrations in various subcellular fractions (vertical axis) in *Anguilla anguilla* (left panels) and *Anguilla rostrata* (right panels) for detoxified metal fractions (panels A and C) and for metal-sensitive fractions (panels B and D). Each point represents an individual eel. Fraction abbreviations: HSP, heat-stable proteins; GRAN, NaOH-resistant granules; MITO, mitochondria; M+L, microsomes + lysosomes; HDP, heat-denatured proteins. Lines represent significant regressions ($P < 0.05$).

Total Tl accumulation in the detoxified-metal compartment (slope: 0.41 ± 0.04) was slightly higher than that observed for the metal-sensitive compartment (slope: 0.31 ±

0.04) in *A. rostrata* yellow eels. On the other hand, for *A. anguilla* the response of the detoxified-metal compartment (slope: 0.32 ± 0.04) was slightly lower than that for the metal-sensitive compartment (slope: 0.38 ± 0.03).

Discussion

Subcellular metal partitioning - general considerations

Protocols for determining the subcellular partitioning of metals are useful for evaluating the metal-handling strategies employed by aquatic animals living in metal-contaminated environments. These protocols normally involve a series of differential centrifugation steps and they are inherently sensitive to potential artifacts, including breakage or aggregation of organelles, leakage of soluble constituents from organelles, and overlap among subcellular fractions (De Duve, 1975; Giguère et al., 2006; Hinton and Mullock, 1997). For a detailed discussion of these potential problems, and a general assessment of the method used in the present study, see Rosabal et al. (2014).

The designation of some subcellular fractions as "granule-like" or "metallothionein-like" should be considered with caution. The operational grouping of fractions into a detoxified-metal compartment (DMF; granule-like and HSP) and a potentially metal-sensitive compartment (MSF; HDP, mitochondria, microsomes + lysosomes fractions), as has been done in the present study, is likely an oversimplification (Wallace et al., 2003). Metal accumulation in some fractions, notably that designated as microsomes + lysosomes, is inherently ambiguous; these organelles have different roles in handling non-essential metals (lysosomes: element detoxification; microsomes: potentially metal-sensitive targets), and the proportion to which microsomes and lysosomes contribute to metal accumulation in this fraction is difficult to determine. Considering the wide range of key biochemical processes that take place in the liver, where the Golgi apparatus, endoplasmic reticulum and ribosomes play important roles, and the limited secretory functions of this organ, we placed the microsomes + lysosomes fraction in the metal-sensitive compartment. It should be mentioned, however, that if this fraction is considered as neutral (excluding the microsomes + lysosomes fraction from the MSF compartment), or considered as a part of the detoxified metal compartment, the partitioning results of metals between MSF and DMF can be affected for As (in both eel

species), Ni (in *A. rostrata*) and Pb (in *A. anguilla*) – see Table SI-1 in the Supporting Information. Metal accumulation in the nuclei + debris fraction is also difficult to interpret, as mentioned in section 3.2. According to the results obtained in the assessment of our protocol (Fig. SI-7, Supporting Information), a good balance between cell breakage and the desired maintenance of organelle integrity was achieved in the application of the subcellular fractionation procedure to yellow eel livers.

Ag (silver)

The predominant role of the HSP fraction in sequestering Ag was observed for both eel species, although for *A. anguilla* such cytosolic ligands appear to exhibit a plateau response in their binding to Ag at high total Ag concentrations in the liver. The association of Ag with thermostable, cysteine-rich, low molecular weight proteins and peptides is consistent with the preference of this "soft" metal for the sulfhydryl groups of MT and MTLP (Mason and Jenkins, 1995). The significant increase in Ag concentrations in the HSP fraction (for both species), and in the relative contribution of this fraction (for *A. rostrata*) to increasing total hepatic Ag concentrations, suggests Ag detoxification in American and European yellow eels by MT and MTLP. This interpretation is consistent with the results of Langston et al. (2002), who reported that the partitioning of Ag among pools of cytosolic ligands showed an association of this element with the MT fraction in livers of field-collected *A. anguilla*. These authors also reported that the proportion of Ag found in the MT pool, which represented up to 92% of the total cytosolic Ag burden, increased as a function of Ag concentrations in the surrounding sediment and water. As has been suggested for freshwater rainbow trout, *Oncorhynchus mykiss* (Hogstrand et al., 1996), MT induction as a result of exposure to Ag could contribute to cellular protection against Ag-induced toxicity in yellow eels (Langston et al., 2002).

In spite of the increase in Ag concentrations in the HSP fraction, significant increases also occurred in metal-sensitive fractions in *A. anguilla* (mitochondria, microsomes + lysosomes and HDP) and *A. rostrata* (HDP fraction). According to Mason and Jenkins (1995), accumulation of a nonessential metal such as Ag in sensitive subcellular compartments, where this element can block functional groups, displace essential metals or modify the active conformation of biomolecules, could be indicative

of chronic Ag toxicity. Note too that in European eels, Ag concentrations in the nuclei + debris fraction were significantly correlated with those of the three metal-sensitive fractions and increased along the Ag bioaccumulation gradient, suggesting that Ag could be bound to nuclear ligands such as DNA or thiol-containing ligands.

From a toxicological perspective, American eels appeared to be somewhat more effective in preventing Ag binding to metal-sensitive biomolecules (e.g., mitochondria, microsomes + lysosomes) than European eels. In this regard, the response of the detoxified metal fractions in sequestering Ag (as estimated from the regression slopes) was approximately four-times higher than that of the metal-sensitive fractions in *A. rostrata*, whereas for the European eels the Ag concentration increase in the fractions involved in metal detoxification was only two-times higher than that in the metal-sensitive compartments. In addition, among metal-sensitive fractions in *A. rostrata*, the only significant increase in Ag concentrations was in the HDP fraction, compared to *A. anguilla*, for which significant increases were observed in all three metal-sensitive fractions as well as for the nuclear + debris fraction.

As (arsenic)

Although the arsenic bioaccumulation range differed markedly between the two eel species (e.g., the maximum As concentration reported in *A. anguilla* was 7-fold higher than that for *A. rostrata*; Table 1), subcellular As partitioning showed a similar pattern in the three potentially metal-sensitive fractions (mitochondria > microsomes + lysosomes > HDP fraction) in each fish species. To our knowledge, there are no previous data available on As subcellular partitioning in eels, but our results do agree with the subcellular distribution of As in the polychaete *Nereis diversicolor* collected from metal-impacted estuaries in England, for which the "organelles" fraction (including mitochondria and the microsomes + lysosomes fraction) was reported as the major As binding component (Dang et al., 2012). In the same study, the marine fish *Terapon jarbua* fed with these As-contaminated worms also showed high As accumulation in the hepatic "organelle" fraction. In the present study, As accumulation in the metal-sensitive fractions has the potential to cause toxicity at the cellular level. However, since As toxicity is speciation-dependent (Thomas et al., 2001), and since we have not determined

the oxidation state of the As species found in the various subcellular fractions, we cannot speculate on the likelihood of As-induced deleterious effects.

Both eel species sequestered some As in the HSP and granule-like fractions. In marine juvenile grunt (*Terapon jarbua*) exposed to waterborne and diet-borne As(III) and As(V) at environmentally relevant concentrations for 10 d, As was shown to be sequestered by binding to cytosolic, thermostable proteins and to granule-like structures (Zhang et al., 2012). High proportions of As were also found in the HSP fraction of muscle tissues of the marine fish *Lateolabrax japonicus* and *Pagrosomus major* of various sizes, suggesting the involvement of MT in As detoxification throughout the life cycle of these animals (He et al., 2010). Induction of MT by As was reported in hepatocytes of the freshwater teleost *Channa punctatus* exposed for 14 d to nonlethal but very high As concentrations (19 and 38 μmol L^{-1}; (Roy and Bhattacharya, 2006)). The relative potency of individual As species for inducing MT production is reported to be a function of the relative toxicity of the species (Kreppel et al., 1993).

Judging from the relative difference in As partitioning between the metal-sensitive and metal-detoxified compartments, European yellow eels were more effective in sequestering As than their American congeners, that is, the slope of the HSP fraction in *A. anguilla* (slope: 0.40 ± 0.1) was significantly higher than for *A. rostrata* (slope: 0.09 ± 0.03). In addition, the granule-like fraction was more involved in As detoxification in the European eels compared to the American eels, where no As accumulation in the NaOH-resistant fraction was observed and where the increase in As concentrations in the HSP fraction was lower than the increases observed for the three metal-sensitive fractions.

Cd (cadmium)

Although maximum Cd concentrations in *A. anguilla* livers were 4-fold higher than those in *A. rostrata*, the partitioning of this metal among the subcellular fractions was remarkably similar in both species. This similarity suggests that these species use the same handling strategies to cope with incoming hepatic Cd. For example, the majority of the Cd was found in the HSP fraction in both European and American eels (58 ± 9% and 62 ± 8%, respectively). In addition, in both species, the slopes of Cd in the detoxified fraction compared to total liver Cd concentrations was higher than those for the other

subcellular fractions, suggesting that Cd is mainly detoxified by MT. This interpretation is consistent with the reported Cd-induced induction of such cytosolic, cysteine-rich, thermostable proteins in eels (Langston et al., 2002). Rodriguez-Cea et al. (2003) also demonstrated Cd-binding to MT isoforms in *A. anguilla*, but since these eels were exposed to very high Cd concentrations (100 μg L^{-1}) the environmental relevance of these results is unclear. In a field study on European eels, Langston et al. (2002) reported a high proportion of this metal in the MTLP pool and noted that this fraction increased as a function of both the accumulated Cd burdens and the ambient Cd concentrations (sediment and water). Similar results were obtained by Van Campenhout et al. (2008), who determined Cd partitioning among various cytosolic pools in *A. anguilla* collected at sites along a metal-contamination gradient. They reported that Cd was largely sequestered in the MT pool and that the proportion of Cd bound to this ligand also increased with increasing Cd exposure.

The predominant role of the HSP fraction in sequestering Cd in yellow eels agrees with the results of studies on other fish, both those exposed to Cd under laboratory conditions (Ng and Wood, 2008; Zhang and Wang, 2006) and those collected from natural environments (Giguère et al., 2006; Kraemer et al., 2005; Oyoo-Okoth et al., 2012). However, in our study, this metal-handling strategy was not completely efficient in preventing Cd from binding to metal-sensitive biomolecules. Thus, in both eel species, Cd concentrations increased significantly along the Cd bioaccumulation gradient in the three metal-sensitive fractions. Note that the magnitude of the increase in Cd concentrations in these fractions followed the same order (mitochondria > microsomes + lysosomes > HDP) for each eel species. Cadmium binding to these metal-sensitive fractions has been also reported in zebrafish (*Danio rerio*) fed with Cd-contaminated prey, in which Cd concentrations decreased in the order HDP > microsomes + lysosomes (Bechard et al., 2008). Likewise, in mummichogs (*Fundulus heteroclitus*) collected from metal-polluted salt marshes, significant increases in Cd concentrations were measured in the HDP and microsomes + lysosomes fractions (Goto and Wallace, 2010). Giguère et al. (2006) also reported non-specific binding of Cd to potentially sensitive subcellular targets in field-collected yellow perch (*Perca flavescens*).

As a "soft" metal, Cd interacts preferentially with thiol and amine functional groups, such as those found in nucleic acids, enzymes and other proteins (Mason and Jenkins, 1995). Cadmium binding to these molecules can potentially provoke functional deficits in organelles such as nuclei, the endoplasmic reticulum and mitochondria (Cannino et al., 2009; Jacobson and Turner, 1980; Moulis, 2010). Some of these effects result from the interaction of Cd with metalloenzymes, leading to conformational changes or to the displacement of physiologically essential elements including Zn^{2+}, Cu^{2+}, Fe^{3+}, $Fe^{/2+}$, Mg^{2+} and Ca^{2+} (Moulis, 2010).

Ni (nickel)

The important role of granule-like structures in detoxifying Ni in European eels is consistent with the subcellular partitioning of this element in the fathead minnow *Pimephales promelas*, in which more than 50% of the total Ni concentration in the whole fish was associated with mineral concretions (Lapointe et al., 2009). The sequestering of metals by such mineralized structures containing Ca, Mg, Cu or Fe as major cationic components has been studied mostly in terrestrial (Hopkin, 1989) and aquatic invertebrates (Mason and Jenkins, 1995), but it appears that metal-rich granules are not limited to invertebrates. In fish cells, such metal-rich structures have also been reported to play a complementary metal-detoxification role in the gills and guts of the goby *Neogobius melanostomus,* where the NaOH-resistant fraction was reported to be the major site of Ni bioaccumulation (Leonard et al., 2014). In American eels, Ni accumulation in the granule-like fraction also increased along the Ni bioaccumulation gradient, but in contrast to European eels the response in this fraction was similar to that observed in the HSP fraction.

With regards to Ni binding to MT and its ability to induce MT biosynthesis, published results for vertebrates are inconsistent. On the one hand, Ptashynski et al. (2002) reported that exposure to Ni induced the production of MT in the intestines of lake whitefish (*Coregonus clupeaformis*) but not in their kidneys, liver and gills. The association of Ni and cytosolic thermostable peptides has been also suggested in aquatic organisms such as *P. promelas* (Lapointe et al., 2009), *O. mykiss* (Leonard et al., 2014) and *P. flavescens* (Giguère et al., 2006). On the another hand, Van Campenhout et al.

(2008) reported that the cytosolic concentration of Ni in the liver of field-collected *A. anguilla* increased significantly as a function of total hepatic Ni concentrations, but that this element was not associated with the MT pool. This latter result is consistent with the classification of Ni as a borderline class A cation (Mason and Jenkins, 1995) with low affinity for the sulfhydryl groups of MT to which other soft metals such Ag, Cd and Cu(I) are bound.

Despite the increase in Ni concentrations in both detoxified fractions, significant Ni concentrations were also found in the metal-sensitive fractions of both eel species, suggesting that metal detoxification was not entirely successful. Similar subcellular Ni distributions were reported by Giguère et al. (2006) for wild yellow perch, where liver Ni concentrations were higher in the HDP fraction than in any other metal-sensitive fraction. For rainbow trout and round goby fed with Ni-contaminated prey, Leonard et al. (2014) also reported high Ni concentrations in these subcellular compartments. As suggested by Mason and Jenkins (1995), the presence of Ni in metal-sensitive fractions, where this element can interact with physiologically important biomolecules, could be associated with potential toxic effects.

Comparison of the subcellular distribution of Ni for the same bioaccumulation gradient (0 – 25 nmol g^{-1}) in the two eel species suggests that Ni detoxification strategies in the European yellow eels were somewhat more efficient than their American counterparts in protecting against potential metal toxicity at the cellular level (i.e., a higher increase in Ni concentrations was found in the detoxified-metal compartment in *A. anguilla*).

Pb (lead)

As hepatic Pb concentrations increased, the granule-like fraction showed the strongest increase in Pb concentrations among all subcellular fractions in both fish species. As mentioned above, mineral concretions associated with metal-detoxification or elimination roles have been reported mostly in invertebrate species (Marigómez et al., 2002; Mason and Jenkins, 1995). However, Goto and Wallace (2010) reported that in mummichogs (*Fundulus heteroclitus*) collected at a Pb-impacted site the majority of their Pb was stored in the NaOH-resistant fraction, and that Pb concentrations in this fraction

increased linearly with the whole body Pb burdens. Although these fish also sequestered Pb in cytosolic thermostable ligands, their contribution to whole body Pb burdens did not increase consistently along the contamination gradient (Goto and Wallace, 2010). The HSP fraction has also been reported to be important for binding Pb in the intestinal cells of the marine fish *Terapon jurbua* (Dang et al., 2012). In contrast, Van Campenhout et al. (2008) reported that MTs played only a limited role in Pb handling in livers of field-collected European eels. This latter analysis is consistent with the fact that Pb, as a borderline metal, interacts less readily with thiol groups than do Cd and Hg (Mason and Jenkins, 1995).

Although, in our study, Pb partitioning slightly favoured the detoxified fractions, Pb concentrations in the three metal-sensitive fractions did increase along the Pb bioaccumulation gradients for both *A. rostrata* and *A. anguilla*. The presence of Pb in these fractions, and the increase in Pb concentrations along the bioaccumulation gradient, could be indicative of potential toxic effects. Thus, our results differ from those reported by Goto and Wallace (2010) for *F. heteroclitus* since the proportion of Pb in the HDP and organelle fractions did not increase with total Pb burdens in this species (however, their two-fold Pb bioaccumulation gradient was rather modest). In contrast, Dang et al. (2012) identified the HDP fraction as the major Pb-binding compartment in liver cells of the marine fish *T. jurbua*. Given the capacity of Pb to mimic other metals involved in important biochemical processes, including Ca, Fe and Zn (Vallee and Ulmer, 1972), its accumulation in such metal-sensitive fractions could result in serious disturbances of cellular functions.

Tl (thallium)

In contrast to the previously considered elements, the subcellular partitioning of Tl has been reported for very few aquatic animals (e.g., Lapointe et al. (2009) and Dumas and Hare (2008)). In both eel species, the HSP pool proved to be the most responsive fraction along the Tl bioaccumulation gradient, indicating a potential interaction of Tl with MTLP. This observation is consistent with the results obtained for *Chironomus riparius* and *Daphnia magna* exposed to Tl-contaminated diets, where >55% and >40%, respectively, of the total thallium burden was found in this fraction (Dumas and Hare,

2008). Like other soft trace metals (Ag, Cd), Tl in its reduced state is likely bound to cysteine residues in MTLP; Tl(I) is known to form stable complexes with reduced sulphur-containing ligands (Mulkey and Oehme, 1993). Accumulation of Tl in this cytosolic fraction was also reported in the oligochaete *Tubifex tubifex*, but in this annelid Tl was also present in comparable quantities in the granule-like fraction (Dumas and Hare, 2008). In both yellow eel species, Tl concentrations in the granule-like fraction increased up to a Tl threshold, above which this metal detoxification strategy seems to become less efficient. The decrease in Tl accumulation in the granule-like fraction at high hepatic Tl concentrations could indicate that this sequestration and detoxification strategy is overwhelmed, leading to more Tl accumulation in metal-sensitive fractions, especially the mitochondrial fraction (Figs. 7 B,D). In juvenile fathead minnows (*P. promelas*), a large proportion of the bioaccumulated Tl was also associated with granule-like structures (Lapointe et al., 2009).

As with the other non-essential elements, Tl accumulation in the metal-sensitive compartment could be a harbinger of toxic effects in the intracellular environment. Among the metal-sensitive fractions, the mitochondrial fraction was the major Tl-binding fraction. High proportions of Tl in the "organelles" fraction (including mitochondria) were also reported in the oligochaete *Tubifex tubifex* (Lapointe et al., 2009).

