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Évaluation de la métallothionéine comme biomarqueur d'effets toxiques chez le bivalve d'eau douce *Pyganodon grandis* dans un environnement aquatique contaminé par les métaux traces

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Résumé

À l'heure actuelle, l'un des défis majeurs en écotoxicologie est de prédire, à l'aide de réponses précoces mesurées à l'intérieur d'organismes (i.e., réponses biochimiques, cellulaires, physiologiques) en réaction à l'exposition à des polluants toxiques, des effets biologiques à des niveaux écologiques pertinents. La métallothionéine (MT) est une protéine de faible poids moléculaire synthétisée par de nombreux organismes, intervenant dans l'homéostasie des métaux essentiels et participant à la détoxication de certains métaux non-essentiels. En théorie, la toxicité des métaux au niveau cellulaire résulterait de réactions se produisant dans le cytosol, entre le métal et des ligands autres que la MT (p. ex., enzymes). Peu d'études se sont intéressées aux relations entre la réponse de la MT et la manifestation d'effets délétères à des niveaux élevés de l'organisation biologique. L'objectif de cette thèse est d'évaluer, en milieu naturel, la MT comme biomarqueur d'effets toxiques chez le bivalve d'eau douce *Pyganodon grandis*, à l'aide d'une approche holistique comprenant des mesures de métaux traces dans le milieu environnant et dans les organismes, l'examen de la distribution du métal à l'intérieur des cellules, et la détection de réponses toxiques à différents niveaux (i.e., individus, populations) en tenant compte de l'influence potentielle de variables physico-chimiques et écologiques confondantes sur ces réponses.

Dans une étude préliminaire, nous avons sélectionné, à l'aide de méthodes statistiques multivariables, un groupe de lacs ayant des niveaux de contamination métallique très différents (notamment pour le Cd) mais présentant des caractéristiques d'habitat semblables: le but était de minimiser l'influence des variables confondantes sur l'accumulation du Cd et la synthèse de MT chez *P. grandis*, et ainsi de faciliter l'identification non-équivoque des réponses toxiques au niveau des individus et des populations. Dans cette étude, nous avons montré que l'influence relative des variables limnologiques confondantes (principalement le pH et la concentration de calcium dissous

dans la colonne d'eau) sur l'accumulation du Cd (effet direct) et la synthèse de MT (effet indirect) dans les populations naturelles de *P. grandis* pouvait être réduite de façon significative par une procédure de sélection des lacs. Dans l'ensemble des lacs sélectionnés, la densité, la biomasse totale, la production annuelle, le taux de renouvellement (ratio P/B), ainsi que le succès reproducteur des populations indigènes de *P. grandis* diminuaient avec l'augmentation des concentrations de l'ion Cd^{2+} libre à l'interface eau-sédiments, et du Cd lié à des ligands cytosoliques de haut poids moléculaire (HPM), représentatifs des métallo-enzymes, dans les branchies des bivalves. Une étude expérimentale *in situ* des compromis énergétiques entre la croissance et la mortalité nous a permis d'expliquer, en partie, les réponses observées au niveau des populations, en mettant en évidence un lien entre l'augmentation des concentrations de Cd dans les fractions de HPM et la diminution des taux de survie individuels chez des organismes transférés d'un milieu propre vers des sites présentant des niveaux de contamination par le Cd très élevés. Il semble cependant difficile dans le cadre de cette thèse d'assigner à la MT ou à d'autres biomarqueurs potentiels (Cd-HPM), un rôle prédictif d'effets écotoxicologiques sur l'état de santé des populations de bivalves, à cause des effets confondants des facteurs écologiques (i.e., chaleur accumulée dans la zone littorale des lacs) sur les réponses des populations. L'étude des réponses à long terme de la MT dans notre organisme modèle suggère une diminution dans le temps du stress anthropique exercé par le Cd sur *P. grandis* dans les lacs étudiés, et nous supposons un remplacement du Cd par des facteurs naturels comme agent structurant des populations de bivalves. Les résultats de cette thèse mettent en évidence la nécessité de considérer les caractéristiques de l'habitat, composantes intrinsèques des écosystèmes, en même temps que les composantes reliées aux activités anthropiques dans les protocoles d'échantillonnage des études environnementales utilisant les biomarqueurs.

Mots clés: Biomarqueurs, approche holistique, contaminants métalliques, cadmium, métallothionéine (MT), répartition sub-cellulaire des métaux, populations, bivalves, lacs.

Summary

One major challenge for ecotoxicologists is to predict population, community and ecosystem responses to contaminant exposure from early-warning measurements made inside organisms. Metallothionein (MT) is a low molecular-weight metal-binding protein synthesized by a wide variety of organisms as a defense mechanism against excess metals in the surrounding media. It has been hypothesized that metal toxicity at the cellular level could arise from reactions in the cytosol, through the non-specific binding of metals to ligands other than MT (non-thionein ligands) that are physiologically important. To date, few attempts have been made to relate sub-cellular metal partitioning measurements to biological effects at ecologically significant levels (e.g., populations, community). In the present thesis, we evaluate the use of MT as a biomarker for toxic effects in the freshwater bivalve *Pyganodon grandis* living in lakes impacted by anthropogenic metal inputs, using a hierarchical approach based on the determination of metal concentrations in the environment and organisms, the examination of sub-cellular distribution of accumulated metals, the detection of toxicological responses at various levels of the biological organization (i.e., individuals and populations), and the evaluation of potential confounding factors that may affect these responses.

In a preliminary study, we proposed a selection procedure to reduce the background variability in screening for cadmium contamination in lakes: nine lakes were selected from an original set of 20, and were chosen so as to have water bodies with similar limnological characteristics but contrasting Cd levels. Our intention was to minimize the differences in habitat quality for the bivalves, and in this way facilitate the unequivocal identification of the biological responses of *P. grandis* to Cd contamination. In this study, we notably showed that the relative influence of limnological confounding factors (primarily pH and dissolved Ca) on Cd bioaccumulation and MT synthesis in natural populations of *P. grandis* was successfully reduced by the lake selection procedure. In the selected lakes,

density, total biomass, production, turnover ratio (P/B) and reproductive success of *P. grandis* populations decreased with increasing concentrations of the free-cadmium ion concentration in the environment. Overall, the concentrations of Cd in the gill cytosolic high molecular weigh pool (presumably representative of metallo-enzymes) of the individual bivalves were the biomarker that was the most strongly correlated with population variables. Consistent with this, we observed a marked increase in mortality rates of *P. grandis* specimens translocated from an uncontaminated site to cadmium-contaminated sites, concomitant with an increase of the Cd associated with high molecular weight proteins (>25 kDa) in gill cytosols of bivalves. However, it is not possible from the present thesis, to positively assign to sub-cellular metal partitioning measurement any predictive role for population health, notably because of the influence of environmental confounding factors (i.e., the number of degree-days available in the littoral zone of the lakes). Based on the results of a long-term study of the variations in MT concentrations in indigenous bivalves of the studied lakes, we determined that metal contamination of our lakes had markedly decreased in the past 13 years, and consequently we hypothesized that the toxic effects of Cd might have been replaced by some natural factors as the main agent for structuring the clam populations in these lakes. This thesis casts legitimate doubt on environmental studies that do not consider habitat characteristics in their sampling protocols along with components related to anthropogenic activities.

Keywords: Biomarkers, hierarchical (multi-level) approach, trace metals, cadmium, metallothionein (MT), sub-cellular metal partitioning, populations, bivalves, lakes.

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Metal-induced stress in bivalves living along a gradient of Cd contamination: relating sub-cellular metal distribution to population-level responses

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Linking changes in subcellular cadmium distribution to growth and mortality rates in transplanted freshwater bivalves (*Pyganodon grandis*)

Aux femmes de ma vie...

ma mère, Michèle P.

ma sœur, Marie-Charlotte P.

et sa fille Joséphine R.

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Chapitre 1

Introduction générale

1.1 Généralités sur les biomarqueurs

Les flux de certains métaux traces dans l'environnement ont fortement augmenté à cause des activités industrielles de l'homme (Nriagu et Pacyna, 1988). Parmi ces métaux, le cadmium est considéré comme étant l'un des plus toxiques pour les organismes aquatiques (Sadiq, 1992; Malley, 1996; Borgmann et al., 2005). En milieu terrestre, la toxicité du cadmium a été mise en évidence dans certaines populations naturelles de vertébrés (Larison et al., 2000), et cet élément trace semblerait être en partie responsable du déclin des forêts du nord-est des États-Unis (Gawel et al., 1996). D'importantes contributions ponctuelles de ce métal proviennent de l'extraction et du raffinage des métaux, ainsi que de leur transformation. Au Canada, chaque année, la fonte et le raffinage des métaux communs sont responsables de 82 % des rejets totaux de cadmium dans l'air et dans l'eau, soit environ 130 tonnes (Environment Canada and Health Canada, 1994). S'ajoutent à ces contributions des apports diffus de cadmium lié aux retombées atmosphériques et aux précipitations acides qui entraînent sa mise en circulation à partir du milieu terrestre (Tessier et al., 1994). Selon des processus physiques, chimiques et biologiques complexes, le cadmium introduit dans l'environnement aquatique se retrouve associé aux sédiments de fond (selon Lawrence et al., 1996, 93% du Cd ajouté expérimentalement à un lac non perturbé se retrouve dans le compartiment sédimentaire) où il devient une menace directe pour les organismes benthiques, et où il constitue également une menace indirecte pour les communautés pélagiques, par l'intermédiaire des réseaux trophiques. Le récent déclin des populations de bivalves d'eau douce en Amérique du Nord pourrait ainsi être attribué, en partie, à l'exposition chronique de ces populations à de faibles concentrations de métaux toxiques, dont le Cd (Naimo, 1995).

Traditionnellement, les méthodes utilisées pour évaluer la toxicité des métaux traces sur les organismes aquatiques comportent des expériences en laboratoire (tests de toxicité) et, dans une moindre mesure, des observations *in situ* des populations indigènes exposées. Les tests de toxicité sont généralement conçus pour déterminer la concentration à partir de laquelle une substance chimique est toxique chez une espèce animale ou végétale donnée, de façon à évaluer la toxicité des eaux ou des sédiments naturels et comprendre les

mécanismes déterminant la toxicité. Ils présentent de nombreux avantages sur les études en milieu naturel. Ils sont rapides, peu coûteux et peuvent être facilement répétés. Cependant, leur capacité à prédire, seuls, les effets d'un stress anthropique sur des environnements naturels a souvent été remise en cause (Kimball et Levin, 1985; Cairns et McCormick, 1992; Luoma, 1995). En effet, dans la plupart des études en laboratoire, le contexte expérimental est très éloigné des conditions réelles d'exposition des populations. D'un autre côté, dans les études en milieu naturel, la présence de nombreuses variables confondantes rend difficile l'interprétation des variations de l'abondance relative des organismes, de la diversité des espèces et de la structure des communautés. Dans ce contexte, le conseil national de recherches Canada (CNRC) recommandait en 1985 une approche alternative et complémentaire impliquant l'utilisation d'indicateurs biochimiques ou « biomarqueurs » pour évaluer les effets des polluants chimiques sur les organismes aquatiques. Plus récemment, le programme d'évaluation des techniques de mesures d'impacts en milieu aquatique (AETE), préconisait l'utilisation de biomarqueurs (conjointement avec d'autres outils) dans le cadre spécifique de la surveillance des effets des effluents miniers sur les écosystèmes aquatiques (Couillard, 1997).

Depuis ces dix dernières années, la recherche de biomarqueurs pour l'évaluation de la qualité de l'environnement s'est fortement intensifiée. Cependant, encore aujourd'hui, il semble exister des divergences sur la définition du terme biomarqueur. Huggett et al. (1992), définissent les biomarqueurs comme des indicateurs biochimiques, physiologiques ou histologiques d'exposition à des (ou d'effets de) xénobiotiques au niveau de la cellule ou de l'organisme. McCarty et Munkittrick (1996) élargissent la définition de biomarqueur à une variation inductible par l'action de l'homme de composants ou processus, structures ou fonctions biochimiques, physiologiques ou écologiques qui peut être mesurée dans un échantillon ou un système biologique. Par souci de clarification des termes, van Gestel et van Brummelen (1996) proposent la définition suivante: *« un biomarqueur est une réponse biologique, à un niveau inférieur à celui de l'individu, induite par une substance chimique présente dans l'environnement, mesurable à l'intérieur de l'organisme ou dans ses produits (urine, fèces, etc.), et qui indique une déviation du fonctionnement normal de l'individu ».* Bien que l'utilisation des métabolites comme biomarqueurs soit généralement peu

recommandée (Peakall et Walker, 1996), c'est cette dernière définition que nous adopterons dans le cadre de cette étude. On distingue habituellement trois types de biomarqueurs: les biomarqueurs d'exposition, les biomarqueurs d'effets et les biomarqueurs de sensibilité aux effets provoqués par l'exposition. Les premiers témoignent de l'exposition antérieure et/ou présente de l'organisme à une ou plusieurs formes biodisponibles de polluant. Les seconds indiquent, en plus, que le polluant a exercé un effet, toxique ou non, sur une cible critique dans l'organisme. Enfin, les derniers, dont l'usage est moins courant, font référence à la variation d'origine génétique de la réponse à la contamination par les polluants qui se traduit par une variation de la sensibilité des organismes (p. ex., la résistance, qui est une diminution de la sensibilité d'origine génétique) (Lagadic et al., 1997a).

L'approche des biomarqueurs se base essentiellement sur les connaissances du fonctionnement des organismes en réaction au stress (Depledge, 1989, 1994). On suppose qu'un individu sain soumis à des concentrations croissantes de polluant verra son état de santé se détériorer progressivement. Ainsi, pour des doses de polluants toxiques peu élevées et/ou des temps d'exposition assez courts, des réponses biochimiques et physiologiques peuvent intervenir pour compenser l'action des contaminants, notamment en limitant leur toxicité. Quand les doses internes et/ou les temps d'exposition augmentent, l'intervention de ces mécanismes de compensation devient insuffisante pour limiter l'action des polluants toxiques. L'état de santé des individus contaminés évolue alors vers une dégradation irréversible dont l'issue est généralement fatale.

Dans une approche holistique, la toxicité d'un polluant peut être considérée comme un continuum complexe de réponses biochimiques, physiologiques, d'organismes, de populations et de communautés (Luoma, 1995; Munkittrick et McCarty, 1995; Engel et Vaughan, 1996) (Fig. 1.1). Chaque niveau de l'organisation biologique comprend une étape de détoxication/compensation. Les effets délétères se produisent quand les mécanismes de détoxication sont débordés ou lorsque les processus de détoxication/compensation entraînent des coûts secondaires. Il en résulte qu'une réponse à un niveau dans l'organisation biologique n'est pas forcément suivie d'une réponse au niveau supérieur. De

plus, au fur et à mesure que l'on progresse au sein de l'organisation biologique, il devient de plus en plus difficile de mettre en évidence des relations de cause à effet (Luoma, 1995).

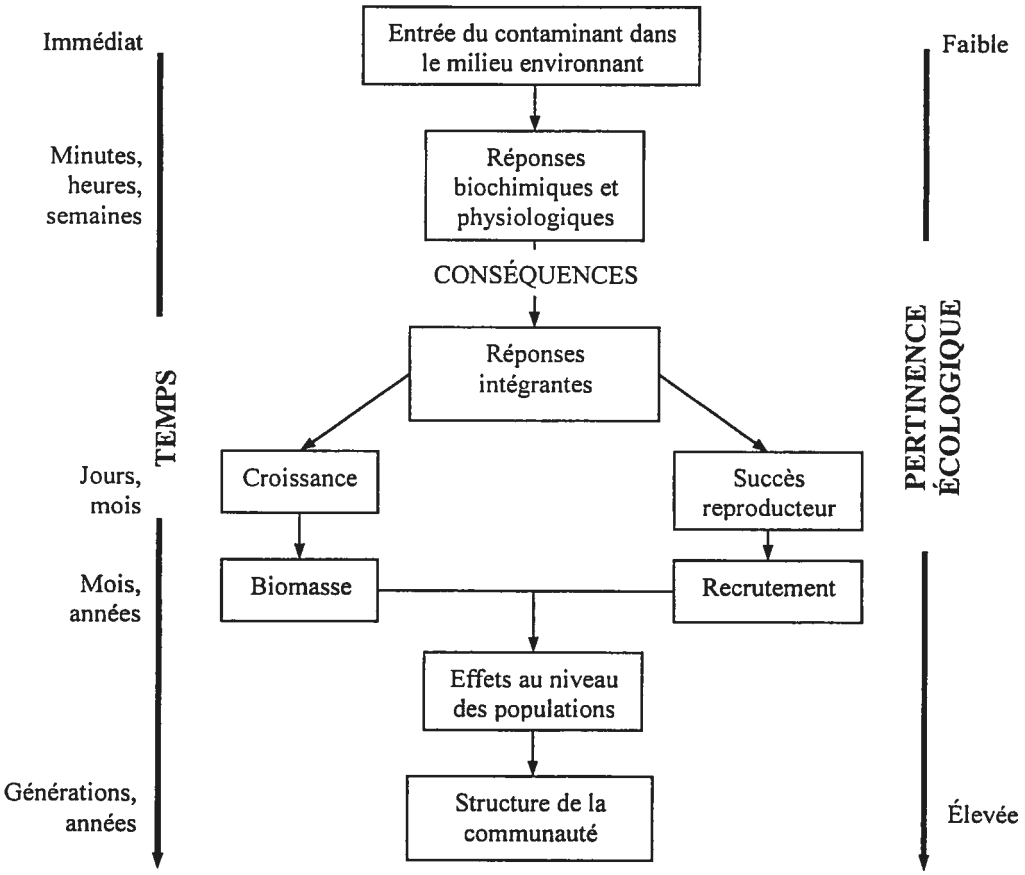

Figure 1.1. Illustration des relations entre le temps, les effets de l'introduction d'un polluant dans un système et la pertinence écologique de la détection et de la quantification de tels effets (d'après Engel et Vaughan, 1996).

Dans le concept d'indicateur biochimique/biomarqueur, les effets biologiques des polluants présents dans l'environnement sont initiés par l'interaction d'une substance chimique toxique avec un récepteur biologique à l'intérieur d'un organisme vivant. On suppose que les concentrations de polluant requises pour déclencher ces réactions sont bien inférieures à celles provoquant une crise dans l'organisme cible ou une dégradation visible dans l'écosystème. Ainsi, idéalement, la détection et la quantification des réactions

biochimiques à l'intérieur de l'organisme pourraient être utilisées comme indicateur précoce, sensible et spécifique d'un stress environnemental (CNRC, 1985).

Plusieurs critères ont été proposés pour déterminer la valeur potentielle d'un biomarqueur (Haux et Förlin, 1989; Stegeman et al., 1992). D'une manière générale, un biomarqueur doit être sensible comparativement aux réponses habituellement mesurées comme la mortalité, et les diminutions du potentiel reproducteur et de la croissance (la notion de sensibilité du biomarqueur rejoint ici celle de précocité). Il doit être spécifique d'un polluant ou d'une classe de polluants. Il doit répondre aux variations des concentrations ambiantes des polluants de façon dose-dépendante. De plus, la réponse du biomarqueur doit autant que possible persister durant toute la durée de l'exposition au polluant. Les variations du biomarqueur indépendantes de la contamination (variations saisonnières, cycles biologiques, autres stress) ainsi que les valeurs de bases en l'absence de contaminants (fluctuations naturelles) doivent être connues. Une relation claire entre la réponse du biomarqueur et des effets plus ou moins durables des polluants sur les organismes, les populations et les communautés doit être établie. Enfin, l'utilisation d'un biomarqueur doit être validée en milieu naturel.

Qu'ils interviennent dans le maintien de l'homéostasie ou qu'ils traduisent des perturbations fonctionnelles, la possibilité d'utiliser les biomarqueurs comme marqueurs précoces de dysfonctionnement ultérieur au niveau des populations et des communautés apparaît comme une approche particulièrement attrayante. Cependant, en l'état des connaissances actuelles, les biomarqueurs ne peuvent être généralement considérés que comme des indicateurs de présence (actuelle ou passée) de polluants dans le milieu et dans les organismes, leur validation pour la prédiction d'effets sur les niveaux d'organisation biologique élevés n'étant pas encore clairement établie (Depledge et Fossi 1994; Lagadic et al., 1994). Seule une approche « multiparamétrique » ou hiérarchique comprenant des mesures de contaminant dans le milieu, l'utilisation d'un ou de plusieurs biomarqueurs, et la détection d'effets toxiques à différents niveaux de l'organisation biologique est capable de fournir une image suffisamment réaliste de l'état de santé écologique des écosystèmes (Lagadic et al., 1997b; Adams et Greeley, 2000; Adams et al., 2002). Dans cette

perspective, de nombreux auteurs ont insisté sur la nécessité de relier (au minimum par une relation statistique, au mieux par une relation de causalité) les réponses biochimiques à l'intérieur des organismes à la fois à des mesures réelles d'exposition et à des effets spécifiques à des niveaux élevés de l'organisation biologique (population, communauté) (Engel et Vaughan, 1996; McCarty et Munkittrick, 1996; Adams et al., 2001).

1.2 Les métallothionéines (MTs)

En ce qui concerne la pollution par les métaux traces, les métallothionéines (MTs) sont les biomarqueurs ayant reçu le plus d'attention. Les MTs sont des métalloprotéines de faible poids moléculaire (6000-8000 Da chez les mammifères), riches en cystéine et présentant une forte affinité pour les ions métalliques des groupes IB et IIB (Roesijadi, 1992). Elles sont présentes sous forme soluble dans le cytoplasme et le noyau de la cellule (Kägi et Schaffer, 1988). L'agencement en séquences spécifiques des cystéines, permet de classer les MTs en trois catégories selon leur homologie avec les MTs mammaliennes (Fowler et al., 1987). Des MTs ont été mises en évidence chez de nombreux organismes appartenant au règne animal (vertébrés et invertébrés) ou végétal, chez des micro-organismes eucaryotes et chez des procaryotes. Toutes les MTs caractérisées chez les vertébrés appartiennent à la classe I. Les MTs des mollusques et des crustacés appartiennent aussi à cette classe (Amiard et Cosson, 1997).

La spécificité de leur structure et leur ubiquité ont contribué à forger l'idée que la MT devait jouer un rôle essentiel dans plusieurs processus fondamentaux. Chez certains organismes aquatiques, les MTs semblent intervenir dans la régulation des concentrations internes de métaux essentiels tels que le zinc et le cuivre et dans le processus de détoxication des métaux essentiels quand ils sont présents en excès dans la cellule et des métaux non essentiels tels que le cadmium et le mercure (Roesijadi, 1992). La MT constituerait, d'une part, une réserve de métaux essentiels utilisable dans de nombreux processus cellulaires (réplication, respiration, transcription, synthèse et dégradation des protéines, métabolisme énergétique): le cuivre et le zinc en excès dans la cellule seraient stockés sous une forme non toxique (Cu-MT et Zn-MT), puis redistribués par des

métalloenzymes selon les besoins physiologiques de l'organisme (Kelly et al., 1996). D'autre part, en séquestrant les métaux non essentiels, la MT limiterait les liaisons non spécifiques toxiques avec d'autres ligands à l'intérieur de la cellule (Mason et Jenkins, 1995). Cependant, selon Cosson et al. (1991), la participation de la MT aux mécanismes de détoxication ne relèverait que d'interactions fortuites entre des cations exogènes et le mécanisme normal de l'homéostasie du zinc et peut-être du cuivre. Quand les métaux toxiques pénètrent à l'intérieur de la cellule, il y a une compétition entre tous les ions métalliques présents pour tous les ligands intracellulaires, dont la MT, et des substitutions ioniques peuvent alors se produire. Par ordre décroissant d'affinité, la MT se lierait préférentiellement aux ions Hg^{2+}, Cu^{2+}, Cd^{2+} et Zn^{2+} (Viarengo, 1989). Un autre mécanisme permettant d'expliquer la capacité de la MT à prendre en charge des métaux différents a récemment été proposé par Dallinger et al. (1997). Les auteurs ont en effet mis en évidence l'existence de deux isoformes de MTs chez un gastéropode terrestre, l'une étant impliquée dans la régulation du cuivre, l'autre dans les processus de détoxication du cadmium. Les MTs semblent également jouer un rôle déterminant dans la réponse au stress oxydant chez certains bivalves marins (Viarengo et al., 1999a). Enfin, chez le rat, des études cliniques ont montré que de nombreux stress physiologiques (exposition au froid et à la chaleur, exercice intense) impliquant une redistribution du zinc au niveau cellulaire, stimulent la synthèse de MT (Oh et al., 1978).

Un autre mécanisme de détoxication entraînant l'insolubilité des métaux *via* la formation de concrétions minérales a déjà été mis en évidence chez de nombreux invertébrés aquatiques (George, 1983; Mason et Jenkins, 1995; Langston et al., 1998; Marigomez et al., 2002). Ce mécanisme semble être le processus de détoxication prédominant, entre autres, chez le bivalve *Crassostrea gigas* pour des métaux toxiques comme l'argent et le cadmium (Martoja et al., 1988; Mouneyrac et al., 1999), ainsi que chez les bivalves *Chlamys varia*, *Scrobicularia plana*, *Dreissena polymorpha* et *Macoma balthica* pour l'argent (Berthet et al., 1992; Mouneyrac et al., 2000). Chez les bivalves, l'importance relative de la MT et des concrétions métalliques dans le processus de séquestration des métaux ne dépend pas seulement de l'espèce (Shi et Wang, 2004), mais également de la durée de l'exposition au métal et de la taille de l'organisme cible (Wallace

et al., 2003a). La distinction entre les deux mécanismes de détoxication est importante lorsque l'on considère le transfert des métaux bioaccumulés au sein de la chaîne trophique (Wallace et al., 1998; Wallace et Luoma, 2003b), les métaux liés aux MTs étant plus disponibles pour les prédateurs que les métaux insolubles.

Le rôle de la MT dans l'acquisition d'une tolérance dans les populations exposées de façon chronique aux métaux a été mis en évidence à la fois chez les mammifères, les poissons et les invertébrés (Roesijadi, 1992). Typiquement, l'exposition antérieure à de faibles concentrations de cuivre, de cadmium, de mercure ou de zinc confère à certains organismes une tolérance accrue à ces métaux pour des niveaux d'exposition plus élevés. Les processus impliqués dans la tolérance acquise pourraient être liés soit à l'existence d'une réserve de MT immédiatement disponible dans les organismes, soit à la mobilisation de la machinerie cellulaire pour une synthèse plus rapide de la MT, soit à des phénomènes d'amplification du gène de la MT (Roesijadi, 1992). Unger et Roesijadi (1996) ont ainsi mis en évidence une synthèse *de novo* de MT plus importante dans des individus préalablement exposés à de faibles concentrations de cadmium chez le bivalve marin *Crassostrea virginica*. Cependant, il existe certaines exceptions. Pour des poissons (*Salmo gairdneri*) acclimatés à des concentrations élevées de zinc, de cuivre et de cadmium, Roch et McCarter (1984) ont constaté que l'induction de la MT n'était pas accompagnée d'une augmentation à la tolérance pour ces métaux. Plus récemment, chez le bivalve marin *Macoma balthica*, Mouneyrac et al. (2000) n'ont pas constaté d'effet significatif de l'origine géographique des organismes (bivalves provenant d'une population exposée de façon chronique aux métaux *vs* bivalves provenant d'une population de référence) sur les niveaux de MT dans les tissus mous, en réponse à l'exposition contrôlée à l'argent et au mercure.

Les mécanismes régulateurs de la biosynthèse des MTs sont complexes. Chez les mammifères les gènes codant pour la MT sont inductibles et leur régulation différentielle est assurée au niveau de la transcription par les métaux (cadmium, cuivre, mercure, argent, zinc), les hormones glucocorticoïdes et les cytokines (Palmiter, 1987). Chez les poissons, le promoteur du gène de la MT présente certaines similitudes avec celui des mammifères

(Olsson et al., 1995). La synthèse de la MT chez les poissons est induite par les métaux, mais elle peut également être influencée par les fonctions endocrines liées à la reproduction (Viarengo et al., 1999b). Récemment, certains travaux ont montré l'existence d'une relation entre l'exposition aux œstrogènes et la régulation de la synthèse de MT chez la truite de lac (*Salvelinus namaycush*) (Palace et al., 2000; Werner et al., 2003). Toujours chez les poissons, l'induction de la MT en réponse à des agents de stress autres que les métaux semble être modulée par les glucocorticoïdes (Roesijadi, 1992). Chez les invertébrés aquatiques, la grande majorité des études portant sur l'induction de la MT est consacrée à l'influence des métaux. Les organismes modèles les plus fréquemment étudiés sont les bivalves marins et les métaux considérés sont généralement le cadmium et le cuivre. De nombreux exemples mettant en évidence l'augmentation des niveaux de MT en réponse à une exposition contrôlée à des métaux peuvent être trouvés dans Bordin et al. (1997). Pour les invertébrés dulcicoles, l'induction de la MT par le cadmium a été démontrée expérimentalement, entre autres, pour les bivalves *Anodonta cygnea* et *Anodonta anatina* (Hemelraad et al., 1986), *Anodonta grandis grandis* (Malley et al., 1993), *Dreissena polymorpha* (High et al., 1997) et *Corbicula fluminea* (Doherty et al., 1988; Baudrimont et al., 1997a).

À l'heure actuelle, pour les invertébrés aquatiques, on cherche de plus en plus à déterminer l'influence des facteurs biotiques (p. ex., facteurs endogènes) sur la biosynthèse de la MT (revue dans Isani et al., 2000). Chez le bivalve d'eau douce *Corbicula fluminea*, par exemple, les variations saisonnières des concentrations de MT semblent être directement reliées au cycle biologique des organismes en l'absence de contamination métallique, impliquant, selon les auteurs, un contrôle hormonal de la synthèse de la MT chez cette espèce (Baudrimont et al., 1997b). Parallèlement, Serra et al. (1999) ont noté l'existence d'une relation entre les concentrations individuelles de MT et le cycle reproducteur du bivalve marin *Mytilus galloprovincialis*. Chez un autre bivalve marin, *Ruditapes decussatus*, les concentrations de MT dans la glande digestive d'organismes prélevés au niveau de sites de référence et de sites contaminés semblaient varier au moment de la période de développement des gonades (Serafim et Bebianno, 2001). Cependant, l'interprétation de ces relations est souvent ambiguë car chez certains bivalves, les

variations des niveaux de MT et des concentrations de métaux bio-accumulés sont également reliés aux variations du taux de croissance, de la taille et de la masse des individus (Bordin et al., 1997; Mouneyrac et al., 1998; Amiard-Triquet et al., 1998; Mouneyrac et al., 2000; Leung et al., 2001).

1.3 La MT comme biomarqueur d'exposition métallique et d'effets toxiques

Grâce à son caractère inductible, à ses propriétés moléculaires, et à son rôle dans l'assimilation, le transport, le stockage et l'excrétion des métaux, les MTs constitueraient des biomarqueurs d'exposition et de stress métallique prometteurs (Roesijadi, 1992; Stegeman et al., 1992; Langston et al., 1998). Dans une perspective écotoxicologique, deux approches sont possibles quant à l'utilisation de la MT. Dans une première approche, des mesures directes de MT dans les organismes pourraient être utilisées comme indicateurs d'une exposition antérieure ou/et actuelle à des métaux toxiques. Dans ce cas, on suppose que l'augmentation de la concentration de MT au-dessus d'un niveau basal est provoquée par une induction de cette protéine en réponse à l'assimilation de métaux toxiques. En principe, une telle approche fournirait une mesure de la fraction significativement toxique des métaux à l'intérieur de la cellule (Olafson et al., 1979). Une seconde approche consisterait à examiner la répartition des métaux parmi les différentes classes de ligands cytosoliques à l'intérieur de la cellule. Certains auteurs suggèrent en effet l'existence d'un phénomène de « débordement cellulaire » qui se manifesterait quand la vitesse de bioaccumulation du métal dépasse le taux de biosynthèse de la MT, et que le métal se lie non-spécifiquement à d'autres ligands cytosoliques ayant d'importantes fonctions physiologiques (Brown et Parsons, 1978; Mason et Jenkins, 1995). En principe, cet état pourrait être considéré comme symptomatique d'un stress métallique et détectable par l'analyse de la répartition intracellulaire des métaux (Stegeman et al., 1992). D'autre part, on pourrait envisager l'existence de coûts secondaires associés à la synthèse de MT: les mécanismes de résistance aux stress, qu'ils soient le produit d'une acclimatation ou d'une adaptation, sont en effet consommateurs d'énergie (Postma et al., 1995). Ainsi, même si le système de détoxication impliquant la MT n'est pas débordé, et que les métaux n'exercent pas directement de toxicité, les coûts métaboliques afférents à la production de MT

pourraient entraîner des réductions de la croissance et/ou de l'effort de reproduction des organismes (i.e., diminution du fitness) (Morgan et al., 1999). Dans cette perspective, le coût métabolique ne représente pas en soi un effet toxique, mais il est l'expression de l'activité de détoxication sans laquelle un organisme ne pourrait survivre dans un environnement contaminé.

En milieu naturel, on trouve de plus en plus d'exemples de validation de la MT comme biomarqueur d'exposition aux métaux traces (i.e., première approche) (Campbell et Tessier, 1996; Couillard, 1997; Viarengo et al., 1999b). Récemment, mentionnons les études de Pedersen et al. (1997) sur le crabe *Carcinus maenas*, de Bebianno et Machado (1997), Mourgaud et al. (2002) et Irato et al. (2003) sur le bivalve marin *Mytilus galloprovincialis*, de Hamza-Chaffai et al. (1999) sur le bivalve marin *Ruditapes decussatus*, de Geffard et al. (2002) sur l'huître marine *Crassostrea gigas*, de Temara et al. (1997) sur l'étoile de mer *Asterias rubens*, de Aspholm et Hylland (1998) sur l'oursin de mer *Strongylocentrotus droebachiensis*, de Hamza-Chaffai et al. (1997) sur le poisson marin *Scorpaena porcus*, de Croteau et al. (2002) sur la larve d'insecte *Chaoborus*, de Baudrimont et al. (1999) et Gagné et al. (2002) sur les bivalves d'eau douce *Corbicula fluminea* et *Elliptio complanata*, et de Laflamme et al. (2000), Olsvik et al. (2001), Palace et al. (2003) et Doebel et al. (2004) respectivement sur la perchaude (*Perca flavescens*), la truite brune (*Salmo trutta*), le meunier noir (*Catostomus commersoni*) et le mulet perlé (*Semotilus margarita*). Cependant, dans la majorité de ces études, la réponse de la MT était reliée à des concentrations de métaux dans les organismes mais pas à des mesures d'exposition dans le milieu.