Overall subcellular metal partitioning

From a biochemical point of view, the subcellular metal partitioning of Ag, Cd, Pb and Tl in the livers of *A. anguilla* and *A. rostrata* differed from those of As and Ni (Fig. 8). The affinity of Ag, Cd, Pb and Tl for subcellular sulfhydryl-containing ligands (Mason and Jenkins, 1995) likely explains the grouping of these four elements. In contrast to these metals, the subcellular partitioning of Ni in both eel species is influenced by the fact that it is a class A – borderline cation with an affinity for bioligands containing O and P that are found in both detoxified (Fig. 8A,C) and metal-sensitive compartments (Fig. 8B,D). With regards to As, its proximity to Ni in the PCA plots is surprising, given their chemical differences.

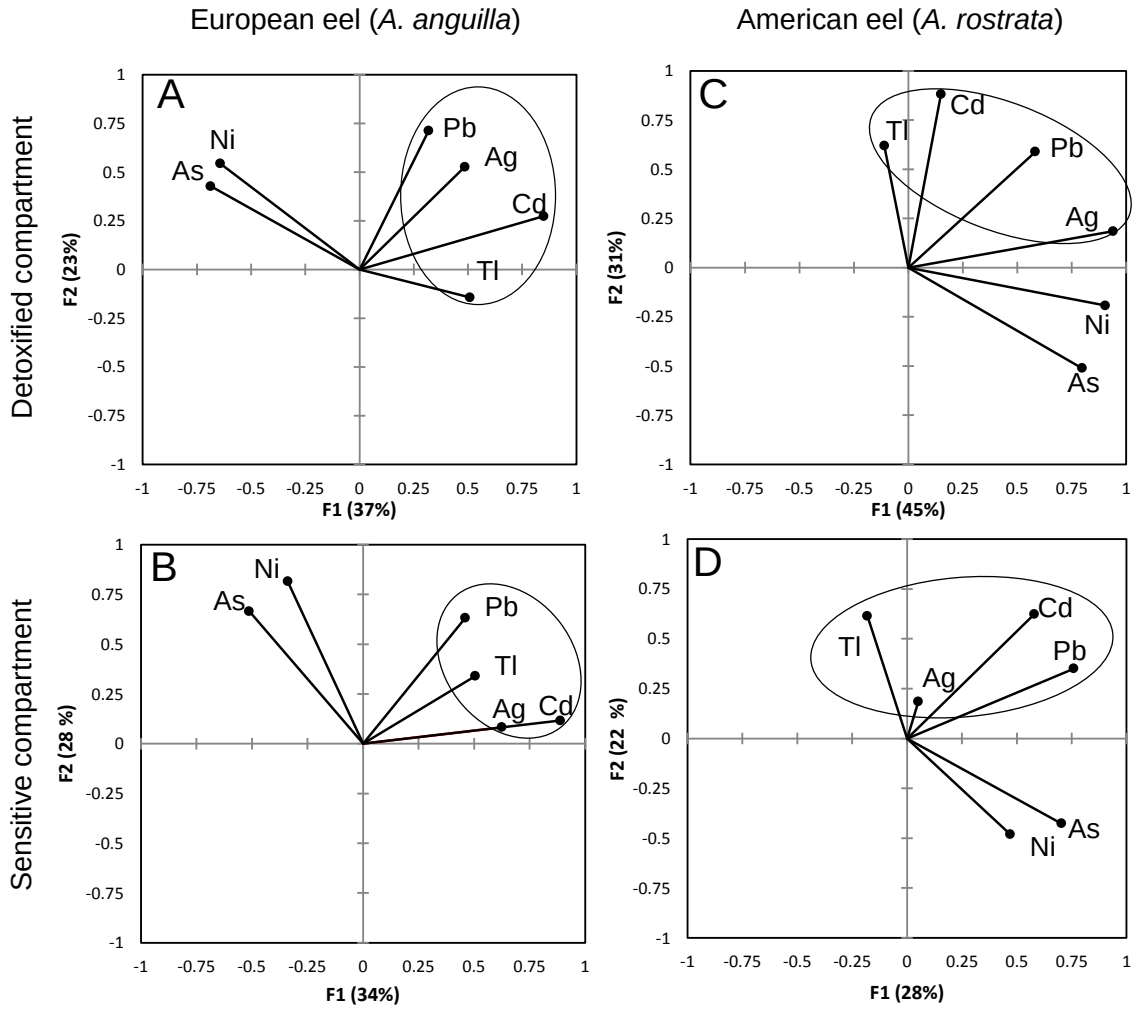

Fig. 8. Principal Component Analysis (PCA) performed using metal-concentrations in detoxified (A, C) and sensitive compartments (B, D) of the livers of Anguilla anguilla (left panels) and Anguilla rostrata (right panels).

We speculate that the biochemical transformation (mainly methylation) of this metalloid into less-toxic forms (Thomas et al., 2001), a process known to occur in hepatocytes, could contribute to its distinctive partitioning compared to that of Ag, Cd, Pb and Tl (Figs. 8A, B, C, D).

From an ecotoxicological perspective, *A. anguilla* and *A. rostrata* were able to activate detoxification responses to the metals to which they had been chronically exposed. Metal sequestering by cytosolic, thermostable ligands, presumably including MT, MTLP, GSH and free amino acids, was the most consistent metal-detoxification response observed for all of the metals studied. In the cases of Ni, Pb and Tl, the

formation of mineral concretions also appears to be an important strategy in both eel species for limiting the binding of non-essential metals to physiologically important biomolecules.

Despite the metal detoxification responses in both eel species, they were not completely effective. Inappropriate binding of these contaminants to physiologically sensitive compartments such as mitochondria, heat-denatured cytosolic proteins and other organelles was consistently measured for all the studied elements. Accumulation in the putative metal-sensitive fractions increased along the metal bioaccumulation gradient and in the most-contaminated individuals the concentrations of some elements in these sensitive fractions exceeded those observed in the detoxified fractions (Pb and Tl in *A. anguilla*, As and Ni in *A. rostrata*). Among the metal-sensitive fractions, the mitochondrial fraction, where key biochemical processes occur, appears to be a major binding compartment for As, Cd, Pb and Tl. Although we did not measure direct metal-induced effects in the present study, our results are consistent with those reported on eels, where perturbations or impairments of biological functions have been reported as a result of metal exposure (Pierron et al., 2014; Pierron et al., 2007; Santos and Hall, 1990). In this context, Baillon et al. (2014) identified specific effects of As and Cd on the transcriptome profile of field-collected Atlantic eels; the transcription level of genes previously described to be involved in As vasculotoxicity in mammals were affected in As-contaminated eels, and significant changes in the transcription level of genes encoding for proteins that play important roles in the metabolism of lipids and proteins were associated with Cd exposure.

Based on the trends in metal concentrations in metal-sensitive fractions, the Cd- and Ni-handling strategies observed in both eel species was not as efficient as those reported in larvae of the insect *Chaoborus* (Rosabal et al., 2012) and the floater mollusc *Pyganodon grandis* (Bonneris et al., 2005), but reasonably similar to that of the yellow perch *Perca flavescens* (Giguère et al., 2006). In both invertebrates, Cd concentrations in the HDP fraction did not increase as function of the Cd bioaccumulation gradient, whereas for the two fish species, Cd in the three metal-sensitive fractions did significantly increase as the total Cd concentration increased. With regards to Ni, in both

eel and yellow perch accumulation of this metal in their sensitive compartments was somewhat higher than those reported for the insect *Chaoborus*.

Based on our results, the accumulation of non-essential metals observed in the mitochondria, microsomes and other metal-sensitive fractions could increase the toxicological risk to eels inhabiting the Saint Lawrence and Gironde environments.

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Supporting information for:

Subcellular partitioning of non-essential trace metals (Ag, As, Cd, Ni, Pb, and Tl) in livers of American (*Anguilla rostrata*) and European (*Anguilla anguilla*) yellow eels

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Three supplementary sections

Aquatic Toxicology, *2015*, 160, 128-141.

Section 1: Relationship between total hepatic metal concentrations and the percentages of the metal in the detoxified metal fractions (panels A and C) and in the potentially metal-sensitive and nuclei/debris fractions (panels B and D). This information complements that the metal concentration data (nmol g^{-1}) presented in Figures 2-7 in the paper. Only the significant regressions ($P < 0.05$) are represented by lines.

- Ag
- As
- Cd
- Ni
- Pb
- Tl

Section 2: Distribution of the two marker enzymes among the various subcellular fractions (CS = citrate synthase; LDH = lactate dehydrogenase).

Section 3: Comparison of the slopes of the relations between the detoxified metal compartment (∑ detoxified metal fractions, DMF) or the metal-sensitive compartment (∑ metal-sensitive fractions, MSF) and the total hepatic metal concentrations, for the cases where the 'microsomes + lysosomes' fraction is considered to be MSF, neutral or DMF, for each metal studied.

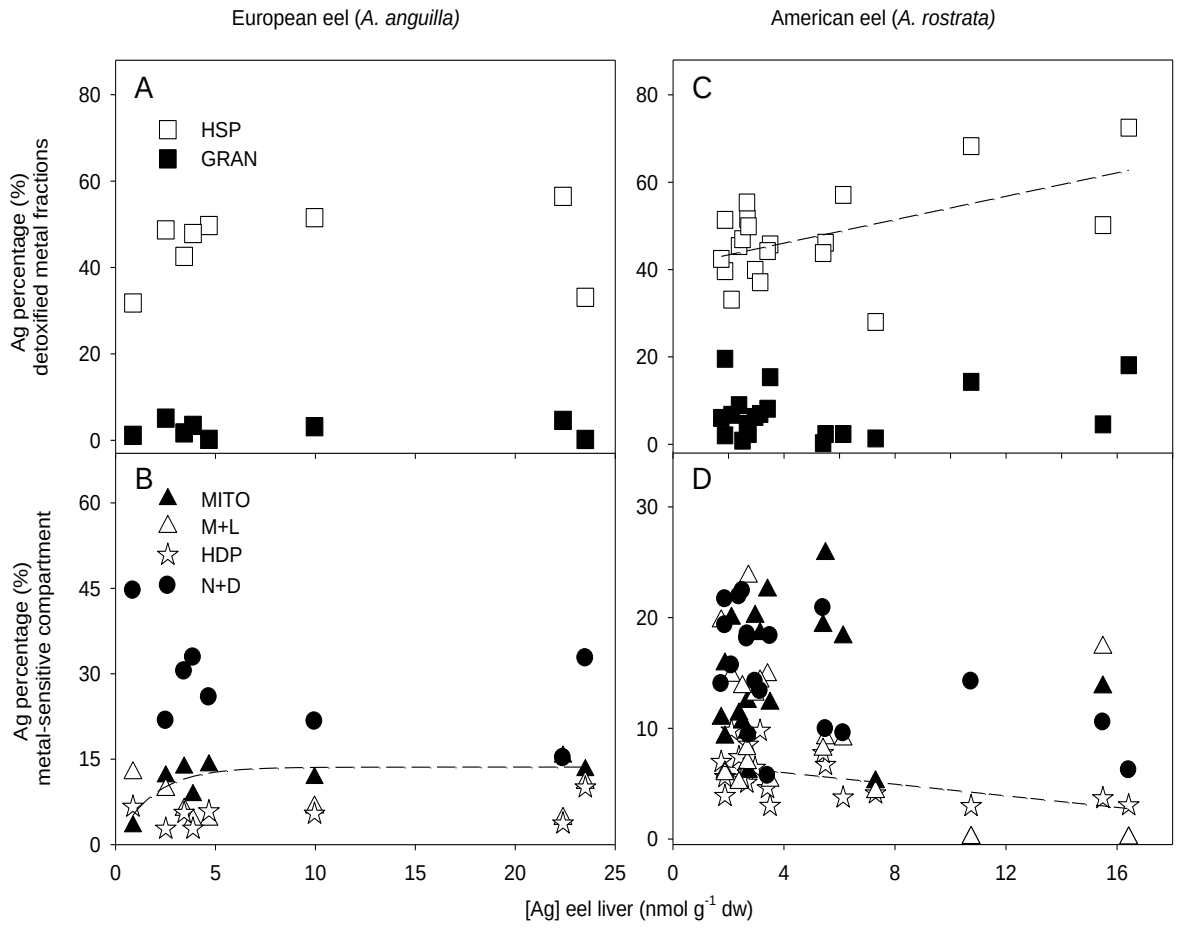

Figure SI-1. Relationship between total hepatic Ag concentrations (horizontal axis) and Ag percentages in *Anguilla anguilla* (left panels) and *Anguilla rostrata* (right panels) for detoxified metal fractions (panels A and C) and for potentially metal-sensitive and nuclei/debris fractions (panels B and D). Each point represents an individual eel. Fraction abbreviations: HSP, heat-stable proteins; GRAN, NaOH-resistant; MITO, mitochondria; M+L, microsomes + lysosomes; HDP, heat-denatured protein; N+D, nuclei + debris. The dashed lines represent significant regressions ($P < 0.05$) for the MITO (B), HSP (C) and HDP (D) fractions.

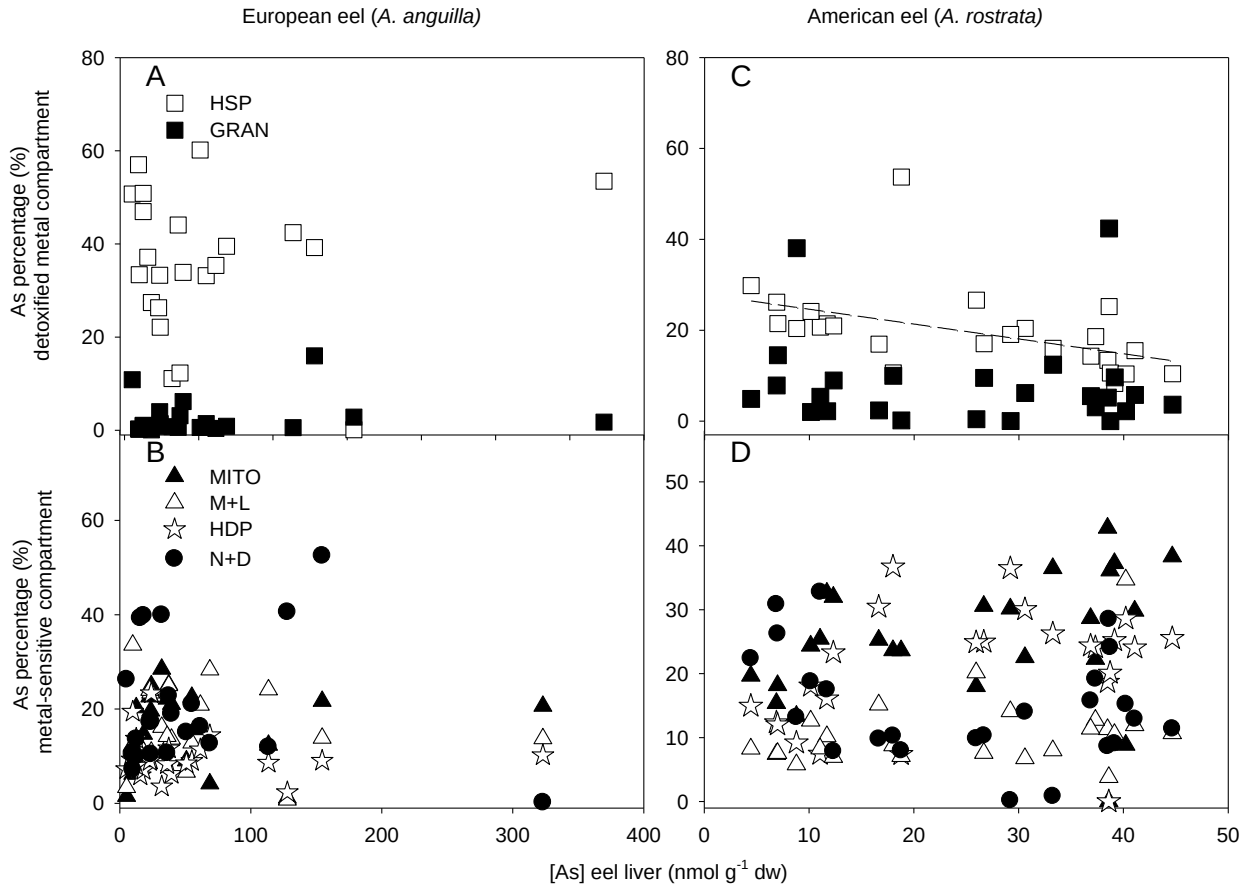

Figure SI-2. Relationship between total hepatic As concentrations (horizontal axis) and As percentages *Anguilla anguilla* (left panels) and *Anguilla rostrata* (right panels) for detoxified metal fractions (panels A and C) and for potentially metal-sensitive and nuclei/debris fractions (panels B and D). Each point represents an individual eel. Fraction abbreviations: HSP, heat-stable proteins; GRAN, NaOH-resistant; MITO, mitochondria; M+L, microsomes + lysosomes; HDP, heat-denatured protein; N+D, nuclei + debris. The dashed line C represents a significant regression ($P < 0.05$) for the HSP fraction.