Contrairement à la première approche, il ne semble pas y avoir de réel consensus en ce qui concerne l'utilisation de la MT comme indicateur de stress métallique (i.e., seconde approche). L'existence du phénomène de débordement cellulaire a déjà été mise en évidence dans des expériences en laboratoire (Engel et Fowler, 1979; Kito et al., 1986; Carpenè et al., 1987) et en mésocosme (Brown et Parsons, 1978). À l'opposé, Jenkins et Sanders (1986) ont démontré que pour des polychètes marins (*Neanthes arenaceodentata*) exposés à des concentrations d'ion cadmium libre susceptibles d'entraîner un état de stress,

il n'y avait pas de redistribution du cadmium entre les différents « pools » de ligands intracellulaires. Les résultats de l'étude expérimentale de Hamilton et al. (1987) sur l'omble de fontaine (*Salvelinus fontinalis*) contredisent également l'hypothèse du débordement cellulaire. En effet, les auteurs ont noté que l'exposition des poissons à des concentrations élevées de cadmium provoquait des effets toxiques chez les organismes, mais que ces effets étaient indépendants du niveau de saturation de la MT. Enfin, plus récemment, Deeds et Klerks (1999) montraient dans une étude en laboratoire que le concept de débordement cellulaire était totalement inadéquat pour l'oligochète d'eau douce *Limnodrilus udekemianus*.

Il existe peu d'études réalisées en milieu naturel montrant soit l'existence du phénomène de débordement cellulaire, soit l'apparition de « perturbations dans la répartition intracellulaire des métaux » ou même éventuellement la « sursaturation des MTs » (Johansson et al., 1986; Klaverkamp et al., 1991; Couillard et al., 1995b; Wang et al., 1999). Dans la plupart de ces exemples, le débordement des mécanismes de détoxication impliquant la MT coïncidait avec l'apparition d'effets délétères au niveau des organismes (augmentation de la peroxydation des lipides membranaires, diminution de l'indice de condition des organismes, aspect nécrotique de certains organes). De la même manière, Baudrimont et al. (1999) suggèrent l'existence d'un phénomène de débordement cellulaire chez des bivalves d'eau douce (*Corbicula fluminea)* transférés d'un site de référence vers un site contaminé: les fortes mortalités observées pour les niveaux de contamination les plus élevés semblaient correspondre à un plafonnement des concentrations de MT dans les organismes. Dans toutes ces études, les effets biologiques au niveau des populations étaient plus souvent suggérés que formellement démontrés (Johansson et al., 1986; Couillard et al., 1995b). De plus, dans la grande majorité de ces études, les caractéristiques de l'habitat susceptibles d'influencer les réponses biologiques n'étaient pas considérées.

Dans une revue de littérature, Couillard (1997) a abordé la question des coûts métaboliques associés à la production de MT. L'auteur a rapporté quatre études en laboratoire et une étude en milieu naturel traitant de ce sujet. Parallèlement, dans une étude

en laboratoire sur un gastéropode marin (*Nucella lapillus*), Leung et Furness (1999) suggèrent une utilisation accrue des réserves énergétiques par les organismes pour les mécanismes de détoxication tels que la production de MT, durant une intoxication par le cadmium. Chez cette espèce, l'augmentation des concentrations individuelles de MT semblait être associée à une réduction de l'indice de condition des organismes. Cependant, dans la majorité de ces études, il n'existe pas de démonstration rigoureuse que les mécanismes de détoxication impliquant la MT ne sont pas débordés pour les niveaux d'exposition utilisés: les effets délétères observés pourraient aussi bien être la manifestation de la toxicité directe des métaux (après débordement cellulaire) que l'expression de coûts métaboliques liés à la synthèse de MT. L'étude de Cattani et al. (1996) sur la relation entre la production de MT et le métabolisme énergétique chez le poisson *Dicentrarchus labrax* ne fait pas exception. Les auteurs ont en effet constaté que la diminution des réserves de glycogène dans le muscle des poissons, durant l'exposition contrôlée à des concentrations élevées de cadmium, était associée, à la fois, à une augmentation de la concentration de MT et à une augmentation de la concentration du Cd lié à des ligands cytosoliques de haut poids moléculaire.

1.4 L'organisme modèle *Pyganodon grandis*

Les bivalves sont souvent employés comme organismes sentinelles dans des études environnementales sur la contamination des systèmes aquatiques par des polluants organiques et inorganiques (Goldberg et al., 1978). Cette classe d'invertébrés possède, en effet, plusieurs caractéristiques biologiques et écologiques qui favorisent son utilisation dans ce type d'études: ils ont une répartition géographique étendue et une espérance de vie assez longue; leur relative sédentarité assure une prise d'information spécifique au site de récolte; les populations abondantes et stables permettent des prélèvements répétés; les spécimens sont faciles à récolter et à manipuler en raison de leur taille; ils sont facilement identifiables; leur tolérance aux polluants est relativement élevée en comparaison à d'autres organismes aquatiques; ils ont la capacité de concentrer les polluants; étant des consommateurs primaires, ils fournissent une information sur la biodisponibilité du contaminant à la base des chaînes trophiques; on peut les utiliser dans des expériences de

transplantation et les délocaliser vers des sites où on ne les retrouve pas forcément (Metcalfe-Smith, 1994; Malley et al., 1996).

En Amérique du Nord, le bivalve d'eau douce *Pyganodon grandis* (Fig. 1.2) se retrouve à travers tout le bassin intérieur canadien, du centre de l'Alberta au centre de l'Ontario, au niveau du système Grands Lacs – St. Laurent à l'est de Montréal, et au États-Unis (Clarke, 1981). On le rencontre dans les étangs permanents, et les lacs et les rivières

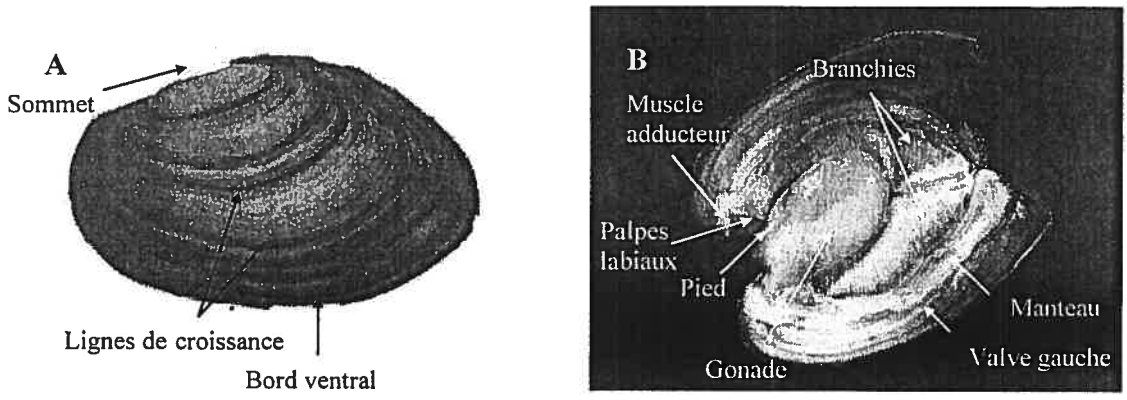

Figure 1.2. L'organisme modèle *Pyganodon grandis* (*Anodonta grandis grandis*). A) Vue extérieure, B) vue intérieure montrant la disposition des principaux tissus et organes.

de différentes tailles. Cette espèce semble tolérer des eaux relativement peu productives et faiblement alcalines, avec une concentration de calcium dissous aussi faible que 2-3 $mg \cdot L^{-1}$ (Huebner et al., 1990). On la retrouve associée à une très grande variété de substrat, mais elle préfère les zones de végétation éparse ou modérément dense (Clarke, 1981). Le cycle de reproduction des Unionidae est complexe. Chez *P. grandis*, les sexes sont généralement séparés (bien que certains cas d'hermaphrodisme soient rapportés). La maturité sexuelle est atteinte vers l'âge de 4 à 5 ans (Hanson et al., 1989). La fertilisation, et le développement des larves ont probablement lieu assez tôt au cours de l'été (Huebner, 1980). Au nord de leur aire de distribution, les spécimens de cette espèce sont des reproducteurs bradytictiques (i.e., les larves sont conservées dans les marsupia branchiaux maternels durant tout l'hiver) alors que plus au sud, ils sont typiquement tachytictiques (i.e., les larves sont relâchées dès

le début de l'automne qui suit la fertilisation) (Lewis, 1985). Chez les Unionidae, le cycle de développement passe généralement par un stade parasitaire obligatoire. *P. grandis* est considéré comme un parasite généraliste: Watters (1994) a en effet recensé plus d'une trentaine d'espèces différentes de poissons comme hôtes potentiels. Les prédateurs connus sont le rat musqué (*Ondatra zebithicus*), la loutre (*Lutra canadensis*), le vison (*Mustela vison*) et certains poissons (Tyrrell et Hornbach, 1998). Bien qu'à l'heure actuelle les moules d'eau douce soient en voie d'extinction à travers toute l'Amérique du Nord (Ricciardi et Rasmussen, 1999), la distribution et l'abondance de *P. grandis* apparaissent stables (Williams et al., 1993; Metcalfe-Smith et al., 1998).

De précédentes études sur la bioaccumulation des métaux et l'induction de la MT dans des populations naturelles de *P. grandis* (Tessier et al., 1993; Couillard et al., 1993; Couillard et al., 1995a,b; Wang et al., 1999) ont permis de mettre en évidence que:

1) les concentrations de MT dans les organismes variaient le long d'un gradient spatial de contamination en Cd^{2+} dissous, tel qu'estimé à partir des équilibres d'adsorption à l'interface eau-sédiment

2) les concentrations de MT dans les organismes étaient corrélées avec le Cd accumulé dans les tissus mous (il n'y avait pas de corrélation significative pour Cu et Zn)

3) les variations saisonnières des concentrations de MT dans les organismes étaient modestes relativement à celles enregistrées le long du gradient environnemental en Cd

4) des perturbations dans la répartition intracellulaire des métaux apparaissaient dans des branchies d'individus exposés à des concentrations de Cd^{2+} dissous supérieures à $\approx$ 1 nM; des symptômes d'effets toxiques étaient associés à cette anomalie biochimique.

Le premier et le deuxième point montrent que la MT répond de façon dose-dépendante aux variations des concentrations d'un contaminant spécifique (i.e., le cadmium). Le troisième point suggère que les facteurs endogènes sont des sources de variation moins importantes que la biodisponibilité des métaux toxiques, en ce qui concerne la production de MT. Ces observations sont en accord avec l'étude de Kalhok et Cyr (1997) sur des populations de *P. grandis* situées dans la même région. En effet, les auteurs démontrent qu'à l'intérieur d'un même lac, l'âge et la taille des moules sont des variables

peu susceptibles d'influencer les concentrations de MT dans les organismes, et qu'entre les lacs, c'est la biodisponibilité des métaux qui détermine la concentration de MT. Enfin, le quatrième point semble indiquer l'existence d'un phénomène s'apparentant au phénomène de débordement cellulaire pour des populations exposées à des concentrations élevées de Cd. Selon ces résultats, la MT constituerait un excellent biomarqueur d'exposition au Cd, et un biomarqueur d'effets toxiques prometteur.

1.5 Objectifs et hypothèses de recherche

Cette thèse s'inscrit dans le cadre d'un projet global visant à évaluer, en milieu naturel, la MT comme biomarqueur d'exposition métallique et d'effets toxiques chez l'organisme modèle *P. grandis*. Pour répondre à cet objectif, nous avons adopté une approche hiérarchique comportant, à la fois, des mesures d'exposition dans le milieu et de concentrations tissulaires de contaminant, la détection et la quantification de réactions biochimiques à l'intérieur des organismes, et l'étude d'effets biologiques spécifiques à différents niveaux hiérarchiques. Bien que cette étude soit réalisée en milieu naturel, dans un ensemble de lacs d'une même région géographique, elle s'apparente également à l'approche expérimentale, en ce sens que nous cherchons à contrôler l'influence des variables confondantes (en minimisant le gradient trophique des lacs) tout en faisant varier les niveaux d'exposition métallique (en maximisant le gradient de contamination des lacs). Dans la présente étude, nous nous sommes intéressés, plus spécifiquement, aux relations entre la répartition intracellulaire du métal entre les différents ligands cytosoliques, incluant la MT, et les effets biologiques à un niveau de pertinence écologique élevée, i.e. la population.

Cette étude a été réalisée dans un ensemble de lacs de la région minière de Rouyn-Noranda, située à environ 600 km au nord-ouest de Montréal. Les sources de contamination en métaux dans cette région proviennent de mines abandonnées, des opérations minières courantes, des résidus des procédés de raffinage et de la déposition atmosphérique des émissions liées au raffinage. La grande majorité des lacs étudiés dans cette thèse est uniquement influencée par les émissions diffuses des opérations de raffinage de la fonderie

Horne de Rouyn-Noranda, en activité depuis 1927. En 1995, la fonderie Horne rejetait dans l'atmosphère 123 t de cuivre, 100 t de zinc, 355 t de plomb, 1,4 t de nickel, 39 t d'arsenic et 4.7 t de cadmium (Doyle et al., 2003). Les facteurs d'enrichissement du Cu, du Zn, du Pb et du Cd dans les sédiments des lacs environnants étaient respectivement de 166, 118, 130 et 13,2 (Arafat, 1985).

Bien que les populations de bivalves étudiées dans cette thèse soient soumises à des contaminations simultanées de plusieurs métaux traces, nous avons considéré le Cd comme étant le seul contaminant cible dans cette étude. Ce choix repose essentiellement sur des études antérieures menées dans la région de Rouyn-Noranda (p. ex., Couillard et al., 1993; Couillard et al., 1995a; Wang et al., 1999; Giguère et al., 2003) qui montrent que parmi l'ensemble des métaux considérés (i.e., Cd, Cu et Zn), le Cd est le principal agent inducteur de la synthèse de MT chez *P. grandis* (i.e., existence de relations dose-réponse, r = 0.76-0.96, entre les concentrations de MT dans les bivalves et la quantité de Cd bioaccumulé dans l'organisme). Le mercure, qui sous sa forme inorganique serait également capable de promouvoir la production de MT (Roesijadi, 1992), n'a pas été considéré dans cette étude. En effet, dans l'ensemble des lacs échantillonnés, les concentrations totales de mercure dans les sédiments variaient très peu le long du gradient spatial de contamination ($\{Hg\}_T$ max./$\{Hg\}_T$ min. = 3 comparativement à une valeur de 21 pour le ratio $\{Cd\}_T$ max./$\{Cd\}_T$ min. pour le même groupe de lacs) (Couillard et al., 1993). Certains auteurs (p. ex., Harrison et al., 1987; Baudrimont et al., 1997) ont également remis en cause le potentiel inducteur du Hg dans la synthèse de MT chez les mollusques.

Dans une première partie (chapitre 2), nous avons étudié les variations de l'accumulation du cadmium et de l'induction de la MT dans des populations de *P. grandis* situées le long d'un gradient spatial de contamination en Cd. Cette étude préliminaire devait servir de base aux études intensives des réponses biologiques au niveau des populations (chapitre 3), ainsi qu'aux expériences de transplantation (chapitre 5). Les objectifs spécifiques de cette étude étaient 1) de sélectionner, à l'aide de méthodes statistiques multivariables, un sous-ensemble de lacs ayant des statuts trophiques semblables et présentant des degrés très différents de contamination par le Cd (le but ultime étant de

minimiser l'influence de variables confondantes sur les réponses biologiques des organismes et des populations), 2) de quantifier l'influence relative des variables limnologiques confondantes sur les processus de bioaccumulation du Cd et de synthèse de MT par rapport à la variable d'exposition elle-même, et 3) de valider, dans le sous-ensemble de lacs sélectionnés, les relations entre la réponse de la MT dans les organismes et les mesures d'exposition dans le milieu. *On veut tester l'hypothèse selon laquelle l'influence des variables limnologiques confondantes sur l'accumulation du Cd et la synthèse de MT chez notre organisme modèle peut être minimisée par une procédure de sélection des lacs.*

Dans le chapitre central de cette thèse (chapitre 3), nous avons 1) évalué l'état de santé des populations indigènes de *P. grandis* des lacs sélectionnés, 2) déterminé la nature des relations entre cet état de santé et la répartition intracellulaire du Cd au niveau d'individus de ces populations et 3) testé l'influence de facteurs écologiques confondants (caractéristiques de l'habitat, régime thermique des lacs, présence de poissons hôtes potentiels pour le stade parasitaire obligatoire des larves de *P. grandis*) susceptibles de modifier ces relations. Plusieurs approches complémentaires ont été utilisées pour diagnostiquer l'état de santé des populations. Nous avons caractérisé, tout d'abord, les réponses numériques des populations (densité, biomasse, production secondaire, etc.) ainsi que les taux de croissance individuels à long terme en fonction du gradient de contamination métallique. Nous avons également évalué le succès reproducteur des populations de *P. grandis* en fonction du même gradient. *L'hypothèse générale à tester est qu'il existe des relations statistiques significatives entre la liaison non-spécifique du Cd à des ligands intracytosoliques autres que la MT et l'apparition d'effets délétères au niveau des populations.*

En rapport direct avec l'étude sur les populations, nous nous sommes intéressés aux variations temporelles à long terme (sur une période de 13 ans) des concentrations de cadmium et de MT dans les populations de *P. grandis* dans notre groupe de lacs (chapitre 4). Dans le cadre spécifique de cette thèse, cette étude descriptive avait pour objectif de déterminer si le stress anthropique exercé par le Cd sur les populations de bivalves avait

varié au cours du temps. *Nous voulions répondre à l'hypothèse avancée à la fin du chapitre 3, stipulant un changement des facteurs structurants des populations de* P. grandis *dans les lacs étudiés (i.e., un déplacement des facteurs structurants purement anthropiques vers des facteurs naturels)*. Dans un cadre appliqué à l'évaluation du risque environnemental, cette étude devait nous permettre de déterminer si les variations temporelles des concentrations de MT dans les organismes modèles pouvaient être utilisées pour suivre les changements à long terme des niveaux de contamination des lacs par les métaux traces.

Enfin, nous avons testé expérimentalement les relations entre la répartition intracellulaire du Cd et les taux (individuels) de croissance et de mortalité de *P. grandis* en transférant des individus de cette espèce d'un milieu non-contaminé vers des sites lacustres présentant des niveaux croissants de contamination métallique (chapitre 5). Bien que l'étude des populations puissent fournir une description des effets du stress associé au Cd (chapitre 3), elle ne peut cependant que fournir une information très partielle sur l'origine de ces effets. L'étude des compromis énergétiques entre la croissance et la mortalité au niveau de l'individu devaient nous permettre de faire le lien entre les réactions biochimiques à l'intérieur des organismes en réponse au stress, et les stratégies démographiques adoptées par les populations. Dans cette expérience, nous avons utilisé des individus issus d'une même population, nous permettant ainsi de minimiser l'influence des mécanismes d'adaptations génétiques développés par les populations en réponse au contaminant (acquisition d'une tolérance), et de réduire la variabilité associée à des différences dans l'historique d'exposition au contaminant. *L'hypothèse à tester est que l'apparition d'anomalies dans la répartition intracellulaire du Cd (p. ex., augmentation des concentrations de Cd lié non-spécifiquement à des ligands cytosoliques autres que la MT) correspond à une réduction de la croissance des individus ainsi qu'à une augmentation de leurs taux de mortalité dans les sites de transplantation situés à l'extrémité du gradient de contamination.*

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Chapitre 2

Cadmium accumulation and metallothionein synthesis in freshwater bivalves (*Pyganodon grandis*): relative influence of the metal exposure gradient versus limnological variability

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2.1 Abstract

The relative influence of limnological confounding factors on cadmium (Cd) bioaccumulation and metallothionein (MT) synthesis was quantified in natural populations of freshwater bivalves (*Pyganodon grandis*) living in lakes along a Cd concentration gradient. During the ice-free period, we measured 15 environmental variables in the water compartment and determined total concentrations of Cd and MT in the gills of bivalves at 37 littoral stations in 20 lakes distributed across the mining area of Rouyn-Noranda in northwestern Québec. A multiple linear regression model including pH (+), dissolved Ca concentrations (–) and free Cd^{2+} concentrations at the sediment-water interface (+) explained 74 % of the variability in Cd concentrations in the bivalve gills. Dissolved Ca (–) and free Cd^{2+} (+) together explained 62 % of the variation in MT concentrations in the bivalve gills. Partial linear regression analyses indicated that the limnological factors' pure and shared effects together accounted for 48 and 45 % of the total variation in Cd and MT concentrations in the gills respectively. A lake selection procedure that could be applied in monitoring programs is proposed to minimise the relative influence of these confounding variables.

Keywords: Cadmium; Bioaccumulation; Metallothionein (MT); Biomonitoring; Multivariate statistical analysis

2.2 Introduction

Bivalves have been widely used as sentinel organisms for monitoring changes in environmental levels of trace metals because of their high bioconcentrating capacities (review in Elder and Collins, 1991; Stewart and Malley, 1997; Boening, 1999). A possible use of bivalves involves measuring total concentrations of metals in organisms derived from natural populations, which represent a time-integrated response to bioavailable metal in food and water (bioaccumulation). Another approach is to determine tissue concentrations of metallothionein (MT), a metal-binding protein synthesised by animals as a defence mechanism against excess metals in the surrounding media. In principle, measurement of MT is superior to the determination of total tissue metal, since it allows one to distinguish between the toxicologically significant intracellular fraction of metals and metals bound in unavailable form (Olafson et al., 1979). The use of MT as a biomarker for metal exposure has been proposed for aquatic organisms such as fish and bivalves (Couillard, 1997; Viarengo et al., 1999). However, in these *in situ* monitoring approaches, results are often difficult to interpret because of the numerous confounding variables that may affect metal accumulation in the field.

Among the metals that are released into aquatic ecosystems, cadmium (Cd) is considered to be one of the most toxic to the biota (Malley, 1996). In freshwaters, environmental factors such as pH, temperature, total organic carbon and dissolved calcium are known to influence Cd accumulation in bivalves (e.g. Graney et al., 1984; Campbell and Evans, 1991; Wang and Evans, 1993; Markich and Jeffree, 1994). More recently, Stuijfzand et al. (1999) have demonstrated that the toxicity of Cd could be amplified by humic acids for the zebra mussel *Dreissena polymorpha*. In this species, Cd bioaccumulation is probably correlated to food quality (expressed as seston C and N content) (Roditi et al., 2000a) and greatly increased in the presence of high-molecular-weight dissolved organic carbon (DOC) (Roditi et al., 2000b). In *Pyganodon grandis*, trends for lower Cd accumulation were observed under eutrophic conditions, suggesting that metal bioavailability is influenced by particle concentrations (Currie et al., 1998). For MT, the vast majority of the studies utilizing freshwater bivalves as sentinel organisms

have focused on the influence of metal exposure (e.g. Couillard et al., 1993; Baudrimont et al., 1999; Wang et al., 1999) and seasonal factors (Baudrimont et al., 1997). However, Viarengo et al. (1999) suggested that changes in some physico-chemical factors (temperature, salinity and oxygen) could also increase intracellular concentrations of MT in some marine species. To our knowledge, there has not yet been a good assessment of the relative importance of environmental confounding factors and metal exposure *per se* (or of their interaction) in controlling metal bioaccumulation and metallothionein synthesis in freshwater bivalves. Most studies have been conducted in the laboratory under unrealistic environmental conditions and have considered few confounding variables at the same time.

The present research is part of a larger project on the field validation of the use of MT as a biomarker of metal exposure and toxic effects in the freshwater bivalve *Pyganodon grandis*. In a study of 20 lakes in a mining region impacted by anthropogenic Cd inputs, we have quantified the relative contributions of limnological factors and Cd field contamination to the observed variations in the concentrations of Cd and MT in our sentinel organism. To be an effective biomonitor, an organism must accumulate contaminants proportionately to field exposure, with minimal influence of limnological factors on the bioavailability and bioaccumulation of the contaminants. Similarly, a good candidate biomarker should be sensitive to the contamination variables but not be too affected by limnological conditions. We hypothesised that the influence of limnological confounding factors on both Cd accumulation and MT synthesis in our sentinel organism could be minimised by a lake selection procedure. We therefore developed an approach to select a subset of ten lakes having similar limnological characteristics but differing markedly in the degree of environmental Cd concentration as well as in the Cd and MT concentration ranges in indigenous bivalves. We used partial linear regression analysis (Legendre and Legendre, 1998) to compare the relative contributions of limnological factors and field Cd contamination to the variations of Cd and MT in bivalves from the 20 original lakes and from the subset of 10 selected lakes. Our intention was to use this subset of lakes for further intensive or transplantation studies, to facilitate the unequivocal identification of the biological responses to metal contamination in our sentinel organisms.

2.3 Materials and methods

2.3.1 Sampling area

Sampling was conducted in the mining region of Rouyn-Noranda (Abitibi, northwestern Québec) where a large smelter has been operating since 1927 and where many surrounding lakes have been contaminated by metals (Couillard et al., 1993). Twenty lakes (Fig. 2.1) representing a Cd concentration gradient (from 0.005 to ≈ 1 nM) were sampled during the ice-free season between May and September of 1997. The number of sampling sites per lake was roughly proportional to the lake area with the exception of lakes Caron, Dasserat and Vaudray, in each of which only one area presenting *P. grandis* densities high enough to sustain collection was found. In lakes with more than one sampling site, sites were separated by a distance of at least 2 km to minimise spatial autocorrelation. Sediments, overlying water and organisms were collected at 37 sampling sites at depths varying between 1 and 3 m.

2.3.2 Data collection and analytical procedures

2.3.2.1 Environmental variables

To evaluate the habitat quality for *P. grandis*, fourteen limnological parameters (Table 2-I) were measured at least three times (usually four) at each sampling site. On each sampling date, water transparency was measured using a Secchi disk. Water samples at ≈ 10 cm above the sediments were collected by SCUBA divers using brown opaque plastic bottles for pH and chlorophyll *a* (Chl *a*) measurements (respectively one 500-mL sample and one 2-L sample), one clear acid-washed polyethylene container (4 L) for conductivity (Cond), dissolved calcium (Ca), dissolved organic carbon (DOC), humic and fulvic acids (HA and FA), and total phosphorus (TP) measurements, and three clear acid-washed polyethylene bottles (250 mL each) for sestonic carbon and nitrogen (Sest C and Sest N) measurements. Before filtration, water samples for chlorophyll *a* and sestonic C and N analyses were run through a Nitex sieve to remove the > 80 µm fraction; the < 80 µm fraction of particles is

representative of that ingested by *P. grandis* (Tessier et al., 1984). Measurements of dissolved oxygen (O_2) and temperature (Temp) were made ≈ 0.5 m above the sediments using a YSI Model 57 field instrument. Lakewater pH was determined in the field with a Fisher Accumet model 1001 pH meter.

Filtrations of all water samples were performed on the day of collection. Water samples for Ca, DOC, HA and FA measurements were filtered through polycarbonate membranes (0.4 µm, Nuclepore), and a filtrate sub-sample for Ca was acidified with HNO_3 (final acid concentration 0.5 %, v/v). All water samples were stored at 4 °C until analysis. Calcium was determined by flame atomic absorption spectrophotometry with the addition of a La-Cs ionic suppressor. DOC was measured by a combustion-infrared method (APHA, 1995). HA and FA were determined by spectrophotometry using 285 and 326 nm wavelengths (Buffle et al., 1982). Calibration curves were produced using commercial humic substances (Suwannee River Fulvic Acid Standard 1S101F and 1HSS; Suwannee River Humic Acid Reference 1R101H). For chlorophyll *a* and phaeopigment (Phaeo) measurements, 500 mL of water were filtered through Whatman GF/C filters. These filters were then folded and wrapped in aluminium foil and stored at -20 °C in plastic petri dishes until extraction. Chlorophyll *a* was extracted using hot 90 % ethanol (Sartory and Grobelaar, 1984) and absorbance was measured spectrophotometrically before and after acidification to correct for phaeopigments. For sestonic C and N determinations, three replicate water samples (100 mL each) were filtered through lyophilised and pre-weighed glass fibre filters (1 µm pore size, Gelman). Filters were folded once and wrapped in aluminium foil and then placed individually in vials and kept at –20 °C until analysis. Frozen filters were then lyophilised before final weighing, and C and N contents were determined with a CNS analyser (Carlo-Erba Instruments, model NA 1500). Total phosphorus was measured using the ascorbic acid colourimetric method after autoclaving samples with sulphuric acid and potassium persulphate (APHA, 1995). Conductivity (Cond) was measured in the laboratory at room temperature using a Cole Parmer conductivity meter (Model 1484-10).

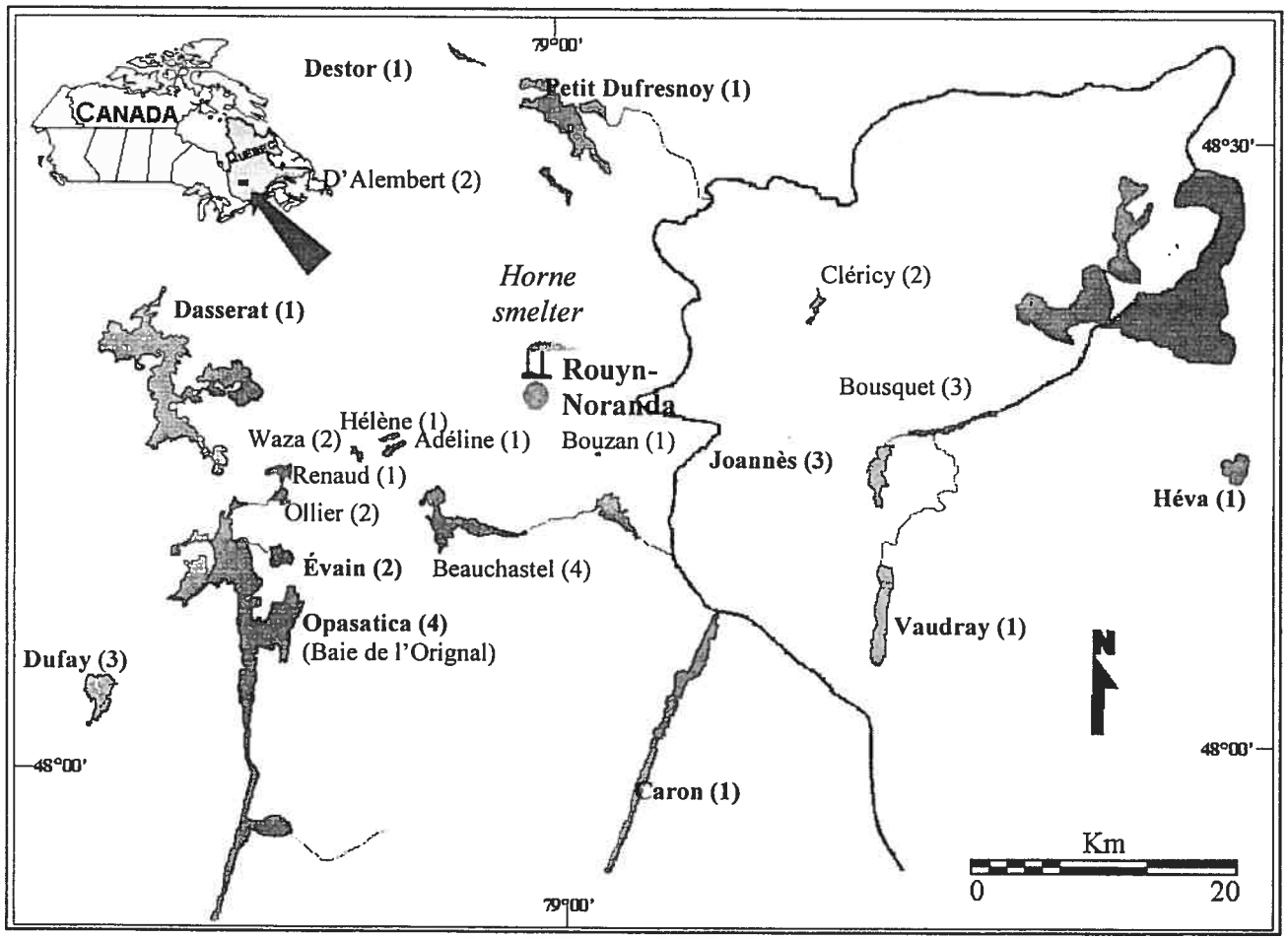

Figure 2.1. Location of the 20 lakes sampled in the Rouyn-Noranda mining area in northwestern Québec. The number of sampling sites for each lake is indicated in parentheses. Lakes selected by the program are in bold.

To quantify the degree of Cd contamination at each sampling site, surficial oxic sediments and the overlying water were collected during summer 1997 (once for sediment samples and monthly, on four occasions, for water samples). Sediment sample collection and procedures for metal extractions and analyses are described in detail in Couillard et al. (1993). To obtain an integrated estimate of Cd exposure, and to circumvent the difficulty of actually measuring free metal ion concentrations, we have derived an estimate of free Cd^{2+} concentrations based on the sorptive partitioning of Cd between water and the surficial oxic sediments. The mathematical development of the model, a summary of the field-derived equilibrium constants built into the model, a discussion of the simplifying assumptions that underlie its use and examples of model calculations are provided in Tessier et al. (1993). In a field study conducted in 14 lakes of the Rouyn-Noranda area in summer 1998, Fortin et al. (2000) compared estimated values of the free Cd^{2+} concentration, as calculated with the sorptive equilibrium model, to actual measurements of Cd^{2+}; the latter were obtained by *in situ* dialysis followed by equilibrium ion-exchange (Fortin and Campbell, 1998). The authors found good agreement between the estimated and measured values ($R^2 = 0.66$).

2.3.2.2 Toxicological variables

Bivalves were obtained from all 37 sampling sites between 14 and 30 July 1997. At each site, 12 mussels of similar size (nominal shell length ≈ 80 mm; actual mean shell lengths ranged between 66 and 86 mm for each site) were collected by SCUBA divers and kept in coolers filled with lake water until processing in the laboratory. Gills from each mussel were obtained within 12 h of collection, and gill tissues from four individuals were pooled (yielding three replicate samples per site), frozen in liquid nitrogen, sealed in plastic bags filled with nitrogen, and stored at –80 °C until the homogenisation step.

To minimise disruption of subcellular organelles, partially thawed gill tissues were gently homogenised, under a nitrogen atmosphere, with a motor-driven 50-mL glass tissue grinder (Duall Co., 80 rpm rotation speed), and the homogenised tissues were kept on ice. Homogenisation was performed with 3 portions of ice-cold 25 mM Tris buffer adjusted to pH 7.2 to 1 portion of tissue (w/w). Subsamples of the tissue homogenate were allocated to

measurements of Cd (gill Cd) and metallothionein (gill MT) concentrations and to the determinations of the dry to wet weight ratios.

For total Cd concentration in the gills, tissue homogenate (100 µg dry wt) was dried in an oven at 65 °C for 24 h and then transferred to a Teflon digestion bomb. Ultrapure concentrated nitric acid (3 mL) was then added and the digestion carried out in a microwave oven (700 W, ≤ 2 min) up to a pressure of 6900 kPa. Cooled digestates were diluted with ultrapure deionised water to a final volume of 25 mL and the Cd concentration was determined by flame atomic absorption spectrophotometry (AAS; Varian Spectra AA20). Procedural blanks and two certified reference materials (TORT-I, lobster hepatopancreas, Marine Analytical Chemistry Standards Program, National Research Council of Canada, Ottawa, ON; SRM No. 1566, oyster tissue, U.S. National Institute of Standards and Technology, Gaithersburg, MD) were analysed during every analytical run. Blanks indicated negligible inadvertent contamination and recoveries of Cd were consistently close to the certified values: 82.9 ± 5.1 % (oyster tissue) and 96.2 ± 2.6 % (TORT-I).

For MT analyses, a subsample of the homogenate was centrifuged at 30,000 × g for 30 min at 4 °C, and the supernatant was analysed for metallothionein with a Hg saturation assay adapted slightly from Dutton et al. (1993), and described in detail in Couillard et al. (1993). As a quality control, recovery of a MT standard (MT from rabbit liver, Sigma Chemical Co.) was determined with every assay; the mean recovery for 19 separate determinations was 101.8 ± 2.8 % (SE).

2.3.3 Lake selection procedure and statistical analyses

One of our main objectives was to select, from the 20 lakes sampled in 1997, a subset of 10 lakes having similar limnological characteristics but differing markedly in their degree of Cd contamination and in the concentrations of Cd and MT in the gills of indigenous bivalves. For this purpose, we calculated for each combination of 10 lakes among 20 (i.e. C_{20}^{10} = 184,756 possible combinations) the ratio of the variance of the geochemical and

toxicological variables together to the variance of the limnological variables, and selected the combination having the highest ratio. Calculations were made on the standardised data tables using a FORTRAN program. We used whole lake averages instead of site averages to limit the number of possible combinations and therefore the time of calculation.

The relationships between both gill Cd and gill MT and the 15 environmental (limnological and geochemical) parameters were examined using multiple linear regression for the full set of lakes (20 lakes = 37 sites). First, principal component analysis (PCA) was used to reduce the dimensionality of the environmental data matrix. The PCA based upon the correlation matrix was performed on the normalised data (all $\log_{10}$-transformed except pH). Principal components (PCs) having eigenvalues of less than one were discarded (Legendre and Legendre, 1998), and varimax rotation maximising the loading of a variable on one component was then applied on the retained PCs. Multiple regression analyses with forward selection of the explanatory variables were used with gill Cd or gill MT for each site as the dependent variable, and PCA scores for each site on each principal component as independent variables, to identify environmental variables correlated with Cd and MT concentrations in the gills of *P. grandis*. Forward selection procedure was stopped whenever the additional effect of the chosen variable had a P-value > 0.15. Variables were removed from the environmental data matrix if they loaded most strongly onto a rotated component which was shown to be uncorrelated with gill Cd or gill MT. Finally, the adequacy of the interpretations of the principal components was tested by multiple linear regression using the variables loading on the principal components as independent variables against both gill Cd and gill MT. Environmental and toxicological data were $\log_{10}$-transformed ($y'_i = \log_{10}(1000 \cdot y_i)$) when assumptions in multiple linear regression analysis (i.e. normality and homoscedasticity of residuals) were not satisfied. The Kolmogorov-Smirnov test was used to verify that regression residuals were normally distributed and homoscedasticity was checked by examination of the biplots of residuals against predicted values. Analyses were performed using SYSTAT for Windows version 8.0.

To determine the relative importance of environmental factors in Cd accumulation and MT production we used the method of variation partitioning proposed by Borcard et al.

(1992). We partitioned the total variation in our toxicological variables gill Cd and gill MT into four independent components ("pure" geochemical, "pure" limnological, "shared" variation between geochemical and limnological, and unexplained) by multiple regression and partial linear regression analyses. This procedure involves the following steps: (1) compute the variation accounted for by the geochemical variable; (2) compute the variation accounted for by the limnological variables; (3) compute the variation explained by the geochemical variable after removing by partial regression analysis the effect of the limnological variables; (4) compute the variation explained by the limnological variables after removing the effect of the geochemical variable by partial regression analysis. The total explained variation is the sum of explained variations in (1) and (4) or in (2) and (3). The pure geochemical variation that is not related to the limnological variables is given by step (3), and the pure limnological variation that is not related to the geochemical variable is given by step (4). The variation shared by the geochemical and limnological variables, is obtained by subtracting (3) from (1) or (4) from (2). Finally, the unexplained portion of variation is obtained by subtracting the pure geochemical variation, the pure limnological variation, and the shared variation from the total variation. To check whether the lake selection procedure described earlier had succeeded in minimising the influence of limnological variables on Cd accumulation and MT production, variation partitioning was performed on two sets of analyses for each toxicological variable, one including the 37 sampling sites in the 20 original lakes and the second including the 18 sampling sites in the 10 lakes selected by the lake selection program. To avoid an artificial increase in the explained variation due to chance (Borcard et al., 1992), the environmental factors were chosen from the set of explanatory variables included in the simplified models of previous multiple regression analyses. The statistical significance of pure geochemical and limnological components was evaluated by Monte Carlo permutation tests (9999 unrestricted permutations under the reduced model) of the sum of all canonical eigenvalues. Statistical significance was set at a Bonferroni-corrected level of $0.05/4 = 0.0125$. All computations were done using the computer program CANOCO 4.0 (ter Braak and Šmilauer, 1998). It should be noted that, although CANOCO is primarily a program for multivariate data analysis, multiple and partial linear regressions can be implemented in

CANOCO respectively as a redundancy analysis (RDA) and partial RDA with only one response variable (cf. Borcard et al., 1992; Birks, 1996).

2.4 Results

2.4.1 Lake selection procedure

Limnological, geochemical and toxicological variables exhibited appreciable variability among the 37 sites (Table 2-I). Dissolved free Cd^{2+} concentrations, pH (on H^+ concentration basis) and Cd concentrations in the gills varied by two orders of magnitude over the study area. MT concentrations in the gills varied by an order of magnitude, as did TP and sestonic N. The range in Ca concentrations was more than 8-fold. Lakes selected by the program (indicated in bold in Fig. 2.1) were lakes Caron, Destor, Dasserat, Dufay, Évain, Héva, Joannès, Opasatica, Petit Dufresnoy and Vaudray, yielding 18 sampling sites. For this selection, the value for the ratio of the variance of the geochemical and toxicological variables together to the variance of the limnological variables was 0.58. The minimum value for this ratio (corresponding to the worst selection of 10 lakes among 20) was 0.06. The lake selection procedure noticeably reduced the dispersion of the values for all limnological variables without affecting the geochemical (i.e. contamination) and toxicological (i.e. response) variables (Table 2-I).

2.4.2 Multiple regression models

Table 2-II shows the individual loadings of the 15 environmental variables on the four derived principal components (PCs) produced in the principal component analysis. The first four PCs accounted for more than 80 % of the total variance in the data. The first principal component (PC1) explained 22 % of the total variance in the data. The loadings for this component were mostly influenced by the concentrations of O_2 (negatively), and DOC, HA and FA (positively). The second principal component (PC2) explained a further 19 % of the remaining variance in the data. The loadings for this component were

Table 2-I. Summary statistics of environmental variables measured in the water compartment and of *P. grandis* biological data for all the sampling sites and the subset of sampling sites selected by the program. (Secchi = Water transparency; Temp = Bottom temperature; O_2 = Dissolved oxygen ; Cond = Conductivity; Ca = Dissolved calcium; Chl *a* = Chlorophyll *a* fraction <80 µm; Phaeo = Phaeopigments fraction <80 µm; Sest C = Sestonic carbon; Sest N = Sestonic nitrogen; TP = Total phosphorus; DOC = Dissolved organic carbon; HA = Dissolved humic acid; FA = Dissolved fulvic acid; Cd^{2+} = Dissolved free Cd ion; Gill Cd = Cd concentration in the gills of bivalves; Gill MT = MT concentration in the gills of bivalves).

	All sites (N = 37)			Selected sites (N = 18)		
Characteristic	Mean	SD	Min-Max	Mean	SD	Min-Max
Limnological variables						
Secchi (m)	1.5	0.49	0.6-2.8	1.5	0.31	1.1-2.1
Temp (°C)	18.2	1.19	15.4-20.7	18.4	0.90	16.8-20.0
O_2 (mg·L^{-1})	8.7	0.71	6.6-10.2	8.9	0.62	8.1-10.2
Cond (µmhos·cm^{-1})	105.4	52.7	34.7-248.3	85.0	32.4	34.7-141.3
pH	7.2	0.51	6.0-8.6	7.1	0.42	6.0-7.7
Ca (mg·L^{-1})	8.5	4.35	2.2-18.5	6.6	2.65	2.2-12.2
Chl *a* (µg·L^{-1})	2.4	1.00	1.0-5.2	2.2	0.74	1.2-3.9
Phaeo (µg·L^{-1})	5.9	2.96	2.2-15.0	5.2	2.08	2.2-10.7
Sest C (mg C·L^{-1})	0.55	0.24	0.28-1.45	0.43	0.12	0.28-0.70
Sest N (mg N·L^{-1})	0.08	0.07	0.02-0.39	0.05	0.02	0.02-0.10
TP (µg·L^{-1})	9.3	6.99	1.8-39.9	6.7	3.12	1.8-13.9
DOC (mg·L^{-1})	10.9	3.62	7.3-21.7	9.5	1.46	7.6-12.6
HA (mg·L^{-1})	0.9	0.45	0.5-2.2	0.8	0.19	0.5-1.0
FA (mg·L^{-1})	9.0	4.49	4.7-22.2	7.7	1.95	5.0-10.4
Geochemical variable						
Cd^{2+} (nM)	0.277	0.27	0.005-0.964	0.263	0.27	0.025-0.964
Toxicological variables						
Gill Cd (nmol·g^{-1} dry wt)	512.6	487.1	18.7-2374.3	603.6	566.8	23.4-2374.3
Gill MT (nmol sites·g^{-1} dry wt)	115.5	69.0	17.9-344.4	134.6	78.5	59.4-344.4

Table 2-II. Principal component analysis (PCA) on the environmental data matrix: loadings (varimax rotation) of environmental variables on the first four PCs and percentage of the overall variance accounted for by each component. Loadings of absolute value greater than 0.50 are underlined.

Variable	PC1	PC2	PC3	PC4
Secchi	−0.23	−0.68	−0.23	−0.19
Temp	−0.29	−0.07	−0.32	−0.57
O_2	−0.68	−0.30	0.01	−0.10
Cond	−0.23	−0.12	−0.92	0.11
pH	−0.42	0.09	−0.83	−0.21
Ca	−0.05	−0.07	−0.97	0.07
Chl *a*	−0.08	0.94	0.04	0.09
Phaeo	0.13	0.88	0.05	0.11
Sest C	0.14	0.36	0.03	0.84
Sest N	0.16	0.17	−0.06	0.89
TP	0.14	0.72	−0.23	0.45
DOC	0.87	0.07	0.25	0.24
HA	0.86	0.02	0.42	0.17
FA	0.86	0.02	0.42	0.17
Cd^{2+}	0.39	0.05	0.75	0.28
% of total variance	22.2	19.5	24.4	15.6

influenced by the Secchi depth (negatively) and by the concentrations of Chl *a*, phaeopigments and TP (positively). The third principal component (PC3) accounted for a further 24 % of the remaining variance and the loadings for the component were affected negatively by the conductivity, pH and Ca concentration, and positively by the concentration of free Cd^{2+}. The fourth component (PC4) accounted for a final 16 % of the variance and water temperature (negatively) and concentrations of sestonic C and N (positively) influenced this component. PC3 was the only principal component significantly correlated with both Cd and MT concentrations in the gills of *P. grandis* (Table 2-III). Thus, the original data matrix of 15 environmental variables could be reduced to 4 variables (i.e. conductivity, pH, and Ca and free Cd^{2+} concentrations) loading on one principal component. A multiple linear regression model including all sites showed that pH (+) and

Ca (–) and free Cd^{2+} (+) concentrations explained 74 % of the variation in the Cd in the gills (Table 2-IV, Fig. 2.2A). Low values of gill Cd were slightly overestimated by the model (Fig. 2.2A). For the multiple regression analysis with the concentration of MT in the gills as the dependent variable, 62% of the total variation was explained by Ca (–) and free Cd^{2+} (+) (Table 2-IV, Fig. 2.2B). One sampling site (Lake Vaudray) was excluded from the model (N = 36) to achieve homoscedasticity of the residuals. For gill MT concentrations greater than about 125 nmol sites·g^{-1}, the model tends to underestimate the observed values (Fig. 2.2B). Both models were highly significant ($P < 0.0001$), and tolerance levels did not indicate severe collinearity among explanatory predictor variables despite the fact that all of them loaded into a single PC.

Table 2-III. Results of forward selection in multiple linear regressions with $\log_{10}$-transformed Cd concentrations and MT concentrations in the gills of *P. grandis* as dependent variables and PCA scores as independent variables. Only variables with P-values < 0.15 are shown.[a]

Variable	Coefficient	SE	t	$p(t)$	R^2
$\log_{10}$Gill Cd					
Constant	5.461	0.062	88.53	<0.0001	0.54
PC3	0.402	0.063	6.42	<0.0001	
$N = 37$	$F = 41.23$	$p(F) < 0.0001$			
Gill MT					
Constant	115.47	7.36	15.69	<0.0001	0.59
PC3	53.03	7.46	7.11	<0.0001	
$N = 37$	$F = 50.50$	$p(F) < 0.0001$			

[a]Abbreviations: Coefficient, estimate of partial regression coefficients; SE, standard error on estimate; t, t statistic for estimate of partial regression coefficients; $p(t)$, significance level of t statistic; R^2, coefficient of multiple determination; N, number of observations; F, overall variance ratio from analysis of variance; $p(F)$, significance of F value.

Table 2-IV. Best regression models predicting $\log_{10}$-transformed Cd concentrations and MT concentrations in the gills of *P. grandis* with environmental factors from the reduced data matrix as independent variables.

Variable	Coefficient	SE	Tolerance	*t*	*p*(*t*)	R^2
$\log_{10}$Gill Cd						
Constant	2.746	1.772		1.550	0.131	0.74
pH	0.960	0.250	0.15	3.840	<0.001	
$\log_{10}$Ca	−1.679	0.352	0.35	−4.773	<0.0001	
$\log_{10}Cd^{2+}$	1.062	0.186	0.24	5.718	<0.0001	
$N = 37$	$F = 31.25$	$p(F) < 0.0001$				
Gill MT						
Constant	125.09	21.07		5.937	<0.0001	0.62
Ca	−5.23	1.73	0.70	−3.029	0.005	
Cd^{2+}	112.79	29.13	0.70	3.872	<0.001	
$N = 36$	$F = 26.70$	$p(F) < 0.0001$				

2.4.3 Partial correlations and variance partitioning

Cadmium concentrations in the gills and free Cd^{2+} concentration at the sediment-water interface were positively correlated across the 37 sites when the effect of limnological confounding variables was taken into account (Fig. 2.3A). A similar linear relationship was observed for the subset of sites selected by the lake selection procedure with a partial correlation coefficient *R* increasing from 0.71 to 0.77 (Fig. 2.3B). The pure geochemical component (i.e. Cd contamination) accounted for 26 % of the variation in the Cd in the gills when all sites were considered (Fig. 2.4). The pure limnological effects accounted for 19 % of the variation in gill Cd. Shared variation that could not be partitioned into pure effects totalled 29 % (Fig. 2.4). The pure geochemical effect due to Cd field contamination substantially increased when the selection of sites was limited to the 18 sampling sites where the ranges in limnological variables were reduced. Concurrently, the proportion of the variation in gill Cd explained by the limnological component (pure and

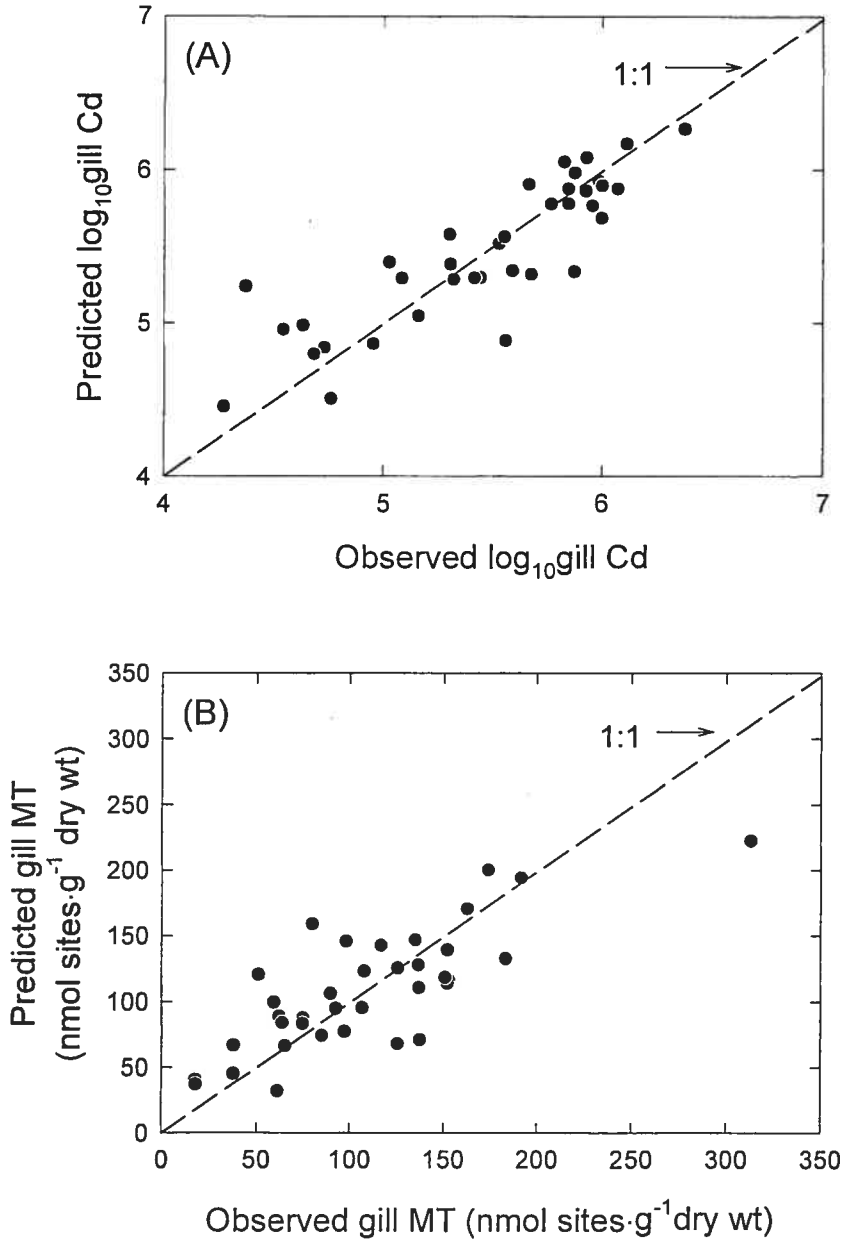

Figure 2.2. Observed and predicted values of A) log_{10}[Cd] and B) [MT] in the gills of *P. grandis* for multiple linear regression models presented in Table 2-IV.

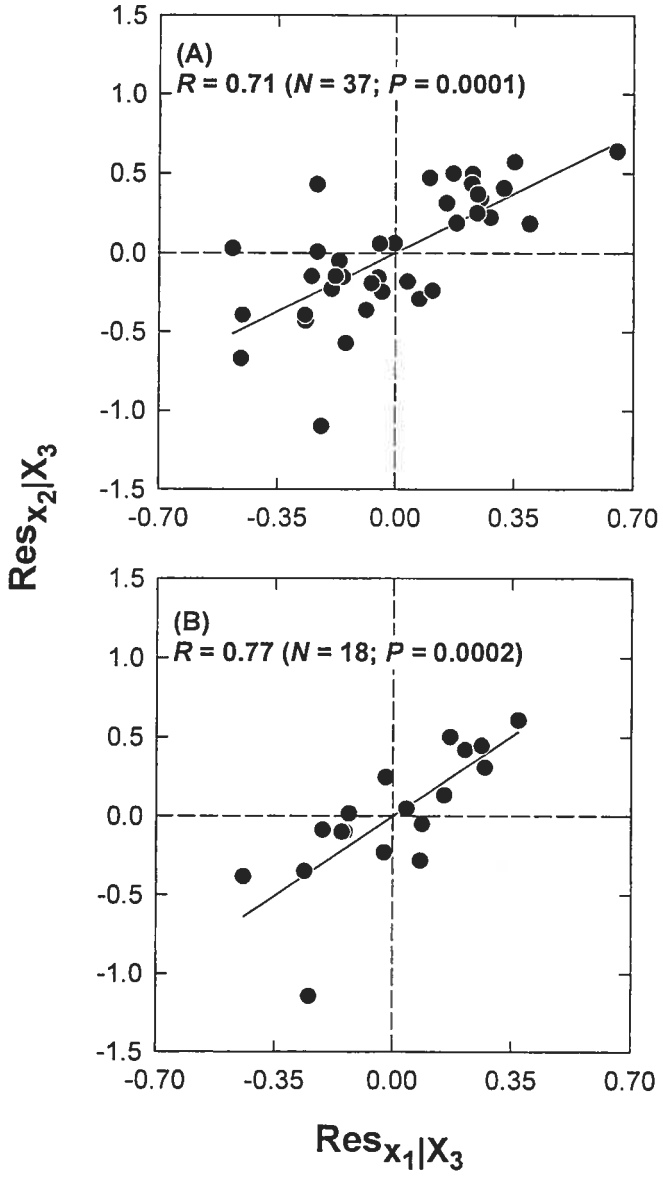

Figure 2.3. Scatter diagram of partial correlation between $\log_{10}$[Cd] in the gills of *P. grandis* ($\mathbf{x_2}$) and $\log_{10}[Cd^{2+}]$ at the sediment-water interface ($\mathbf{x_1}$) holding the limnological data matrix ($\mathbf{X_3}$) containing pH and $\log_{10}$[Ca] constant for A) all the 37 sampling sites, and B) 18 sampling sites selected by the program. The statistical significance of the partial correlation coefficients was obtained by permutation tests (9999 permutations).

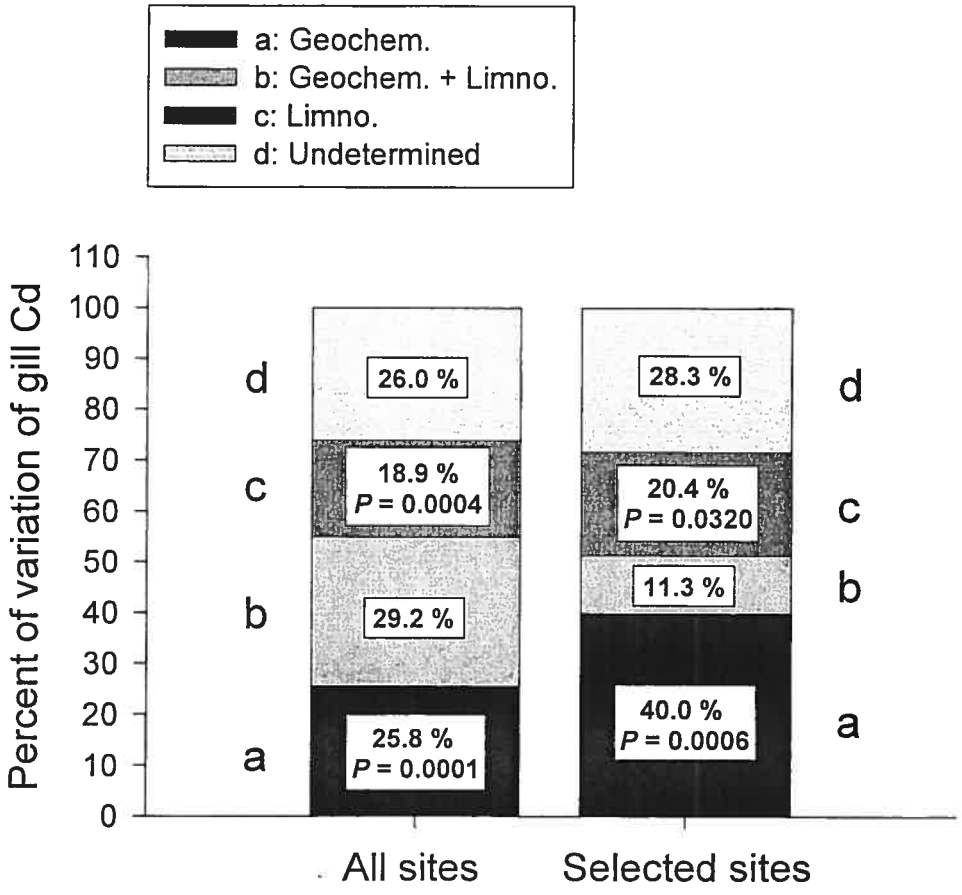

Figure 2.4. Variation partitioning of [Cd] in the gills of *P. grandis* among geochemical ([Cd^{2+}]) and limnological (pH and [Ca]) variables for all the 37 sampling sites and 18 sampling sites selected by the program. The statistical significance of pure geochemical and limnological components was assessed by Monte Carlo permutation tests (9999 unrestricted permutations under the reduced model). Statistical significance was accepted at the $P < 0.0125$ level.

shared effects together) was reduced by the lake selection procedure (48 % for all sites and 32 % for the subset of sites selected by the program) (Fig. 2.4). The pure limnological effects accounted for 20 % of the variation in gill Cd for the subset of 18 sampling sites, but the Monte Carlo permutation test on the trace statistic of this partial regression was not significant at the Bonferroni-corrected level ($P > 0.0125$). The amount of unexplained

variation was relatively low and did not vary between the two sets of data (26 and 28 % for the set of 37 sites and the subset of 18 sites respectively).

Partial correlations between the concentration of MT in the gills and dissolved free Cd^{2+} concentration, holding limnological variables constant, were significant for both the set of 36 sites and the subset of sites selected by the program (Fig. 2.5). The partial correlation coefficient was noticeably increased by the lake selection procedure (Fig. 2.5). In the two sets of analysis, the pure limnological component accounted for a minor proportion of the variation in gill MT (11 and 3 % of the total variation for the set of 36 sites and the subset of 18 sites respectively) (Fig. 2.6). The proportion of the variation in gill MT explained by pure Cd contamination increased from 17 to 55 % when only the selected lakes were considered, whereas shared variation between contamination and confounding factors remained constant (Fig. 2.6). The amount of unexplained variation varied greatly between the two sets of data (38 and 8 % for the set of 36 sites and the subset of 18 sites respectively).

2.5 Discussion

2.5.1 Predictors of Cd concentrations in *P. grandis*

The range of Cd concentrations in the gills of *P. grandis* observed in this study (19 to 2370 $nmol \cdot g^{-1}$ dry wt) was comparable to previously reported values for the same species. Tessier et al. (1993) reported gill Cd concentrations ranging from 52 to 3200 $nmol \cdot g^{-1}$ dry mass for specimens collected from 38 lakes distributed over a 350,000 km^2 study area in Québec and Ontario; of the 38 lakes sampled by Tessier et al., 10 were re-sampled in the present study. In another study, Pip (1990) reported much lower values (9-89 nmol $Cd \cdot g^{-1}$ dry weight) for bivalves collected from a large, non-contaminated lake in Manitoba. Similar ranges of Cd concentrations (4-67 $nmol \cdot g^{-1}$ dry weight) were obtained for the freshwater bivalve *Elliptio complanata* in a survey of 21 small lakes located in south

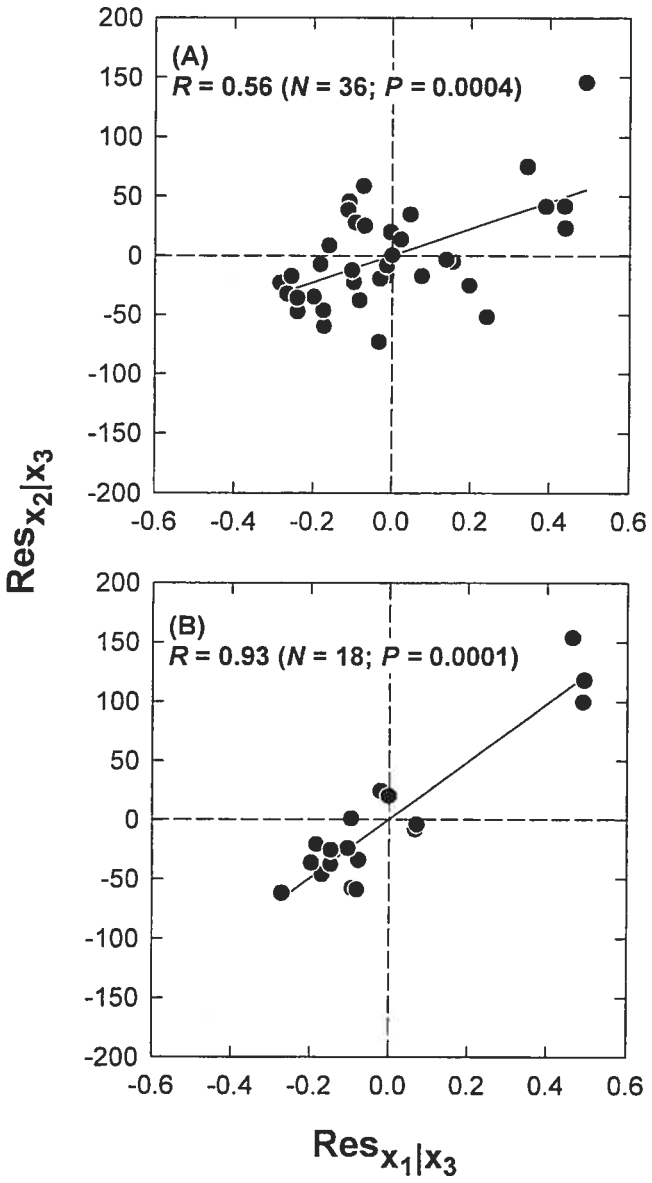

Figure 2.5. Scatter diagram of partial correlation between [MT] in the gills of *P. grandis* (x_2) and [Cd^{2+}] at the sediment-water interface (x_1) holding the limnological variable [Ca] (x_3) constant for A) 36 sampling sites, and B) 18 sampling sites selected by the program. The statistical significance of the partial correlation coefficients was obtained by permutation tests (9999 permutations).

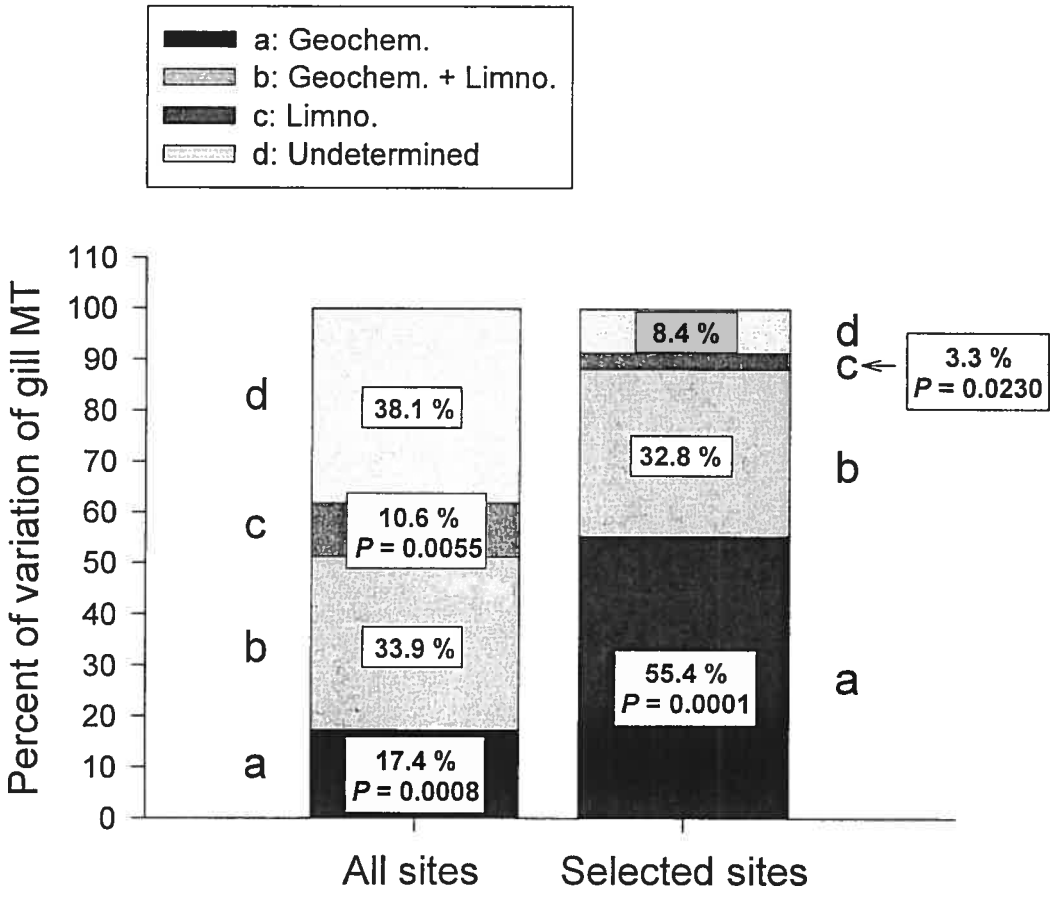

Figure 2.6. Variation partitioning of [MT] in the gills of *P. grandis* among geochemical ($[Cd^{2+}]$) and limnological ([Ca]) variables for 36 sampling sites and 18 sampling sites selected by the program. The statistical significance of pure geochemical and limnological components was assessed by Monte Carlo permutation tests (9999 unrestricted permutations under the reduced model). Statistical significance was accepted at the $P < 0.0125$ level.

central Ontario (Campbell and Evans, 1991). These values were comparable to the lowest Cd concentrations found in our specimens (Table 2-I).

Metal uptake by aquatic organisms is generally described as involving an initial reaction of the free metal ion or one of its kinetically labile complexes with a membrane-embedded-transport system that takes the metal across the external membranes of the exchange surface (Simkiss and Taylor, 1989). Hence, variations in metal uptake are usually better explained on the basis of changes in free metal ion concentration rather than changes

in total metal concentration (Campbell, 1995). Tessier et al. (1993) demonstrated that the free Cd^{2+} ion concentration at the sediment-water interface was a good predictor of the amount of Cd accumulated by *P. grandis*: a simple regression equation including data from 19 lacustrine sites indicated that free Cd^{2+} ion concentration alone explained 61 % of the variance of the total Cd concentration in the gills. Results of the present study demonstrate that the predictive power of this simple model can be substantially increased when water quality parameters such as pH and dissolved calcium concentration are taken into account.