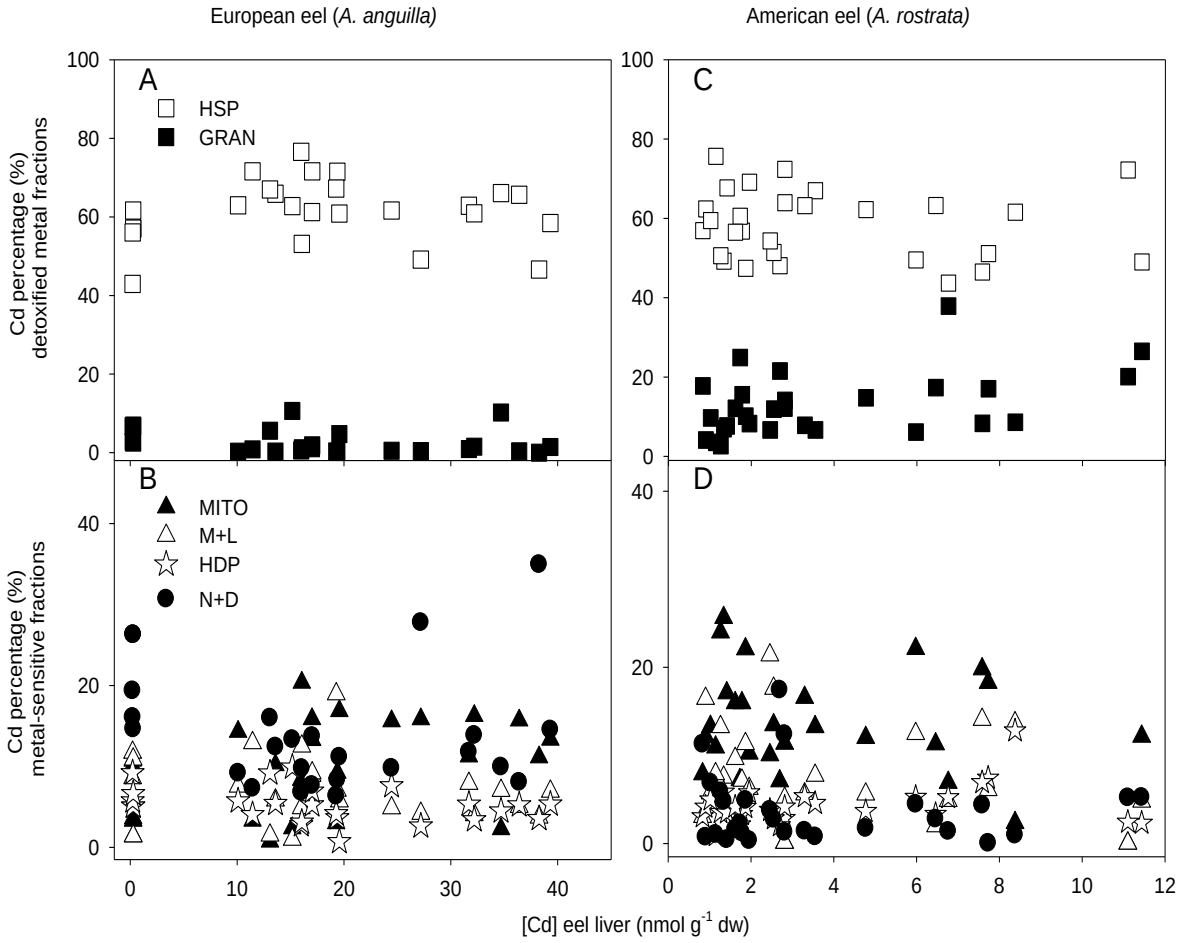

Figure SI-3. Relationship between total hepatic Cd concentrations (horizontal axis) and Cd percentages in *Anguilla anguilla* (left panels) and *Anguilla rostrata* (right panels) for detoxified metal fractions (panels A and C) and for potentially metal-sensitive and nuclei/debris fractions (panels B and D). Each point represents an individual eel. Fraction abbreviations: HSP, heat-stable proteins; GRAN, NaOH-resistant; MITO, mitochondria; M+L, microsomes + lysosomes; HDP, heat-denatured protein; N+D, nuclei + debris.

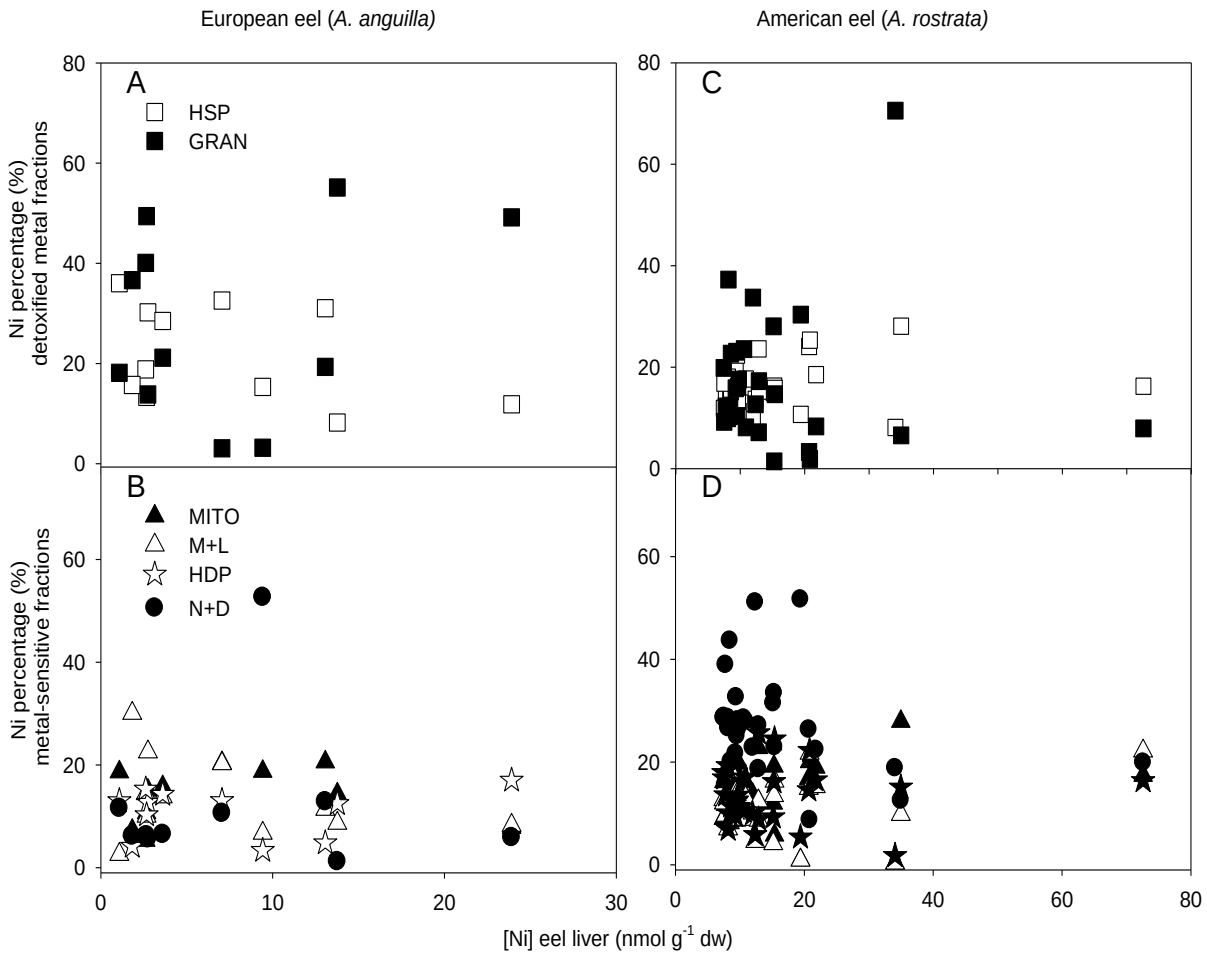

Figure SI-4. Relationship between total hepatic Ni concentrations (horizontal axis) and Ni percentages in *Anguilla anguilla* (left panels) and *Anguilla rostrata* (right panels) for detoxified metal fractions (panels A and C) and for potentially metal-sensitive and nuclei/debris fractions (panels B and D). Each point represents an individual eel. Fraction abbreviations: HSP, heat-stable proteins; GRAN, NaOH-resistant; MITO, mitochondria; M+L, microsomes + lysosomes; HDP, heat-denatured protein; N+D, nuclei + debris.

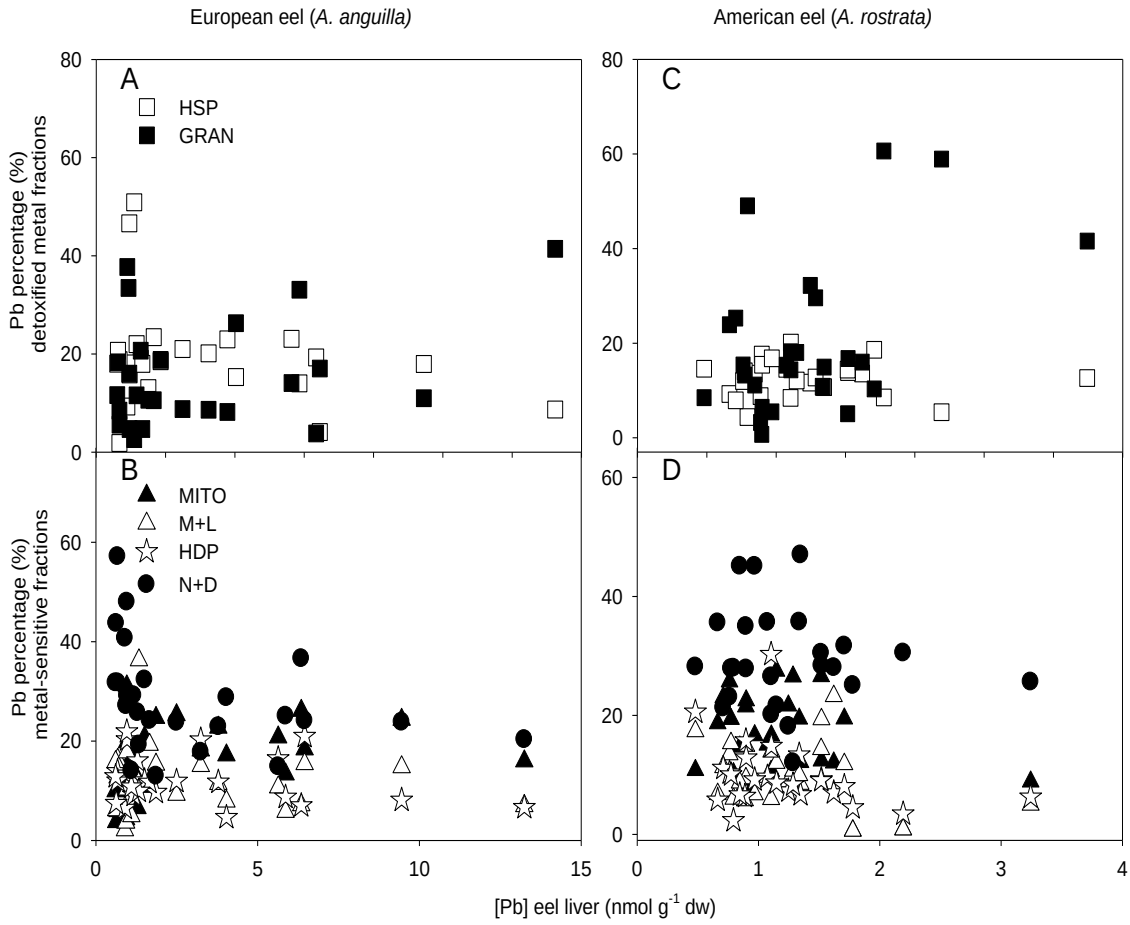

Figure SI-5. Relationship between total hepatic Pb concentrations (horizontal axis) and Pb percentages *Anguilla anguilla* (left panels) and *Anguilla rostrata* (right panels) for detoxified metal fractions (panels A and C) and for potentially metal-sensitive and nuclei/debris fractions (panels B and D). Each point represents an individual eel. Fraction abbreviations: HSP, heat-stable proteins; GRAN, NaOH-resistant; MITO, mitochondria; M+L, microsomes + lysosomes; HDP, heat-denatured protein; N+D, nuclei + debris.

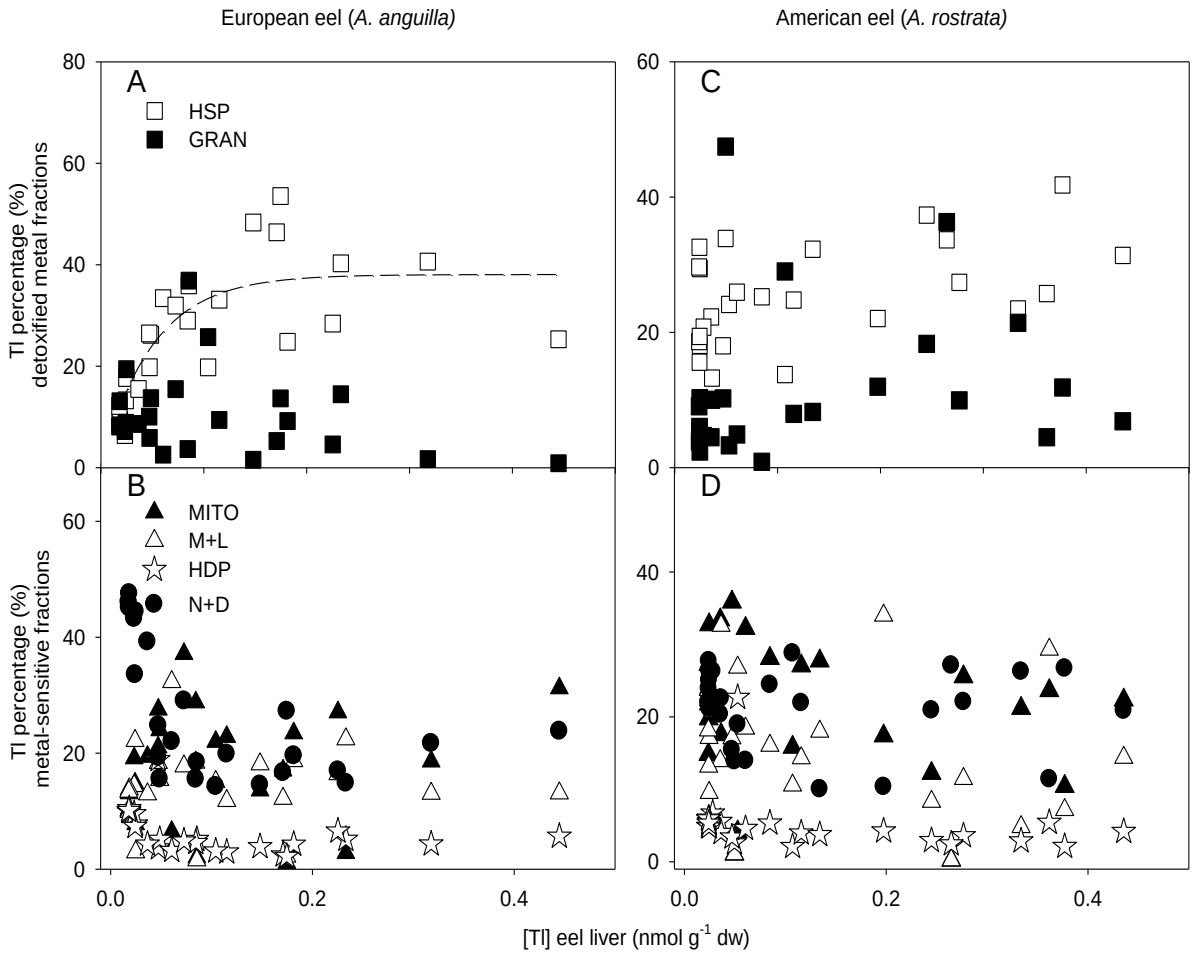

Figure SI-7. Figure SI-6. Relationship between total hepatic Tl concentrations (horizontal axis) and Tl percentages in *Anguilla anguilla* (left panels) and *Anguilla rostrata* (right panels) for detoxified metal fractions (panels A and C) and for potentially metal-sensitive and nuclei/debris fractions (panels B and D). Each point represents an individual eel. Fraction abbreviations: HSP, heat-stable proteins; GRAN, NaOH-resistant; MITO, mitochondria; M+L, microsomes + lysosomes; HDP, heat-denatured protein; N+D, nuclei + debris. The dashed line in A represents a significant regression ($P < 0.05$) for the HSP fraction

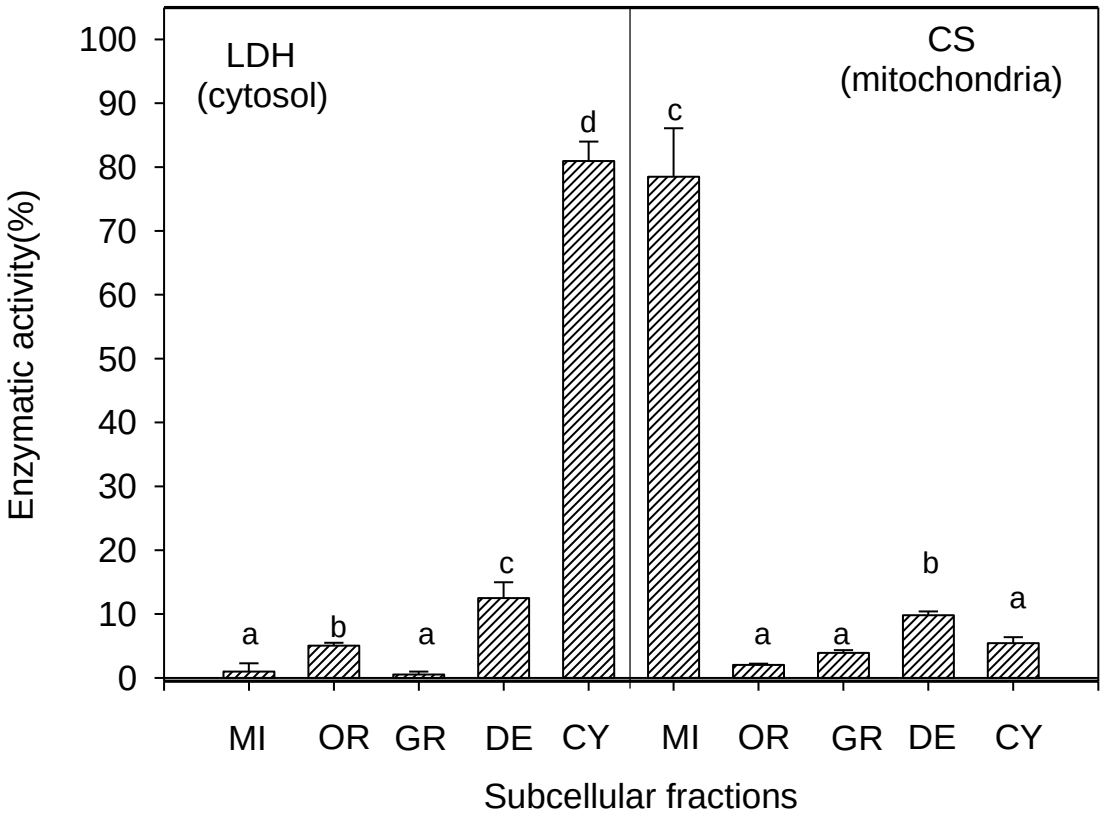

Figure SI-8. Percentage (%) of LDH and CS enzymatic activity in each subcellular fraction obtained from livers of *A. rostrata*. Error bars are standard deviations (n = 3).The same lowercase letter indicates a non-significant difference, whereas different letters indicate that differences are significant (ANOVA followed by Tukey test, $P < 0.05$). MI: mitochondria; OR: microsomes/lysosomes; GR: NaOH-resistant fraction; DE: nuclei/debris; CY: cytosol.

Table SI-1. Comparison of the slopes of the relationships between the detoxified metal compartment (∑ detoxified metal fractions, DMF) or the metal-sensitive compartment (∑ metal-sensitive fractions, MSF) and the total hepatic metal concentrations, for the cases where the 'microsomes + lysosomes' fraction is considered to be MSF, neutral or DMF, for each metal studied.