In our multiple regression analysis, increasing dissolved Ca concentrations had a negative effect on the Cd concentration in *P. grandis* (Table 2-IV). This observation is consistent with results of previous field studies encompassing a wide variety of freshwater organisms (Stephenson and Mackie, 1988; Amyot et al., 1994; van Hattum et al., 1996). We hypothesise that calcium ions might compete with Cd ions at gill binding sites. Competition by Ca ions might be particularly effective for bivalves since these organisms have a high metabolic requirement for calcium, notably for the deposition of new shell material. Calcium has been shown to reduce Cd bioaccumulation in freshwater bivalves in the laboratory (Wang and Evans, 1993; Markich and Jeffree, 1994), probably a consequence of the fact that Cd is reported to pass through animal membranes via channels used for calcium (Simkiss and Taylor, 1989). Consistent with this explanation are the observations that Ca channel blockers inhibit Cd entry into the gills of both freshwater (Holwerda et al., 1989) and marine bivalve species (Roesijadi and Unger, 1993; Vercauteren and Blust, 1999).

The coefficient of the simple correlation between pH and gill Cd concentrations indicated a negative relationship between these two variables across the sampling sites ($r = -0.60$, $P < 0.0001$; results not shown). To a certain extent, this relationship may be explained by the lower bioavailable Cd levels generally associated with increasing values of water pH in our lakes (free Cd^{2+} and pH were negatively correlated; $r = -0.87$). However, in the multiple regression model predicting Cd concentrations in the organisms, the partial regression coefficient for pH was positive (Table 2-IV), suggesting that the amount of Cd bioaccumulated by *P. grandis* was greater in more alkaline lakes. Several

explanations for these apparent inconsistencies are possible. First, the relationship between pH and the dependent variable gill Cd in our multiple regression might be spurious. Change in the algebraic sign of the regression coefficient for pH might be an informal indication of the presence of multicollinearity among independent variables (Neter et al., 1990). In multiple linear regression analysis, multicollinearity among explanatory variables is known to cause unstable estimation of the regression coefficients. In the present case, however, tolerance levels in the multiple regression model were systematically > 0.10. Alternatively, the positive relationship between pH and Cd concentration in the gills in our model (or negative, if we use the H^+ concentration instead of pH) might result from the competitive interaction between hydrogen and cadmium ions at binding sites on the outer gill membrane, as was suggested earlier for Ca. Unless the concentration of available metals is increased sufficiently by geochemical mobilisation or by pH-induced changes in speciation (Nelson and Campbell, 1991), the decreased pH may actually lead to reduced metal uptake by the aquatic biota (Campbell and Stokes, 1985). Competition by H^+ has already been reported for aquatic insect larvae (*Chaoborus*) in highly acidic (pH < 5.5) lakes (Hare and Tessier, 1998; Croteau et al., 1998) and for the freshwater amphipod *Gammarus fasciatus* in circumneutral lakes (Amyot et al., 1994).

2.5.2 Predictors of MT concentrations in *P. grandis*

Results obtained in this study corroborate previous models of MT induction developed for *P. grandis* in the same geographical area (Couillard et al., 1993; Wang et al., 1999): spatial variations in the concentration of MT in the gills were strongly related to changes in the ambient free Cd^{2+} concentration, as estimated from sediment-water sorptive equilibria (Table 2-IV). The predictive power of the simple regression models reported in Couillard et al. (1993) and Wang et al. (1999) was generally high (R^2 = 0.62 and 0.88 respectively), presumably because of low limnological variability among the lakes, especially for parameters related to the hardness-alkalinity of the water (mean dissolved Ca concentration = 6.0 ± 3.1 $mg \cdot L^{-1}$; values for individual lakes ranged between 2.4 and 13.8 $mg \cdot L^{-1}$). As shown in our multiple regression analysis (Table 2-IV), the steady-state concentrations of MT in *P. grandis* tend to decrease as the aqueous Ca concentration increases. Since the

entry of toxic metals into the organism is thought to be the key triggering event for MT biosynthesis leading to an increase in [MT] over and above basal levels, the negative effect of dissolved Ca on MT concentration in the gills is probably due to the competitive interaction between Cd^{2+} and Ca^{2+} at biological uptake sites. This result is consistent with the pattern of Cd bioaccumulation in *P. grandis* (see previous section).

Metallothionein synthesis can be induced not only by metals but also by a wide range of endogenous and exogenous factors (Kägi, 1993). In freshwater bivalves (*Corbicula fluminea*) collected from an unpolluted site, Baudrimont et al. (1997) found that seasonal variations in MT concentrations in the soft tissues could be correlated with the phases of the bivalves' reproductive cycle. In their study, the increase in MT concentrations coincided with the gonadal maturation period, whereas minimum values for MT concentrations corresponded to the post-spawning period. In the marine clam *Ruditapes decussatus*, the concentrations of MT in the digestive gland varied significantly during the period of sexual differentiation (June-September), in specimens collected from unpolluted and metal contaminated sites (Serafim and Bebianno, 2001). These natural variations are of great importance, since they can lead to incorrect data interpretation: differences in MT concentrations may be due not only to the gradient in ambient metal concentrations but also to the variations in the timing of the organisms' reproductive cycle at each sampling site. Despite the fact that all mussels were collected within a 17 day period in our study, the proportion of gravid mussels varied considerably from one lake to another, suggesting that the biological cycles of the different populations were not fully synchronised across the geographical area. However, the influence of reproductive status, based on the presence/absence of larvae in the gills, probably accounted only for a small proportion of the unexplained variation in MT concentrations in our study since we selected only non-gravid specimens for tissue metal and MT analyses. In a field study, Wang et al. (1999) monitored gill MT concentrations in *P. grandis* monthly, from June to September, in two lakes located towards the lower end of the Cd environmental gradient in the Rouyn-Noranda area. The seasonal variations in MT concentrations in molluscs from a given lake were lower than inter-lake (spatial) variations, suggesting that characteristics related to the

basic biology and physiology of *P. grandis* are less important than changes in metal bioavailability as sources of variation in MT concentrations in this species.

In the present study, a deliberate attempt was made to collect organisms that were similar in size. Despite this selection process, mean shell length of bivalves for each site ranged between 66 and 86 mm. This disparity is simply a reflection of the fact that in some lakes most individuals were small and in others most individuals were large. Size and body weight of organisms are known to influence MT concentration (Bordin et al. 1997; Amiard-Triquet et al., 1998) and tissue metal concentrations (Hinch and Stephenson, 1987 and literature cited therein; Metcalfe-Smith et al., 1996) in clams and mussels. However, size differences were probably less important than changes in metal bioavailability as sources of variation in Cd and MT concentrations in the present study. Indeed, the predictive power of both multiple regression models was not significantly increased by the addition of mean shell length or gill dry weight as explanatory variables (results not shown). Results obtained by Kalhok and Cyr (1997) in a field survey of *P. grandis* populations in the Rouyn-Noranda area are consistent with this conclusion. They demonstrated that, within a given lake, MT concentrations in the soft tissues did not vary with length or age of the organisms.

2.5.3 Implications for the design of biomonitoring studies

The lake selection procedure we have proposed was successful in increasing the amount of variation due to the ambient level of contaminant in both Cd and MT concentrations in bivalves (1.6- and 3.2-fold for Cd and MT in the gills respectively). At the same time, the proportion of variation in these two variables accounted for by the pure limnological component was reduced and/or became insignificant. The use of this site selection procedure could be extended to many monitoring programs. Currently, a regulatory program is being implemented in Canada for the assessment of the aquatic effects of mining; the biological monitoring of receiving environments is an important component of the proposed programme (AQUAMIN, 1996). Typical study designs would include multiple sampling periods before and after mine development at both exposed and reference locations. A key objective of the programme is the unequivocal identification of

the causes of unacceptable mine-related effects. To attain this objective, environmental factors that may confound results must be identified and taken into account (AQUAMIN, 1996). In this context, a variant of the lake selection procedure described here could be used to minimise the influence of these confounding factors.

However, at least for gill MT levels, the interaction between Cd exposure and the limnological confounding factors was not minimised by the lake selection procedure (Fig. 2.6). Furthermore, the pure and shared effects of the confounding factors were still responsible for more than 30 % of the variation of both toxicological variables (Figs. 2.4 and 2.6). These results have an important implication from a biomonitoring perspective. They unambiguously demonstrate how important it is to examine basic limnological parameters in conjunction with the determination of internal concentrations of Cd and MT when evaluating bioavailable Cd levels in water. Minimally, dissolved Ca concentrations and pH should be determined during each sampling event. Standardising for these factors, or accounting for them in multiple regression models, would therefore greatly improve accuracy in biomonitoring programs that use bivalves to determine the spatial and temporal trends in metal pollution.

Another key result of the present study is the general linear increase observed for both Cd and MT concentrations in our sentinel organism in response to the metal concentration gradient after controlling for limnological confounding variables (Figs. 2.3 and 2.5). Indeed, one of the most important features of an effective biomonitor organism is that a quantitative relationship should exist between its trace metal content and the concentration of contaminant in its environment (Phillips and Rainbow, 1993). In the same way, a good biomarker should respond in a concentration-dependent manner to changes in ambient levels of the contaminant (Haux and Förlin, 1989). It is tempting to conclude that *P. grandis* could be used effectively as a biomonitor for Cd contamination and that MT is a valuable biomarker for Cd exposure. However, as we have emphasised, the partial correlation results suggest that the responses of the biomonitor and the biomarker to Cd contamination in the field are also sensitive to limnological variations across the study area:

reliability is markedly improved (especially for MT) when sampling sites are carefully selected.

2.6 Acknowledgements

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Chapitre 3

Metal-induced stress in bivalves living along a gradient of Cd contamination: relating sub-cellular metal distribution to population-level responses

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3.1 Abstract

The use of biomarkers to assess the impacts of contaminants on aquatic ecosystems has noticeably increased over the past few years. Few of these studies, however, have contributed to the prediction of ecologically significant effects (i.e., at the population or community levels). The present field study was designed to evaluate the potential of metallothionein (MT) and sub-cellular metal partitioning measurements for predicting toxic effects at higher levels of the biological organization in freshwater bivalves (*Pyganodon grandis*) chronically exposed to Cd. For that purpose, we quantitatively sampled *P. grandis* populations in the littoral zone of nine lakes on the Precambrian Canadian Shield during two consecutive summers (1998 and 1999); lakes were characterized by contrasting Cd levels but similar trophic status. We tested relationships between the population status of *P. grandis* (i.e., growth parameters, density, biomass, secondary production, turnover ratio and cumulative fecundity) and (*i*) ambient Cd concentrations, (*ii*) sub-organismal responses (MT concentrations in the gill cytosol of individuals and Cd concentrations in three metal-ligand pools identified as M-HMW, the high molecular weight pool, M-MT, the metallothionein-like pool and M-LMW, the low molecular weight pool) and (*iii*) ecological confounding factors (food resources, presence of host fishes for the obligatory parasitic larval stage of *P. grandis*). Our results show that littoral density, live weight, dry viscera biomass, production and cumulative fecundity decreased with increasing concentrations of the free-cadmium ion in the environment (Pearson's r ranging from –0.63 to –0.78). On the other hand, theoretical maximum shell lengths (L_∞) in our populations were related to both the dissolved Ca concentration and food quality (sestonic C and N concentrations). Overall, Cd concentrations in the gill cytosolic HMW pool of the individual molluscs were the biomarker response that was most frequently and most strongly correlated with the population variables (Pearson's r ranging from –0.58 to –0.80). Our findings demonstrate, however, the difficulty of currently assigning to sub-cellular metal partitioning measurements (mainly Cd bound to the HMW fraction) any predictive role for population health, notably because of the influence of ecological confounding variables (e.g., the cumulative number of degree-days in the littoral zone, as is the case here). Metal

contamination of our lakes has decreased markedly in the past 10 years and consequently we believe that the toxic effects of metals may have been replaced by some natural factors as the main agent for structuring the clam populations in these lakes.

Keywords: Metals; Chronic toxicity; Population-level responses; Sub-cellular partitioning; Ecological confounding factors; Bivalves

3.2 Introduction

Over the last two decades, biomarkers have received considerable attention in the field of ecotoxicology (e.g., NRCC, 1985; McCarthy and Shugart, 1990; Huggett et al., 1992; Peakall et al., 1999). Biomarkers can be defined as "biochemical, physiological, histological and morphological responses to an environmental chemical, that are measured inside an organism and that indicate a departure from its normal status" (van Gestel and van Brummelen, 1996). In principle, the detection and quantification of these sub-organismal responses could be developed as early-warning and specific indicators of environmental stress, notably for aquatic ecosystems (NRCC, 1985); however their application in nature is still fraught with difficulties. One major problem in the use of biomarkers comes from the cause-effect linkages issue (McCarty and Munkittrick, 1996; McCarty et al., 2002). So far, the vast majority of biomarkers used in field monitoring studies indicate current or past exposure to a contaminant, and contribute little to the prediction of the direct consequences for the organism or population in question. In this context, some authors (e.g., Adams et al., 2001) have stressed recently that research on biomarkers should focus on establishing relationships to effects at different levels of the biological organization, notably for ecologically significant endpoints at the reproductive, population and community levels.

For metals, much of the work in the area of biomarkers has focused on metallothioneins (MTs). These low-molecular weight (6-10 kDa), cysteine-rich metal-binding proteins are reported to play a key role in the binding and transport of cadmium (Cd) and other trace metals in aquatic animals (Roesijadi, 1992). Direct measurement of [MT] has been proposed as a simple indicator of exposure to toxic metals in freshwater and marine environments (Couillard, 1997; Langston et al., 1998), and several field studies have demonstrated the usefulness of its application in this context. Another possible use of MT, i.e., as a biomarker of toxic effects, involves the examination of the intracellular distribution of metals among cytosolic ligands, including MT. It has been hypothesized that metal toxicity at the cellular level could arise from reactions in the cytosol, through the non-specific binding of metals to ligands other than MT (i.e., non-thionein ligands) that are physiologically important (Brown and Parsons, 1978; Mason and Jenkins, 1995). Some

attempts have been made to relate sub-cellular metal partitioning and MT synthesis to deleterious effects in aquatic organisms, most frequently in time-course experiments in which animals were subjected to abrupt changes in metal exposure (usually Cd or Cu). In these studies, binding of the metal to ligands outside the MT pool was associated, among other things, with lipid peroxidation (Couillard et al., 1995), growth reduction (Sanders and Jenkins, 1984), reproduction decrease (Jenkins and Mason, 1988), behaviour modification (Wallace et al., 2000) and mortality (Baudrimont et al., 1999); toxicological effects at higher levels of the biological organization were at best suggested (e.g., Couillard et al., 1995).

For more realistic chronic exposure tests (i.e., organisms exposed to elevated metal concentrations in their natural habitat during their entire lifetime), results are often conflicting and observations of effects related to metal accumulation are lacking. In the few field investigations in which biomarker and population-level responses have been examined simultaneously, MT expression has been successfully linked to decreases in density and biomass in fishes (Farag et al., 2003) and shifts in age structure in bivalves (Blaise et al., 2003). Conversely, other reports (e.g., Schlenk et al., 1996) have failed to correlate increases in MT levels with the occurrence of deleterious effects at the population level. In most of these studies, however, metal exposure has not been adequately characterized, and none of them have considered the intracellular distribution of the metal(s). Moreover, site-specific natural factors likely to influence the characteristics of the studied populations were seldom, if ever, evaluated.

The present study is part of a larger project on the evaluation of the use of MT as a biomarker for metal-induced stress in the bivalve *Pyganodon grandis* living in lakes impacted by anthropogenic metal inputs. We have studied a suite of biological endpoints ranging from sub-organismal to population level responses to address this issue; approaches that integrate responses across levels of organization are especially valuable because they help to understand the mechanistic linkages between the biomarkers responses and the ecologically relevant responses. In a first complementary study (Giguère et al., 2003), we measured steady-state Cd and MT concentrations and determined the sub-cellular

partitioning of Cd in excised gills of *P. grandis* in a series of lakes with similar trophic status but differing markedly in the degree of environmental Cd exposure. We showed that Cd was present in non-thionein ligand pools even for low chronic exposure concentrations, suggesting an imperfect detoxification of the metal in this species. Consistent with this observation, the increased accumulation of Cd in the gill cytosol was associated with symptoms of cellular toxicity. We hypothesized that these toxic effects observed at the cellular level could affect higher levels of the biological organization. In the present paper, we therefore investigate the characteristics of *P. grandis* populations (abundance, standing biomass, secondary production, production-biomass ratios, cumulative fecundity) in the same group of lakes. Our aim was to establish statistical correlations between Cd binding to various ligands and the onset of adverse effects in the populations. We also looked for the effects of natural confounding factors (both biotic and abiotic) that may affect the population responses.

3.3 Materials and methods

3.3.1 Study site and sampling design

Our study was conducted in 9 lakes located in the mining region of Rouyn-Noranda, in northwestern Québec (approximately 48°00'N, 79°00'W). The lakes were selected from an original set of 20, sampled during the previous summer (1997), and were chosen so as to have water bodies with similar trophic status but contrasting metal levels (especially Cd) (this thesis, chapter 2). Our intention was to minimize the differences in habitat quality for the bivalves, and in this way facilitate the unequivocal identification of the biological responses of *P. grandis* to metal contamination. *Pyganodon grandis* populations were quantitatively sampled in the entire littoral zone of the nine lakes. A depth of 6 m was chosen as the lower limit of the littoral zone since *P. grandis* is rarely found in deeper waters in these and other Canadian lakes (Hanson et al., 1988a; Huebner et al., 1990). To characterize habitat quality and determine Cd exposure, we also collected water samples and sediments in the littoral zone of the selected lakes, at one to four stations, between 0.5 and 3 m depth. We sampled *P. grandis* individuals at the same littoral stations for the

determinations of cytosolic Cd and metallothionein (MT) concentrations and sub-cellular partitioning of Cd. Bivalves used for sub-organismal measurements in the present study were the same specimens analysed in the study of Giguère et al. (2003).

In most of the studied lakes, the littoral zone was characterized by a gentle slope and heterogeneous substrate type. In shallow waters (<3 m), the dominant macrophyte species were *Potamogeton robbinsii* and *Potamogeton richardsonii* with *Vallisneria americana, Nitella* sp., *Myriophyllum* sp. and *Isoetes* sp. occurring less frequently. We did not notice any signs of predation on our bivalve populations, with the exception of Lake Ollier, where piles of empty shells (middens) were found at many locations on the shore.

3.3.2 Environmental characteristics

3.3.2.1 Cd exposure

To determine the degree of exposure of *P. grandis* to ambient Cd, SCUBA divers collected sediment cores at each littoral station during the summers of 1997 and 1998. The sediment cores were extruded in the boat and the uppermost 0.5 cm, containing the oxidised sediments, was transferred into acid-cleaned high-density polyethylene (HDPE) bottles half filled with lake water. To account for local (within-site) spatial variability, each bottle contained three to four slices of oxidised sediments, each slice coming from a different core. These bottles were kept at ~4°C during transport to the laboratory where they were stored at –20°C until analysis. The sediments were subjected to a partial extraction procedure as described in Couillard et al. (1993). Based on the sediment Cd, organic carbon and iron oxyhydroxide concentrations together with the time-averaged pH values (for the two summer periods) of the overlying water, we estimated free Cd^{2+} concentrations at the sediment-water interface using the geochemical model described in Tessier et al. (1993). This model assumes that the free-metal ion concentration at the water-sediment interface is controlled primarily by competitive sorption of the metal on the various sediment phases present in oxic sediments. This approach has the advantage of integrating the exposure of bivalves to Cd over time; given that the sedimentation rate of the average (undisturbed) lake in the Rouyn-Noranda area is approximately 1.5 $mm \cdot yr^{-1}$ (Kliza and Temler, 2001),

and that the first 0.5 cm of the sediment cores were used for metal analyses, we can reasonably assume that our free Cd ion concentration estimates represent the mean exposure to the contaminant for at least three consecutive years.

3.3.2.2 Limnological variables

To evaluate the habitat quality for *P. grandis*, water samples were collected at each station, four times during the summer of 1997 and two times during the summer of 1998. Samples were taken by SCUBA divers 10 cm above the lake bottom. Measured variables included pH and chlorophyll *a* (Chl *a*), dissolved calcium (Ca), dissolved organic carbon (DOC) and sestonic carbon and nitrogen (Sest C and Sest N) concentrations. Before filtration, water samples for chlorophyll *a* and sestonic C and N analyses were run through a Nitex® sieve to remove the >80 µm fraction; analysis of gut contents of another freshwater bivalve, *Elliptio complanata*, showed that >90 % of the particles were smaller than 80 µm (Tessier et al., 1984). Methods for the analyses of all limnological variables are described in detail in Perceval et al. (2002). Water temperature at the bottom was recorded at each station every two hours between June and September of 1998 using miniature data loggers (Onset Computer Corp.) placed in waterproof containers. Degree-days available for *P. grandis* were calculated by the rectangular method as described by Young and Young (1998). Briefly, one takes the average of the daily maximum and minimum temperatures and subtracts a lower threshold temperature to determine the degree-day accumulation for 24 h. The number of degree-days is then the cumulative total for all days between June and September. In this calculation, we used 10°C as the lower temperature threshold based on our underwater observations that bivalves became active when the water temperature reached roughly 10°C.

3.3.3 Littoral zone fish community

Because unionids require a fish host for their specialized larval stage (i.e., glochidia), their distributions are intimately tied to the distributions of their host-fishes (Watters, 1992; Haag and Warren, 1998; Vaughn and Taylor, 2000). To evaluate the relative importance of fish community composition and fish abundance on *P. grandis* densities and biomass, we

quantitatively sampled littoral zone fishes in the studied lakes during June and July 1999 with a 50×3 m bag seine (9-mm knotless mesh). For each lake, one seine haul was made at each of ten different seining sites between 9h30 and 16h00. Sampling sites were chosen where clams are known to occur. The seine was deployed to enclose an area of 400-600 m^2 varying in depth from 0.5 to 3.0 m. Sampling efficiency was assessed by a diver who followed the seine during net hauls. Fish escaped from the net only when it became snagged on rocks or logs; all such samples were excluded from our analyses. For each seine haul, fish were sorted to species and enumerated. In all the statistical analyses, we retained only fish species that have been reported to be potential hosts for *P. grandis* on the basis of the list given in Watters (1994).

3.3.4 *P. grandis* biological variables

3.3.4.1 Responses at the sub-organismal level

Methods for bivalve collection, gill preparation and determinations of Cd and MT concentrations and sub-cellular partitioning of Cd are described in detail in Giguère et al. (2003). Briefly, a sub-sample of 12 individuals of similar size (shell lengths ranging between 75 and 85 mm) was collected at each littoral zone station in our series of lakes in June 1998. MT concentrations in the gill cytosol were measured by a ^{203}Hg saturation assay described in detail in Couillard et al. (1993). Cd partitioning among the various cytosolic protein pools was determined by size exclusion chromatographic separation (HPLC). The separation technique is fully described in Wang et al. (1999) with the difference that the eluting fractions were collected every 2 min in the present study. The metal burdens measured in each eluting fraction were then combined into three metal-ligand pools: a high molecular weight (HMW) pool (245-18 kDa), a metallothionein-like pool (18-1.8 kDa), and a low molecular weight (LMW) pool (<1.8 kDa). Total Cd concentrations in the cytosol were measured by inductively coupled plasma mass spectrometry (ICP-MS: Fisons model VG PQII) and each fraction from the HPLC separation was analysed for Cd by flameless atomic-absorption spectrophotometry (THGA graphite tube atomizer, Perkin-Elmer model SIMAA 6000).

3.3.4.2 Responses at the population level

DENSITY AND BIOMASS ESTIMATES – *P. grandis* populations were sampled by SCUBA divers, following a stratified random method (Cochran, 1977). A first sampling was done from July to August 1998. From the equation given in Downing and Downing (1992), we determined that the requisite number of samples per lake needed to achieve a level of precision (SE/mean) of ~30 % for density estimates was between 14 and 33. Six to twenty-one transects were then randomly set up in each of the littoral zones of the selected lakes. Each transect line was perpendicular to the shoreline and consisted of buoys indicating the 2-, 4-, and 6-m depths. There were three depth strata for each transect, 0-2, 2-4, and 4-6 m. Within each depth strata, we used an echo sounder to position one 1×1 m quadrat along the transect, at a depth selected at random with a table of random numbers. SCUBA divers removed all the sediments up to a depth of ~30 cm within the quadrats using an airlift sampler to ensure the collection of both endo- and epibenthic animals. The sediments collected were gently washed in the boat on a 5 mm mesh screen and the unionid clams were removed. All *P. grandis* specimens were measured along the axis of maximum growth, using a digital vernier caliper. The screen retained clams > 8-10 mm in length. In each lake, 18 to 51 m^2 of substrate were excavated with this method. A second sampling was done in September 1999. During that campaign, seven to fourteen transects were chosen at random in the littoral zone of the lakes. In each of the 0-2, 2-4, and 4-6 m depth strata, divers swam 10 m segments perpendicular to the transect line and, using a metre stick, collected all the bivalves visible or found by excavating the top 5 cm of substrate within 1 m of each side of the segments. All individuals were placed in dive bags, enumerated and measured in the boat and returned to their habitat. For each 2×10 m segment, two divers also recorded information about the vegetation cover and substrate type to test the influence of these variables on the distribution of clams. Plant cover was noted following a numerical code system (Dushenko et al., 1988) ranging from 0 to 4 where 0 = no vegetation, 1 = scarce (occasional plants), 2 = moderate (beds occurring in regular patches), 3 = dense (extensive beds but well below water surface) and 4 = very dense (extensive beds reaching the water surface and posing difficulties for navigation). Substrate type was divided into 4 categories (silt, sand, gravel to pebble, and cobble to

boulder) and the resulting qualitative variable was turned into a simple ordinal index, for which each unique combination of sediment particle sizes in quadrats is ranked (see Brim Box and Mossa, 1999 for a full description of this index). In 1999, between 420 and 840 m^2 of substrate were inspected in each of the studied lakes. Unbiased estimates of the population mean density and variance were made for each sampling year using the method described in Cochran (1977). When we compared the results of the two sampling campaigns, we found that the two methods of bivalve collection yielded comparable density estimates for the nine lakes: 95% confidence intervals around mean estimates for 1998 and 1999 consistently overlap each other for each lake. We therefore decided to pool the 1998 and 1999 data for the determination of bivalve density, biomass, secondary production and cumulative fecundity.

From 17 to 675 *P. grandis* specimens were collected and measured during the two sampling periods. In Lake Caron, where bivalves were less abundant, an additional collection of about a hundred individuals was made outside the quadrats. For each lake, we randomly selected a subsample of ~50 individuals of various shell lengths to best represent the size range in the different populations for the determination of weight-length relationships. Individuals were taken alive to the laboratory where the debris encrusting the outside of the shell was removed. Live weight was measured to the nearest 0.01 g. Dry weight of the viscera was determined by removing the body from the shell, drying the clam body to a constant weight at 60 °C for 36 h, and weighing it to an accuracy of 0.001 g. Weight-length regressions were done on the $\log_{10}$-transformed data using the ordinary least-squares equation. For all models, coefficients of determination (r^2) were consistently high and ranged from 0.898 to 0.996. These relationships were then used to determine total live and dry viscera weights of each collected clam, on the basis of its length, allowing us to estimate the standing biomass for each population.

BIVALVE AGE ESTIMATION – The inversion of the von Bertalanffy growth equation (see Anthony et al., 2001 for the mathematical development of the equation) was applied to *P. grandis* in the nine lakes to calculate the ages of all bivalves collected. Since the vast majority of clams were returned to their habitat after measurement, we used this equation to

derive age-frequency distributions from length-frequency data. For each lake, measurements for the determination of the growth equation parameters were made on a subsample of ~100 individuals of various sizes collected in 1998 and 1999. Following McCuaig and Green (1983), maximum lengths at successive external growth rings (or annuli) were determined for each specimen using a digital vernier caliper (± 0.01 mm). Only specimens with clearly visible growth annuli were used. Individuals produced between one and nine pairs of consecutive growth rings. A bivariate plot of length at one interval versus length at the next consecutive interval (a Walford Plot) was established for each lake, and simple linear regression analysis of this relationship allowed us to estimate the von Bertalanffy equation parameters L_∞ and K. In the equation, L_∞ (asymptotic length) represents the theoretical maximum length an organism would reach at infinite age, and K (Brody's growth constant) defines the rate at which the organism's size approaches L_∞. The other remaining parameter, L_0 (length at $t = 0$), was determined for each clam population by measuring the size of ~50 mature glochidia under a dissecting microscope. Mean lengths of glochidia ranged from 0.35 to 0.37 mm, and were comparable to published estimates for this species (see Lefevre and Curtis, 1910).

In the present study, growth-ring analyses of shells depend on the assumption that external annuli are formed annually. These rings are assumed to mark growth cessation during the winter months, under low temperatures. The annual nature of external rings has been validated in the past for several unionid species, utilising mark and recapture techniques (e.g., Negus, 1966). However, the assumption that external annuli are produced on an annual basis has been questioned recently: if annuli were in fact produced less frequently, the traditional annulus-based method of estimating bivalve growth would yield erroneously high estimates of the growth constant K and thus, lower age estimates (Anthony et al., 2001). In a separate field experiment conducted in the study area, in which we followed the growth of ~150 *P. grandis* specimens of various sizes over 400 days, we found that the parameters of the von Bertalanffy growth equation calculated from direct measurement of changes in size of the transplanted bivalves over that period were comparable to those obtained by the examination of external shell annuli in indigenous organisms. A test of common slope conducted on the Walford plot regressions revealed that

the two lines did not differ significantly ($F_{1,212}$ = 0.076, P = 0.783), yielding comparable estimates for K (0.159 and 0.173 yr^{-1} respectively). Furthermore, the technique used in the present study requires that the rings be produced at regular intervals and, above all, that growth cycles be consistent across lakes; given the marked seasonal changes that occur within the study area and the fact that all study lakes routinely freeze in winter, this is likely the case.

SECONDARY PRODUCTION – Total live and dry viscera production of *P. grandis* were estimated for each lake by a method similar to that of Hanson et al. (1988a). First, the number of clams of age n was calculated by multiplying the mean density of clams in the population by the proportion of clams of age n. Mean weight for each age-class was estimated from the weight at age n for each clam in the population (calculated from the appropriate weight-length relationships). The mean weight increment for clams of age n was then determined as mean weight of clams in the population at age n minus mean weight at age n–1. This method estimates the biomass produced over the past year by the present population and is an underestimate because mortality is not accounted for (Hanson et al., 1988a).

FECUNDITY ESTIMATES – Reproductive output was estimated on the basis of the number of glochidia present in the outer gills of each gravid female. In September 1999, eighty to ninety individuals of various sizes were collected in the littoral zone of each lake. Immediately after collection, each clam was sealed in an individual plastic bag (Whirl-pak™) to avoid loss of glochidia by spontaneous abortion (Lefevre and Curtis, 1910). Bivalves were then transported to the laboratory and kept at –20°C until processing. The sex of each individual was determined by two criteria: gravid females were identified by the presence of eggs or glochidia within the outer demibranchs and non-gravid females possessed swollen outer demibranch walls. The rest of the specimens were assumed to be males. Age at first reproduction (i.e., age when females had glochidia in their gills for the first time) was also recorded. To count glochidia, partially thawed gills were separated from the rest of the body with a scalpel, cut into small (0.5×0.5 cm) pieces and placed in a 1-L

glass container. The 100 µm filtered contents of the sampling bags were added to collect any glochidia that might have been released during transport to the laboratory and the glass container was filled to 200 mL with tap water. This solution was agitated vigorously with a blunt probe for 10 min to break gill structures and release the larvae. The volume of water and glochidia was brought up to 500 mL (or 1 L for large samples) and mixed thoroughly, and five 2-mL subsamples were collected with a Hensen-Stempel pipette and placed in a zooplancton counting chamber where they were counted. The total number of glochidia per clam was determined by multiplying the mean number of glochidia per mL by the appropriate dilution factor. Size-specific fecundity was determined by linear regression (ordinary least-squares equation) of glochidia number ($\log_{10}$-transformed) *versus* total shell length ($\log_{10}$-transformed). For the different models, shell length explained between 50 and 75% of the variation in the number of fertilized eggs found in gravid females. These relationships were then used to estimate the number of glochidia produced by the various age-classes of clams in the selected lakes. Cumulative fecundity, expressed on a areal basis, is the sum of all glochidia produced by the gravid females in the different age-classes during their reproductive life span. In this calculation, we accounted for the variation in the percentage of females carrying glochidia across the various age classes in each clam population.

3.3.5 Statistical procedures

We used one-tailed Pearson correlation analysis to test the bivariate relationships between each of the *P. grandis* population variables and *i*) the ambient free-cadmium ion concentration, *ii*) the total gill cytosolic Cd concentration, *iii*) the gill cytosolic MT concentration, *iv*) the Cd concentration in the high molecular weight (HMW) pool, *v*) the Cd concentration in the metallothionein-like pool and *vi*) the Cd concentration in the low molecular weight (LMW) pool. Each time simple correlation coefficients between population variables and potential confounding factors (i.e., limnological and fish community variables) were found to be statistically significant, these relationships were re-tested after controlling for the effects of confounding factors using partial correlation analysis (Legendre and Legendre, 1998). In each correlation analysis, we used lake means

for limnological, geochemical (ambient metal levels) and sub-organismal data for lakes with more than one sampling station. With the exception of density estimates, for which we used the square-root transformation, all data were $\log_{10}$-transformed to linearize the relationships between variables. The significance levels of simple and partial correlation coefficients were tested using the permutation methods described in Legendre and Legendre (1998). Permutation testing is recommended for very small samples, as is the case here (n = 9 for each correlation). The Bonferroni correction method for multiple testing was used when several tests of significance were carried out simultaneously.

3.4 Results

3.4.1 Lake characteristics

The estimated free Cd^{2+} concentration in the most contaminated lake (L. Héva) was 20-fold higher than that in the least contaminated habitat (Table 3-I). Lake water pH also exhibited appreciable variation among the nine lakes. The range in Ca concentration was more than 5-fold and that in DOC concentration only 2-fold. Variables indicative of the quantity and quality of food available for *P. grandis* (i.e., Chl *a*, and sestonic carbon and nitrogen concentrations) also varied from lake to lake, but the maximum:minimum ratios were markedly lower than that found for Cd exposure (Table 3-I). In large and deep lakes, water temperature was generally lower than that in small and shallow ones: the cumulative degree-days in the littoral zone were negatively related both to lake area (r = –0.64; P two-tailed = 0.065) and to maximum depth (r = –0.76; P two-tailed = 0.018).

A total of twenty different littoral zone fish species were found in our lakes; of these 20 species, seven have been identified as hosts for *P. grandis* (i.e., yellow perch (*Perca flavescens*), golden shiner (*Notemigonus crysoleucas*), rock bass (*Ambloplites rupestris*), pumpkinseed sunfish (*Lepomis gibbosus*), lake whitefish *(Coregonus clupeaformis*), common shiner (*Notropis cornutus*) and white sucker (*Catostomus commersoni*)). The number of fish species was maximum in lakes Caron, Dufay, Ollier and Opasatica and

Table 3-I. Morphometric, geochemical, limnological and ecological characteristics of the studied lakes.

Lake (number of sampling sites)	Lake area (km^2)	Littoral zone area (% lake area)	Littoral slope (%)	Maximum depth (m)	Cd^{2+} (nM)	pH	Ca ($mg\ L^{-1}$)	Chl *a* ($\mu g\ L^{-1}$)	Sest C ($mg\ C\ L^{-1}$)	Sest N ($mg\ N\ L^{-1}$)
Bousquet (*n*=3)	2.32	50	11.1	22	0.61±0.11	6.50	4.3±0.2	1.5±0.2	0.67±0.19	0.169±0.088
Caron (*n*=1)	12.25	15	34.2	75	0.30	6.97	11.6	1.3	0.32	0.081
Dufay (*n*=3)	4.36	67	5.8	12	0.28±0.03	6.81	3.0±0.1	2.2±0.3	0.55±0.04	0.061±0.006
Évain (*n*=2)	2.05	57	4.9	8	0.05±0.01	7.48	6.8±0.0	2.1±0.5	0.39±0.08	0.056±0.011
Héva (*n*=1)	2.37	92	1.6	7	0.81	6.15	2.1	2.2	0.74	0.092
Joannès (*n*=3)	4.45	45	4.6	21	0.23±0.02	7.24	6.9±0.1	2.0±0.0	0.44±0.02	0.050±0.006
Ollier (*n*=2)	0.79	82	2.8	11	0.05±0.01	7.52	12.4±0.1	1.7±0.4	0.51±0.08	0.063±0.012
Opasatica (*n*=4)	9.75	61	2.5	14	0.04±0.01	7.60	8.4±0.0	1.4±0.1	0.38±0.04	0.053±0.008
Vaudray (*n*=1)	7.37	43	5.7	30	0.66	6.65	3.3	1.1	0.34	0.033
Max:Min	15.5	6.1	21.4	10.7	20.8	28.2	5.9	2.0	2.3	5.1

Note: The slope of the bottom in the littoral zone was determined from bathymetric maps and is the mean of individual slopes (n = 41-671, according to lake area) calculated from the difference in elevation between the shoreline and the 6-m isobath (i.e., 6 m) divided by the shortest distance separating them. For $[Cd^{2+}]$ estimates, surficial oxic sediments were collected on two occasions (June 1997 and June 1998) and water samples on six occasions (June, July, August and September 1997, June and August 1998). Values for all limnological data (with the exception of degree-days) are means based on six samples taken in June, July, August and September 1997, June and August 1998. For lakes with more than one sampling station, means are followed by ± 1 SE, indicating within-lake variability. We used temperature data from three littoral stations (not one) to calculate degree-days available for *P. grandis* in lakes Caron and Vaudray (values are followed by ±1 SD, for lakes with more than one littoral station). Values for host-fish abundance are means (± SD) based on 10 seine hauls.

Table 3-I. (concluded).

Lake	DOC (mg L^{-1})	Degree-days	No. fish species as potential hosts for *P. grandis*	Host-fishes abundance (CPUE)
Bousquet	16.2±0.3	715±61	3	19±18
Caron	10.4	669±55	4	6±4
Dufay	9.6±0.0	752±42	4	32±41
Évain	7.7±0.0	847±21	3	70±141
Héva	9.9	755	2	60±49
Joannès	11.1±0.2	758±6	2	10±13
Ollier	7.8±0.1	817±58	4	53±42
Opasatica	7.9±0.2	771±17	4	22±20
Vaudray	9.0	706±20	1	69±94
Max:Min	2.1	1.3		11.4

minimum in lake Vaudray (Table 3-I). Yellow perch was the dominant fish species in almost all lakes, representing 30 to 90% of the captured fish.

3.4.2 Bivalve abundance and size-frequency distributions

We used multiple linear regression (MLR) analysis to evaluate the relative influence of substrate type and vegetation cover on the number of clams found in the quadrats (Table 3-II). Inter-lake variation in bivalve abundance due to differences in Cd exposure was also taken into account by forcing the ambient Cd^{2+} concentration at the sediment-water interface into the MLR model. Overall, our model explained 35% of the variability in the number of *P. grandis* individuals collected by divers (Table 3-II). The free Cd ion concentration was negatively correlated with bivalve abundance and accounted for a major proportion (~20%) of the explained variation. Water depth (z and z^2 terms combined) explained a further 9% of the remaining variation. For this variable, coefficients of the two monomials were negative, indicating a curvilinear decrease in bivalve abundance with increasing depth. The relative influence of both substrate type and vegetation cover on clam distribution was weak. Particle size category of the sediments accounted for only 5% of the variation in the number of clams; the positive sign of the coefficient suggests that *P.*

Table 3-II. Results of forward selection (p-to-enter value ≤ 0.05) in multiple linear regression with the abundance of *P. grandis* as the dependent variable and ambient free-cadmium ion concentration (Cd^{2+}), depth of the water column (z), particle size category of sediments (PSC) and vegetation cover as explanatory variables. Only variables with p-values < 0.05 are shown. The depth variable (z) was centred on its mean to alleviate the problem of multicollinearity. The ± symbols denotes the standard errors of the regression parameters. n is the total number of 2×10 m segments searched by SCUBA-equipped divers for all lakes studied.

$Log_{10}(abundance+1) =$	$0.377 \pm 0.056 - 0.553 \pm 0.059 \log_{10}(Cd^{2+}) - 0.060 \pm 0.012 z^2 + 0.290 \pm 0.081 \log_{10}(PSC) - 0.050 \pm 0.018 z$			
$p(t)$	< 0.0001	< 0.0001	< 0.001	< 0.01
$r^2_{partial}$	0.203	0.069	0.053	0.019
$R^2 = 0.344$	$SE_{est} = 0.449$	$F = 35.27$	$P < 0.0001$	$n = 274$

Note: In our model, we used lake means reported in Table 1 for Cd^{2+} concentrations.

grandis was more abundant in coarse sediments. On the other hand, the addition of vegetation cover as an explanatory variable in our MLR model did not increase significantly its predictive power (partial $r^2 = 0.003$; $p(t) = 0.247$).

The size-frequency distributions of *P. grandis* populations differed between lakes (*G*-test, $\chi^2 = 750.7$, df = 48, $P < 0.0001$) (Fig. 3.1). Mean shell lengths ranged between 59.7 and 82.6 mm. In most lakes, the majority of clams were between 60 to 80 mm long. There was a high frequency of clams greater than 90 mm long in Lakes Évain, Joannès and Caron. In all lakes, the proportion of individuals less than 40 mm long was consistently low (representing less than 10% of all collected clams in each population) and was negatively correlated with ambient free Cd^{2+} concentration and positively related to water pH (Spearman rank correlations: $r_s = -0.66$; $P = 0.052$ and $r_s = 0.74$; $P = 0.024$, respectively).

3.4.3 Population-based metrics and their relationships with sub-organismal responses

The theoretical maximum shell lengths (L_∞) differed considerably among the populations (Table 3-III) and were significantly positively correlated with the observed maximum lengths ($r = 0.60$; P one-tailed = 0.038). High values for the estimates of the Brody's growth constant (K) in Lakes Héva, Dufay, Évain, Opasatica and Caron indicate that bivalves approach their theoretical maximum lengths at fast rates in these lakes. There were no significant relationships between each of the two long-term growth parameters and the ambient free-cadmium ion concentration, and only a weak negative correlation between L_∞ and the concentration of Cd in the gill cytosolic MT pool of individuals (Table 3-IV). For this latter relationship, correlation coefficient was not significant after Bonferroni correction. Nevertheless, this method of correction is generally considered to be overly conservative (Legendre and Legendre, 1998) and often leads to rejecting too few individual null hypotheses (H_0) in the set of all independent tests.

Pyganodon grandis mean densities ranged between 0.04 and 1.56 individuals m^{-2} in our lakes (Table 3-III). Littoral densities decreased with increasing concentrations of both Cd^{2+} in the environment and Cd in the HMW pool of individual gill cytosols (Table 3-IV).

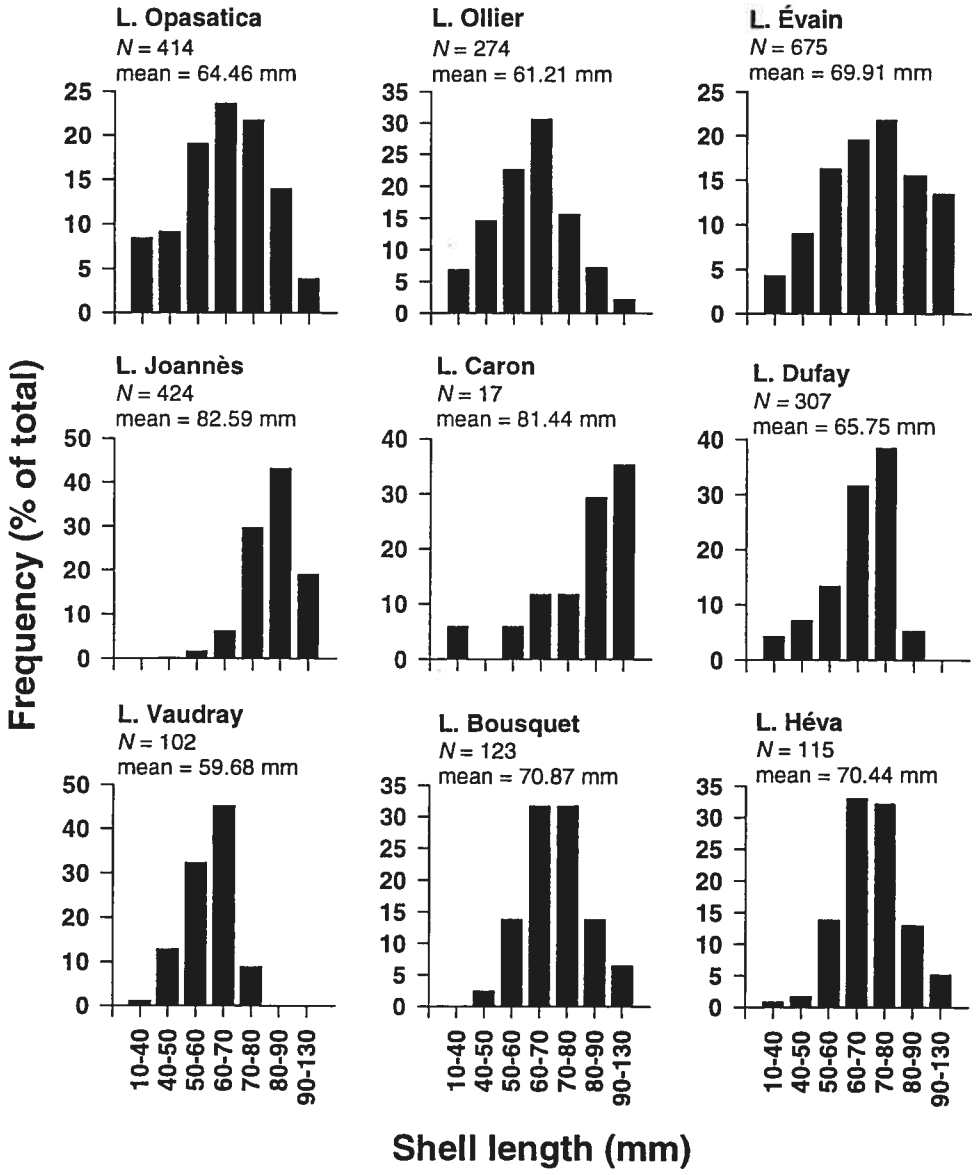


Figure 3.1. Shell length-frequency distributions of *P. grandis* in the studied lakes. The number of individuals collected and mean shell length are given for each lake. Lakes Opasatica, Ollier and Évain are reference lakes (ambient $[Cd^{2+}]$ ranging between 0.04 and 0.05 nM), lakes Joannès, Caron and Dufay exhibit intermediate levels of Cd contamination (ambient $[Cd^{2+}]$ ranging between 0.23 and 0.30 nM) and lakes Vaudray, Bousquet and Héva are the most contaminated lakes (ambient $[Cd^{2+}]$ ranging between 0.60 and 0.81 nM).

Table 3-III. *Pyganodon grandis* population data for the studied lakes.

Lake	L_∞ (mm)	K (yr^{-1})	Density (no. ind m^{-2})	Total live biomass (g m^{-2})	Total live production (g m^{-2} yr^{-1})	P/B (yr^{-1})	Dry viscera biomass (g m^{-2})	Dry viscera production (g m^{-2} yr^{-1})	Cumulative fecundity (no. glochidia m^{-2})
Bousquet	126.6	0.10	0.27 (0.13; 0.41)	9.5 (3.8; 15.2)	1.8	0.20	0.25 (0.10; 0.41)	0.05	1900
Caron	116.6	0.22	0.04 (–0.02; 0.09)	1.6 (–0.5; 3.7)	0.4	0.23	0.03 (–0.01; 0.08)	0.01	1300
Dufay	88.9	0.24	0.54 (0.32; 0.76)	13.2 (8.1; 18.2)	3.2	0.21	0.27 (0.17; 0.37)	0.06	6200
Évain	111.9	0.23	1.56 (1.14; 1.98)	49.8 (33.4; 66.1)	14.4	0.27	1.07 (0.73; 1.42)	0.32	17800
Héva	102.4	0.25	0.15 (0.07; 0.23)	3.4 (1.3; 5.5)	1.3	0.28	0.07 (0.03; 0.11)	0.03	1600
Joannès	111.0	0.15	0.77 (0.31; 1.22)	35.3 (15.5; 55.0)	5.2	0.14	0.73 (0.32; 1.14)	0.09	15300
Ollier	133.6	0.11	0.59 (0.41; 0.78)	10.9 (5.9; 15.8)	4.3	0.31	0.21 (0.12; 0.31)	0.08	6000
Opasatica	110.3	0.22	1.12 (0.66; 1.58)	33.2 (20.7; 45.7)	11.4	0.33	0.56 (0.35; 0.78)	0.19	9200
Vaudray	92.6	0.16	0.18 (0.10; 0.26)	2.7 (1.6; 3.8)	0.7	0.22	0.05 (0.03; 0.06)	0.01	950

Note: For density and biomass estimates, 95% confidence limits of the mean are indicated in parentheses.

Table 3-IV. Pearson correlation coefficients between *P. grandis* population variables and ambient free Cd ion concentration, gill cytosolic Cd and MT concentrations and concentrations of Cd in three metal-ligand pools for the studied lakes ($n = 9$). Sub-organismal biological data used in our correlation analyses are taken from Giguère et al. (2003). All variables were $\log_{10}$-transformed with the exception of density estimates for which we used the square root transformation. Significance level of correlation coefficients was tested by permutation (9999 random permutations). * P (one-tailed) < 0.05; ** P (one-tailed) < 0.05/6 with Bonferroni correction.

	Cd^{2+}	Cd cytosol	MT	Cd-HMW	Cd-MT	Cd-LMW
L_∞	–0.39	–0.55	–0.48	–0.10	–0.57*	–0.54
K	–0.05	0.19	0.28	–0.27	0.26	0.20
Density	–0.78*	–0.36	–0.36	–0.80**	–0.28	–0.18
Total live biomass	–0.67*	–0.25	–0.21	–0.66*	–0.17	–0.14
Total live production	–0.78**	–0.37	–0.35	–0.79**	–0.28	–0.21
P/B	–0.52	–0.57*	–0.59*	–0.58*	–0.53	–0.40
Dry viscera biomass	–0.63*	–0.22	–0.17	–0.60*	–0.14	–0.11
Dry viscera production	–0.76*	–0.36	–0.34	–0.75*	–0.27	–0.18
Cumulative fecundity	–0.74*	–0.34	–0.30	–0.72*	–0.27	–0.23

Ranges in biomass and production estimates (for both total live and dry viscera weights) were more than 30-fold and that for cumulative fecundity 20-fold (Table 3-III). As was the case for density, biomass, production and cumulative fecundity estimates were negatively correlated both with the free-cadmium ion concentration in the water and the Cd concentration in the HMW fraction (Table 3-IV). Even though they were unrelated to environmental free-cadmium ion concentrations, turnover ratios (P/B) of *P. grandis* populations decreased with increasing concentrations of Cd bound to the HMW fraction (Table 3-IV). The P/B ratio was also negatively correlated with the total concentrations of Cd and MT in the gill cytosol of individuals (Table 3-IV).

3.4.4 Influence of natural confounding factors

Multiple linear regression models presented in Table 3-V showed that Ca (+) and sestonic C (+) or N (+) concentrations explained a high proportion (~80%) of the variation in the asymptotic shell length in the bivalve populations. On the other hand, Brody's growth constant (K) was not related to any of these variables or to other limnological factors. Although *P. grandis* population variables were not significantly related to the majority of limnological factors, density, biomass, production and fecundity estimates were all positively correlated with both lake water pH and the number of degree-days available in the littoral zone of the studied lakes (Table 3-VI). On the other hand, P/B ratios for *P. grandis* were best correlated with DOC concentration (Table 3-VI). At the same time, both degree-days and DOC were significantly related to free Cd^{2+} concentration ($r = -0.71$ and $r = 0.68$, respectively, P two-tailed < 0.05 for both relationships). After controlling for the effects of these two variables using partial correlation analyses, we found that the relationships between population-level responses and both ambient Cd^{2+} concentration and sub-organismal responses (mostly Cd concentration in the HMW fraction) were no longer significant (Fig. 3.2). We did not consider the influence of lake water pH in our partial correlations, since the value for cumulative degree-days was the factor that was systematically most strongly correlated with all population variables (with the notable exception of P/B ratio) (Table 3-VI). Furthermore, the role of pH on clam populations

Table 3-V. Multiple linear regression models relating the theoretical shell length of the average individual growth curve in *P. grandis* populations (L_∞) to selected environmental variables. The ± symbols denotes the standard errors of the regression parameters.

(*a*) $\log_{10}(L_\infty)$ =	1.974±0.033	+ 0.227±0.045·$\log_{10}$(Ca)	+ 0.308±0.095·$\log_{10}$(Sest C)
	p_{perm}	0.0021	0.0182
R^2 = 0.810	F = 12.79	P = 0.0068	n = 9 lakes
(*b*) $\log_{10}(L_\infty)$ =	2.117±0.067	+ 0.160±0.039·$\log_{10}$(Ca)	+ 0.166±0.053·$\log_{10}$(Sest N)
	p_{perm}	0.0058	0.0229
R^2 = 0.803	F = 12.25	P = 0.0071	n = 9 lakes

Note: The statistical significance of the equation parameters was obtained by permutation tests (9999 random permutations of raw data).

cannot be dissociated (mathematically or geochemically) from that of Cd since mean summer H^+ concentrations in the overlying water were used for the calculation of the concentrations of the free-metal ion. Finally, neither the number of littoral zone fish species nor their abundance significantly influenced the density or the standing biomass of *P. grandis* in our lakes (Table 3-VI).

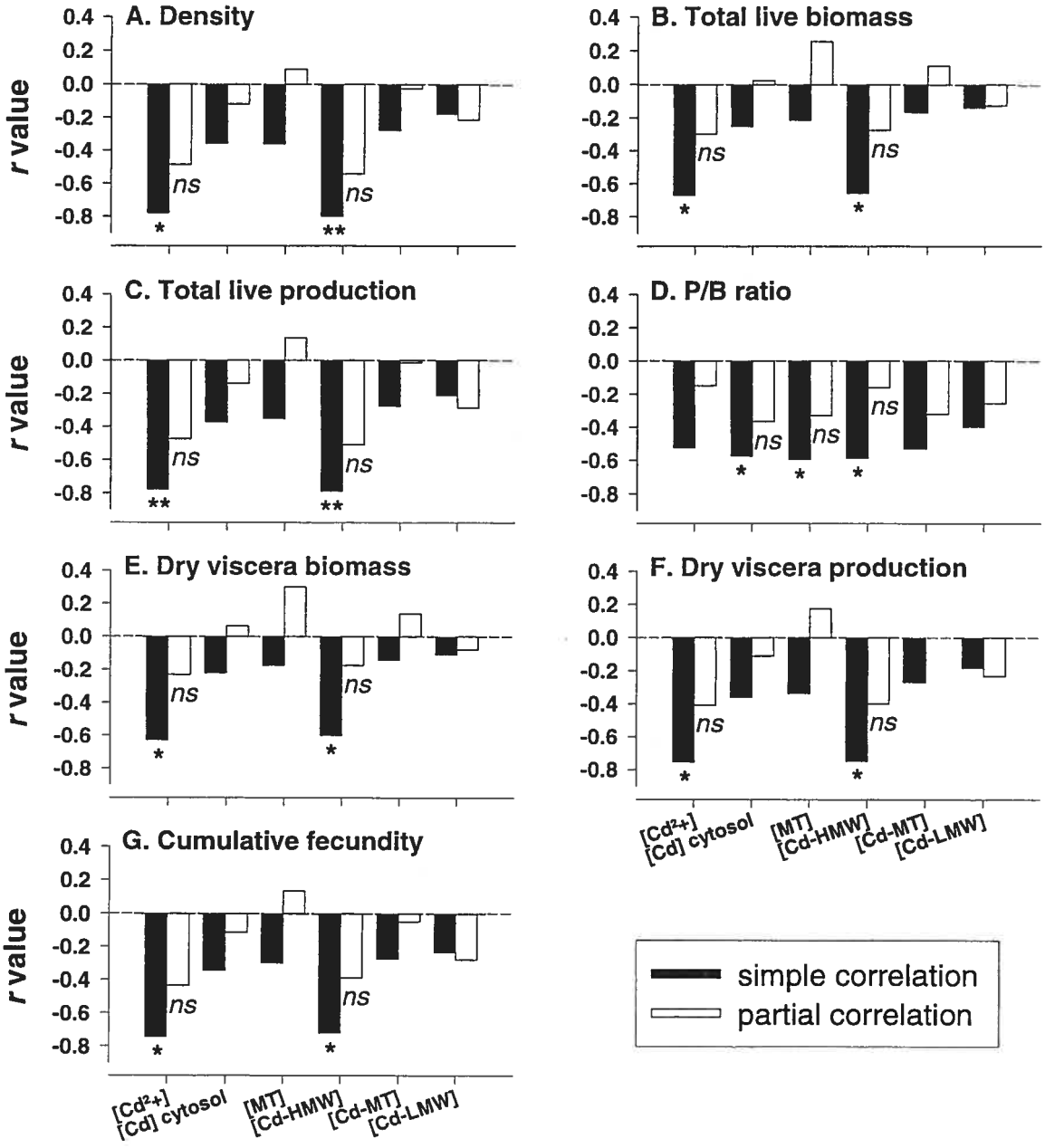
3.5 Discussion

Although great variability exists in the literature, our estimates of population density, biomass and secondary production are generally comparable to those reported for *Pyganodon grandis* (e.g., Huebner et al., 1990) and other unionid bivalves (e.g., Strayer et al., 1981) in North American lakes. Also consistent with our results, Vaughn and Hakenkamp (2001) mentioned turnover ratios for unionids ranging from 0.13 to 0.20 yr^{-1} in their recent review of the functional role of bivalves in freshwater ecosystems. For long-term growth parameters, Morris and Corkum (1999) found theoretical maximum lengths similar to our own estimates (L_∞ between 85 and 135 mm) for *P. grandis* populations from different lotic habitats in Ontario. However, the instantaneous growth rates (K) calculated for these populations were somewhat higher than those observed in the present study, ranging from 0.28 to 0.46 yr^{-1}. On the contrary, *P. grandis* specimens from a large

Table 3-VI. Pearson correlation coefficients between *P. grandis* population variables and potential (natural) confounding factors for the studied lakes ($n = 9$). All variables were $\log_{10}$-transformed with the exception of density estimates for which we used the square root transformation. Significance level of correlation coefficients was tested by permutation (9999 random permutations). * P (one-tailed) < 0.05; ** P (one-tailed) < 0.05/9 with Bonferroni correction.

	pH	Ca	Chl *a*	Sest C	Sest N	DOC	Degree-days	No. fish species	Fish abundance
Density	0.73*	0.27	0.40	–0.13	–0.33	–0.48	0.84**	0.26	0.27
Total live biomass	0.66*	0.23	0.46	0.00	–0.17	–0.24	0.75*	0.28	0.08
Total live production	0.70*	0.25	0.47	0.02	–0.20	–0.43	0.86**	0.34	0.24
P/B	0.27	0.20	–0.10	–0.03	–0.04	–0.65*	0.40	0.36	0.50
Dry viscera biomass	0.61*	0.22	0.50	0.06	–0.08	–0.15	0.73*	0.31	0.05
Dry viscera production	0.66*	0.24	0.50	0.08	–0.09	–0.36	0.86**	0.39	0.24
Cumulative fecundity	0.75*	0.36	0.55	–0.09	–0.27	–0.39	0.78*	0.37	0.01

Figure 3.2. Values of partial correlation coefficients (open bars) measuring the intensity of the (linear) relationships between each of the *P. grandis* population variables and the ambient free-cadmium ion concentrations, the gill cytosolic Cd and MT concentrations and the concentrations of Cd in three metal-ligand pools (Cd-HMW, Cd-MT and Cd-LMW) while controlling for the effects of natural confounding factors (i.e., number of degree-days in the littoral zone for A, B, C, E, F, G and DOC concentration for D). Values of simple correlation coefficients (full bars) are also reported for comparison. All variables were $\log_{10}$-transformed (except for density estimates for which we used the square root transformation). Statistical significance of partial correlation coefficients was assessed by permutation tests (9999 random permutations of residuals of the full regression model). ns: not significant; * P (one-tailed) < 0.05; ** P (one-tailed) < 0.05/6.



oligotrophic lake in Minnesota grew more slowly, with K estimates three to eight times lower than ours (Anthony et al., 2001). Finally, glochidia production by our clam populations generally fell in the lower range of other published estimates for unionids in Canada (e.g., Jansen and Hanson, 1991).

3.5.1 Population-level responses to Cd exposure

Responses of freshwater bivalve populations to metals, other than the acquisition of tolerance, have essentially not been studied in the field. Therefore comparisons of our results with the literature are necessarily limited. Our findings generally indicate a decline in *P. grandis* population health with increasing chronic exposure to Cd, although we acknowledge that causal relationships cannot be unequivocally deduced from correlation. Given our relative small sample (n = 9 lakes), the effect of Cd on clam populations must be rather strong to be observed. Without doubt, the most relevant result is the sensitivity of our secondary production estimates to Cd contamination (especially if we consider the fact that the correlation between secondary production and ambient Cd^{2+} is still significant after the Bonferroni correction was applied). Secondary production is indeed a functional measure of energy flow through a population and it provides insights not only into individual- and population-level processes but also for ecosystem-level ones. Thus, it is generally considered a powerful endpoint for assessing ecosystem degradation in response to human activities (e.g., Carlisle and Clements, 2003).

Because secondary production is essentially the product of individual growth and population density, it may be influenced by any factor that affects these processes. Our data suggest that Cd-induced reductions in population density and biomass appear to be the primary factors regulating variation in secondary production among the lakes since growth parameters seem independent of the level of metal contamination (Table 3-IV). However, as several authors have already done (e.g., Bernard, 1981), we question the validity of using L_∞ and K simultaneously to describe growth rates in animal populations: these parameters describe the endpoint of the growth curves and not necessarily the growth during the life of the organism.

Another key result is the decrease of cumulative fecundity with metal exposure. Because reproductive output is one of the life-history traits that contributes the most to the variations in population growth for freshwater invertebrates coping with metal contamination (Jensen et al., 2001), a reduction in glochidia production by populations in lakes at the high end of our exposure gradient will have tremendous impact on their

population dynamics. At the extreme, a failure to recruit young animals into these populations would cause extinction over several generations. In this context, it is noteworthy that in populations from the most severely impacted lakes (i.e., Vaudray, Bousquet and Héva) we had some difficulty finding small (total shell length < 40 mm) and hence young clams (Fig. 3.1). Several authors (e.g., Hornberger et al., 2000; Brown et al., 2003) have reported reproductive failure for indigenous clams living in moderately metal-contaminated environments; in these studies the proportion of reproductive animals was negatively associated with increasing tissue metal concentrations (predominantly Ag and Cu), a surrogate for metal exposure. Because mean individual fecundity (i.e., total number of glochidia in the two marsupial gills of a reproductive female of a standard size) is independent of Cd contamination in our group of lakes (r = 0.26; P > 0.05, results not shown), we suspect that low glochidia production by populations in lakes with elevated environmental free Cd^{2+} concentrations is simply a reflection of the fact that bivalves are less abundant in these lakes. As shown by Downing et al. (1993) in their field study of the reproductive ecology of the freshwater bivalve *Elliptio complanata*, fertilization success (i.e., the fraction of fertilized ova within a given population) is indeed strongly correlated with spatial aggregation of individuals, suggesting that perturbations altering the density or size distribution of bivalve populations may have serious consequences for the maintenance of viable populations. Although cumulative fecundity decreases with increasing environmental Cd, the viability of glochidia is not influenced by maternal Cd exposure. From a laboratory experiment conducted in parallel, we determined that the viability of glochidia originating from gravid females sampled in our group of lakes was consistently high for all populations, with mean values ranging from 86 to 96 % (Perceval et al., unpublished results).

In the statistical relationships described above, we compared variables representative of the habitat quality for at least two consecutive summers with population-level responses of a long-lived species (> 15 years). Our conclusions thus rely on the assumption that the relative ranking of the lakes along the environmental gradient does not vary much through time. This is likely the case for the metal contamination gradient. For six of the nine lakes that were sampled in the present study (Lakes Bousquet, Dufay, Héva, Joannès, Opasatica and Vaudray), we were able to compare current free Cd^{2+} concentration

estimates with estimates from 1989 (Couillard et al., 1993); we found good agreement between the two variables (Spearman correlation on rank-ordered data $r_s = 0.83$; $P < 0.05$).

3.5.2 Sub-cellular metal partitioning as a potential monitoring tool

From a statistical standpoint, our results suggest that steady-state Cd concentration in the HMW fraction of *P. grandis* gill cytosol is quite valuable as a predictor for the variations in population density, biomass, production, turnover ratio and reproductive output (i.e., all population-level responses but long-term growth parameters). In response to the hypothesis that metal toxicity at the cellular level could arise through the non-specific binding of metals in the cytosol to non-thionein ligands that are physiologically important, it seems that examination of sub-cellular metal distribution in organisms could be used as an indicator of population impairment. However, it is noteworthy that not much predictive power is gained by using [Cd-HMW] instead of the ambient metal concentration. Although statistical correlation does not prove causality and is not necessarily reliable as an indication of mechanistic links, nevertheless this result can be considered a preliminary step in the validation of the biomarker (see a discussion on this subject in McCarthy and Munkittrick, 1996).

In our study, the HMW pool is operationally defined as the fraction of metal ligands between 245 and 18 kDa, which includes metalloenzymes. In their comprehensive review, Mason and Jenkins (1995) identified metalloenzymes as potential targets for Cd when this metal is accumulated in excess in the cell. Our results depart somewhat from this "spillover" model in that Cd was found in the HMW fraction even for low environmental exposures (Giguère et al., 2003). Given that toxic effects at the cellular level likely result from a change in metalloenzyme structure when essential metals such as Cu and Zn are displaced by nonessential metals (e.g., Brown and Parsons, 1978 and literature cited therein), and that these enzymes are involved in the majority of important cellular processes (e.g., respiration, protein synthesis and degradation, transcription), our observation of apparent deleterious effects of Cd at the cellular and higher level of biological organization are not surprising. Indeed, according to the continuum concept for metal toxicity described in Luoma (1995), the initial reaction of Cd with HMW ligands in the cell will be followed

by responses at the physiological, whole organism and population levels of the biological organization, unless compensatory/detoxification mechanisms at each of these levels are sufficient to attenuate the propagation of the original Cd "signal".

In our earlier study (Giguère et al., 2003), we reported that malondialdehyde (MDA), an indicator of oxidative stress, was best related to cytosolic Cd bound to the LMW pool, whereas in the present analysis Cd-HMW proved to be the best predictor for population impairment. For comparison, we computed the correlation coefficients between MDA levels in *P. grandis* and each of the population-level variables for the 9 study lakes, and found that there were no significant relationships between the two sets of variables (Pearson's r ranging from –0.004 to –0.56; $P > 0.05$ for all relationships, results not shown). Mention should be made, however, that contrary to our first study, we used here lake averages for all sub-organismal measurements (including MDA levels) for the statistical comparisons. In addition to the fact that all the sub-cellular Cd fractions are correlated among themselves, and that our statistical population is small, we cannot rule out the possibility that the apparent difference between the two studies is a statistical artefact. Since most population-level responses are driven by the environmental Cd^{2+} concentrations (Table 3-IV), and considering that all cytosolic metal complexes exhibit significant correlations with this variable (e.g., $r = 0.95$, $P < 0.05$ for Cd-HMW), one might well anticipate correlations between population level responses and Cd-HMW in the present study.

3.5.3 Ecological factors as structuring agents for bivalve populations

In the present study, we identified several natural factors that influenced the responses of *P. grandis* populations at different scales (Tables 3-II, 3-V and 3-VI), adding complexity to the interpretation of our results. The distribution of clams was significantly affected by substrate type at a local (i.e., quadrat) scale. Although the effect of substratum was of minor importance compared with that of Cd or water depth (and thus probably accounts for negligible background variability in our population density, biomass and total live, dry viscera and glochidia production estimates), *P. grandis* was nevertheless more abundant in coarse sediments. This result confirms several field observations that have suggested that

freshwater bivalves prefer sand and gravel over muddy habitats (Cvancara, 1972; Huehner, 1987) but contrasts markedly with that of Downing et al. (2000) who demonstrated that *P. grandis* specimens preferably move toward muddy sediment patches under controlled conditions. These authors speculate that by these movements, individuals might inhabit areas that are richer in organic matter or more easily penetrated when they become threatened by a predator. In agreement with this result, Michaelson and Neves (1995) observed in a habitat-suitability experiment conducted in the laboratory that the dwarf wedgemussel (*Alasmidonta heterodon*) consistently chose fine sediments over coarse ones. The discrepancies among experimental and field results indicate that the abundance and persistence of clams in the field must be regulated by factors other than sediment composition (as shown by the low predictive power of our MLR model in Table 3-II).

The correlations between lake water pH and population density, biomass, production and cumulative fecundity are likely spurious, for at least two reasons. Firstly, Økland and Økland (1986) reported a lower threshold pH value for clam distribution of 4.7 in freshwater ecosystems which is much lower than the pHs measured in our series of lakes. Secondly, although there is a significant variation in *P. grandis* shell weights among the lakes (ANCOVA: $F_{8,388} = 20.267$; $P < 0.001$), we found no evidence of shell thinning with the decrease in water pH ($P > 0.05$ for the correlation between the adjusted means of shell weight for a standard size and pH). Shell thinning is generally considered one of the first physiological indices of acid stress in bivalves (Pynnönen, 1990), and is attributed to the mobilization of the $CaCO_3$ reserves from the shell.