Trace metal	microsome + lysosome fraction as:	European eel (*A. anguilla*)			American eel (*A. rostrata*)		
		Detoxified metal fraction (DMF)	Metal-sensitive fraction (MSF)	DMF or MSF?[d]	Detoxified metal fraction (DMF)	Metal-sensitive fraction (MSF)	DMF or MSF?[a]
Ag	MSF[a]	slope : 0.42 ± 0.07 r^2 = 0.87	slope : 0.28 ± 0.03 r^2 = 0.93	DMF	slope : 0.70 ± 0.05 r^2 = 0.90	slope : 0.12 ± 0.05 r^2 = 0.23	DMF
	Neutral[b]	slope : 0.42 ± 0.01 r^2 = 0.87	slope : 0.21 ± 0.02 r^2 = 0.97		slope : 0.70 ± 0.05 r^2 = 0.90	slope : 0.06 ± 0.03 r^2 = 0.24	
	DMF[c]	slope : 0.50 ± 0.05 r^2 = 0.94	slope : 0.21 ± 0.02 r^2 = 0.97		slope : 0.75 ± 0.04 r^2 = 0.94	slope : 0.06 ± 0.03 r^2 = 0.24	
As	MSF[a]	slope : 0.45 ± 0.06 r^2 = 0.81	slope : 0.37 ± 0.04 r^2 = 0.88	=	slope : 0.15 ± 0.05 r^2 = 0.27	slope : 0.55 ± 0.07 r^2 = 0.73	MSF
	Neutral[b]	slope : 0.45 ± 0.06 r^2 = 0.81	slope : 0.25 ± 0.02 r^2 = 0.88	DMF	slope : 0.15 ± 0.05 r^2 = 0.27	slope : 0.35 ± 0.05 r^2 = 0.72	MSF
	DMF[c]	slope : 0.57 ± 0.05 r^2 = 0.87	slope : 0.25 ± 0.02 r^2 = 0.88		slope : 0.34 ± 0.03 r^2 = 0.79	slope : 0.35 ± 0.05 r^2 = 0.72	=
Cd	MSF[a]	slope : 0.59 ± 0.04 r^2 = 0.89	slope : 0.22 ± 0.04 r^2 = 0.83	DMF	slope : 0.63 ± 0.03 r^2 = 0.94	slope : 0.21 ± 0.03 r^2 = 0.65	DMF
	Neutral[b]	slope : 0.59 ± 0.04 r^2 = 0.89	slope : 0.17 ± 0.02 r^2 = 0.77		slope : 0.63 ± 0.03 r^2 = 0.94	slope : 0.13 ± 0.02 r^2 = 0.55	
	DMF[c]	slope : 0.64 ± 0.03 r^2 = 0.90	slope : 0.17 ± 0.02 r^2 = 0.77		slope : 0.69 ± 0.03 r^2 = 0.96	slope : 0.13 ± 0.02 r^2 = 0.55	

Ni	MSF[a]	slope : 0.55 ± 0.08 r^2 = 0.86	slope : 0.30 ± 0.03 r^2 = 0.93	DMF	slope : 0.33 ± 0.07 r^2 = 0.46	slope : 0.49 ± 0.05 r^2 = 0.81	MSF
	Neutral[b]	slope : 0.55 ± 0.08 r^2 = 0.86	slope : 0.23 ± 0.02 r^2 = 0.95		slope : 0.33 ± 0.07 r^2 = 0.46	slope : 0.30 ± 0.03 r^2 = 0.80	=
	DMF[c]	slope : 0.62 ± 0.08 r^2 = 0.87	slope : 0.23 ± 0.02 r^2 = 0.95		slope : 0.51 ± 0.06 r^2 = 0.76	slope : 0.30 ± 0.03 r^2 = 0.80	DMF
Tl	MSF[a]	slope : 0.38 ± 0.03 r^2 = 0.87	slope : 0.32 ± 0.04 r^2 = 0.75	DMF	slope : 0.41 ± 0.04 r^2 = 0.83	slope : 0.31 ± 0.04 r^2 = 0.67	DMF
	Neutral[b]	slope : 0.38 ± 0.03 r^2 = 0.87	slope : 0.25 ± 0.03 r^2 = 0.79		slope : 0.41 ± 0.04 r^2 = 0.83	slope : 0.19 ± 0.02 r^2 = 0.76	
	DMF[c]	slope : 0.44 ± 0.04 r^2 = 0.84	slope : 0.25 ± 0.03 r^2 = 0.79		slope : 0.53 ± 0.03 r^2 = 0.91	slope : 0.19 ± 0.02 r^2 = 0.76	
Pb	MSF[a]	slope : 0.31 ± 0.03 r^2 = 0.87	slope : 0.34 ± 0.03 r^2 = 0.86	=	slope : 0.6 ± 0.1 r^2 = 0.60	slope : 0.19 ± 0.05 r^2 = 0.35	DMF
	Neutral[b]	slope : 0.31 ± 0.03 r^2 = 0.87	slope : 0.24 ± 0.02 r^2 = 0.89	DMF	slope : 0.6 ± 0.1 r^2 = 0.60	slope : 0.13 ± 0.04 r^2 = 0.32	
	DMF[c]	slope : 0.42 ± 0.02 r^2 = 0.93	slope : 0.24 ± 0.02 r^2 = 0.89	DMF	slope : 0.7 ± 0.1 r^2 = 0.64	slope : 0.13 ± 0.04 r^2 = 0.32	

[a]: In these regressions, the 'microsomes + lysosomes' are included in the ∑MSF compartment, as is the case in the manuscript.
[b]: Neutral signifies that the 'microsomes + lysosomes' are not included in either the ∑DMF or ∑MSF compartment.
[c]: In these regressions, the 'microsomes + lysosomes' are included in the ∑DMF compartment.
[d]: In this column, the compartment (∑DMF or ∑MSF) showing the higher slope in each simulation is identified.

13. Metal (Ag, Cd, Cu, Ni, Tl, Zn) binding to cytosolic ligands in field-collected larvae of the insect *Chaoborus* as determined by Size-Exclusion Chromatography coupled to Inductively Coupled Plasma-Mass Spectrometry

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Metallomics (in preparation)

Abstract

A hyphenated fractionation approach based on size-exclusion chromatography coupled to inductively-coupled plasma mass spectrometry (SEC-ICPMS) was applied to the cytosol and the heat-stable protein and peptide (HSP) fraction of larvae of the phantom midge (*Chaoborus punctipennis*) collected from five mining-impacted lakes. We determined the distribution of Ag, Cd, Cu, Ni, Tl and Zn among three molecular weight pools (HMW: high molecular weight, "2400" kDa – 40 kDa; MMW: medium molecular weight, 40 kDa – "0.7" kDa; LMW: low molecular weight, < "0.7" kDa). Appreciable concentrations of non-essential metals were found in the potentially metal-sensitive HMW (Ag, Ni) and LMW (Tl) pools, whereas the MMW pool, which includes metallothioneins (MTs) and metallothionein-like proteins (MTLPs), appeared to be involved in Ag and Cd detoxification. Higher-resolution fractionation of the HSP fraction using a SEC column revealed further differences in the subcellular partitioning of nonessential metals (i.e., Ag = Cd ≠ Ni ≠ Tl). These results provide unprecedented details on the metal-handling strategies employed by a metal-tolerant, freshwater animal in a field situation

Résumé

Une approche de fractionnement basée sur la chromatographie d'exclusion stérique couplée à l'ICPMS (SEC-ICPMS) a été appliquée aux cytosols complets provenant de larves de l'insecte *Chaoborus punctipennis* récoltées dans cinq lacs contaminés en métaux. Nous avons déterminé la distribution de l'Ag, du Cd, du Cu, du Ni, du Tl et du Zn parmi trois groupes de poids moléculaire (*HMW*: poids moléculaire élevé, "2400" kDa - 40 kDa; *MMW*: poids moléculaire moyen, 40 kDa - "0,7" kDa; *LMW*: faible poids moléculaire, < "0,7" kDa). Des concentrations appréciables de métaux non essentiels ont été trouvées dans les groupes *HMW* (Ag, Ni) et *LMW* (Tl), tandis que le groupe *MMW*, qui comprend la MT et les MTLP, semblent être impliqué dans la détoxication de l'Ag et le Cd. Un fractionnement plus poussé de ces métaux, dans la fraction cytosolique thermostable (fraction *HSP*), a été réalisé à l'aide d'une colonne d'exclusion stérique avec une plus grande résolution; ce fractionnement a révélé des différences importantes dans la répartition subcellulaire des métaux non essentiels (Ag = Cd ≠ Ni ≠ Tl). Ces résultats fournissent des détails sans précédent sur les stratégies de « manipulation » (*handling*) de métaux utilisées par un organisme dulcicole, tolérant aux métaux traces, et récolté sur le terrain.

Introduction

Measurements of trace metals in the whole bodies or organs of aquatic animals are used to assess metal exposure in aquatic systems.[1] However, such measurements do not reveal whether or not the accumulated metal is likely to cause toxic effects.[2] To link metal bioaccumulation and metal toxicity, information on whether metals in animal cells are detoxified or in biochemically active pools is required.[3, 4] In the cytosol of cells, non-essential metals can bind to either mid-range molecular-weight targets such as metallothioneins (MT) or metallothionein-like proteins or peptides (MTLP)[5-7], which allows them to be detoxified, or to high or to low molecular-weight entities (e.g., enzymes) that could lead to toxic effects.

A protocol involving differential centrifugation and heat denaturation steps was used to determine the subcellular partitioning of metals many aquatic organisms.[8] This approach provides valuable information concerning the distribution of cytosolic metals between the heat-stable protein or peptides (HSP) fraction (e.g., MT and MTLP) and the heat-denatured proteins (HDP) fraction, which includes cytosolic enzymes.[3, 9] However, such methods do not reveal the proteins or peptides involved in metal sequestration or that represent metal-sensitive targets. To achieve this goal, size-exclusion chromatography can be used to separate the cytosol into various molecular weight pools and then metals can be measured in each pool by ICPMS.[9, 10] This SEC-ICPMS approach has been refined with the development of "hyphenated" systems[11], whereby high-resolution separation by chromatographic techniques (with metal free components and low-interactivity columns) or electrophoresis is coupled on line to an ICPMS.[12-14] Such hyphenated techniques provide more detailed information on metal-binding entities such as MT or MTLP (e.g., molecular weight and composition of the metal-binding entity; stoichiometry of metal-ligand binding).[15] Additionally, the use of this analytical strategy to identify sensitive biomolecules targeted by metals in the HMW and LWM fractions could give valuable information on potential metal effect biomarkers.[16]

In the present study, we applied a hyphenated approach based using SEC-ICPMS to investigate metal-handling strategies used by larvae of the phantom midge *Chaoborus punctipennis*, which has been used previously to estimate Cd, Ni and Se exposure in the

pelagic zone of mining-impacted lakes.[17-19] Previous studies on field-collected *Chaoborus* larvae have demonstrated the role of the HSP and HDP fractions in the handling of essential (e.g., Cu, Zn and Se) and non-essential metals (Cd, Ni), but nothing has been reported regarding the nature of the metal-binding proteins or peptides found in the cytosol.[20] In the present work, we determined the cytosolic distribution of Ag, Cd, Cu, Ni, Tl and Zn in field-collected *Chaoborus punctipennis* larvae. Metal distributions in the whole cytosol were studied using a column with a broad molecular weight separation range, whereas a narrower molecular weight range column was chosen for the subsequent study of the heat-stable protein fraction. Based on the ligand-binding preferences of trace elements[7], we hypothesized that "softer" elements such as Ag and Cd would be mainly associated with MMW ligands (e.g., MT or MTLP), unlike the harder elements (e.g., Ni).

Experimental procedures

Chemicals and standards

Unless stated otherwise, all analytical grade chemicals were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Solutions of 100 mM ammonium acetate (LC grade) were prepared in water and then sonicated before adjustment of the pH with ammonium hydroxide (pH = 8.0). Given that bio-ligands such as MT are susceptible to oxidation, the sonication step was included to remove dissolved oxygen. A 10 mM solution of EDTA (ethylenediamine-tetraacetic acid) in 100 mM ammonium acetate (pH = 8.0) was used as the column cleaning solution. A rabbit liver Cd-MT_2 isoform standard was obtained from Enzo Life Sciences (Villeurbanne, France). Trace-metal grade nitric acid (Fischer Scientific, Illkirch, France) was used for the preparation of samples, standards and blanks. All dilution and solutions were prepared with ultrapure water (18 MΩ cm) obtained from a Milli-Q water purification system (Millipore, Bedford, MA).

Collection of *Chaoborus* larvae

Chaoborus larvae were collected in 2011 from five lakes located on the Canadian Precambrian Shield near the mining areas of Sudbury, Ontario (Lakes Swan, Tilton, Hannah and Lohi) and Rouyn-Noranda, Quebec (Lake Opasatica). These lakes are

contaminated to various degrees by historical emissions from metal smelters.[21, 22] For example, larvae from Lake Opasatica (considered as a reference site) are reported to have lower total concentrations per unit dry weight (mean value (± SD, n = 3)) of Ni (1.0 ± 0.5 nmol g^{-1}), Zn (1500 ± 180 nmol g^{-1}), Cu (140 ± 14 nmol g^{-1}) and Cd (3.1 ± 0.6 nmol g^{-1}), compared to those from highly metal-contaminated lakes (ranges for [Ni]: 20 – 64 nmol g^{-1}; [Zn]: 1800 – 2500 nmol g^{-1}; [Cu] : 190 – 820 nmol g^{-1}; [Cd]: 18 – 88 nmol g^{-1}).[20] *Chaoborus* larvae were collected after sunset by hauling a plankton net with a mesh aperture of 164 µm horizontally in the water column and then transported in lake water to the laboratory for sorting.[23] Around 30 similar-sized, fourth-instar, larvae of the widespread species *Chaoborus punctipennis* were pooled, placed in 1.5-mL polypropylene microcentrifuge tubes and then immediately frozen and kept in liquid nitrogen until analysis.

Preparation of subcellular fractions

Larval samples collected from the five lakes were thawed on ice in 100 mM ammonium acetate buffer that had been adjusted to pH 7.4 in the ratio 1: 2 (insect wet weight : buffer volume; mg : mL) and each sample was homogenized using a Pellet Pestle (Kontes, Vineland, NJ, USA) for 2 s at 30-s intervals for 5 min.[20] An aliquot was collected from the larval homogenate for total metal determinations and the remainder was centrifuged at 100,000 × *g* for 45 min at 4 ^{o}C. The resulting pellets (nuclei + debris + granule-like fraction) were kept at -80 $^{\circ}$C and analyzed for total metals (see *Metal quantification and mass balance*) and the supernatant was retained as the cytosol. For three lakes (Lakes Opasatica, Swan and Lohi), 100-µL samples were removed from the larval cytosol for hyphenated analysis (n = 3). After removal of these 100-µL portions, the remaining cytosols plus the cytosols from the other two lakes (Lakes Hannah and Tilton) were held at 80 ºC for 10 min, left on ice for 30 min and then centrifuged at 100,000 × *g* for 45 min at 4 ^{o}C to obtain a pellet comprising the heat-denatured protein (HDP) fraction and a supernatant containing heat-stable proteins and peptides. For all lakes studied, larval samples were collected and analysed in triplicate, with the exception of Lake Swan for which duplicate larval samples were analysed.

SEC-ICPMS analysis

Chromatographic separation of the whole cytosol and the HSP fraction were conducted with an Agilent 1200 series bio-inert HPLC system (Agilent, Wilmington, DE) equipped with an integrated 4-channel degassing pump (G5611A), an autosampler with 100 µL injection loop (G5667A) and a Multiple Wavelength Detector (MWD, Agilent G1365B). The chromatographic equipment contained metal-free components (mainly PEEK; polyether ether ketone) in the sample flow-path so as to ensure the integrity of bio-molecules and minimize unwanted surface interactions. For elemental detection, a 7500 cs ICP-MS model (Tokyo, Japan) fitted with a micromist nebulizer and platinum cones was used. The instrument was tuned daily with a solution containing 1 µg L^{-1} Li, Y, Tl, and Ce in 2% HNO_3.

Separations of cytosolic fractions (100 µL) were carried out using a Superdex 200 (SEC_{200}) HR 10/30 size exclusion column (linear mass separation range between 10 – 600 kDa; GE Healthcare, Uppsala, Sweden), whereas the 100-µL HSP fractions were fractionated on a Superdex peptide (SEC_{pep}) 10/30 size exclusion column (linear mass separation range between 0.1 – 7 kDa; GE Healthcare, Uppsala, Sweden). The Superdex 200 column was previously calibrated with thyroglobulin (670 kDa), ferritin (474 kDa), transferrin (80 kDa), Mn-SOD (40 kDa), myoglobulin (16 kDa), Cd-MT_2 (6.8 kDa) and vitamin B_{12} (1.3 kDa), whereas the Superdex peptide column was calibrated with carbonic anhydrase (10 kDa), Cd-MT_2 (6.8 kDa), vitamin B_{12} (1.3 kDa) and cysteine (0.12 kDa). As we obtained metal-binding proteins with MW values outside the optimal separation range for the two columns (as provided by the suppliers), we used data from our MW standard calibration curve (plotting time versus log MW) to infer values for some MW fractions for the SEC_{200} column (e.g., "2400" kDa; "0.7" kDa) and the SEC_{prep} column (e.g., "27" kDa); inferred values are presented between quotation marks. For each column, fractionation was carried out with 100 mM ammonium acetate (pH = 8.0) as the mobile phase at a flow rate of 0.7 mL min^{-1}. Before each analysis, the column was flushed with 100 µL of the mobile phase containing 10 mM EDTA. Three injections of the EDTA solution (100 µL) were necessary between each SEC analysis to ensure low metal carry-over from the stationary phase. After the EDTA injections, the column was conditioned with the mobile phase. The chromatographic effluent was directly introduced via PEEK tubing into the MWD for evaluation of protein absorbance at 280 nm and at

254 nm and subsequently delivered to the ICPMS for ^{60}Ni, ^{64}Zn, ^{65}Cu, ^{107}Ag, ^{114}Cd and ^{205}Tl measurements. Identification of potential metal-binding proteins in the cytosol and in the HSP fraction that may be targeted by the metals studied was performed using the UniProt Knowledgebase (UniProtKB) at http://www.uniprot.org/uniprot/.

Metal quantification and mass balance

Metal concentrations in the cytosol and in the HSP fractions are expressed as the total metal burden (nmol) divided by the total larval dry weight (g, dw). A mass balance calculation was carried out by comparing the total element burdens, estimated from the 100-µL aliquots removed from the first larval homogenate, with burdens calculated from the sum of metals measured in the various subcellular fractions (nuclei + debris + granule-like; HDP; HSP). Good mass balance percentage values (mean value ± SD, n = 15) were obtained for Ni (111% ± 36%), Zn (107% ± 35%), Cu (107% ± 31%), Ag (92% ± 24%), Cd (111% ± 20%), and Tl (111% ± 23%). Metal column recoveries (n = 6) were also determined by measuring metal burdens in the collected chromatographic effluent after injecting cytosolic samples with known metal concentrations and then comparing these burdens with the amounts of metal injected. Acceptable column recoveries were obtained for Ni (106% ± 22%, n = 6), Zn (137% ± 7%, n = 5), Cu (126% ± 9%, n = 5), Ag (101% ± 38%, n =5), Cd (110% ± 3%, n = 6), and Tl (97% ± 9%, n = 6).