Not surprisingly, most *P. grandis* population-level responses were significantly correlated with the thermal regime that prevailed in the littoral zone of the lakes. Although correlation coefficients are relatively high and often statistically significant even after drastic correction for multiple testing, it could be argued (as was the case for ambient free Cd^{2+}) that we are comparing here a variable that was measured during a single season with long-term population responses. Nevertheless, given that all lakes are situated in the same geographical area and that water temperature is also related to the morphological characteristics of the lakes, which do not change significantly through time, one might reasonably expect the temperature differences among lakes to reoccur over the years.

It has long been recognized that metabolic processes are temperature dependent in poikilothermic animals such as bivalves. For example, Hanson et al. (1988b) demonstrated in a field experiment that a variation with depth of 1.2°C in mean water temperature during the summer season was sufficient to affect individual growth of *P. grandis simpsoniana* (for comparison, the maximum difference in mean summer water temperatures among our lakes was 2.3°C). Together with decreases in growth, authors also noticed lower densities and biomass in the deepest portion of the littoral zone where temperatures were markedly lower, suggesting, without clearly demonstrating, that this factor might also play a role in the regulation of the biotic properties of clam populations. Several empirical models have indeed shown that water temperature can be a good predictor for secondary production in freshwater invertebrate populations, including bivalves (Plante and Downing, 1989; Morin and Bourassa, 1992). In contrast to the results of Morin and Bourassa (1992), however, we did not find any significant increase in turnover rates (P/B) with increasing thermal inputs.

Simple (Table 3-VI) and partial (Fig. 3.2) correlation analyses suggest that the influence of temperature on bivalve populations exceeds that of ambient Cd levels. This result is not incompatible with the rest of the data collected during our multi-year research programme conducted in the region of Rouyn-Noranda. Between 1989 and 1997, the group of six lakes for which we monitored the environmental levels of metal exhibited an overall decrease of ~55% in the ambient free Cd^{2+} concentration, following a reduction in atmospheric emissions of Cd by the smelter located in Rouyn-Noranda (this thesis, chapter 4). In parallel, there was a 30% decrease in the concentration of Cd in the gills of *P. grandis* specimens collected from those lakes. Concomitant with the declines in metal exposure and accumulation, we also noticed indices of better physiological condition in indigenous clams. In 1994, individuals collected from one of the most severely impacted lake (L. Vaudray) systematically presented necrotic gills; by 1998, gills were regular in shape. Taken together, these results tend to show that natural factors are now replacing anthropogenic ones as structuring agents of clam populations in lakes in the Rouyn-Noranda area. However, it cannot be inferred from this simple observation whether or not our candidate biomarker (i.e., Cd concentrations in the HMW pool) is valid. We do not have sufficient evidence to determine if the absence of correlation between the biomarker and population-level responses, after controlling for the temperature effects, is due to 1) an

improvement of environmental conditions (i.e., present-day levels of Cd exposure are relatively low), 2) the development of compensatory mechanisms in the populations (e.g., acquired genetic tolerance) that counteract the toxicity under low levels of Cd exposure, or 3) a lack of sensitivity of the biomarker itself (in the sense that the biomarker is not predictive of effects at higher levels of the biological organization).

3.5.4 Implications

Based on our results, it is difficult to currently assign to sub-cellular metal partitioning measurements any predictive role for toxic effects at higher levels of organization, mainly because of the influence of confounding variables. Although our lake selection procedure (see chapter 2) did an adequate job of minimizing the relative contribution of most limnological variables to the variability of the clam population-level responses, it did not succeed in eliminating the effect of temperature. Given the close relationships that exist between this variable and lake size and maximum depth, we should in retrospect have considered lakes with similar morphological characteristics in our initial selection; more recent programmes for the monitoring of impacts of man-induced perturbations on lacustrine ecosystems have integrated the study of lake-basin morphometry into their protocols.

More importantly, our findings cast legitimate doubt on the validity of those environmental studies that do not explicitly consider habitat characteristics, which are specific constituents of ecosystems, along with components related to anthropogenic activities. For a proper use of biomarkers within an ecological risk assessment framework, and as a corollary of this, for extrapolating from lower to higher levels of organization, we need a better identification and understanding of the modifying factors that influence the biological responses to stress at the different levels.

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Chapitre 4

Long-term trends in accumulated metals (Cd, Cu and Zn) and metallothionein in bivalves from lakes within a smelter-impacted region

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4.1 Abstract

Temporal monitoring studies are needed to detect long-term trends in ecosystem health. In the present study, we tested metallothionein (MT) as a potential biomarker for long-term variations in trace metal levels in lakes subject to atmospheric metal inputs from a nearby copper smelter. Over a 13-year period, we estimated on several occasions ambient free Cd^{2+}, Cu^{2+} and Zn^{2+} concentrations in six lakes with contrasting metal levels, and measured metal and metallothionein concentrations in gills of bivalves (*Pyganodon grandis*) living in these lakes. All but one of the study lakes had comparable drainage ratios, so inter-lake differences in hydrological export of metals from contaminated watersheds to receiving waters were likely minimal. Declines in the metal emissions from the smelter (especially for Cd and Zn) during the 1980s led to appreciable decreases in both calculated free Cd^{2+} ion concentrations in the study lakes (–59 ± 21% between 1989 and 1998) and accumulated Cd levels in their clam populations (–46 ± 12% between 1989 and 2002). Taking all lakes into account, MT concentrations in bivalves have comparatively dropped by 44% (± 10%) since 1989. In contrast to what we found for Cd, there were no significant reductions in the calculated free Cu^{2+} and Zn^{2+} concentrations in the various lakes during our study period (–2 and –10%, respectively, with 95% confidence intervals spanning zero). Overall, observed decreases in MT in bivalves over time were best correlated with similar decreases in both environmental and accumulated Cd levels ($r = 0.77$, $P = 0.0003$ and $r = 0.79$, $P < 0.0001$, respectively, both P-values corrected for temporal autocorrelation). Results from the present study demonstrate that changes in MT in the freshwater bivalve *P. grandis* reflect long-term variations in Cd contamination in lakes.

Keywords: Metal contamination; Atmospheric deposition; Lakes; Long-term monitoring; Metallothionein (MT); Bivalves

4.2 Introduction

Historical records obtained from ice sheets (Hong et al., 1994 and 1996), lake sediments (Renberg et al., 1994) and peat bogs (Shotyk et al., 1998) combined with recent quantitative assessments (Nriagu and Pacyna, 1988) show that human activities have greatly increased the fluxes of certain trace metals to the atmosphere, well above their natural levels. Problems caused by the subsequent deposition of potentially toxic metals to freshwater habitats have been extensively studied in Canada, either in manipulated lakes, in which metals were experimentally added (see Malley, 1996 and literature cited therein; Hintelmann et al., 2002) or in lakes in smelter-impacted areas (e.g., Nriagu, 1984; Yan and Welbourn, 1990). Over the last two decades, however, atmospheric emissions of trace metals from all anthropogenic sources have declined in both North America and Europe (Pacyna and Pacyna, 2001), due to a combination of industrial technological developments and legislated controls. As a result, the focus of current research has shifted from assessing the effects of metal pollution on freshwater ecosystems to detecting their chemical and biological recovery from past damage (e.g., Havas et al., 1995; Woodfine and Havas, 1995; Keller and Gunn, 1995; Yan et al., 1996; Croteau et al., 2002a; Belzile et al., 2004).

Traditionally, the evaluation of the biological impacts of trace metals in aquatic systems has involved the use of biomonitors (Phillips and Rainbow, 1993; Luoma, 1996). In this approach, metal concentrations in some "sentinel" species are often used as a surrogate measurement of the pollutant's availability to the indigenous biota. Analysis of metallothioneins (MTs) in the tissues of such sentinel organisms is an other potential tool for monitoring trace metal contamination in both freshwater (Couillard, 1997) and marine (Langston et al., 1998) environments. Metallothioneins are low-molecular weight, cysteine-rich, heat-stable metal-binding proteins involved in cellular essential metal (Cu and Zn) ion regulation and non-essential metal (Cd, Hg and Ag) detoxification (Roesijadi, 1992; Kägi, 1993). Theoretically, the measurement of MT is superior to the direct determination of total tissue metal since it allows one to distinguish between the toxicologically significant intracellular metal fraction and metals bound in unavailable form (Olafson et al., 1979).

The use of MT as a biomarker for metal exposure for freshwater organisms has been spatially validated in field situations. Indeed, site-to-site variations in steady-state MT concentrations in insect larvae (Croteau et al., 2002b), bivalves (Couillard et al., 1993; Perceval et al., 2002) and fish (Laflamme et al., 2000; Olsvik et al., 2001, Van Campenhout et al., 2003) have been shown to respond in a concentration-dependent manner to increases in environmental metal levels (or at least in bioaccumulated metals). Some field and laboratory studies using freshwater invertebrates (mostly bivalves) have also examined the temporal variations of MT concentrations during contamination (i.e., uptake) (Couillard et al., 1995; Baudrimont et al., 1999) or recovery (i.e., depuration) phases (Baudrimont et al., 2003; Gillis et al., 2004), but only through experiments in which animals were subjected to environmentally unrealistic step-changes in metal exposure, and always for a limited period of time (<400 days). To our knowledge, long-term responses of MT in organisms exposed to progressive variations in metal concentrations throughout their lives have not yet been studied.

During a 13-year study, we evaluated the use of metallothionein measurements in natural populations of bivalves (*Pyganodon grandis*) to monitor changes in metal levels in lakes from a smelter-impacted area. The lakes are located in the vicinity of Rouyn-Noranda, Quebec, Canada, where a large copper smelter has been operating since the mid-1920s. Although atmospheric emissions from the smelter are presently largely controlled, prior to 1985 many local lakes were subjected to relatively high metal deposition. Over the entire study period, we determined on several occasions ambient Cd, Cu and Zn concentrations in water overlying the sediments in lakes with contrasting metal levels, together with metal and MT concentrations in *P. grandis* specimens collected from these lakes. Our objectives were to determine 1) if the observed declines in the smelter's metal emissions had led to corresponding decreases in the degree of exposure of *P. grandis* to Cd, Cu and Zn as well as in bioaccumulated metals and 2) if long-term variations in both environmental and accumulated metal levels were, to some extent, related to similar changes in MT concentrations in our sentinel organism.

4.3 Material and methods

4.3.1 Study area

The study lakes are located in a 3500 km^2 area around the city of Rouyn-Noranda in Abitibi, Quebec (48°00′N, 79°00′W). All lakes lie at elevations of <300 m, on a bedrock that is composed essentially of volcanic and sedimentary rocks and is irregularly covered by glaciolacustrine deposits (rich in clay) left by the paleo-glacial Lake Ojibway (surficial geology map No. 1639A, Geological Survey of Canada, Natural Resources Canada). Lake watersheds are largely forested, and the vegetation encountered is typical of the coniferous boreal forest. Based on preliminary field observations by our research team in this region, we chose six lakes that differed markedly in their degree of Cd, Cu and Zn contamination. The relative metal contamination of these lakes is determined primarily by their proximity to the smelting operations in Rouyn-Noranda and by wind direction, since no point sources of metal inputs are evident in their watersheds. Lakes Joannès, Vaudray, Bousquet and Héva are located 26 to 51 km downwind from the smelter (Table 4-I), whereas L. Opasatica and Dufay are 29 and 39 km upwind of the smelter, respectively.

4.3.2 Sample collection and preparation

Sampling was done by SCUBA-equipped divers during the ice-free season (May to October) on several occasions between 1989 and 2002. Sediments, water and bivalves were collected at a single littoral site in each lake, at depths varying between 1.5 and 3 m, over a sediment surface area of ~ 1500 m^2. We repeatedly sampled the same stations throughout the entire study period. Field protocols remained consistent across all sampling events, with only minor deviations as noted below.

To determine the degree of exposure of *Pyganodon grandis* to ambient metals, we collected surficial bottom sediments at each littoral station during the summers of 1989, 1997 and 1998, using hand-held polypropylene corers (7.8-cm diameter). Each core was extruded in the boat and the uppermost 0.5 cm, containing the oxidised sediments, was

Table 4-I. Locations and morphometric characteristics of the six study lakes. LA = lake area; ΣLA = total lake area in the catchment; Z_{max} = maximum depth; WA = watershed area; CA = terrestrial portion of the watershed; DR = drainage ratio.

Lake	Lake code	Location	Distance from the smelter§ (km)	LA (km^2)	ΣLA† (km^2)	Z_{max} (m)	WA (km^2)	CA‡ (km^2)	DR*
Upwind of the smelter									
Dufay	DU	48°03′N, 79°28′W	39	4.20	5.03	12	42.48	37.45	7.45
Opasatica	OP	48°05′N, 79°18′W	29	53.62	65.12	~ 60	500.18	435.06	6.68
Downwind from the smelter									
Bousquet	BO	48°14′N, 78°43′W	30	2.41	15.58	22	302.17	286.59	18.39
Héva	HE	48°11′N, 78°19′W	51	2.37	2.52	7	23.43	20.91	8.30
Joannès	JO	48°11′N, 78°41′W	26	4.38	4.47	21	45.82	41.35	9.25
Vaudray	VA	48°07′N, 78°42′W	30	7.64	8.45	30	62.40	53.95	6.38

Note: LA, ΣLA and WA were measured on 1:50 000 topographic maps using SigmaScan Pro 5.0 for image analysis.

§Data obtained from Borgmann et al. (2004).

†Some watersheds had more than one lake.

‡CA = WA–ΣLA.

*DR = CA/ΣLA.

removed using a Teflon sheet and placed in an acid-cleaned high-density polyethylene (HDPE) bottle half filled with lake water. To account for local spatial variability, and in order to obtain an amount of sediments sufficient for analysis, each bottle contained three to four slices of oxidised sediments, each slice coming from a different core. These bottles were kept at ~ 4°C during transport to the laboratory where they were stored at –20°C until analysis.

At the same time, water samples for the determination of pH, dissolved calcium (Ca) and dissolved organic carbon (DOC) were collected at ~ 10 cm above the bottom sediments in acid-cleaned polyethylene bottles rinsed with the lake water at the time of collection. Samples for pH measurements were taken on several occasions during each sampling campaign (at least two times, usually four) in order to obtain time-averaged pH values. Water pH was immediately measured on shore using a portable pH meter. Samples for Ca and DOC measurements were filtered through polycarbonate membranes (0.4 µm, Nuclepore) on the day of collection, and filtrate sub-samples for Ca were acidified with HNO_3 (final concentration 0.5%, v/v). All water samples were stored at 4°C until analysis.

Bivalves were collected during the summers of 1989, 1994, 1997, 1998 and 2002. During each sampling campaign, divers collected between twelve and twenty-one individuals of similar size at each site (nominal shell length ~ 80 mm; actual mean shell lengths ranged from 70.9 to 89.5 mm for each lake, all sampling events taken into account). From age-length relationships established for *P. grandis* specimens from the same group of lakes (Perceval et al., unpublished results), we estimated that depending on the sampling year, the collected clams were between 9 and 10 years old in L. Bousquet, between 8 and 9 in L. Dufay, between 5 and 6 in L. Héva, between 8 and 10 in L. Joannès, between 4 and 6 in L. Opasatica and between 9 and 13 in L. Vaudray. These differences among the lakes are simply a reflection of the fact that shell growth rates varied from one population to another. To minimise differences in MT concentrations due to variations in the animals' reproductive cycle (Baudrimont et al., 1997), molluscs were collected from all lakes at the same time of the year, just before the onset of gametogenesis (usually in early June). *P. grandis* specimens were maintained alive at field temperature in coolers filled with lake

water during their transportation to the laboratory where they were dissected at most within 12 h after collection. Gills from each collected clam were manually isolated with a scalpel. We chose gills as our target organ based on a previous study conducted in the Rouyn-Noranda area, in which we had demonstrated that gills contained the greatest proportion of the total metal burden (40 ± 13% for Cd) in *P. grandis* (Tessier et al., 1993). Gill tissues from four to five individuals were pooled (yielding three to four replicate samples per lake according to the year of sampling), frozen in liquid nitrogen, sealed in plastic bags filled with nitrogen, and stored at -80°C for at most 6 months until the homogenisation step. Prior to 1994, tissues were directly sealed in polyethylene bags filled with nitrogen and kept at –40°C until homogenisation.

4.3.3 Laboratory analyses

To minimise inadvertent trace metal contamination, all lab-ware was soaked in 15% nitric acid and rinsed in ultrapure water (>18 MΩ) before use.

4.3.3.1 Water and sediments

In water samples, dissolved Ca was determined by flame atomic absorption spectrophotometry (AAS; Spectra AA20, Varian) with the addition of a La-Cs ionic suppressor. DOC was measured by a combustion-infrared method (APHA, 1985). Sediment samples were thawed and centrifuged to remove excess water, and sub-samples were analysed using a sequential extraction procedure designed to partition particulate trace metals into operationally defined fractions (namely F1: exchangeable metals; F2: metals bound to carbonates or specifically adsorbed; F3: metals bound to iron or manganese oxides; F4: metals bound to organic matter and sulfides) (Tessier et al., 1979). Depending on the metal to be analysed and the concentration range, we used either flame AAS, graphite furnace atomic absorption spectrophotometry (GFAAS; SIMAA 6000, Perkin-Elmer) or inductively-coupled plasma atomic emission spectroscopy (ICP-AES; Atom Scan 25, Thermo Jarrell Ash) for the determination of metal concentrations in the sediment extracts. Sediment organic C concentrations were measured with a CNS analyser (Carlo-Erba Instruments, model NA 1500) after removal of inorganic C by acidification with 0.5

M H_2SO_4 (15 min, 100 mL per g sediment dry wt). Based on the sediment metal, organic carbon and iron oxyhydroxide concentrations together with the time-averaged pH values of the overlying water, we have derived estimates of free Cd^{2+}, Cu^{2+} and Zn^{2+} concentrations at the sediment-water interface using a geochemical model based on the sorptive equilibrium of the free-metal ions between water and oxic sediments. This approach allowed us 1) to obtain an integrated estimate of exposure of *P. grandis* specimens to potentially available metals and 2) to circumvent the difficulty of actually measuring free metal ion concentrations. The model for Cd^{2+} is described in Tessier et al. (1993) and the one for Cu^{2+} and Zn^{2+} can be found in Tessier (1992).

4.3.3.2 Bivalves

Partially thawed gill tissues were gently homogenised under a nitrogen atmosphere with either a Brinkmann Kinematica Polytron homogeniser (in 1989) or a glass tissue grinder (Duall Co.) operated manually (in 1994) or mechanically (in 1997, 1998 and 2002), and the homogenised tissues were kept on ice. For bivalves collected after 1989, homogenisation was performed with ice-cold 25 mM Tris buffer (OmniPur) adjusted to pH 7.2; the tissue-to-buffer ratio was adjusted to either 1:1 (wet weight tissue:volume of buffer) (in 1998), 1:2 (in 1994), 1:3 (in 1997) or 1:19 (in 2002). In 1989, gill tissues were homogenised in an equal weight of ice-cold 0.9% NaCl solution. Sub-samples of the tissue homogenates were allocated to measurements of total gill metal (gill Cd, gill Cu and gill Zn) and metallothionein (gill MT) concentrations and to the determination of the dry to wet weight ratios. Bivalves collected in 1998 were not analysed for total metal concentrations.

For the determination of gill metal concentrations, tissue homogenates were either oven- or freeze-dried, transferred to a Teflon bomb and digested in ultrapure concentrated HNO_3 (Aristar grade). For samples collected before 2002, the digestion was carried out in a microwave oven (700 W, ≤2 min) up to a pressure of 6900 kPa. Samples collected in 2002 were digested in an autoclave at 240°C for 3 h. Cooled digestates were then diluted with ultrapure water and analysed for Cd, Cu and Zn by either flame AAS (Spectra AA20, Varian) or plasma atomic emission spectrometry (Atom Scan 25, Thermo Jarrell Ash or Vista, Varian). To monitor analytical performance, we digested samples of similar weight

of at least one (usually two) certified reference material (TORT-1 or 2, lobster hepatopancreas, National Research Council of Canada; SRM No.1566, oyster tissue, US National Institute of Standards and Technology) during each run; recoveries of reference materials were consistently within the certified range for each metal. Procedural digestion blanks were also run, and they indicated low to negligible inadvertent contamination.

For MT analyses, homogenate sub-samples were centrifuged at 30,000×*g* for 30 min at 4°C, and the supernatants were divided into four analytical replicates that were analysed the same day for metallothionein with a Hg-saturation assay (Dutton et al., 1993). As a quality control, recovery of a MT standard (MT from rabbit liver, Sigma Chemical Co.) was determined with every assay; the mean recoveries were 96 ± 1% in 1989 (N = 4 separate determinations), 99.6 ± 2.7% in 1994 (N = 12), 101.8 ± 2.8% in 1997 (N = 19), 94.3 ± 2.0% (N = 10) in 1998 and 95 ± 3% in 2002 (N = 4). Metallothionein concentrations are mean values of both field and analytical replicates and are expressed as nanomoles of Hg-binding sites per gram of dry tissue weight. Since the exact number of Hg-binding sites per MT molecule is unknown for this species, MT concentrations cannot be expressed in moles MT·g^{-1}.

In 2002, we evaluated the potential methodological artefact of using different dilution factors during gill homogenisation. For that purpose, we obtained two composite samples of bivalve gills from each of a reference (L. Opasatica) and a metal-contaminated (L. Héva) lake, and separated them into three equivalent portions. Each portion of gill tissue was then homogenised in either one, three or nineteen portions (w/w) of Tris buffer and the resulting 2×3×2 homogenates were analysed the same day for MT. Results of two-way non-parametric ANOVA (Scheirer-Ray-Hare extension of the Kruskal-Wallis test for ranked data; Sokal and Rohlf, 1995, pp. 446-447) with lake category, homogenate dilution and the lake×dilution interaction as fixed factors indicated that differences in metal exposure were significantly ($P < 0.05$) more important than changes in the dilution factor as sources of variation in MT concentrations (Fig. 4.1).

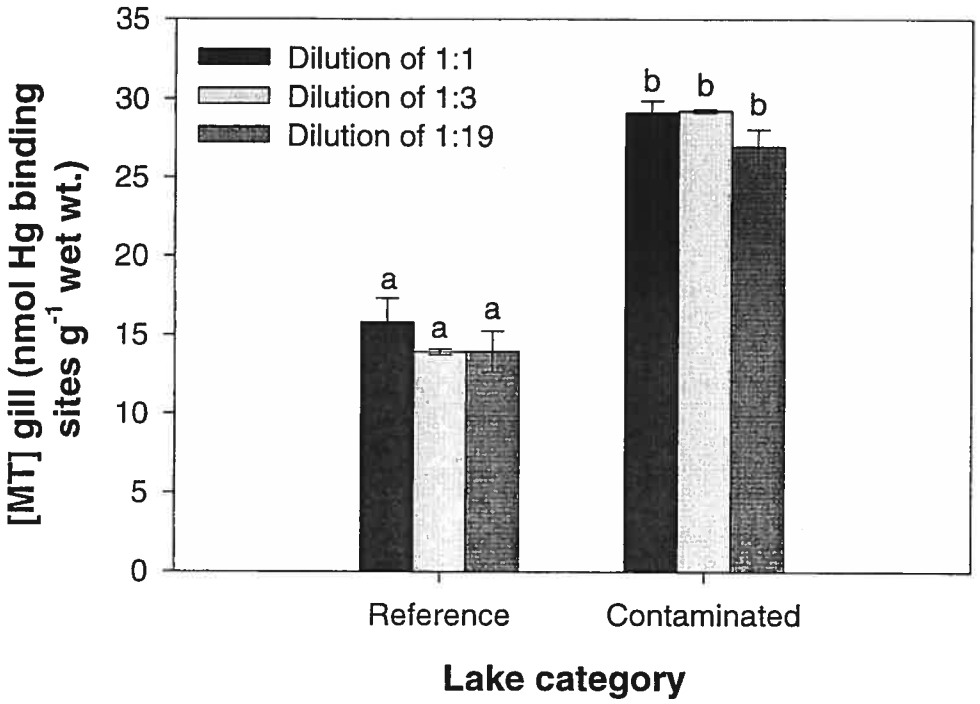


Figure 4.1. Metallothionein (MT) concentrations (mean ± standard error) in the gills of *Pyganodon grandis* at dilutions of 1:1, 1:3 and 1:19 for specimens collected from a reference (L. Opasatica) and a metal-contaminated (L. Héva) lake. $N = 2$ composite samples of gill tissues for each dilution factor in each lake category. Histogram bars with the same lowercase letter do not differ significantly at $P < 0.05$ (two-way ANOVA for ranked data: Scheirer-Ray-Hare extension of the Kruskal-Wallis test).

4.3.4 Statistical analyses

In the present study, we repeatedly sampled the same populations of *P. grandis* over time. Such repeated sampling introduces a considerable risk of temporal non-independence that often leads to increased or decreased probability of type I error in the assessment of differences among times of sampling (Underwood, 1997, pp. 404-408). The ANOVA and correlation analyses we used here were therefore modified in order to take this temporal autocorrelation into account. Firstly, the possible differences in bivalve gill metal and metallothionein concentrations between the lakes and the years of sampling were tested

using repeated-measures multivariate analysis of variance (MANOVAR), with gill Cd, Cu, Zn or MT concentrations for each of the four years of sampling (i.e., 1989, 1994, 1997 and 2002) as the dependent variables, "lake" as the between-subjects factor and "year of sampling" as the within-subjects factor. In MANOVAR, the number of dependent variables (k) that can be analysed usually depends on the total number of samples (N) and the number of between-subjects treatment levels (i.e., the number of lakes, M) (Potvin et al., 1990); the main condition that $N - M > k$ was satisfied in each analysis ($18 - 6 > 4$). Since the interaction between "lake" and "year" was significant in all MANOVAR and because our main interest was to study the temporal changes in total metal and metallothionein concentrations in *P. grandis*, we also compared the means for gill Cd, Cu, Zn and MT among the various sampling years in every lake separately, using univariate repeated-measures analysis of variance (ANOVAR). Each time compound symmetry of the variance-covariance matrix for the ANOVAR failed, we adjusted the probabilities using the Huynh-Feldt statistic (Wilkinson, 1998). When F tests revealed a significant "year" effect, the Tukey-Kramer test for multiple comparisons (with the number of degrees of freedom and the mean square error corrected for repeated measures) was used to assess where differences lay. When necessary, total metal and metallothionein data were $\log_{10}$-transformed to achieve normality and homogeneity of variances. Analyses were performed using SYSTAT for Windows version 8.0.

Secondly, the relationships between gill MT and both gill and environmental metal concentrations were examined using correlation analysis (Pearson's r). We used the spatio-temporal version of Dutilleul's modified t-test (Dutilleul and Pinel-Alloul, 1996) for all correlations. The Kolmogorov-Smirnov-Lilliefors test was used to verify that all variables were normally distributed and logarithmic transformation was applied when such assumptions were not satisfied. All computations were done using the computer program "Modified t-test" (Legendre, 2000). Although this program is primarily used for computing t-tests corrected for spatial autocorrelation, it can be adapted to temporally dependent data by replacing one of the two coordinate vectors by a temporal variable (i.e., the year of sampling, as is the case here) and the other by a constant (Pierre Legendre, Université de Montréal, personal communication).

4.4 Results

4.4.1 Metals in the environment

During the 1980s, the atmospheric emissions of Cd and Zn from the Horne smelter declined drastically (Fig. 4.2), whereas Cu emissions exhibited considerable variability throughout the entire study period. Temporal trends for Cd and Zn emissions are closely related because Cd is generally associated with zinc ores. Since the beginning of our work in 1989, releases of Cd and Zn have been reduced by 75% and 90%, respectively.

Distance from the smelter and wind direction are probably not the only variables that affect metal concentrations in the lakes: free metal-ion concentration estimates from L. Joannès were consistently lower compared to estimates from L. Héva, a more distant lake, and free Cd^{2+} concentration estimates from L. Dufay, located upwind of the smelter, were higher than estimates from L. Joannès, which is downwind from the smelter (Tables 4-I and 4-II).

Nevertheless, following the decline in Cd atmospheric emissions, we observed decreases in the estimated free Cd^{2+} concentrations of the lakes between 1989 and 1998 (Table 4-II): the most important decline was observed in Lake Opasatica (–90%) and the least important one in Lake Héva (–35%). Temporal trends were not so evident for the variations in both free $[Cu^{2+}]$ and $[Zn^{2+}]$ (Table 4-II). The relative ranking of the lakes along the Cd and Zn contamination gradient did not vary much during our study period (Spearman correlation on rank-ordered data: $r_s = 0.77$, $P = 0.072$ for the correlation between $[Cd^{2+}]$ estimates from 1989 and those from 1998; $r_s = 0.94$, $P = 0.005$ for the correlation between $[Zn^{2+}]$ estimates from 1989 and those from 1998). Lake-to-lake variations in environmental metal concentrations (all sampling years taken into account) were also significantly positively correlated between metal pairs (Spearman correlation coefficients and associated probabilities corrected for temporal autocorrelation: $r_s = 0.45$, $P = 0.0416$; $r_s = 0.84$, $P < 0.0001$; $r_s = 0.55$, $P = 0.0210$ for the correlation between $[Cd^{2+}]$ and $[Cu^{2+}]$, $[Cd^{2+}]$ and $[Zn^{2+}]$ and $[Cu^{2+}]$ and $[Zn^{2+}]$, respectively) reflecting their common

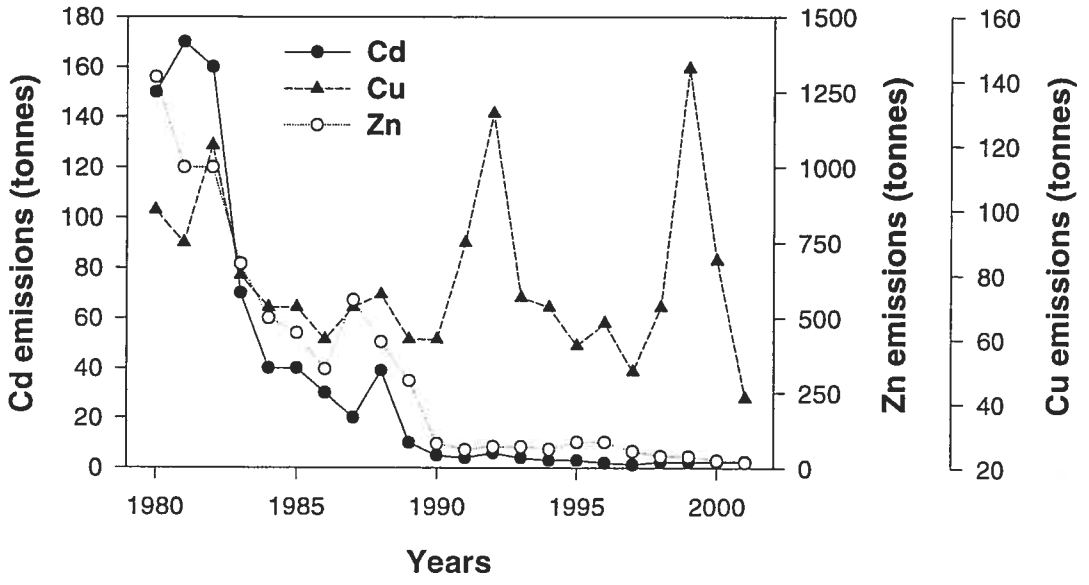


Figure 4.2. Temporal changes in annual atmospheric emissions of Cd (solid circles), Cu (solid triangles) and Zn (open circles) from the Horne copper smelter in Rouyn-Noranda between 1980 and 2001 (data courtesy of Noranda Inc.).

(largely atmospheric) origin. With regard to the other environmental variables, lakewater pH values showed slight increases between 1989 and 1998 (Table 4-II); by 1998, all lakes had reached near-neutral pH values. Over the same period, dissolved Ca concentrations remained constant. In all study lakes, dissolved organic carbon concentrations exhibited little variability among the years, with the exception of L. Bousquet. This latter lake has relatively high DOC concentrations owing to its high drainage ratio (Tables 4-I and 4-II).

4.4.2 Temporal trends of metals and metallothionein in *P. grandis*

Multivariate analysis of variance (MANOVAR) indicated significant differences among lakes and sampling years for mean concentrations of metals (Cd, Cu and Zn) and MT in the gills of *P. grandis* (Table 4-III). The interaction between year and lake was significant ($P < 0.05$) in all MANOVAR analyses, suggesting that the effect of time on metal and MT concentrations in bivalves is lake-specific. However, it should be noted that the amount of

Table 4-II. Temporal changes in the calculated free-metal ion concentrations (Cd^{2+}, Cu^{2+} and Zn^{2+}) in the overlying water of the studied lakes as well as their pH and dissolved organic carbon (DOC) and calcium (Ca) concentrations. Lakes downwind from the smelter are indicated with an asterisk.

Lake	Year	pH[a]	[DOC] (mg C L^{-1})	$[Ca]_{diss.}$ (µM)	$[Cd^{2+}]$ (nM)	$[Cu^{2+}]$ (nM)	$[Zn^{2+}]$ (nM)
Dufay	1989§	6.51	7.2	85	0.88	15.4	43.8
	1997	6.78	9.7	81	0.34	14.5	30.0
	1998	6.93	9.7	70	0.33	10.3	25.5
Opasatica	1989§	7.39	6.5	165	0.28	19.5	2.8
	1997	7.48	8.8	210	0.03	2.5	2.0
	1998	7.60	6.4	206	0.03	2.6	1.8
Bousquet*	1989§	6.34	9.7	105	1.77	9.4	90.4
	1997	6.34	17.2	100	0.85	18.6	121.9
	1998	6.94	12.7	107	0.71	11.2	73.3
Héva*	1989§	6.18	7.1	59	1.43	18.0	138.2
	1997	6.00	10.1	54	0.96	28.8	304.0
	1998	6.88	9.4	50	0.93	27.4	181.3
Joannès*	1989§	7.22	ND	170	0.32	17.7	23.4
	1997	7.18	11.8	174	0.22	17.9	31.0
	1998	7.46	9.1	178	0.20	17.7	26.2
Vaudray*	1989§	6.56	6.5	90	2.22	34.3	72.6
	1997	6.51	9.2	85	0.88	34.8	113.4
	1998	7.26	8.5	75	0.67	54.1	69.3

Note: The free Cd^{2+}, Cu^{2+} and Zn^{2+} concentrations were estimated from sediment metal, organic carbon and amorphous iron oxyhydroxide concentrations, together with the pH of the overlying water. The geochemical model is based on the sorptive equilibrium of free-metal ions between oxic sediments and water (see Tessier, 1992 and Tessier et al., 1993 for details).

[a]Mean pH estimated from summer time series = $-\log_{10}(\Sigma[H^+]/N)$.

ND: not determined.

§Data obtained from Couillard et al. (1993).

total variance explained by the year×lake interaction was consistently low for all dependent variables (i.e., 3.1, 9.1, 9.2 and 5.7% for gill Cd, gill Cu, gill Zn and gill MT, respectively) and thus differences in temporal trends among the various lakes should be minimal.