To identify what proportion of the total SEC-ICPMS metal signature (monitored as intensity) reported in the cytosol was found in each of the SEC_{200} MW pools (HMW, MMW and LMW), a ratio relating the area of the peaks observed in each SEC-ICPMS chromatogram (intensity *versus* time) between the limits of each MW pool to the sum of the total area integrated in the cytosol was determined for each metal. To describe how individual SEC_{pep} fractions tended to behave as a function of the total internalized metal in the HSP fraction, areas of the chromatographic peaks integrated between the limits of each SECpep fraction (normalized by total larval weight) were plotted against the total metal concentrations in the HSP fraction.

For metal analysis of the centrifugation pellets (i.e., nuclei + debris + granule-like structures and the HDP fraction), samples were freeze-dried and digested with 200 µL of 70% nitric acid at 80 *ºC* for 2 h. Aliquots *(*100-µL) of the supernatant subcellular

fractions (i.e., cytosol and HSP fraction) were similarly digested at 80 ºC, but by adding 100 µL of 70% nitric acid. All samples were then diluted with Milli-Q H_2O to reach a final nitric acid concentration of 2% (v/v) for the determination of total metal concentrations by ICPMS. Metals were quantified by external calibration using standard concentrations ranging from 0.1 to 10 µg L^{-1} (Ni, Ag, Cd and Tl) and from 1 to 100 µg L^{-1} (Cu and Zn) in 2% nitric acid.

Statistical analysis

All numerical data are represented by means ± standard deviations (SD). Differences in fraction area among the cytosolic MW pools (% values, after arcsine transformation) were assessed by one-way ANOVA using a non-parametric Kruskal-Wallis test followed by a Tukey honest significant differences (HSD) test. Relationships between total metal concentrations and the SEC_{pep} fraction area of each metal (normalized by total larval weight) in the HSP fraction were initially examined in bivariate scatterplots and tested by simple correlation analysis (Pearson r) after verifying assumptions of normality (Shapiro-Wilk test). When bivariate plots indicated a possible relationship, linear and exponential regression models were tested when the necessary assumptions were satisfied. Correlations reported between fraction areas of metals were obtained by applying non-parametric Spearman rank correlations. Statistical analyses were performed using STATISTICA version 6.1 software (StatSoft, Tulsa, Oklahoma, USA) and *P*-levels of 0.05 were used as the threshold for statistical significance.

Results and Discussion

Metal bioaccumulation gradients

Metal concentrations in the cytosol and the HSP fraction of *C. punctipennis* larvae varied greatly among the lakes studied (Table 1). Higher cytosolic metal concentrations were found for larvae collected from Lake Lohi (Ag, Cd, Cu, Ni and Zn) and Lake Swan (Tl) compared to Lake Opasatica, where the lowest metal concentrations were measured, with the exception of Zn.

Table 1. Metal concentration means (standard deviation, SD, n = 2-3) in the cytosol and in the heat-stable proteins and peptides (HSP) fraction of larvae of *Chaoborus punctipennis* collected from 5 lakes. Ratio $[M]_{max}$ / $[M]_{min}$ for each metal is also given.

Lakes	[M] *Chaoborus* (nmol g^{-1} dw)					
	Ag	Cd	Cu	Ni	Tl	Zn
Cytosol						
Opasatica	0.11 (0.030)	2.3 (0.63)	43 (10)	0.46 (0.11)	0.010 (0.0010)	220 (51)
Swan	0.20 (0.010)	21 (1.5)	56 (4.4)	8.3 (0.57)	0.050 (0.0030)	180 (16)
Lohi	0.39 (0.030)	41 (5.9)	130 (3.8)	15 (0.71)	0.024 (0.0020)	430 (51)
Ratio $[M]_{max}/[M]_{min}$	*3.6*	*18*	*3.0*	*33*	*5.0*	*2.4*
Heat-stable protein fraction						
Opasatica	0.068 (0.0079)	1.5 (0.16)	31 (1.9)	0.36 (0.15)	0.0060 (0.00057)	110 (15)
Swan	0.094 (0.0029)	5.2 (0.16)	34 (13)	3.4 (1.9)	0.028 (0.0020)	110 (13)
Lohi	0.25 (0.18)	24 (3.8)	76 (42)	8.6 (3.8)	0.021 (0.012)	360 (150)
Tilton	0.084 (0.021)	31 (6.7)	47 (9.0)	4.8 (2.4)	0.014 (0.0015)	150 (100)
Hannah	0.069 (0.014)	11 (4.8)	38 (15)	7.9 (3.7)	0.0070 (0.0034)	150 (100)
Ratio $[M]_{max}/[M]_{min}$	*3.7*	*21*	*2.5*	*24*	*4.7*	*3.3*

With regard to the HSP fraction, *Chaoborus* from Lakes Lohi (Ni, Zn, Cu, Ag), Tilton (Cd) and Swan (Tl) had the highest metal concentrations, whereas those from Lake Opasatica again had the lowest metal levels. Ratios of maximum to minimum metal concentrations ($[M]_{max}$ / $[M]_{min}$) for both fractions covered a very broad range, with higher ratios for the non-essential metals (e.g., Ni, Cd) than for the essential elements. As a consequence of the Ni-bearing emissions from the smelters located in Sudbury[21], but not Rouyn-Noranda, the highest $[M]_{max}$ / $[M]_{min}$ ratios were observed for Ni (cytosol: 33; HSP: 24). Large differences between the least and the most contaminated fractions were also found for Cd (cytosol: 18; HSP: 21) and Tl (cytosol: 5.0; HSP: 4.7), whereas the bioaccumulated metal gradient was lower for Cu (cytosol: 3.0, HSP: 2.5) and Zn (cytosol: 2.4, HSP: 3.3). These results are in agreement with previous studies on *Chaoborus* collected in the field along metal exposure gradients, where larval Zn and Cu concentrations vary less than those of the non-essential metals (e.g., Cd; Ni).[20] At the subcellular level, the mean concentrations measured in the HSP fraction represented more than 50% (n = 12-15) of the total larval burden for Ag (50% ± 23%), Cd (80% ± 17%), Cu (75% ± 32%), Ni (65% ± 38%) and Tl (79% ± 19%). These proportions are similar to those previously reported for field-collected *Chaoborus*, for which the majority of the bioaccumulated Cu, Cd and Ni was found in the HSP fraction.[20]

Metal binding to cytosolic ligands

The cytosolic fractions of *Chaoborus* larvae collected from Lakes Opasatica, Swan and Lohi were applied to the SEC_{200}-ICPMS system to perform a screening of the cytosolic metal-containing biomolecules (Figs. 1, 2). Metal recoveries obtained (n = 6) from the HPLC system were good for Ag (133% ± 5%), Cd (110% ± 7%), Cu (132% ± 9%), Ni (108% ± 6%), and Tl (96% ± 8%). In contrast, Zn recovery was inconsistent, suggesting the interaction of this metal with the HPLC components during the chromatographic separation.

The six metals (Ag, Cd, Cu, Ni, Tl, Zn) were associated with different cytosolic biomolecule fractions, operationally defined as HMW (high molecular weight; "2400" kDa – 40 kDa; elution time: 10-22 min), MMW (medium molecular weight; 40 kDa – "0.7" kDa; elution time: 22-32 min) and LMW (low molecular weight; < "0.7" kDa;

elution time: > 32 min) fractions. The UV detection data at 280 nm (data at 254 nm are not shown as they are similar) show that biomolecules elute over the entire molecular mass range of the column, with some overlap with the metal elution peaks. The HMW pool, which includes metalloenzymes, and the LMW pool where free amino acids and small peptides are found, are considered to be potentially metal-sensitive fractions.[5] On the other hand, the MMW pool includes MT and MTLP, which are involved in metal homeostasis (Cu, Zn) and detoxification (Ag, Cd).[24, 25] All elements were found in the HMW and MMW pools (Fig. 2); only Tl was detected in LMW pool (Fig. 1B).

Nickel

For Ni, the mean proportion of Ni binding to HMW ligands did not exceed 30% of the total cytosolic Ni, but for the cytosols from *Chaoborus* collected from Lakes Lohi and Swan these proportions were significantly higher ($P < 0.05$) than that for *Chaoborus* larvae from Lake Opasatica (Fig. 2A). This result is in agreement with observations reported for *Gammarus fossarum*, where Ni accumulated in the pool denoted as " non-MTLP", which included the HMW (255 kDa – 18 kDa) and LMW (< 1.8 kDa) fractions, increased significantly as a function of Ni exposure.[26] The retention times corresponding to the HMW pool covered a MW range from 670 kDa to 200 kDa with a maximum peak at 385 kDa (15.3 min). We speculate that the Ni found in the cytosolic HMW pool could be associated with Mg- or Fe-containing proteins, where Ni could replace one of these essential elements, a reaction that may result in impairment of the biological functions in which these proteins are involved.[27] In this MW range, some cytoplasmic proteins expressed in dipterans (the order to which *Chaoborus* belongs) contain Mg as a cofactor, e.g., the endoribonuclease Dcr-1of 255 kDa [28] and the drop kinase of 225 kDa[29]; these proteins are potential Ni targets.

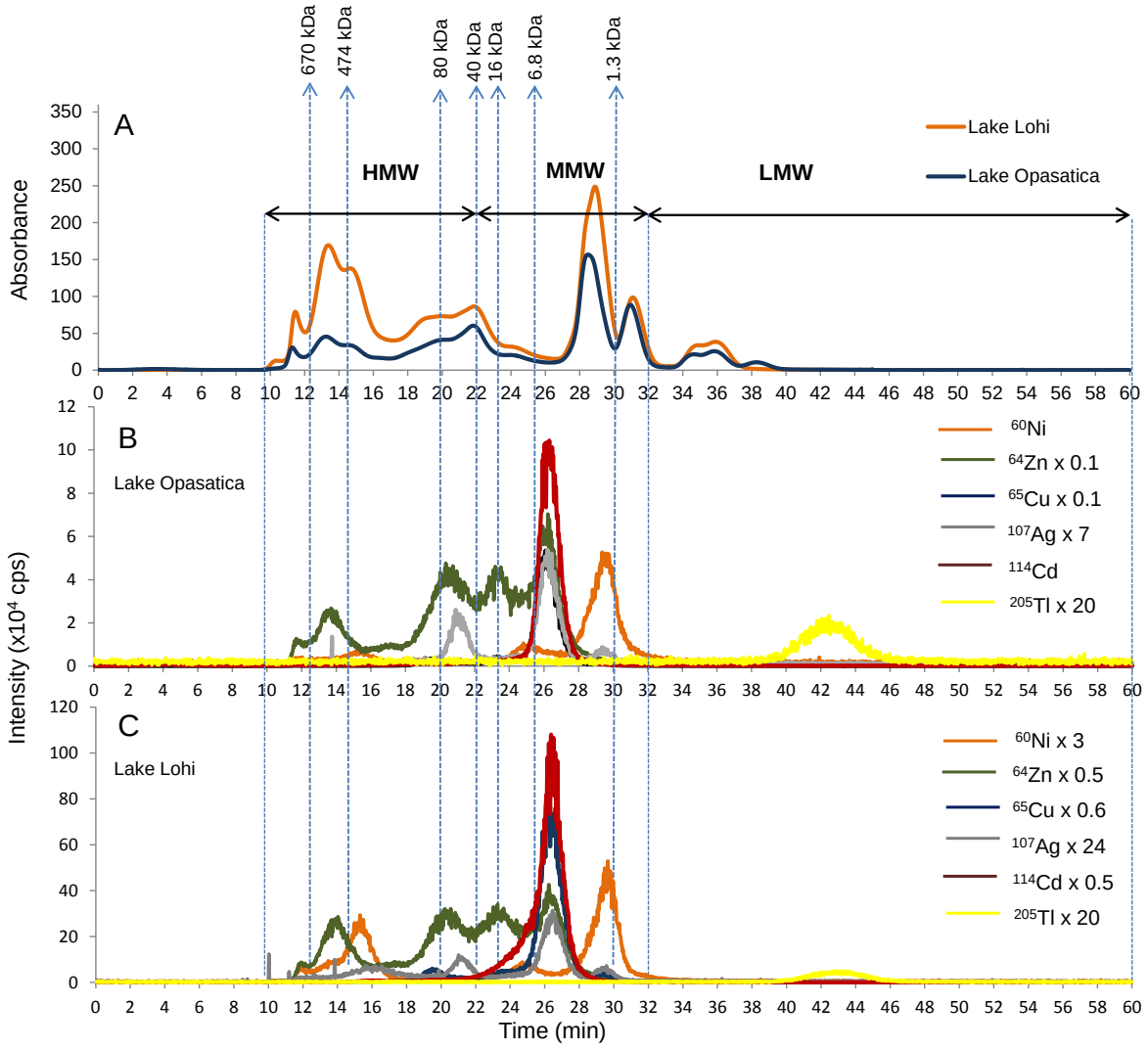

Fig.1. SEC_{200} chromatograms of the cytosol from field-collected *Chaoborus punctipennis* larvae: A) with UV detection at 280 nm for larvae from Lake Opasatica (black line, reference sample) and Lake Lohi (gray line, highly contaminated sample), or with ICPMS detection for larvae from Lake Opasatica (B) or Lake Lohi (C). Vertical dotted lines represent retention times of the MW standards. The three operationally-defined MW fractions comprise: HMW (high molecular weight; "2400 kDa – 40 kDa; elution time: 10-22 min), MMW (medium molecular weight; 40 kDa – "0.7" kDa; elution time: 22-32 min) and LMW (low molecular weight; "0.7" kDa; elution time: > 32 min).

Most of the total cytosolic Ni was found in the MMW pool (from 69 to 78%, Fig. 2A), and these proportions remained unchanged ($P > 0.05$) with increasing total cytosolic Ni (Fig 2A). However, Ni concentrations in the MMW pool for larvae collected from Lake Swan and Lake Lohi were significantly higher ($P < 0.05$) than that for larvae from the reference lake. Considering that Ni has a low affinity for sulphydryl functional groups, its sequestration in the MMW pool is likely determined more by the occurrence of O- and P-containing ligands than by the presence of MT and other sulfhydryl-rich ligands.[7] Based on the ability of Ni to replace essential metals in physiologically important enzymes[27], the Ni intensity observed in the peaks eluting from 23 min (16.5 kDa) to 30 min (1.3 kDa) may reflect thermostable binding of this element to low molecular weight dipteran proteins where Mg acts as a cofactor.

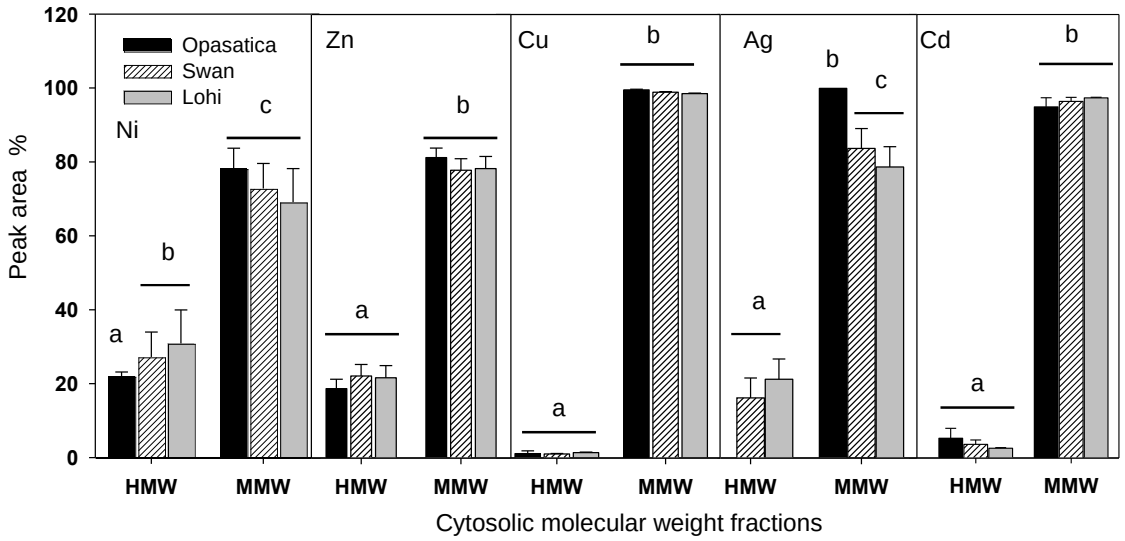

Fig. 2. Peak area percentages (mean ± S.D.; n = 3) of ^{60}Ni, ^{64}Zn, ^{65}Cu, ^{107}Ag and ^{114}Cd in two cytosolic molecular weight pools (HMW, MMW) of *Chaoborus punctipennis* collected from Lakes Opasatica, Swan and Lohi. The cytosols were fractionated by SEC_{200}-ICPMS. Bars with the same letters indicate non-significant differences, whereas different letters indicate that the differences are significant (ANOVA followed by Tukey test, $P < 0.05$).

Copper and zinc

For the two essential metals studied, higher proportions were found in the MMW pool (average for all larvae of 79% ± 1.4% for Zn and 99% ± 0.1% for Cu, n = 9) than in the

HMW pool (Figs. 2B, C). Similarly, in liver of field-collected gibel carp (*Carassius auratus gibelio*), the Cu and Zn concentrations in the MMW pool (defined as ~10 kDa) also increased as a function of the cytosolic metal concentrations.[30] Copper and Zn binding to MMW ligands was also identified in zebrafish gills, where Zn was also associated with higher MW proteins.[31] The appreciable Zn signals reported in the HMW and MMW fractions in *Chaoborus* could be associated with the role of this essential metal in the stability of the zinc-finger structures[32] reported in various dipteran proteins located in the cytosol, including E3 ubiquitin-protein ligase (130 kDa)[33], ubiquitin protein ligase deltex (82 kDa)[34] and translation inhibition factor 2 (35 kDa).[35] With regards to Cu, its cytosolic distribution in the HWM pool likely reflects the association of Cu with physiologically important metalloproteins.[36] On the other hand, both essential metals are largely associated with MT or MTLP (5-4.0 kDa), which are known to be involved in the homeostasis of these elements.[7]

Silver

Higher proportions of total cytosolic Ag were found in the MMW pool than in the HMW pool (Fig. 2D). At a low cytosolic Ag concentration (i.e., larvae from Lake Opasatica), Ag could only be detected in the MMW pool (Figs. 1B and 2D). For the two more contaminated cytosols, Ag was bound to both HMW and MMW bioligands; the proportion of Ag in the MMW pool decreased from 100% to about 79% in the cytosols of larvae collected from the two more contaminated lakes (Fig. 2D). High Ag concentrations in the MMW (10 – 20 kDa) pool compared to the HMW (>20 kDa) pool were also found in livers of *Anguilla anguilla* eels collected from sampling sites differing in their metal contamination levels.[37] In eels, the percentage of Ag found in the MMW fraction, which represented up 92% of the total cytosolic Ag, increased as a function of the Ag concentrations in the surrounding sediment and water. These observations are consistent with the preference of this "soft" metal for the sulphydryl groups found in MT or MTLP. Other sulphydryl-containing proteins in the HMW pool may be targeted by this non-essential metal.

Cadmium

Virtually all of the cytosolic Cd was found in the MMW pool (average for all larval samples of 97 ± 1.2 %, n = 9), irrespective of the actual Cd concentration in the cytosol (Fig. 1B, C and 2E). The association of Cd with MTLP bioligands in *Chaoborus* has been reported previously in a study where MTLP concentrations were determined with a Hg-saturation method.[38] The authors obtained a strong correlation between the measured MTLP and the total larval Cd concentration, reflecting the induction of MTLP by Cd and the subsequent complexation of Cd by the induced peptide. In *Anguilla anguilla*[37] and in *C. auratus gibelio*[30] collected from metal-impacted habitats, high proportions of the total cytosolic Cd were also found in the MMW fraction, and this accumulation increased with the increasing cytosolic Cd concentrations. The predominant role of MTLP in sequestering Cd was also reported for indigenous floater mussels (*Pyganodon grandis*)[39], where cytosolic Cd concentrations in the hepatopancreas were significantly related to the MTLP levels.

Thallium

In contrast to the dominance of the HMW and MMW fractions for the other metals, Tl was only found in the LMW fraction. Considering the potential interaction of Tl(I) with sulphydryl-containing groups[7], the partitioning of this metal could be ascribed to its association with free amino acids containing such chemical groups (e.g., cysteine) or possibly to the occurrence of thallium methylation to form $(CH_3)_2Tl^+$ as has already been reported in bacteria.[40]

Metal binding to metallothionein-like peptides and other thermostable ligands

To further investigate metal-binding to cytosolic ligands, we isolated the HSP fraction of larvae from five lakes by heat treatment of the cytosol. These HSP fractions were subjected to further separation using SECpep-ICP MS (Figs. 3 and 4). The metals studied were associated with various subcellular fractions operationally defined as SEC_{pep} fraction 1 ("31" kDa – 6.2 kDa; elution time: 11-16 min), SEC_{pep} fraction 2 (6.0 kDa – 1.7 kDa; elution time: 16-22 min) and SEC_{pep} fraction 3 (< 1.7 kDa; elution time: > 22 min).

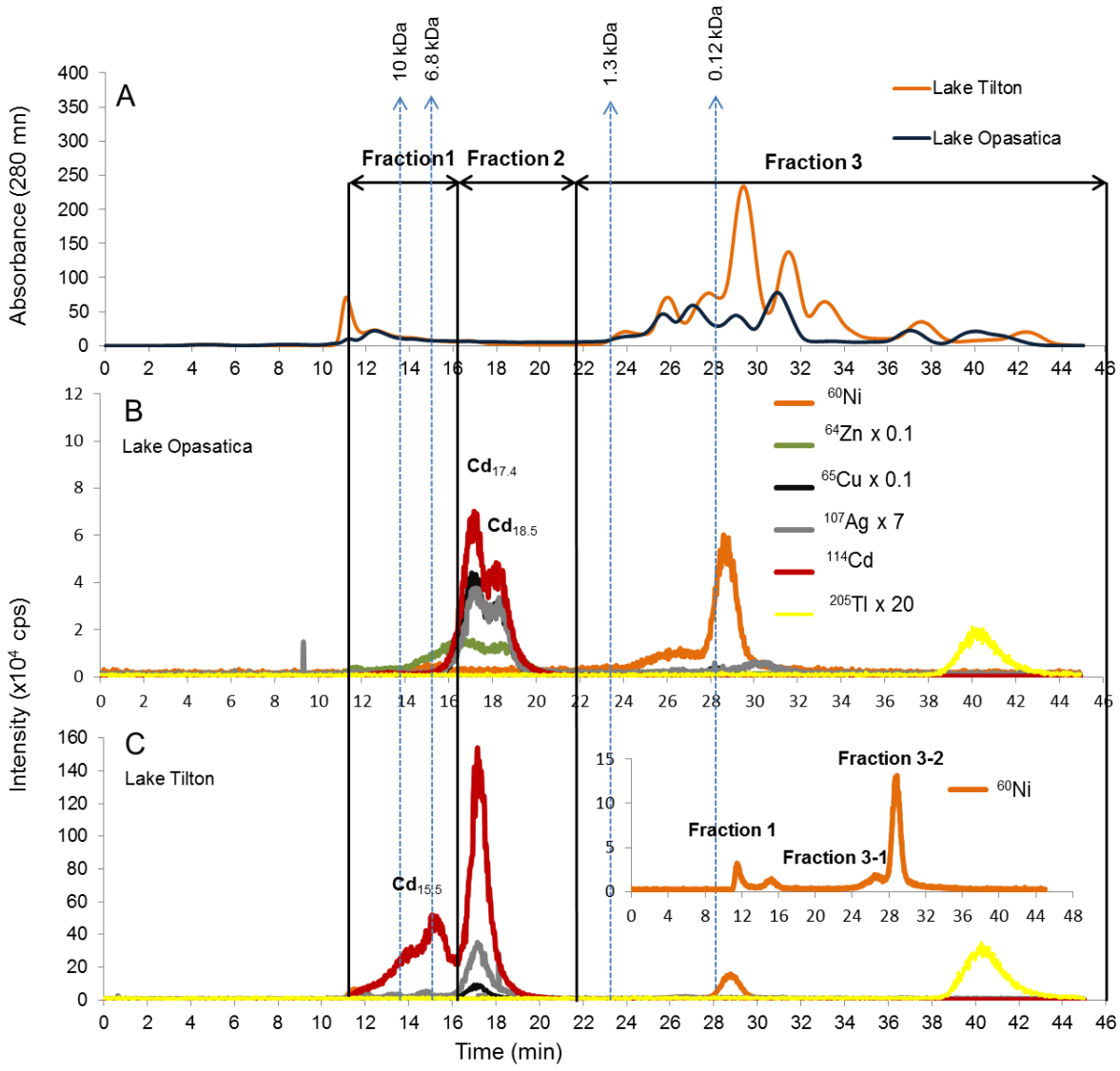

Fig. 3. SEC_{pep} chromatograms of metal-containing ligands in the HSP fraction of *Chaoborus punctipennis* larvae. A) with UV detection at 280 nm for larvae from Lake Opasatica (black line, reference lake) and Lake Tilton (gray line, highly contaminated lake), with ICPMS detection for larvae from Lake Opasatica (B) and from Lake Tilton (C) among three MW SEC_{pep} fractions: fraction 1 ("31" kDa – 6.2 kDa; elution time: 11-16 min); fraction 2 (6.2 kDa – 1.7 kDa; elution time: 16-22 min); fraction 3 (< 1.7 kDa; elution time: > 22 min).

Non-essential metals

The Ni profile of metal-containing biomolecules showed that the partitioning of this element occurred across the entire range from SEC_{pep} fraction 1 ("31" kDa – 6.0 kDa) to SEC_{pep} fraction 3 (< 1.7 kDa) (Fig. 3C and insert). In SEC_{pep} fraction 3, two peaks were

detected: one from 24.4 min to 27.6 min (0.5 kDa – 0.2 kDa; denoted as SEC_{pep} fraction 3-1 in Fig. 3C) and from 27.6 min to 30 min (0.2 kDa – 0.1 kDa; denoted as SEC_{pep} fraction 3-2 in Fig. 3C). Along the Ni bioaccumulation gradient, the areas of the chromatographic peaks found in SEC_{pep} fraction 3-1 (r^2 = 0.83, P = 0.03; n = 12) and SEC_{pep} fraction 3-2 (r^2 = 0.92, P = 0.01; n = 12), both normalized by the total larval weight, increased linearly and towards a plateau (Fig. 4A); the highest areas were reported for SEC_{pep} fraction 3-1 in all cases. None of the Ni-containing biomolecules co-eluted with Ag, Cd, Cu or Zn (Figs. 3B, C), indicating that thermostable bioligands other than MTLPs are associated with this potentially toxic metal in the HSP fractions of *Chaoborus* larvae. This distinctive characteristic for Ni binding is not surprising, given that, as a borderline metal[7], Ni has a weak affinity for sulphydryl-rich ligands in comparison to other class B metals. The biomolecules responsible for Ni binding in the HSP fraction are unknown. We speculate that Mg- (or Fe-) containing enzymes may be potential targets for Ni, but it is unclear why this Ni substitution would confer thermal stability on these proteins.

The SEC-ICPMS chromatogram of Cd in the HSP fraction from Lake Opasatica showed two peaks at 17.4 min (4.7 kDa) and 18.5 min (3.2 kDa) in SEC_{pep} fraction 2, peaks denoted as $Cd_{17.4min}$ and $Cd_{18.5min}$, respectively (Fig. 3B). Similar chromatographic profiles showing co-elution with Cd at these retention times were obtained for Ag, Cu and Zn in the HSP fractions of larvae originating from the reference lake (Fig. 3B). As the total Cd concentration in the HSP fraction increased, the $Cd_{18.5min}$ peak tended to disappear, whereas the $Cd_{17.4min}$ peak signal significantly increased (Figs. 3C and 4E). As was observed for the HSP samples collected from the reference lake, the SECpep-ICP MS chromatograms for HSP samples from the contaminated lakes also showed co-elution of Ag, Cu and Zn under the $Cd_{17.4min}$ peak (Fig. 3C).

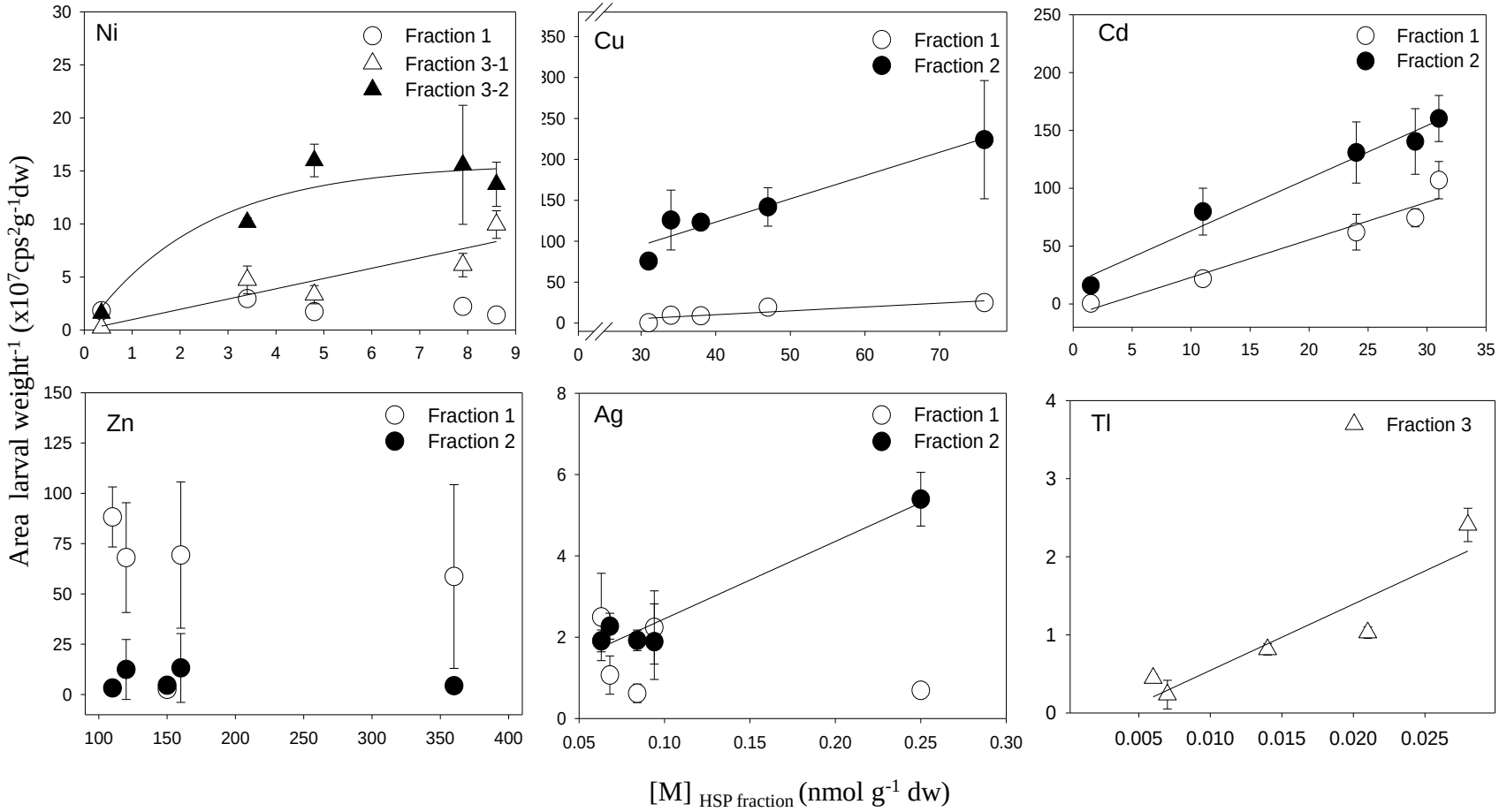

Fig. 4. Relationships between peak area (107 cps2) x larval weight-1(g dw) (mean ± S.D.; n = 2-3) for 60Ni, 64Zn, 65Cu, 107Ag, 114Cd, 205Tl and the total metal concentration in the HSP fraction of *Chaoborus punctipennis* larvae for the three MW SECpep fractions: fraction 1 ("31" kDa – 6.2 kDa; elution time: 11-16 min); fraction 2 (6.2 kDa – 1.7 kDa; elution time: 16-22 min); fraction 3 (< 1.7 kDa; elution time: > 22 min).

In addition to the peaks occurring in SEC_{pep} fraction 2, Cd was also detected in a biomolecule of higher molecular weight in the HSP samples from all lakes except Lake Opasatica, the reference lake. This Cd peak was detected at an elution time of 15.5 min, corresponding to a MW of 6.5 kDa (denoted as $Cd_{15.5min}$ in Fig. 3C). With increasing total Cd concentrations in the HSP fraction, the response of the ligands in SEC_{pep} fraction 2 (slope: 78 ± 4; $P = 0.0005$, n = 14) in sequestering this element was significantly stronger ($P < 0.05$) than that of SEC_{pep} fraction 1 (slope: 54 ± 6; $P = 0.003$, n = 14); as a consequence, Cd concentrations detected in SEC_{pep} fraction 2 were consistently higher than those in fraction 1 for all the larval HSP samples (Fig. 4E).

Our results suggest that at low Cd concentrations in the HSP fraction, this element is sequestered by two constitutive MT isoforms with molecular weights (estimated using data obtained from the calibration column) of ~4.7 kDa and 3.2 kDa, which are very similar to the MW reported for the MtnA and MtnB isoforms of the fruit fly *Drosophila melanogaster*.[41, 42] When more Cd accumulates in the HSP fraction, the concentration of the putative 4.7 kDa MtnB isoform increases but the 3.5 kDa MtnA isoform does not respond in a similar manner. This observation agrees with previous studies[43, 44] in which the existence of distinct MT isoforms playing different roles in metal detoxification was reported for snails. In addition to the putative 4.7 kDa MT isoform, ligands of higher MW (6.5 kDa, possibly MT dimers) also appear to be involved in Cd sequestration as the total Cd concentration increases in the HSP fraction. This metal-handling strategy under high Cd exposure is consistent with an earlier field study on chronically metal-exposed *Chaoborus*, where we demonstrated that the Cd detoxification response became more effective at higher internalized Cd concentrations.[20]

Most of the Ag accumulated in the HSP fraction was found in SEC_{pep} fraction 2 (86% ± 16% for all lakes, n = 12) and the ICPMS signal of this peak increased as the total Ag concentration increased (Fig. 4D). In contrast, Ag in SEC_{pep} fraction 1 did not increase as a function of the Ag accumulated in the HSP fraction (Fig. 4 D).

The distribution of Tl in the HSP fraction differed markedly from that observed for the other metals. The only appreciable Tl peak was observed at an elution time of 40.2 min (Figs. 3B, C), i.e., not at a retention time where proteins would be expected to elute. The

area of the peak responded linearly as a function of the total Tl concentration in the HSP fraction (r^2 = 0.84, *P* = 0.03, n = 14, Fig. 4F). We speculate that the Tl profile may reflect the presence of a methylated species ($(CH_3)_2Tl^+$).[40]

Essential metals

Within the HSP samples, more than 80% of the total Cu was found in MW SEC_{pep} fraction 2, with a peak at 17.4 min, but both fractions 1 and 2 responded significantly along the total Cu bioaccumulation gradient in the HSP fraction (Fig. 4C). In contrast, we observed that Zn measured in both SEC_{pep} fractions did not vary along the Zn gradient in the HSP fraction (Fig. 4B), indicating an important loss of this metal during the chromatographic separations. Because of the low recoveries observed for Zn in the HSP fraction, we cannot interpret the lack of response of SEC_{pep} fractions 1 and 2.

Metal-metal interactions

In the SEC_{pep} separations of the HSP samples, the peak at 17.4 min corresponded to the maximum ICPMS signal for Ag, Cd, Cu and Zn. Strong correlations were reported between the areas of Cd SEC_{pep} fraction 2 and Cu SEC_{pep} fraction 2 (r = 0.89, *P* = 0.01) and between Cd SEC_{pep} fraction 2 and Ag SEC_{pep} fraction 2 (r = 0.74, *P* = 0.03). These results suggest co-sequestration of these metals by MT or MTLP, an interpretation consistent with the known affinity of Ag, Cd and Cu(I) for the sulphydryl groups found in MT or MTLP.[7] In addition, multi-element binding of MT, which has been documented in *in vitro* experiments[45], has been also reported in field-collected organisms. For example, Van Campenhout and co-workers[13] showed that Cd, Cu and Zn were largely bound to MT in field-collected *Anguilla anguilla*. Heteronuclear complexes of MT with various metals such as Cd, Cu, Zn and Hg, as is the case in the present work, were detected and identified in cytosolic fractions of the white-sided dolphin *Lagenorhynchus acutus* by hydrophilic interaction LC coupled with ICPMS and electrospray high resolution mass spectrometry.[46] The multi-elemental binding of MT shown in *Chaoborus* is consistent with the capacity of both MT isoforms of *D. melanogaster* to produce complexes with Cd, Cu and Zn.[41]

Ecotoxicological considerations

The SEC-ICPMS approach revealed the presence of bioligands involved in the handling of both essential (Cu and Zn) and nonessential (Ag, Cd, Ni, Tl) metals in field-collected *Chaoborus* larvae. In the cytosol, metal complexes were observed in potentially metal-sensitive pools (Ag, Ni in the HMW pool and Tl in the LMW pool), whereas the MMW pool appears to be involved in Ag, Cd and Ni detoxification. Within the HSP fraction, different biomolecules are involved in binding Cd and Ni. Cadmium is largely bound to molecules that elute where MTLPs are expected to appear, and the relative importance of these putative MT isoforms varies as a function of the accumulated Cd concentration, which is consistent with a change in the subcellular handling of Cd as metal exposure increases. In this area of the chromatogram, Cd co-elutes with other soft metals (Ag, Cu) but Ni elutes much later in a distinct mono-metallic peak, the molecular nature of which remains to be determined. The majority of peaks detected in the UV chromatograms did not match the Ag, Cd, Cu, Tl and Zn chromatographic peaks, but the UV peak at 28.7 min did overlap with the major Ni peak, suggesting the presence of a Ni-containing complex with a MW of approximately 0.10 kDa and some delocalized electrons (aromatic rings).