Despite little variation between 1989 and 1994, Cd concentrations in the bivalve gills showed a decreasing tendency in all lakes over our whole study period (Fig. 4.3A).

Table 4-III. Results of multivariate analysis of variance using repeated measures (MANOVAR) on total metal (Gill Cd, Gill Cu and Gill Zn) and metallothionein (Gill MT) gill concentrations for *P. grandis* in relation to the lake (BO, DU, HE, JO, OP and VA) and the year of sampling (1989, 1994, 1997 and 2002). All dependent variables were $\log_{10}$-transformed to meet assumptions of normality and homogeneity of variances.

Source of variation	Num. df	Denom. df	Wilks' λ	*F*	*P*
Gill Cd					
Between-subjects					
Lake	5	12	0.0126	188.39	<0.0001
Within-subjects					
Year	3	10	0.0234	139.29	<0.0001
Year×lake	15	28	0.0487	3.71	0.0013
Gill Cu					
Between-subjects					
Lake	5	12	0.0327	71.03	<0.0001
Within-subjects					
Year	3	10	0.0690	44.96	<0.0001
Year×lake	15	28	0.0516	3.60	0.0017
Gill Zn					
Between-subjects					
Lake	5	12	0.0417	55.09	<0.0001
Within-subjects					
Year	3	10	0.2099	12.55	<0.0001
Year×lake	15	28	0.0902	2.60	0.0142
Gill MT					
Between-subjects					
Lake	5	12	0.0202	116.17	<0.0001
Within-subjects					
Year	3	10	0.0503	62.92	<0.0001
Year×lake	15	28	0.0920	2.56	0.0152

However, univariate repeated-measures analysis of variance (ANOVAR) indicated that these temporal changes were not statistically significant for both Lakes Bousquet (P = 0.1458) and Dufay (P = 0.0803), whereas for L. Vaudray, observed reductions were only significant between 1994 and 2002 (Tukey's test, P = 0.0220). Greatest reductions in gill Cd were seen in bivalves from L. Opasatica (–65% between 1989 and 2002) and Joannès (–50% over the same period of time). Except for Lake Vaudray, the time trends for Cu

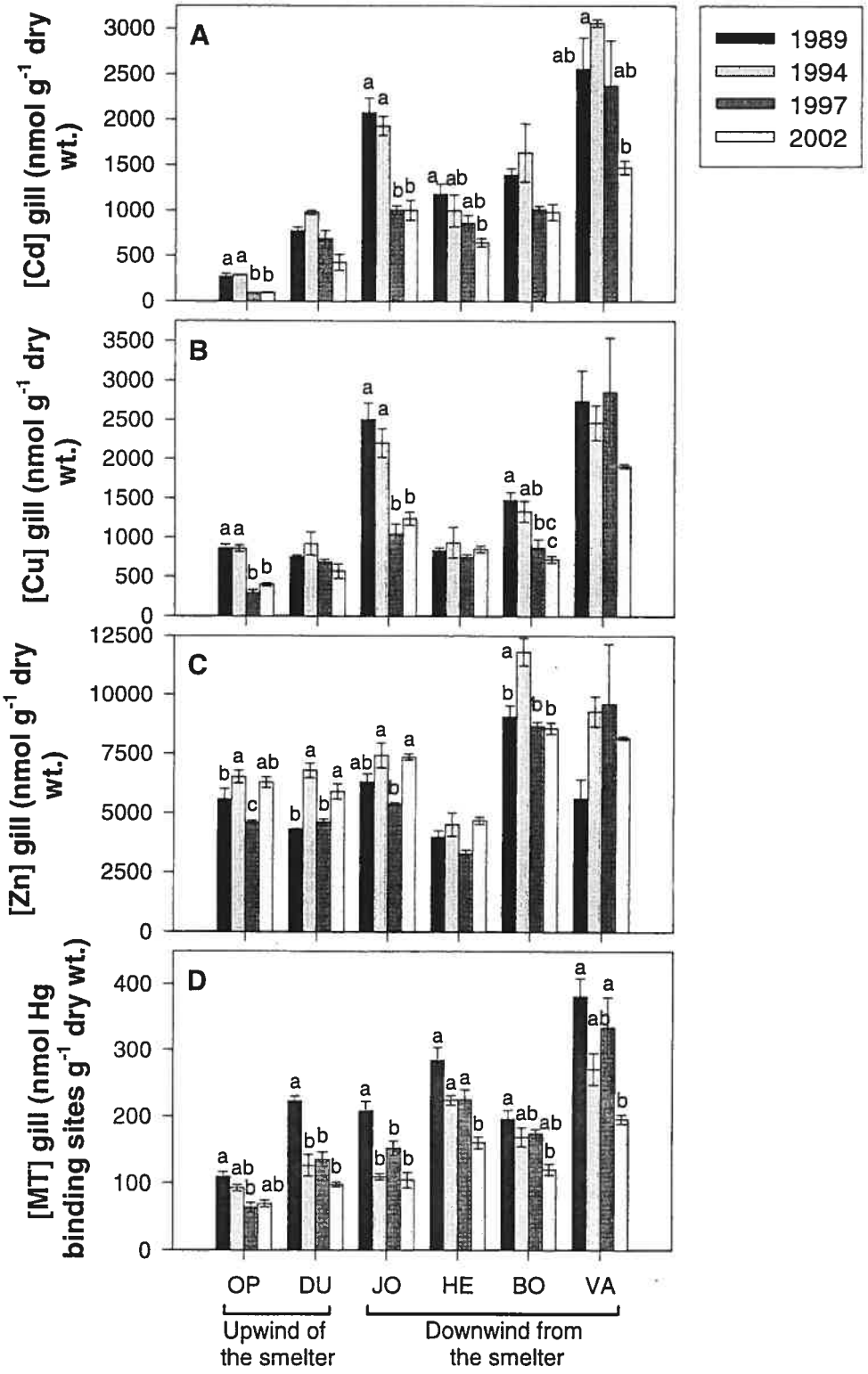
concentrations in *P. grandis* were similar to those observed for Cd (Fig. 4.3B). There were, however, no significant differences in gill Cu concentrations among the years for clams from Lakes Dufay (ANOVAR, $P = 0.1142$), Héva (ANOVAR, $P = 0.5271$) or Vaudray (ANOVAR, $P = 0.2647$). Overall, Zn concentrations in bivalve gills exhibited considerable variability throughout the study period (Fig. 4.3C), but in most of the lakes, mean Zn concentrations measured in specimens collected in 2002 did not differ significantly from those measured in bivalves sampled in 1989. The decreases in MT concentrations were generally more marked between 1989 and 1994 and from 1997 to present (Fig. 4.3D), in contrast to what we found for gill Cd and Cu, for which the greatest declines were observed between 1994 and 1997. In all lakes, MT concentrations in clams in 2002 were significantly lower compared to those measured in 1989 (with the notable exception of the specimens originating from L. Opasatica).

In 2002, ~ 20 years after the control of smelter emissions (Fig. 4.2), Cd levels in bivalves from L. Vaudray, Joannès, Bousquet and Héva had not yet reached those found in clams from L. Opasatica, our least contaminated lake (Tukey's multiple comparison test, $P < 0.05$). Concurrently, *P. grandis* individuals from L. Vaudray, Héva and Bousquet still exhibited significantly higher MT concentrations compared to the specimens from L. Opasatica (Tukey's test, $P < 0.05$).

4.4.3 Relationships between metallothionein concentrations in *P. grandis* and bioaccumulated and environmental metal concentrations

Significant positive correlations were found between MT concentrations in the bivalve gills and their concentrations in both Cd and Cu for the period between 1989 and 2002 (Figs. 4.4A and 4.4B), indicating that the decreases in MT in *P. grandis* over time were related to similar decreases in these accumulated metals. However, stepwise multiple regression showed that the relationship between MT and Cu concentrations was no longer significant when all bioaccumulated metals were considered simultaneously as predictors (Table 4-IV). Instead, we found that MT concentrations in the gills of clams were negatively

Figure 4.3. Temporal variations of A) Cd, B) Cu, C) Zn and D) metallothionein (MT) concentrations (mean ± standard error; $N = 3$ composite sub-samples of four to five individuals each) in the gills of *Pyganodon grandis* from six lakes in the region of Rouyn-Noranda. Different lowercase letters indicate mean differences among years (Tukey-Kramer HSD test, $P < 0.05$) within a particular lake. Data for 1989, 1994 and 1997 are obtained from Couillard et al. (1993), Wang et al. (1999) and Perceval et al. (2002), respectively.


A
B
C
D
1989
1994
1997
2002
[Cd] gill (nmol g-1 dry wt.)
[Cu] gill (nmol g-1 dry wt.)
[Zn] gill (nmol g-1 dry wt.)
[MT] gill (nmol Hg binding sites g-1 dry wt.)
OP
DU
JO
HE
BO
VA
Upwind of the smelter
Downwind from the smelter

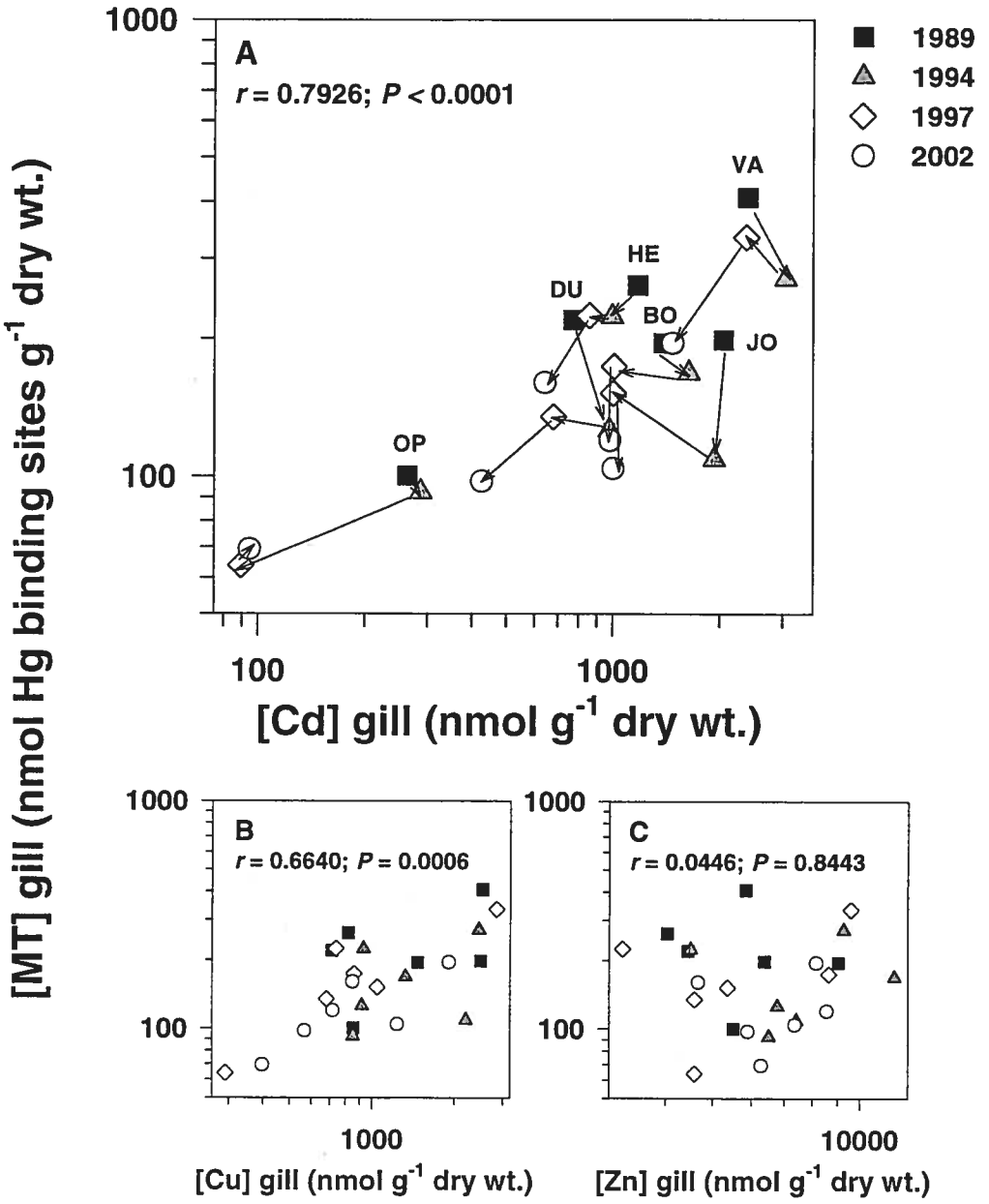


Figure 4.4. Relationships between mean metallothionein concentrations in the gills of *P. grandis* and their A) Cd concentrations, B) Cu concentrations and C) Zn concentrations for the period between 1989 and 2002. Each point corresponds to a particular lake (see Table 1 for lake codes) and arrows indicate the direction of temporal changes for each lake. *P* values of the correlation coefficients (Pearson's *r*) are adjusted for temporal autocorrelation.

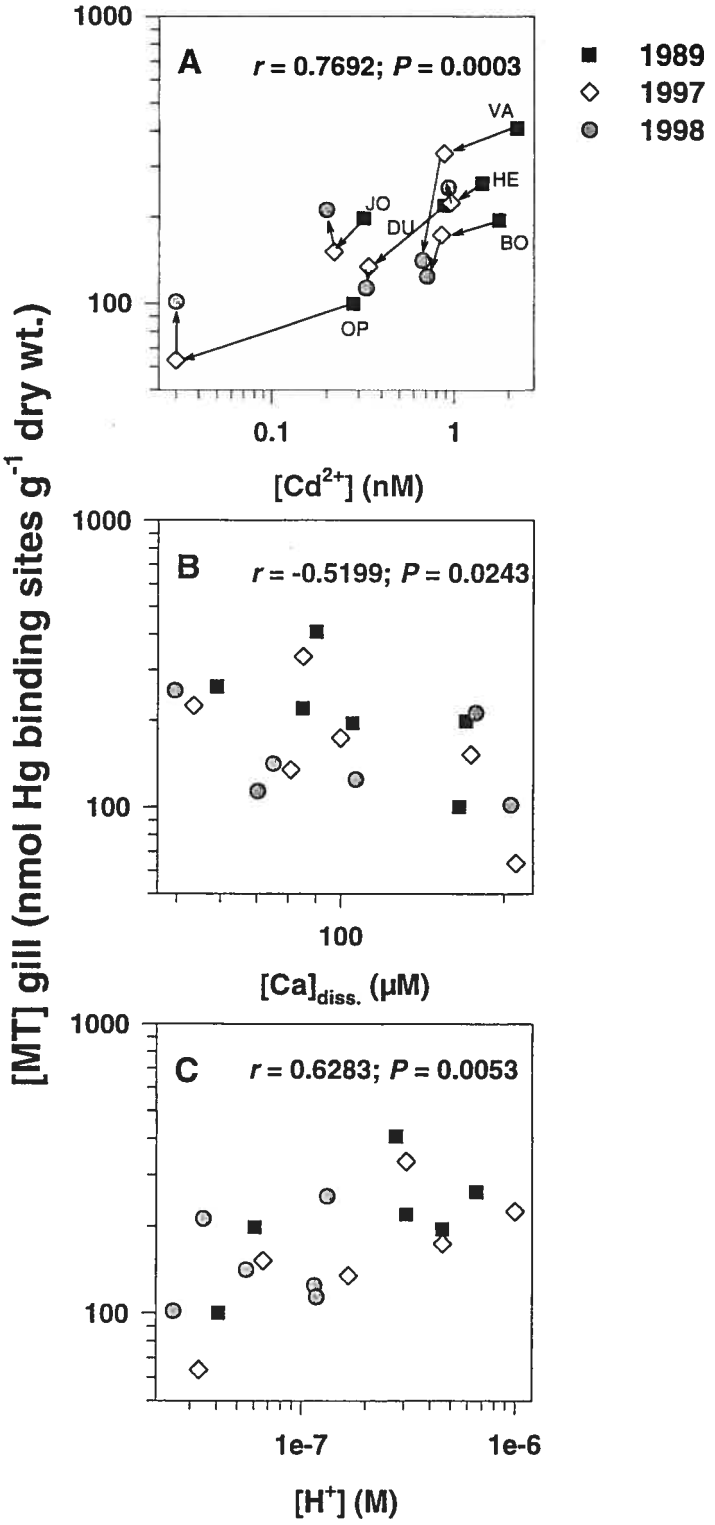
correlated with their Zn concentrations. However, this latter relationship might be spurious since gill Zn and gill MT are not intrinsically linked (Fig. 4.4C). For the data set as a whole, the regression model explained more than 80% of both spatial and temporal variability in gill MT concentrations, with gill Cd accounting for a major proportion (~ 72%) of the total variation.

Table 4-IV. Stepwise linear regression analysis of MT concentrations in the gills of *P. grandis* as a function of their concentrations in metals (from 1989 to 2002). All variables were $\log_{10}$-transformed to meet assumptions of normality and homoscedasticity of the residuals. R^2 and SE correspond to the proportion of the variation explained and the standard error of coefficient estimates, respectively.

Variable	Coefficient	SE	t	$p(t)$	Partial R^2	R^2
Constant	2.430	0.542	4.480	0.0003		0.824
Gill Cd	0.388	0.100	3.873	0.0010	0.724	
Gill Zn	–0.542	0.165	–3.283	0.0039	0.082	
Gill Cu	0.233	0.168	1.384	0.1823	0.018	
$N = 23$	$F = 29.620$	$P < 0.0001$				

Consistent with the results presented above, variations in MT concentrations in clams over the study period (1989-1998) were positively and strongly related to changes in ambient free Cd^{2+} ion concentrations (Fig. 4.5A). Neither free [Cu^{2+}] (partial $r = 0.26$, $p(t) = 0.3080$) nor free [Zn^{2+}] (partial $r = 0.25$, $p(t) = 0.3365$) in water overlying the sediments contributed further to the variation in gill MT (multiple linear regression analysis; results not shown). To a lesser extent, gill MT concentrations were also significantly correlated with both dissolved Ca (–) and hydrogen ion (+) concentrations in lake water (Figs. 4.5B and 4.5C, respectively). No significant relationship was found between gill MT and DOC ($r = -0.08$, $P > 0.05$, results not shown).

Figure 4.5. Relationships between mean metallothionein concentrations in the gills of *P. grandis* and A) free Cd ion concentrations, B) dissolved Ca concentrations and C) hydrogen ion concentrations in water overlying the sediments for the period between 1989 and 1998. Each point corresponds to a particular lake (see Table 1 for lake codes) and arrows indicate the direction of temporal changes for each lake. *P* values of the correlation coefficients (Pearson's *r*) are adjusted for temporal autocorrelation. Metallothionein data for 1998 are obtained from Giguère et al. (2003).


1000
100
A
r = 0.7692; P = 0.0003
VA
HE
JO
DU
BO
OP
0.1
1
[Cd2+] (nM)
1989
1997
1998
B
r = -0.5199; P = 0.0243
100
[Ca]diss. (µM)
C
r = 0.6283; P = 0.0053
1e-7
1e-6
[H+] (M)
[MT] gill (nmol Hg binding sites g-1 dry wt.)

4.5 Discussion

Metals in the atmosphere have a relatively short residence time, estimated to vary between days and weeks (Salomons and Förstner, 1984). Nevertheless, within this short time span, trace metals (mostly in particulate form) are able to travel long distances (generally tens of kilometres from the source of emission), before being removed from the atmospheric compartment by dry or wet deposition. As a result, they can accumulate in a variety of media, such as surface soils and lake sediments.

In their recent assessment of air emissions from copper and zinc production facilities in Canada, Doyle et al. (2003) estimated that in 1997 and 1998, total deposition rates of Cd, Cu and Zn within a 20-km radius of Rouyn-Noranda ranged from 0.4 to 3.6, 12 to 1200 and 8 to 200 $mg \cdot m^{-2} \cdot yr^{-1}$, respectively. Concentrations of emitted metals typically followed an exponential decrease with distance from the smelter. Because most of our lakes are 30 km (or more) away from Rouyn-Noranda (Table 4-I), present-day deposition rates of metals in their surroundings are likely at the lower end of the previous ranges.

Given that no current mining operations or mine-tailing ponds are evident in the lake watersheds, we assume here that the vast majority of metals entering the lakes comes from atmospheric inputs either through direct deposition on the lake surface or through movement from the drainage basins (via release of metals deposited on the land surface, and weathering of bedrock or unconsolidated deposits). In a given geographical area, the relative importance of atmospheric and terrestrial metal loadings to lakes will generally be related to the proportion of land and water in the watershed. In our case, inter-lake differences in metal export from contaminated watersheds should be minimal since all but one (L. Bousquet) of the study lakes have similar catchment area-to-lake area ratios (Table 4-I). In that sense, it is noteworthy that temporal trends for environmental and accumulated metal levels as well as for MT concentrations in Lake Bousquet do not stand out from those observed in the rest of the lakes. Despite its large drainage ratio (~ 2-3 fold higher than those calculated for the other lakes), the recovery of L. Bousquet seemed unaffected by the additional influx of metals from its watershed.

Metal contamination in lake ecosystems in smelter-impacted areas is often accompanied by a problem of acidification within the entire watershed (i.e., soils and surface waters) due to the emission of acidic substances, principally SO_2, from smelters. The study lakes have resisted severe acidification, with pH values remaining above 6 (Table 4-II), probably because they are all located on a clay belt that possesses appreciable acid neutralization capacity. The absence of severe acidification presumably affects the metal budgets for these lakes; indeed, several studies show that the percent of metal load due to terrestrial runoff is greatly reduced in circumneutral lakes (especially for Cd) (see a review in Nelson and Campbell, 1991).

Evidence of recovery from Cd contamination

Overall, our data show that water quality in the sampled lakes improved markedly after atmospheric emissions of trace metals from the smelter were reduced (Fig. 4.6). This is especially true regarding Cd contamination. Indeed, over a decade, we estimate that mean reductions in calculated ambient free Cd^{2+} concentrations and Cd concentrations in bivalves in the entire set of lakes were 59 ± 21% (± 95% confidence interval) and 46 ± 12%, respectively. Since many freshwater organisms are sensitive to Cd, even at moderately elevated environmental concentrations (Malley, 1996), this result augurs well for the recovery of animal communities in many impacted lakes near Rouyn-Noranda. In this regard, our findings are consistent with those of other studies from the same region. For example, Croteau et al. (2002a) observed similar decreases (from –68 to –39%) in Cd concentrations in planktonic insect larvae collected over a 10-year interval (approximately from 1988 to 2000) in lakes within 25-30 km of Rouyn-Noranda. Cattaneo et al. (2004) reported recent shifts in the taxonomic composition of diatom assemblages of L. Dufault, located ~ 7 km north of Rouyn-Noranda, that suggest partial chemical recovery of the lake from metal pollution. In this severely impacted lake, however, evidence of biological recovery has not yet been detected in invertebrate (Borgmann et al., 2004) and fish populations (Sherwood et al., 2002), which remain greatly reduced. Chronic toxicity tests with the amphipod *Hyalella azteca* showed that toxic effects in L. Dufault were probably caused primarily by Cd (Borgmann et al., 2004). Contrary to the study lakes, the watershed of L. Dufault was subjected in the past to intensive mining activities and emissions from

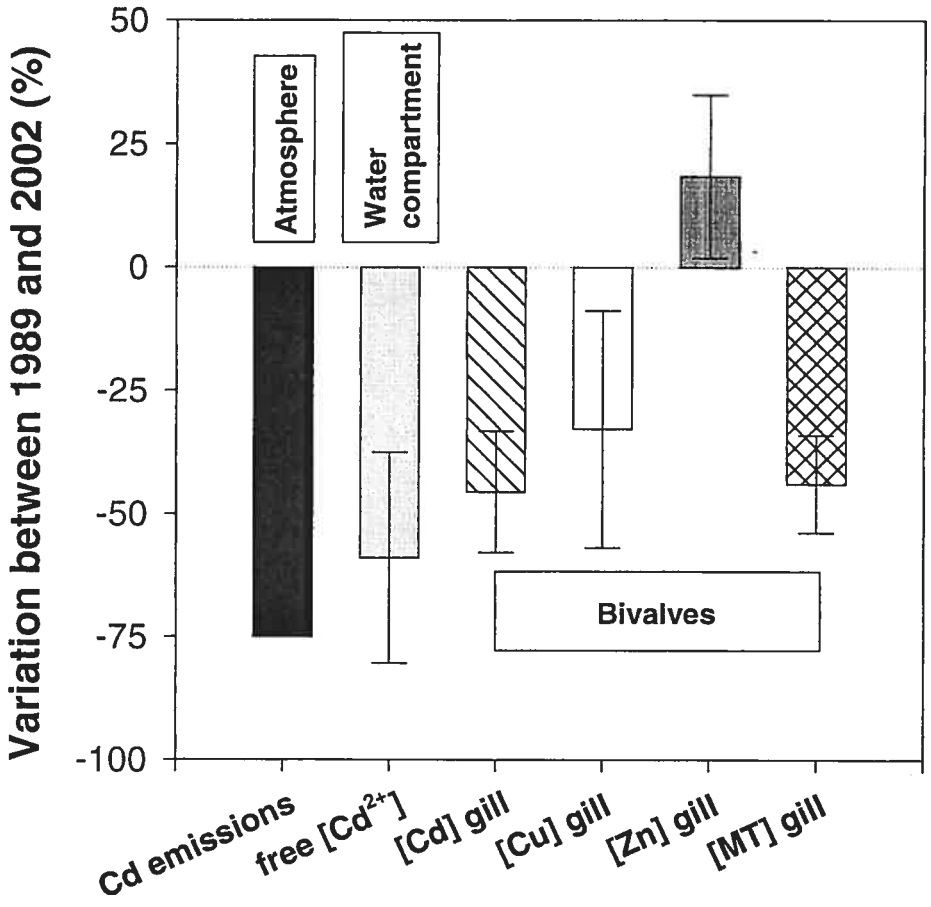


Figure 4.6. Changes in Cd atmospheric emissions, environmental free Cd ion concentrations in the littoral zone of the study lakes (mean ± 95% confidence interval; N = 6 lakes), and metal (Cd, Cu and Zn) and metallothionein (MT) concentrations in the gills of *P. grandis* individuals collected from those lakes (means ± 95% confidence intervals; N = 6 lakes). For ambient free [Cd^{2+}], % of variation was calculated for the period for 1989 to 1998.

the copper smelter contributed less than 30% to the sediment accumulation rates of metals (Cd, Cu, Pb and Zn) (Couillard et al., 2004).

When comparing the relative importance of contaminant declines in the atmospheric, water and biotic compartments (Fig. 4.6), one should keep in mind that the bivalves collected in the various lakes at the start of our survey (in 1989) were

predominantly 8-year-old adults. As a consequence of this, it is highly probable that these specimens were initially exposed to higher free Cd^{2+} ion concentrations than those reported in Table 4-II, and hence the true decrease in Cd^{2+} exposure is probably greater than that indicated in Fig. 4.6. According to the same argument, the extent of reduction of Cd emissions from the smelter is also underestimated.

In contrast to what we found for cadmium, mean calculated free Cu^{2+} concentrations in our group of lakes did not vary significantly over time (–2% between 1989 and 1998, with a 95% confidence interval spanning zero). Given the great variability of the Cu emissions from the Rouyn-Noranda smelter throughout the study period (Fig. 4.2), and because the majority of metal reaching the lakes presumably comes from this aerial source, the above result is not totally surprising. More surprising, given the appreciable decrease in atmospheric Zn emissions, is the fact that we did not observe any significant variation in the calculated free Zn^{2+} concentrations in the various lakes ($-10 \pm 29\%$ between 1989 and 1998). We suspect that the current source of Zn to the lakes is from the catchment soils that have accumulated previously deposited metal and that are being depleted. The above argument inherently assumes that the mobility of Zn in soils differs from that of Cd or Cu.

The temporal trends for Zn concentrations in the bivalve gills (Fig. 4.3C) suggest that *P. grandis* regulates the internal concentrations of this essential metal. This result is consistent with the conclusions of Couillard et al. (1993) and Giguère et al. (2003) who observed that lake-to-lake (i.e., spatial) variations in Zn concentrations in *P. grandis* within the Rouyn-Noranda area were unrelated to the changes in environmental free $[Zn^{2+}]$. Therefore, accumulated Zn levels in these bivalves cannot be used to monitor the temporal changes in Zn contamination that occurred in our group of lakes.

MT as a biomarker for long-term changes in metal levels

Results from the present study demonstrate that changes in MT reflect long-term changes in Cd contamination in lakes. If all lakes are taken into account, the declines in bivalve MT levels are comparable in magnitude to those observed for environmental and accumulated Cd concentrations during the same period (Fig. 4.6). Correlation analysis revealed that gill

MT and gill Cd concentrations were significantly related during the 13-year period of the survey. For nonessential metals such as Cd, binding by MT most likely represents a protection against metal toxicity by limiting metal availability at inappropriate sites (Roesijadi, 1992). Indirectly, MT may be involved in the elimination of Cd through the transfer of Cd-MT complexes to lysosomes (Mason and Jenkins, 1995); the turnover rate of these Cd-laden lysosomes is generally slow, contributing to the long biological half-life of this metal in some organisms. A recent laboratory study conducted on the freshwater clam *Corbicula fluminea* support this hypothesis. During a six month depuration phase, MT concentrations in the soft body of adult specimens of *C. fluminea* pre-exposed to Cd and Zn declined rapidly (i.e., exponentially), whereas Cd levels decreased slowly and progressively with time. In this experiment, the authors determined (using size-exclusion chromatography) that the quantity of Cd sequestered by the MT fraction represented a major portion (~ 40%) of the total Cd accumulated in the whole body of bivalves (Baudrimont et al., 2003).

To determine whether MT levels in *P. grandis* decreased more rapidly than accumulated Cd levels during the chemical recovery of the lakes, we regressed the variations in MT concentrations between 1989 and 2002 against the corresponding variations in Cd concentrations, and interpreted the slope of the regression line. To account for the spatial processes that also influenced this relationship (since we consider six lakes located along a spatial gradient of metal contamination), we used a multiple regression model in which lakes, coded as dummy variables (Legendre and Legendre, 1998), were entered as predictors concurrently with gill Cd. The value of the partial regression coefficient for gill Cd was then assumed to represent the change in the mean response of MT over time per unit increase in accumulated Cd. We found a value of 0.815 (0.320; 1.309) (95% confidence interval of the estimate) for the coefficient, indicating that the deputation kinetics for both MT and Cd are very similar (since the regression coefficient did not differ significantly from 1). The same exercise was repeated for the variations between 1989 and 1997, and the resulting coefficient had a value of 0.437 (0.036; 0.838), suggesting that the depuration rate of MT in bivalves during that period was much slower than that of Cd. The regression coefficient was not significant for the variations between

1989 and 1994 (as shown by the temporal trajectories of the various lakes in Fig. 4.4A). These results contrast markedly with those obtained by Baudrimont et al. (2003) under controlled conditions.

This study suggests that lakes of the Rouyn-Noranda region are recovering from Cd contamination as indicated by the decline in calculated free [Cd^{2+}] at the water-sediment interface and in accumulated Cd levels in indigenous bivalves. This study also provides a strong correlative evidence that long-term trends in gill MT concentrations in *P. grandis* may be useful as predictors of temporal changes in environmental Cd contamination in lakes. Finally, our results reinforce the idea that field and laboratory based studies should be used jointly to properly detect the temporal trends in the health of an aquatic system.

4.6 Acknowledgements

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Chapitre 5

Linking changes in subcellular cadmium distribution to growth and mortality rates in transplanted freshwater bivalves (*Pyganodon grandis*)

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Article en préparation.

5.1 Abstract

The relationships between Cd accumulation and subcellular distribution, and growth and mortality rates were examined in the freshwater bivalve *Pyganodon grandis* in a transplant experiment. Organisms were transferred from a clean lacustrine site to five lakes situated along a Cd concentration gradient in the mining region of Rouyn-Noranda. The bivalves were maintained in open enclosures placed in the bottom sediments of the littoral zone of the lakes for 400 days. At the end of the experiment, metallothionein (MT) was measured in the bivalve gills with a Hg-saturation assay and Cd partitioning among the various cytosolic protein pools was determined by size-exclusion chromatography. Marked differences were observed among the five transplant sites: the range in calculated free-cadmium ion concentrations in water overlying the sediments was 35-fold whereas Cd concentrations in the gill cytosol of the transplanted bivalves varied 3-fold. The distribution of Cd among the various cytosolic complexes in the bivalve gills also varied significantly among sites; at the two most polluted sites, Cd concentrations in the high molecular weight pool (HMW > 25 kDa) were significantly higher than the baseline levels determined from bivalves caged at the reference site, whereas concentrations of the Cd-MT complex were intermediate. As a result, the ratio of nonmetallothionein-bound cytosolic Cd to metallothionein-bound cytosolic Cd, representative of the fraction of the metal available to express toxicity at the cellular level, was higher at these two sites. Reductions in survival were related to this ratio. However, the exact nature of Cd toxicity in *P. grandis* is still unclear and future investigations on the nature of the Cd-HMW complexes are needed.

Keywords: Metallothionein (MT); Cadmium; Subcellular metal partitioning; Transplant experiment; Growth-mortality trade-offs

5.2 Introduction

Contaminants exert their toxicity at all levels of biological organization, from molecules to ecosystems. Undoubtedly, the ultimate levels of concern for ecotoxicologists are populations, communities and ecosystems (Clements and Kiffney, 1994). Indeed, measurements made on higher levels of organization generally provide the best indication of the ecological consequences of exposure to contaminants (Underwood and Peterson, 1988). However, results of ecotoxicological studies on populations or communities are complex and highly variable, and therefore difficult to interpret. One common difficulty in these studies is to distinguish natural changes from those due to pollutants themselves. For example, it can be difficult from a field survey alone to establish the causes of observed changes in the number of species or their abundance, since these latter variables vary in time and space, as a result of density dependence acting through or together with changing levels of food supply, predation, parasitism, intra- or interspecific competition, migration and environmental variables (Sibly, 1999). Conversely, studies at the cellular level can give insights into the mechanistic bases of toxicity, but lower level effects may not be necessarily translated into higher-level effects (see Luoma, 1995). In this context, some authors have recently emphasized the importance of studying organism-level responses, in combination with responses at different levels of organization, to monitor stress in natural environments (e.g., Maltby, 1999). Notably, the examination of the trade-offs between growth and mortality has been shown to be essential to understand how organisms cope with stress (Sibly and Calow, 1989).