From an ecotoxicological perspective, and focusing on the nonessential metals, the presence of Ag and Cd in the MTLP fraction indicates that they have been at least partially detoxified by *Chaoborus*, whereas for both Ni and Tl the evidence for their detoxification is less clear. To our knowledge, the predominant binding of these latter two metals in low molecular weight forms is unprecedented in the metallomics literature.

The observation that these LMW forms survive the chromatographic separation step intact indicates that Ni and Tl are tightly bound (i.e., slow dissociation kinetics). This observation, together with the virtual absence of these elements in the high molecular weight pool, may be taken as evidence that the two metals are relatively unavailable within the cytosol. It clearly will be of interest to determine the molecular nature of these puzzling forms.

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Supporting information for:

Metal (Ag, Cd, Cu, Ni, Tl, Zn) binding to cytosolic ligands in field-collected larvae of the insect *Chaoborus* as determined by Size-Exclusion Chromatography coupled to Inductively Coupled Plasma-Mass Spectrometry

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Metallomics (in preparation)

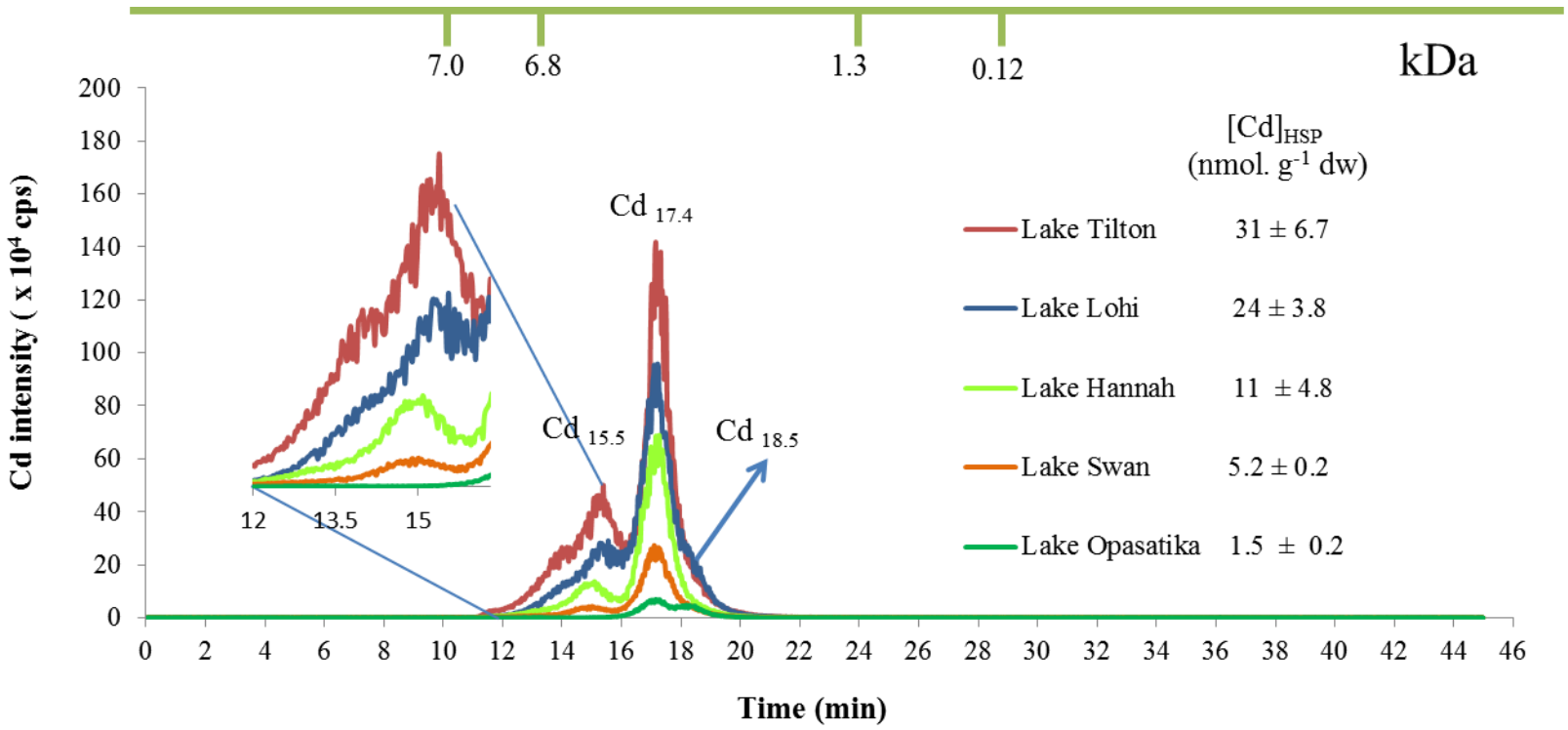

Figure S-1. SEC_{pep} – ICPMS chromatogram of Cd-containing ligands in the HSP fraction of *Chaoborus punctipennis* larvae collected from 5 mining-impacted lakes. Total Cd concentrations in each HSP fraction are also given.

14. APPENDIX

Figure A.1: Simplified illustration of the SEC-ICPMS approach used for the cytosol and the HSP fractions of *Chaoborus* larvae. Metal-ligand complexes were separated as a function of their molecular weight before to be analysed on line by ICP-MS.

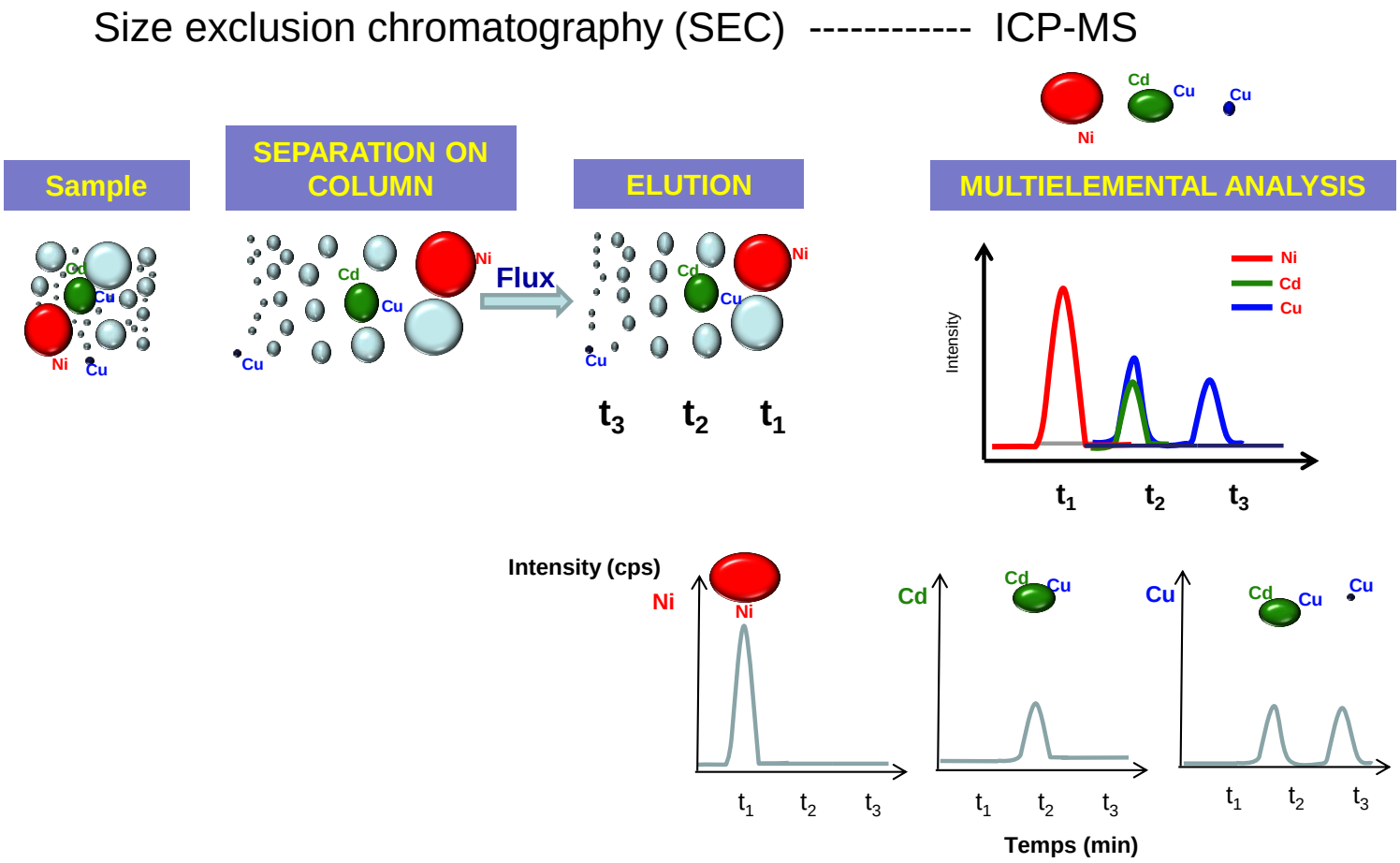

Table A 1 : Comparison of the slopes of the relationships between the detoxified metal compartment (∑ detoxified metal fractions, DMF) or the metal-sensitive compartment (∑ metal-sensitive fractions, MSF) and the total internalized metal concentrations, for the cases where the 'microsomes plus lysosomes' fraction is considered to be MSF, neutral or DMF, for Cd and Ni.

Trace metal	Organisms	Compartments	Microsomes and lysosomes as:		
			Metal-sensitive compartment (MSF)	Neutral[b]	Detoxified metal compartment (DMF)[c]
Cd	*Chaoborus* (whole body)	DMF	slope : 0.69 ± 0.08, r^2 = 0.89	slope : 0.69 ± 0.08, r^2 = 0.89	slope : 0.74 ± 0.03, r^2 = 0.99
		MSF	*P* = 0.2	*P* = 0.3	*P* = 0.3
		MSF or DMF?[a]	DMF		
	Perca flavescens (liver)	DMF	slope : 0.52 ± 0.03, r^2 = 0.98	slope : 0.52 ± 0.03, r^2 = 0.98	slope : 0.63 ± 0.02, r^2 = 0.99
		MSF	slope : 0.33 ± 0.03, r^2 = 0.96	slope : 0.23 ± 0.02, r^2 = 0.95	slope : 0.23 ± 0.02, r^2 = 0.95
		MSF or DMF?[a]	DMF		
	Pyganodon grandis (digestive glands)	DMF	slope : 0.56 ± 0.02, r^2 = 0.99	slope : 0.56 ± 0.02, r^2 = 0.99	slope : 0.79 ± 0.03, r^2 = 0.99
		MSF	slope : 0.08 ± 0.02, r^2 = 0.60	slope : 0.06 ± 0.03, r^2 = 0.48	slope : 0.06 ± 0.03, r^2 = 0.48
		MSF or DMF?[a]	DMF		
	Pyganodon grandis (gills)	DMF	slope : 0.78 ± 0.02, r^2 = 0.99	slope : 0.78 ± 0.02, r^2 = 0.99	slope : 0.74 ± 0.03, r^2 = 0.99
		MSF	slope : 0.011 ± 0.004, r^2 = 0.50	*P* = 0.06	*P* = 0.06
		MSF or DMF?[a]	DMF		
Ni	*Chaoborus* (whole body)	DMF	slope : 0.88 ± 0.13, r2 = 0.92	slope : 0.88 ± 0.13, r2 = 0.92	slope : 0.90 ± 0.12, r2 = 0.94
		MSF	*P* = 0.5	*P* = 0.6	*P* = 0.6
		MSF or DMF?[a]	DMF		
	Perca flavescens (liver)	DMF	slope : 0.19 ± 0.01, r2 = 0.99	slope : 0.19 ± 0.01, r2 = 0.99	slope : 0.36 ± 0.01, r2 = 0.99
		MSF	slope : 0.54 ± 0.01, r2 = 0.99	slope : 0.38 ± 0.01, r2 = 0.99	slope : 0.38 ± 0.01, r2 = 0.99
		MSF or DMF?[a]	DMF		=

[a]: comparing the slopes between DMF and MSF compartments in each simulation
[b]: non including Microsomes + lysosome in the MSF compartment
[c]: including Microsomes + lysosome in the DMF compartment

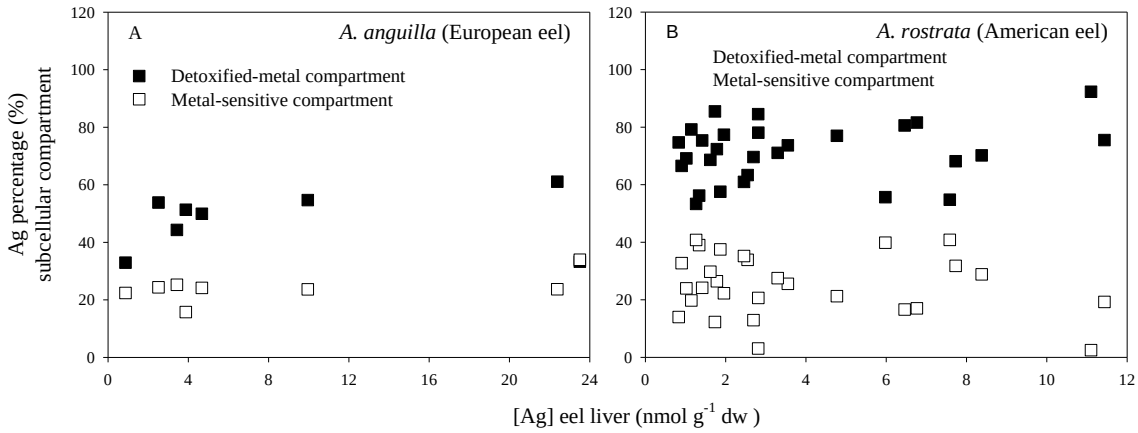

Figure A.2: Relationship between total hepatic Ag concentrations (horizontal axis) and Ag percentage (%) in detoxified-metal compartment (closed squares) and metal-sensitive fractions (open squares) in *Anguilla anguilla* (panels A) and *Anguilla rostrata* (panels B). Each point represents an individual eel.

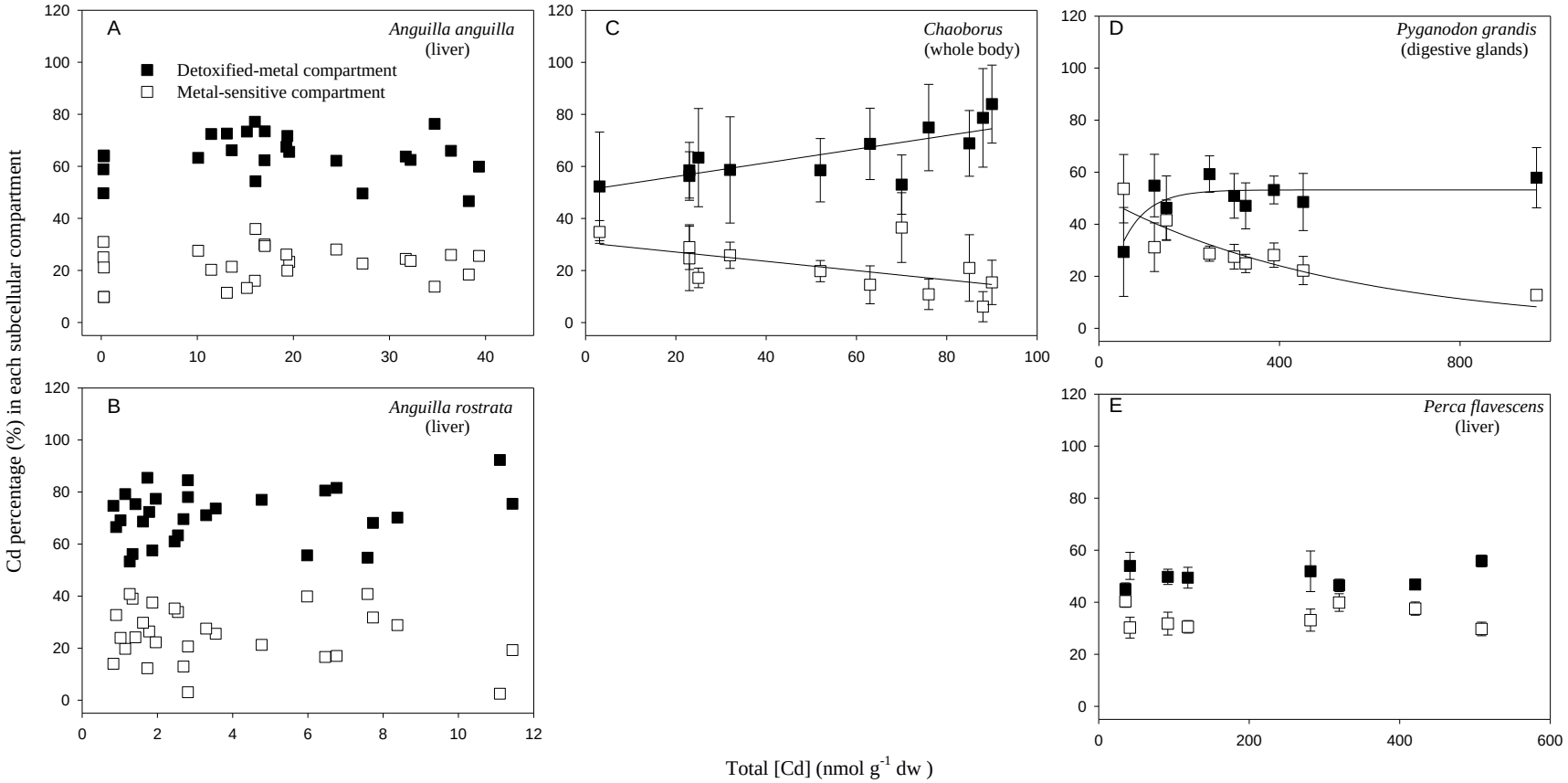

Figure A.3: Relationship between total Cd concentrations (horizontal axis) and Cd percentage (%) in detoxified-metal compartment (closed squares) and metal-sensitive fractions (open squares) in *Anguilla anguilla* (liver, panel A), *Anguilla rostrata* (liver, panel B), *Chaoborus* larvae (whole body, panel C), *Pyganodon grandis* (digestive gland, panel D) and *Perca flavescens* (liver, panel E). Lines represent significant regressions ($P < 0.05$).

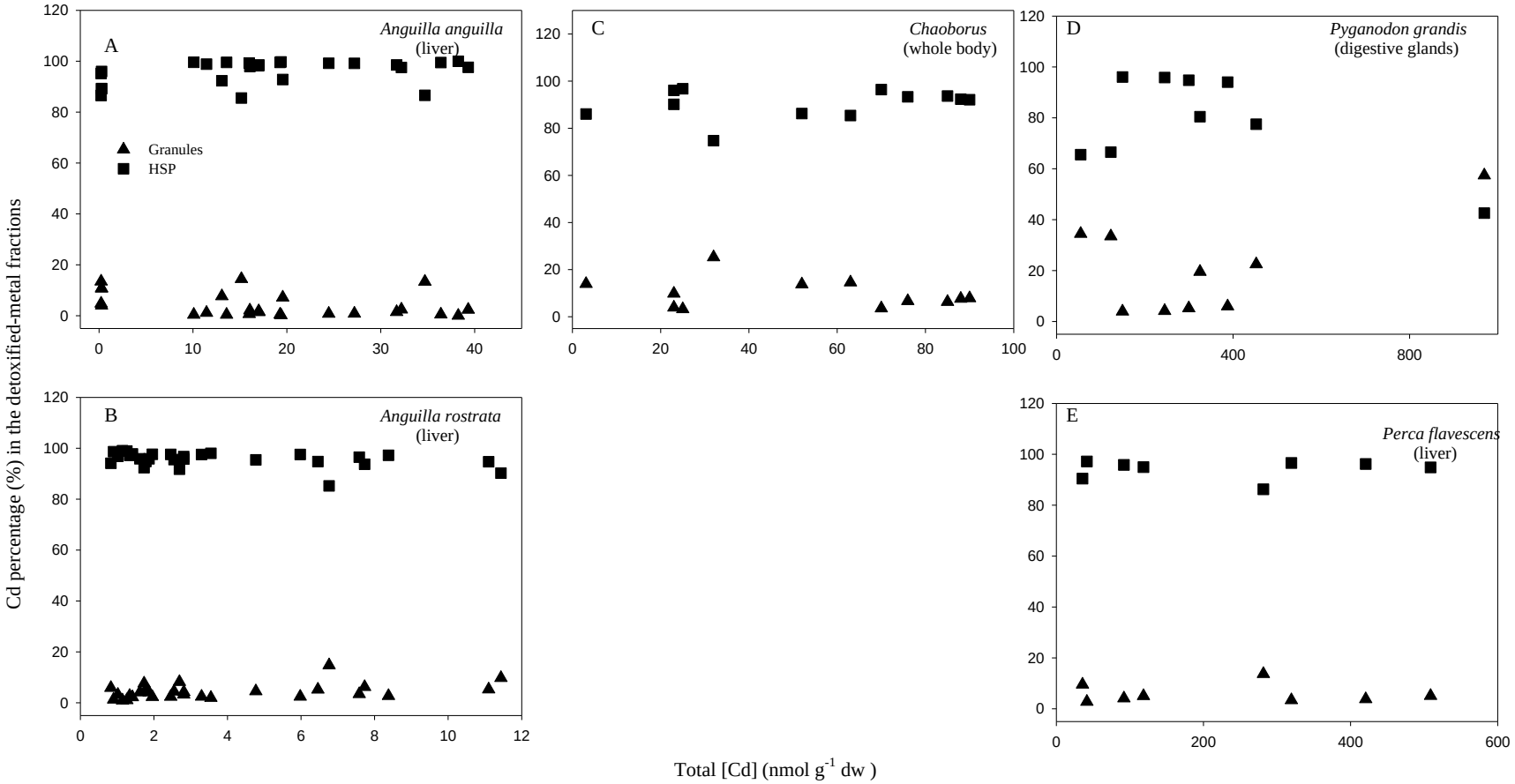

Figure A.4: Relationship between total Cd concentrations (horizontal axis) and Cd percentage (%) in the granules (triangle) and in the HSP fractions (square) in *Anguilla anguilla* (liver, panel A), *Anguilla rostrata* (liver, panel B), *Chaoborus* larvae (whole body, panel C), *Pyganodon grandis* (digestive gland, panel D) and *Perca flavescens* (liver, panel E). Lines represent significant regressions ($P < 0.05$).

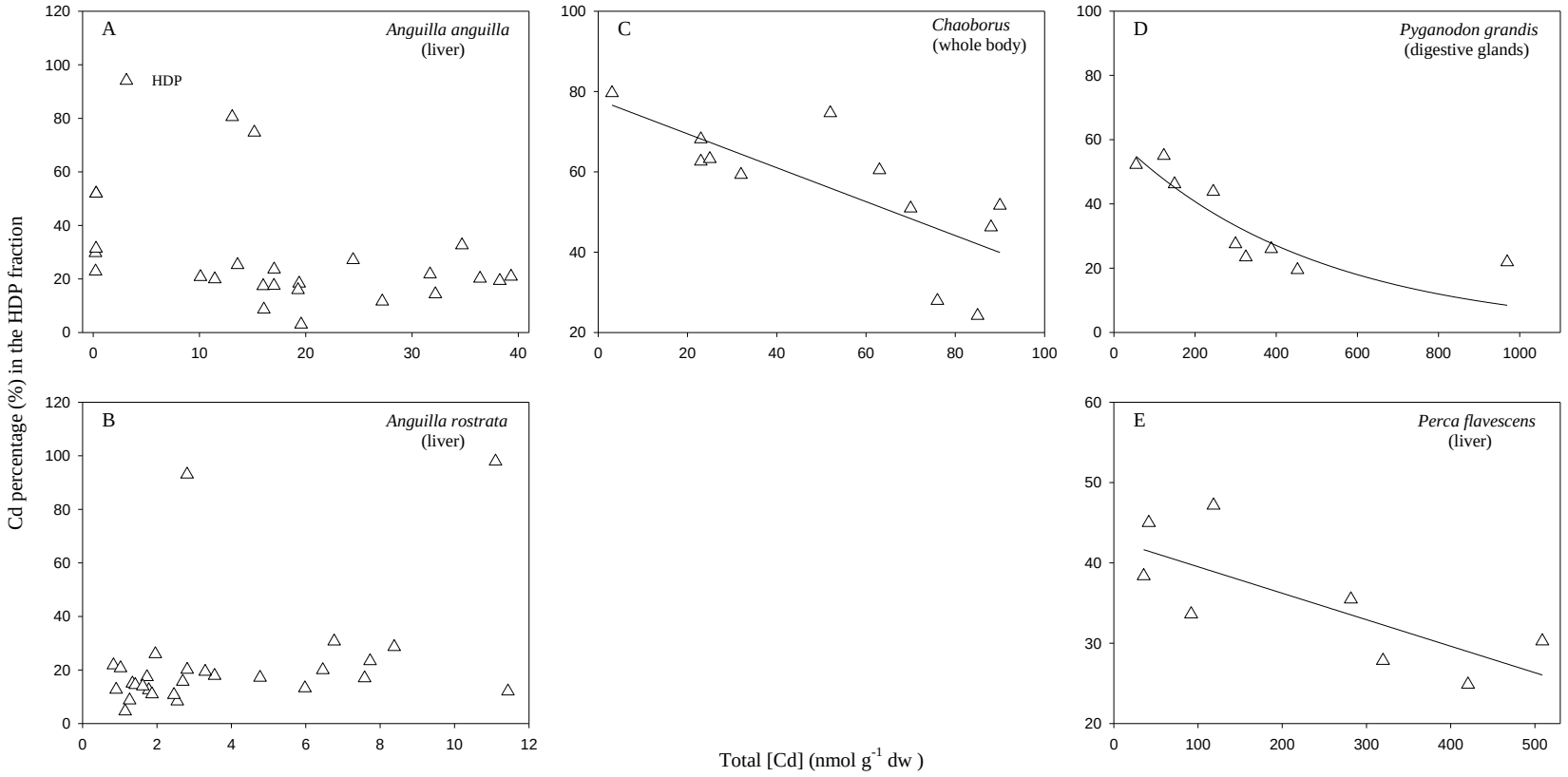

Figure A.5: Relationship between total Cd concentrations (horizontal axis) and Cd percentage (%) in the HDP fractions in *Anguilla anguilla* (liver, panel A), *Anguilla rostrata* (liver, panel B), *Chaoborus* larvae (whole body, panel C), *Pyganodon grandis* (digestive gland, panel D) and *Perca flavescens* (liver, panel E). Lines represent significant regressions ($P < 0.05$).

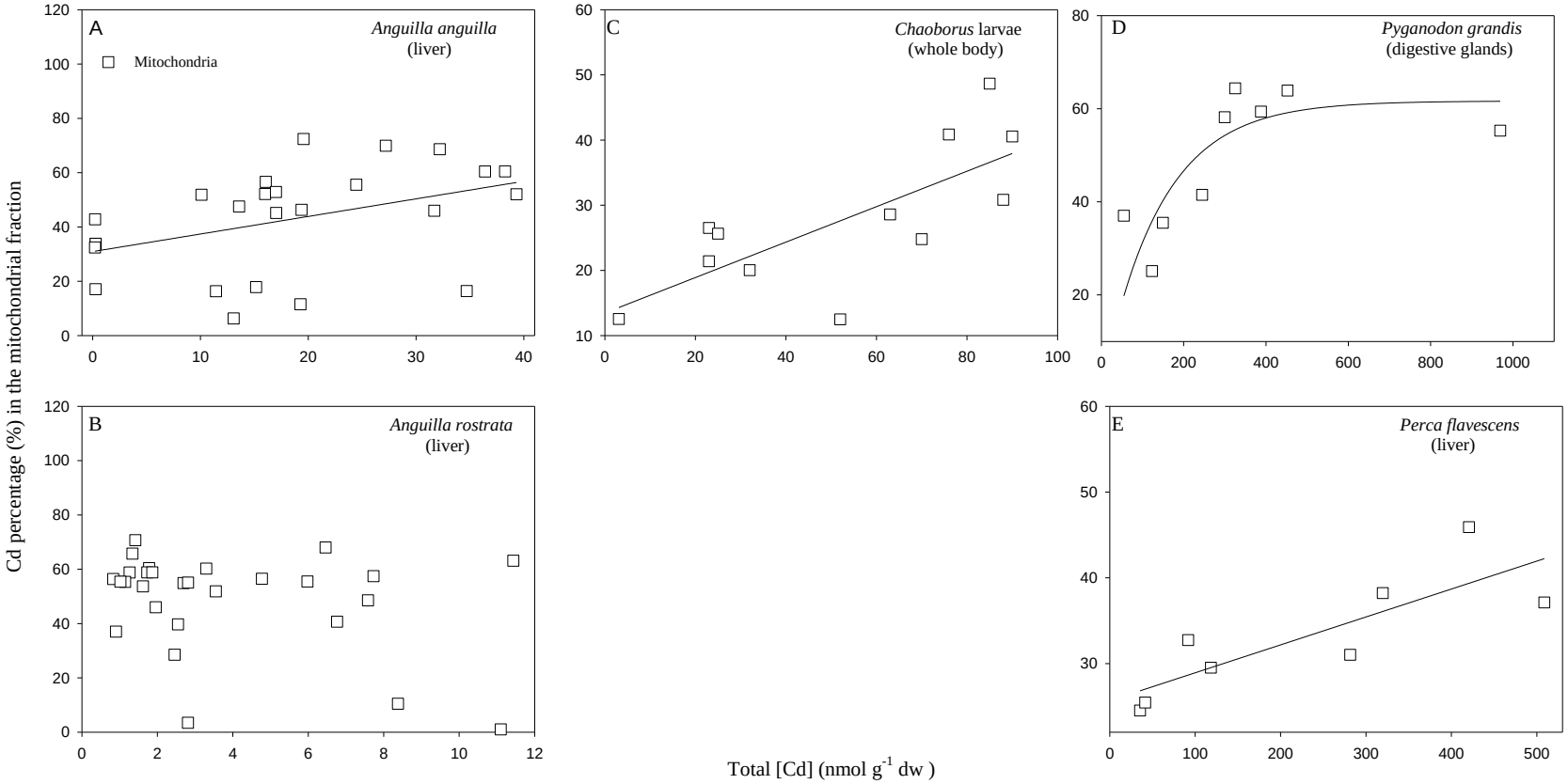

Figure A.6: Relationship between total Cd concentrations (horizontal axis) and Cd percentage (%) in the mitochondrial fractions in *Anguilla anguilla* (liver, panel A), *Anguilla rostrata* (liver, panel B), *Chaoborus* larvae (whole body, panel C), *Pyganodon grandis* (digestive gland, panel D) and *Perca flavescens* (liver, panel E). Lines represent significant regressions ($P < 0.05$).

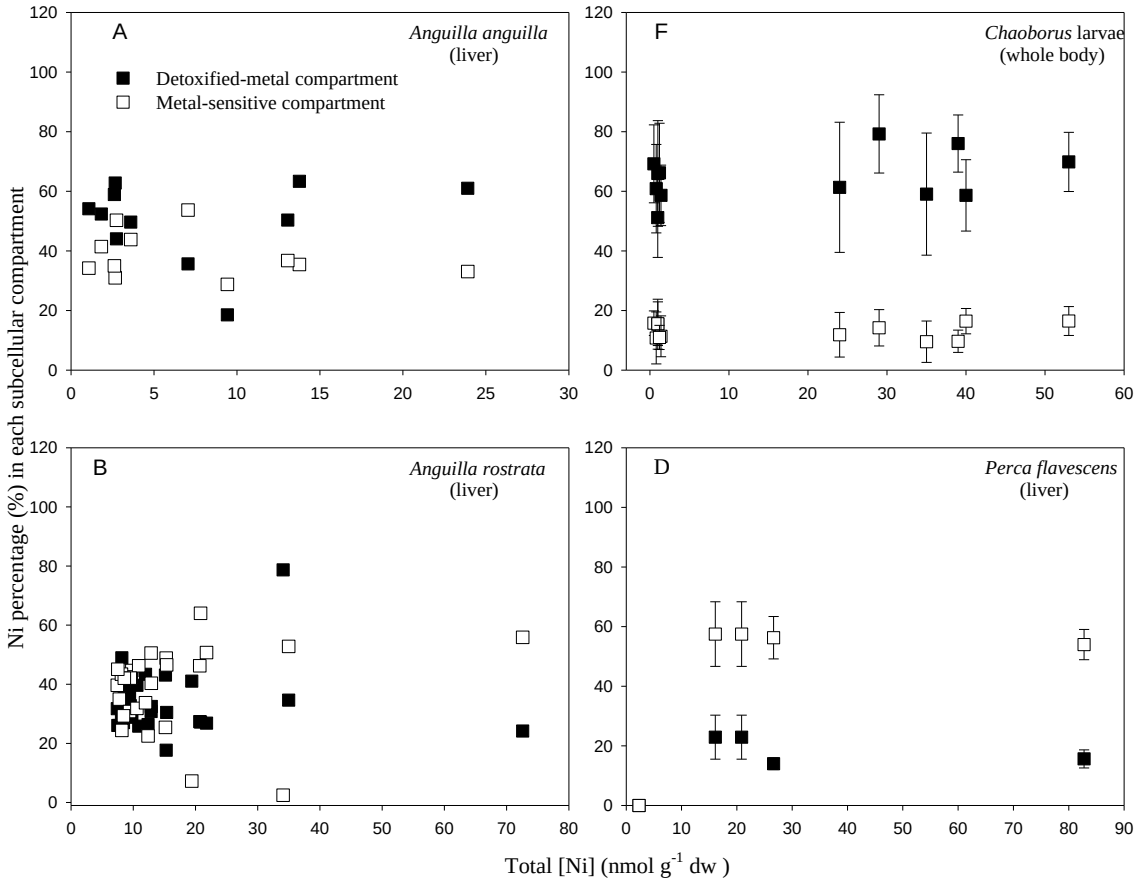

Figure A.7: Relationship between total Ni concentrations (horizontal axis) and Ni percentage (%) in detoxified-metal compartment (closed squares) and metal-sensitive fractions (open squares) in in *Anguilla anguilla* (liver, panel A), *Anguilla rostrata* (liver, panel B), *Chaoborus* larvae (whole body, panel C) and *Perca flavescens* (liver, panel D).

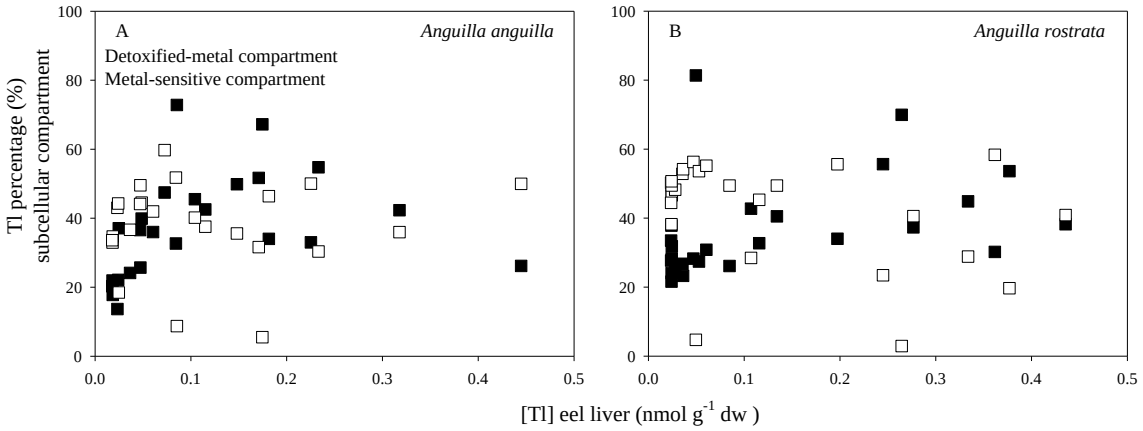

Figure A.8: Relationship between total hepatic Tl concentrations (horizontal axis) and Tl percentage (%) in detoxified-metal compartment (closed squares) and metal-sensitive fractions (open squares) in *Anguilla anguilla* (panels A) and *Anguilla rostrata* (panels B). Each point represents an individual eel.