Many aspects of metal accumulation in aquatic organisms, including toxicity, can be understood by examining the subcellular distribution of accumulated metal. In invertebrates, metal detoxification processes essentially involve the binding of metals with inducible metal-binding proteins named metallothioneins (MTs), and their sequestration into mineralized concretions (Roesijadi, 1992; Langston et al., 1998; Marigomez et al., 2002; Wallace et al., 2003). With regard to the first detoxification pathway, it has been suggested that excessive accumulation of metals beyond the binding capacity of MT should result in their binding to other intracellular ligands (i.e., non-thionein ligands), and that this

non-specific binding results in metal-induced toxicity at the cellular level (Brown and Parsons, 1978; Mason and Jenkins, 1995). Although the relationships between subcellular metal distribution and toxicity have been extensively studied in aquatic invertebrates, the vast majority of this work was conducted in the laboratory (e.g., Sanders et al., 1983; Sanders and Jenkins, 1984; Jenkins and Mason, 1988; Wallace et al., 2000), and as such, results are often difficult to extrapolate to nature (Luoma, 1995).

In the field, we determined that the freshwater bivalve *Pyganodon grandis* chronically exposed to increasing Cd concentrations exhibited an increase of Cd in the non-thionein ligand pool of the gill cytosol, and that this state was associated with symptoms of cellular toxicity (Giguère et al., 2003). For the same bivalve, we showed that population density, biomass, secondary production, turnover ratio and reproductive success decreased with increased Cd concentration bound to high molecular weight ligands in gill cytosols of the individual molluscs (Perceval et al., 2004). However, it was not possible from this study alone to unequivocally assign to subcellular metal partitioning measurements any predictive role for population health because of the influence of environmental confounding factors. Further research is clearly needed to understand the relationships between the intracellular distribution of Cd and the biological responses in our model organism.

In the present study, we have adopted a field manipulation approach to investigate metal exposure → bioaccumulation → effects relationships in *P. grandis*, specifically focusing on manifestations of toxicity at the organism level as evidenced by alterations in growth and survival. The sub-lethal endpoint of growth is particularly relevant from an ecological perspective since it integrates all physiological processes that occur in an organism (Sheehan, 1984), and it has been linked to ecotoxicological impairment in bivalve populations (e.g., Bayne et al., 1985). In this perspective, we performed a transplant experiment with *P. grandis* in a series of lakes characterized by contrasting Cd levels. Our approach was essentially based on the comparison of the chemical and biological properties of individuals that had been collected from a clean bivalve population and, after randomization and translocation, had subsequently been exposed to different environmental conditions at transplant sites. The primary advantage of conducting transplant experiments

rather than monitoring populations is the increased experimental control while maintaining a high level of environmental realism (de Kock and Kramer, 1994). Here, we used statistically similar groups of experimental animals with regard to population, size, and exposure history. We hypothesized that bivalves transplanted to sites along the Cd exposure gradient would exhibit increased concentrations of Cd in the non-thionein ligand pool, and that this condition would coincide with a decrease in organism growth and an increase in mortality rates.

5.3 Materials and methods

5.3.1 Experimental sites and treatments

Five sites were used for the transplant experiment with one site in each of five lakes (Lakes Opasatica, Joannès, Vaudray, Dasserat and Dufault) located in the mining area of Rouyn-Noranda, northwestern Quebec, Canada. Based on a preliminary sampling (Perceval et al., 2002), we selected five lakes having the widest Cd concentration gradient possible (calculated free Cd^{2+} ion concentrations ranging from ~ 0.05 to 1 nM) from a set of lakes presenting water bodies with comparable trophic status. Lake Opasatica, which acted as the source of experimental animals, is a headwater lake with no point sources of metal pollution and is therefore classified as the reference lake. Lakes Joannès and Vaudray have been polluted by metals via atmospheric transport and are classified here as intermediate-contaminated lakes. Lakes Dasserat and Dufault are subject to point-source and atmospheric metal inputs and are classified as highly contaminated lakes. Table 5-I shows Cd concentrations in water and sediment samples obtained from those lakes during recent sampling campaigns. The experimental sites were situated in the littoral zone of the lakes (in the epilimnion), at a distance of ~ 40 m from the lakeshore. Water depth at all sites was approximately 3 m. To minimize the likelihood of human disturbance, sites were not marked by surface floats or buoys; instead, littoral flags were used to locate them. All sites were characterized by a gentle substrate slope (<5 %) and homogenous sediments, and by the absence of dense beds of macrophytes. All sites contained a few resident *P. grandis*, except for those located in Lakes Dasserat and Dufault.

Table 5-I. Cadmium concentrations in water (means ± SD) and sediments of the study lakes.

	Group				
	Reference	Intermediate-contamination		High contamination	
	L. Opasatica (OP)	L. Joannès (JO)	L. Vaudray (VA)	L. Dasserat (DS)	L. Dufault (DT)
Cd in the dissolved phase (nM)	0.05 (0.01)	0.35 (0.13)	0.68 (0.21)	1.04 (0.33)	3.40 (0.85)
Cd in oxic sediments ($nmol \cdot g^{-1}$ dw)	6.8	27.6	22.4	28.6	515
Calculated free Cd^{2+} ion at the sediment-water interface (nM)	0.03	0.25	0.39	1.08	0.76

Note: Water samples for dissolved Cd concentrations were collected from each lake at a littoral station nearby our experimental sites in June and September 1998 and in June 1999 using *in situ* diffusion samplers installed at ~ 10 cm above the sediments. Sediment samples for the determination of Cd concentrations in the upper (0.5 cm) oxidized sediments were collected from the same locations in June 1998 using hand-held polypropylene corers operated by SCUBA-equipped divers. Cd in sediments was extracted for 6 h at 96°C with 0.04 M $NH_2OH \cdot HCl$ in 25 % (v/v) HOAc and the residue was extracted at 85°C for 5 h with 30 % H_2O_2 adjusted to pH 2. Free Cd^{2+} ion concentrations were calculated from the sediment metal, organic carbon and amorphous iron oxyhydroxide concentrations together with the pH of the overlying water, using a geochemical model based on the sorptive equilibrium of free-metal ions between oxic sediments and water (see Tessier et al. (1993) for a detailed description of the model).

At the beginning of July 1999, SCUBA-equipped divers deployed six 0.72-m^2 enclosures at each of the five transplant sites. Each enclosure consisted of plastic borders arranged in circles of 95 cm diameter, inserted 10 cm in the sediments (leaving a 10-cm wall projecting above the sediment surface) to prevent immigration or emigration of experimental animals. Enclosures were deployed around a central point at each site, in order to minimize environmental heterogeneity among enclosures.

The experiment consisted of transferring *P. grandis* specimens from the reference site (Lake Opasatica) to the experimental sites situated along the Cd exposure gradient (Lakes Joannès, Vaudray, Dasserat and Dufault). In order to assess the baseline levels for accumulated Cd and metallothionein in transplanted *P. grandis*, a group of clams was caged in the source lake, Lake Opasatica. Previous experiments using freshwater bivalves

showed that a post-transplant period of more than one year is usually necessary for the influence of the destination environment to surpass that of the source environment (e.g., Hinch and Green, 1989). Therefore, *P. grandis* specimens were exposed for a period of 400 days (from to July 1999 to September 2000) at the various sites.

5.3.2 Bivalve collection, processing and deployment

In this experiment, it was impractical to standardize the transplanted bivalves by genotype. However, care was taken to collect all *P. grandis* specimens from the same site in the same lake and to randomly assign the collected clams to their transplant destinations. Thus, the genetic differences among the source populations are likely reduced, and genetic variation will not be confounded with destination (site) effects.

On July 21, 1999, SCUBA divers collected ~ 900 actively filtering bivalves of a size range representative of the natural assemblage (i.e., shell lengths ranging from 51.15 to 92.87 mm) from a single littoral site in Lake Opasatica, in an area known to abound in *P. grandis*. In this lake, maximum densities of *P. grandis* exceeded 6 individuals·m^{-2}, with an average of one individual·m^{-2} in depths less than 6 m (Perceval et al., 2004). Each collected clam was marked in the boat by attaching a numbered plastic label (Dymo™) to the posterior face of one valve with underwater glue (PC-Marine Putty Epoxy™). Following marking, bivalves were measured along the axis of maximum growth (to the nearest 0.01 mm), using a digital vernier caliper (Mitutoyo™). To minimize the stress associated with air exposure, marked animals were returned to the water within 5 minutes of collection. They were held inside temporary enclosures in the littoral zone of L. Opasatica for one week, until their deployment at the various transplant sites. Twelve bivalves of similar size (70.12 ± 1.03 mm; mean ± 95 % confidence interval) were retained for the determinations of initial metallothionein and accumulated Cd concentrations and subcellular distribution of Cd before deployment; these individuals were transported to the field laboratory in coolers filled with lake water, and sacrificed within 12 h after collection for gill preparation.

A total of 760 bivalves were used in this experiment. Power analyses indicated that about 100 individuals were necessary to achieve the power to detect statistically significant differences in growth, when growth rates differed by only 25 % (e.g., Salazar and Salazar, 1995). Here, we randomly assigned a group of 152 bivalves to be transplanted to each of the five experimental sites: three enclosures received 32 animals each, yielding a density of 44 individuals·m^{-2} (referred to as high density treatments in the rest of the text), and the three others received 8 animals each, yielding a density of 11 individuals·m^{-2} (referred to as natural density treatments) comparable with that of the indigenous populations from L. Opasatica. We manipulated bivalve density in our enclosures primarily to assess the effects of crowding on growth and mortality. Additionally, to test whether maintaining the clams within the enclosure had an influence on their metallothionein and Cd concentrations, and on shell growth and mortality rates, 32 marked *P. grandis* per site were "replanted" in their natural living position outside the experimental cages (i.e., non-caged bivalves). Following the procedures outlined in Salazar and Salazar (1995), size-frequency distributions of bivalves were kept similar across all sites and across all enclosures: a nested analysis of variance (ANOVA) confirmed that there were no significant differences in mean shell lengths among sites ($F_{4,725} = 0.047$; $P = 0.996$) or among enclosures ($F_{30,725} = 0.030$; $P = 1.000$). At the end of bivalve deployment, upper parts of enclosures were covered with a rigid plastic screen (mesh size 5.5×4 cm) to deter muskrat predation.

5.3.3 Water and sediment sampling

To evaluate the habitat quality and monitor food availability for transplanted bivalves, experimental sites were sampled on four occasions in the summers of 1999 and 2000. Water samples were collected in polyethylene bottles at ~ 30 cm above the sediments by SCUBA divers and were analyzed using the methods described in Perceval et al. (2002). Measured properties included Secchi depth, dissolved oxygen at the bottom (O_2), pH, specific conductivity (Cond), dissolved calcium (Ca), dissolved organic carbon (DOC), total phosphorus (TP), chlorophyll *a* (Chl *a*), total suspended solids (TSS) and carbon and nitrogen content in seston (Sest C and Sest N). Before filtration, water samples for chlorophyll *a* and sestonic C and N analyses were run through a Nitex sieve to remove the

>80-µm fraction; analysis of gut contents of another unionid bivalve, *Elliptio complanata*, showed that 90 % of the ingested particles were smaller than 80 µm (Tessier et al., 1984). Water temperature was recorded at each site at 5-h intervals for the duration of the experiment using *in situ* computerized data loggers placed in waterproof containers and fixed to a plastic rod approximately 30 cm from the sediment surface. Degree-days available for bivalves between July 1999 and September 2000 were calculated by the rectangular method as described in Young and Young (1998).

Substrate type has been shown to affect growth and shell allometry of bivalves (Hinch et al., 1986). To evaluate the influence of sediment characteristics on shell growth rates of transplanted bivalves, SCUBA divers collected three sediment cores at each site (July 1999), using hand-held polypropylene corers (20 cm deep, 47.4 cm^2 area). The sediment cores were extruded in the boat and the top 10 cm of each sample was transferred in an individual plastic bag. Sediments were kept at ~ 4°C during transport to the laboratory where they were stored at –20°C until analysis. We used the ASTM method D422-63 to determine the particle-size distribution in each sediment sample (ASTM, 1985).

5.3.4 Bivalve retrieval and analyses

On day 400 (September 2000), bivalves were retrieved from the experimental sites by SCUBA divers, placed in coolers filled with lake water, and transported to the field laboratory where they were processed within 12 h after collection. Of the 760 bivalves used in the experiment, 218 died and 57 were not recovered. The majority (i.e., 53) of missing clams were those initially transplanted outside the experimental cages. Upon return to the laboratory, all surviving specimens were measured for total shell length (0.01 mm accuracy). For each site, a sub-sample of nine *P. grandis* of similar size (nominal shell length 70 mm, actual mean shell lengths: 69.79 ± 0.97 mm, 69.23 ± 1.23 mm, 69.88 ± 1.04 mm, 71.80 ± 2.96 mm and 68.21 ± 7.90 mm for OP, JO, VA, DS and DT, respectively) were randomly selected from enclosures with high densities (whenever possible, we selected three animals from each of the three enclosures with high densities) and three others were chosen among the non-caged bivalves for chemical analyses. Gills from each

selected clam were manually isolated with a scalpel. Assuming that metal uptake and metal partitioning are similar in each of the demibranchs that composed the gills of *P. grandis*, we used one part of these large lamellae for metallothionein (MT) quantification and the other part for determining bioaccumulated Cd concentrations and sub-cellular Cd partitioning. Gill tissues from three individuals were pooled, frozen in liquid nitrogen, sealed in plastic bags filled with nitrogen, and stored at –80°C until the homogenization step. This procedure provided three replicate samples for each site as well as one control for caging effects for all measurements. MT analyses were carried out 9 months after collection whereas measurements of sub-cellular partitioning were done within 16 months of collection.

Partially thawed gill tissues were gently homogenized under a nitrogen atmosphere with a motor-driven glass tissue grinder (Dual Co.). To limit organelle disruption, homogenization was limited to 80 rev/min. Homogenization was performed with ice-cold 25 nM Tris buffer (OmniPur) adjusted to pH 7.2; the tissue-to-buffer ratio was adjusted to 1:19 (wet weight tissue:weight of buffer) for MT measurements and to 1:3 for the determinations of accumulated Cd concentrations and subcellular Cd partitioning. A sub-sample (~ 1 mL) of each gill homogenate was dried in an oven at 65°C for 48 h to determine the dry to wet weight ratio.

MT concentration in gills

Gill homogenates (5 mL) were centrifuged at 30,000×*g* for 30 min at 4°C, and the supernatants were divided into four analytical replicates that were analyzed the same day for MT with a Hg-saturation assay adapted slightly from Dutton et al. (1993) and described in detail in Couillard et al. (1993). As a quality control, recovery of a MT standard (MT from rabbit liver, Sigma Chemical Co.) was determined with every assay; the mean recovery for 4 separate determinations was 87.8 ± 4.7 % (mean ± SD). Metallothionein concentrations are mean values of both field and analytical replicates and are expressed as nanomoles of Hg-binding sites per gram of dry tissue weight.

Cd concentrations in gill cytosols

Homogenate sub-samples (1 mL) were ultracentrifuged at 160,000×*g* for 1 h at 2°C and the supernatants filtered through Acrodisk filters (0.45-µm porosity filters, Gelman, usually used as prefilters before ion chromatography). Filtrate supernatants were then acidified with HNO_3 (final acid concentration 0.5 %, v/v) and analyzed for Cd concentrations by atomic emission spectrometry (ICP-AES: Varian Vista). The recovery of Cd in spiked samples was within 95% of the amount added.

Determination of Cd partitioning in gill cytosols

Sub-samples (155 µL) of the 160,000×*g* gill cytosol supernatants obtained above (non-acidified) were fractionated by high-performance liquid chromatography (HPLC, Waters Action Analyzer Chromatograph, equipped with a Waters 996 photodiode array detector) on a steric exclusion column (BIOSEP-SEC-S 2000; 30 × 0.75 cm). The column was eluted with a mobile phase of 10 nM Tris, 100 mM NaCl, and 0.03 % NaN_3, adjusted to pH 7 at a flow rate of 0.5 $mL \cdot min^{-1}$. The eluant was degassed with helium before each use and the HPLC column was washed periodically with an injection of 1 mM EDTA in the same mobile phase.

The column was calibrated for molecular weight estimations using dextran (2 000 kDa, V_0 = 5.7 mL), bovine albumin (66 kDa), egg albumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa), myoglobin (17 kDa), ribonuclease A (13.7 kDa), vitamin B_{12} (1355 Da) and tryptophan (204 Da) as standard markers. Eluting fractions (1 mL) were collected automatically up to a volume of 20 mL and combined into three metal-ligand pools: a high molecular weight (HMW) pool (255-25 kDa), eluting from 10 to 16 min after injection, a MT-like pool (25-1.3 kDa), eluting from 16 to 24 min after injection and a low molecular weight (LMW) pool (<1.3 kDa), eluting from 24 to 40 min after injection. Pooled fractions were then analyzed for Cd by graphite furnace atomic absorption spectrophotometry (GFAAS: Perkin-Elmer model Simaa 6000 with Zeeman background correction and a Perkin-Elmer model AS-72 automatic sampler). A matrix modifier (0.1% Pd and 0.06% $Mg(NO_3)_2$) was added to the samples before measurements. Analytical procedural blanks and certified reference water samples (NIST 1643d and 1640d, National

Institute of Standards and Technology, Gaithersburg, MD, USA) were analyzed during each analytical run. Procedural blanks indicated no appreciable contamination (N = 52: below the detection limit) and certified samples were quantitatively recovered (N = 36: 108.1 ± 10.3 % and 107.3 ± 7.2 % for NIST 1643d and 1640d, respectively).

As a final quality control measure, we compared the sum of the Cd quantity recovered in the three metal-ligand pools with the initial total metal quantity in the gill cytosol as determined in the 1-mL sub-sample removed from the original homogenate, before the chromatographic separation. Agreement between the two values was consistently good (N = 25: 119 ± 11 %).

5.3.5 Data analyses

We used one-way analyses of variance (ANOVA) to test for differences among transplant sites for MT and bioaccumulated Cd concentrations and for Cd concentrations in each of the three metal-ligand pools in bivalve gills. Following ANOVA, Dunnett's tests were used to identify which sites differed from the reference site (L. Opasatica). We used the $\log_{10}$-transformed values of the response variables for all analyses.

Bivalve growth was calculated from direct measurement of changes in shell lengths during the 400-day experimental period. To evaluate the precision of shell measurements, we determined the 95 % range of all measurement errors by re-measuring 10 *P. grandis* of a range of shell sizes (58 to 89 mm) 20 times each, in random order: the measurement error found in blind repeated trials was ~ 1 mm in 95 % of the 200 trials. An analysis of covariance (ANCOVA) was used to test for differences among transplant sites, density treatments and enclosures in growth of *P. grandis*. Because the growth rates of unionids have been shown to be dependent upon individual size (Hinch et al., 1986) and because all individuals were not the same size at the beginning of the experiment, the initial shell length was used here as the covariate. Following ANCOVA, we used the Scheffé's multiple range test to identify which sites differed from each other. The significance level of Scheffé's test is designed to allow all possible linear combinations of group means to be

tested. Therefore, the Scheffé's test is necessarily more conservative (i.e., a larger difference between means is required for significance) than other post-hoc tests (Day and Quinn, 1989). Both the response variable and covariate were $\log_{10}$-transformed to meet the requirements of normally distributed and homoscedastic residuals.

Some clams in each of the 5 transplant sites exhibited negative growth during the period of the experiment. Although we acknowledge that some decreases in shell length could have arisen because of measurement error, some have been attributed to actual shell shrinkage in *P. grandis* (Downing et al., 1992; Downing and Downing, 1993). We therefore included all such negative measurements in our analysis.

To determine whether the transplant destination and crowding were significant sources of variation in bivalve mortality, we computed a two-way factorial ANOVA with the arcsine-transformed values (Sokal and Rohlf, 1995) of the percentage of individuals that were found dead in each enclosure at the experiment's end as the dependent variable and the transplant site, the initial density of bivalves in enclosures, and the site×density interaction as the fixed factors. Since the fate of too many control bivalves could not be positively confirmed (i.e., animals may have died, been preyed upon or migrated out of the search area), we did not evaluate the effects of maintaining the clams inside the experimental cages on mortality rates.

Relationships between variables were tested using simple ordinary least-squares regression and correlation (Pearson's *r*) analyses.

5.4 Results and discussion

Results of physico-chemical measurements made on water and sediment samples from the various sites during the transplantation phase are shown in Table 5-II. All the lakes selected for transplant destinations have circumneutral pH and low to moderate conductivity. They are all oligotrophic, with mean total phosphorus (TP) concentrations < 5 $\mu g \cdot L^{-1}$ and chlorophyll *a* biomass ~ 1$\mu g \cdot L^{-1}$. Water overlying the sediments was well oxygenated at all

Table 5-II. Physical and chemical properties of water and sediments at the 5 lacustrine sites chosen for the transplant experiment.

	Group				
	Reference	Intermediate-contamination		High contamination	
	L. Opasatica	L. Joannès	L. Vaudray	L. Dasserat	L. Dufault
Water quality variables					
Secchi depth (m)	1.6	1.4	2.1	2.4	2.4
Bottom O_2 ($mg \cdot L^{-1}$)	9.2 (1.0)	9.4 (1.4)	8.9 (1.2)	9.0 (0.4)	8.7 (0.2)
Degree-days	1376	1272	1266	1389	1344
pH[a]	7.25–7.86	7.24–7.71	7.10–7.49	6.90–7.72	7.19–7-43
Conductivity ($\mu S \cdot cm^{-1}$)	97 (14)	63 (10)	36 (7)	83 (15)	159 (27)
Ca ($mg \cdot L^{-1}$)	9.2 (0.7)	7.7 (0.7)	3.5 (0.4)	9.3 (0.4)	19.3 (0.3)
DOC ($mg \cdot L^{-1}$)*	7.0 (0.5)	10.5 (0.6)	8.0 (0.1)	5.5 (0.4)	5.1 (0.4)
TP ($\mu g \cdot L^{-1}$)*	3.0 (0.9)	2.2 (0.4)	1.4 (0.1)	2.8 (0.04)	1.9 (0.4)
Chl *a* ($\mu g \cdot L^{-1}$)	0.68 (0.16)	1.22 (0.89)	0.72 (0.19)	1.13 (0.36)	0.88 (0.29)
TSS ($mg \cdot L^{-1}$)	4.79 (1.09)	3.23 (1.65)	1.52 (0.39)	2.76 (0.38)	2.36 (0.51)
Sest C ($mg\ C \cdot L^{-1}$)	0.35 (0.05)	0.41 (0.11)	0.36 (0.13)	0.43 (0.05)	0.44 (0.03)
Sest N ($mg\ N \cdot L^{-1}$)	0.055 (0.006)	0.057 (0.013)	0.052 (0.027)	0.057 (0.005)	0.058 (0.009)
Sedimentary variables					
% Sand (>53 μm)	34.9 (8.8)	39.8 (2.3)	79.5 (4.1)	62.3 (1.2)	60.7 (5.5)
% Silt (>2 and <53 μm)	52.9 (7.5)	58.0 (3.0)	19.0 (3.0)	17.5 (0.6)	39.3 (5.5)
% Clay (<2 μm)	12.2 (1.4)	2.1 (1.0)	1.5 (1.2)	20.1 (1.1)	0.0 (0.0)

Note: Water samples were collected by SCUBA divers at ~ 30 cm above the bottom sediments in polyethylene bottles. Values for water quality variables are means (± SD) based on 4 sampling dates in the summers of 1999 and 2000. Degree-days were calculated from daily minimum and maximum temperatures recorded continuously (every 5 hours) between July 1999 and September 2000 using miniature data loggers. Particle size for the top 0-10 cm sediments was determined as the mean (± SD) of three replicate samples collected in July 1999.

[a]Range.

*DOC and TP were measured only once during each summer.

sites. Chlorophyll *a*, and sestonic C and N analyses revealed little difference among the five sites with regard to the quantity and quality of food available for bivalves (the ranges in Chl *a*, Sest N and Sest C concentrations were 1.8-, 1.3- and 1.1-fold, respectively). In contrast, dissolved Ca concentrations exhibited appreciable variability among the sites (the range in Ca concentrations was nearly 6-fold). Sediment grain size also varied among the sites (Table 5-II). Although attempts were made to locate areas with similar substrate type, sites in Lakes Opasatica and Joannès had muddy sediments (> 60 % silt and clay) whereas those located in L. Vaudray, Dasserat and Dufault had sandy sediments (60–80 % sand).

Cd accumulation and subcellular distribution in transplanted bivalves

Transplanting bivalves from one place to another implies that they are disturbed (handled, marked and caged) and moved. Therefore, treatments to evaluate the effect of moving and disturbing the animals need be included in transplant experiments. In the present study, effects of caging were evaluated by transplanting a group of clams outside the enclosures in each site. With the exception of clams transplanted to L. Dasserat, gill Cd and MT concentrations in non-caged bivalves were generally comparable with those measured in caged animals (Fig. 5.1), suggesting that caging procedures had only a minor influence on metal accumulation and MT synthesis in *P. grandis* in our experiment. Effects of moving were estimated (indirectly) by collecting bivalves from the reference site in L. Opasatica and then caging them at the same site. Prior to deployment, bivalves from L. Opasatica had mean gill cytosolic Cd and MT concentrations of 39 nmol·g^{-1} dry weight and 74 nmol Hg-binding sites·g^{-1} dry weight, respectively (Fig. 5.1). These concentrations did not differ significantly (*t* test; $P > 0.05$) from those measured in gill samples of bivalves after a 400-day exposure at the same site (mean cytosolic Cd and MT concentrations were 36 nmol·g^{-1} dry weight and 64 nmol Hg-binding sites·g^{-1} dry weight, respectively). Assuming that no major changes in ambient metal concentrations occurred in L. Opasatica during the experiment, these results indicate that moving and disturbing (i.e., caging) the clams had no significant effects on their MT or bioaccumulated Cd concentrations. Similarly, there were no significant effects of moving the animals on their subcellular Cd distribution (*t* test; $P > 0.05$ for each gill cytosolic fraction).

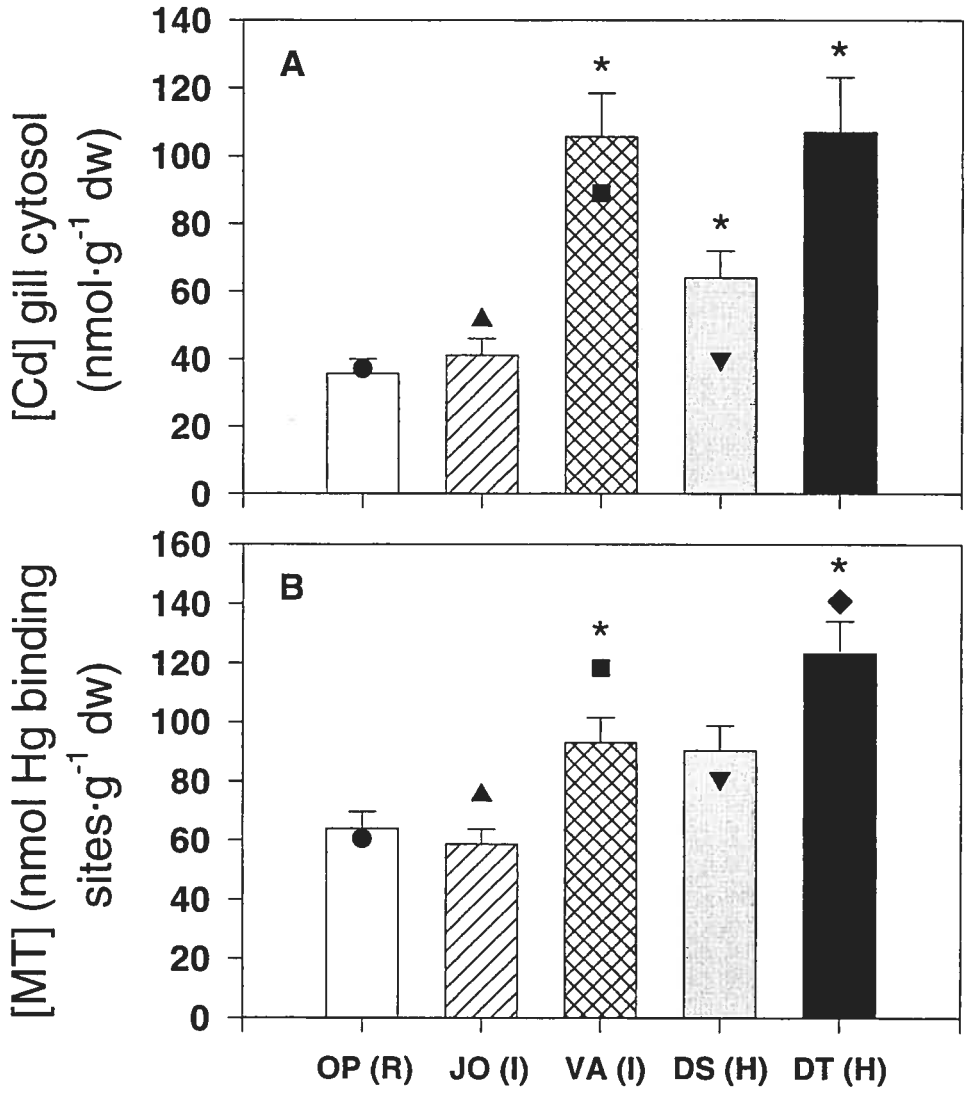

Figure 5.1. A) Cd and B) metallothionein (MT) concentrations in the gill cytosol of *P. grandis* specimens transferred from a clean lacustrine site to five lakes situated along a Cd exposure gradient. Bars represent geometric means (+ 1 SE) calculated from least squares means and back-transformed; $n = 3$ replicate samples of three pooled individuals each for each site. Bars marked with * are significantly different ($P < 0.05$; Dunnett's comparison test) from the control (OP). Black symbols above the bars represent the values of accumulated Cd levels and MT concentrations for bivalves transplanted outside the experimental cages ($n = 1$ composite sub-sample of 3 individuals). Sites were ranked as reference (R), moderately contaminated (I) or highly contaminated (H) according to the concentrations of Cd in water and sediments.

Accumulation of Cd by clams depended upon transplant destination ($F_{5,11}$ = 17.2; P < 0.0001) and generally increased with water and sediment Cd exposure (Fig. 5.1A and Table 5-I). Gill cytosolic Cd concentrations for bivalves transplanted to L. Vaudray, Dasserat and Dufault were respectively 3-, 1.8- and 3-fold higher than the baseline levels of Cd determined from clams caged at the reference site. Surprisingly, bivalves from L. Vaudray exhibited levels of Cd that were comparable with those observed in bivalves from two most contaminated sites despite lower Cd exposure. This result may be partly explained by the remarkably low dissolved Ca concentrations measured in L. Vaudray during the experiment (Table 5-II). Indeed, we know from previous work conducted in lakes of the Rouyn-Noranda region that Cd accumulation in *P. grandis* generally decreased with increasing Ca concentrations in lake water (Perceval et al., 2002), presumably due to the competitive interaction between Ca^{2+} and Cd^{2+} at gill binding sites. Total gill MT concentrations in bivalves after a 400-day exposure were also significantly different among transplant sites ($F_{5,11}$ = 9.3; P = 0.0012) (Fig. 5.1B), and they were directly related to accumulated Cd concentrations (Fig. 5.2). Several previous studies have reported a linear relationship between concentrations of MT and Cd in *P. grandis* (e.g., Wang et al., 1999; Giguère et al., 2003) and other aquatic invertebrates (Mouneyrac et al., 2000; Croteau et al., 2002; Bebianno and Serafim, 2003).

The comparison of our results with those from a recent study by Giguère et al. (2003) indicated that *P. grandis* specimens transplanted to L. Joannès and Vaudray did not reach the Cd and MT concentrations of the indigenous populations even after 400 days, suggesting that steady-state between internal and external media was not reached. This result demonstrates that transplanted bivalves may not be a perfect surrogate for natural populations after relatively short term exposures (compared to the lifetime exposure of natural populations). It should also be mentioned that the low water temperatures prevailing during the winter period in our study lakes must have had an impact on the metabolic activity of the transplanted bivalves, potentially limiting metal accumulation (Fraysse et al., 2000).

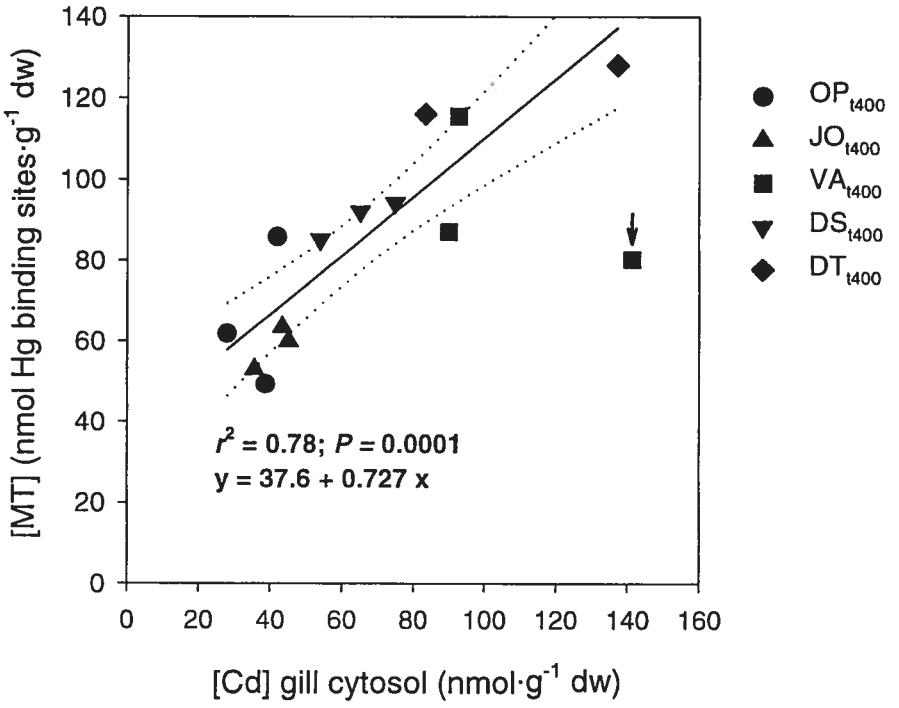

Figure 5.2. Relationships between metallothionein (MT) concentrations and cytosolic Cd in samples of gill tissues of transplanted bivalves. Each point represents data for a composite sub-sample of three individuals. The ordinary least squares regression line is shown with 95 % confidence interval; the data point indicated by an arrow is excluded from the analysis.

Results from subcellular Cd partitioning showed that the majority of Cd in the gill cytosol of transplanted bivalves was always associated with MT-like proteins (Fig. 5.3). Conversely, Cd was never detected in the low molecular weight (LMW) ligand pool. The concentration of Cd found associated with high molecular weight (HMW) fraction varied significantly among sites ($F_{5,11}$ = 14.6; P = 0.0002), and systematically increased as environmental Cd concentration increased (Fig. 5.3, Table 5-I): Cd-HMW concentrations in bivalves from L. Joannès, Vaudray, Dasserat and Dufault were, respectively, 1.5, 3, 3.1 and 5× higher than those measured in clams from the reference site. In contrast, bivalves from the two most contaminated sites did not exhibit the highest concentrations of Cd-MT complex (Fig. 5.3), and as a consequence of this, the non-MT-

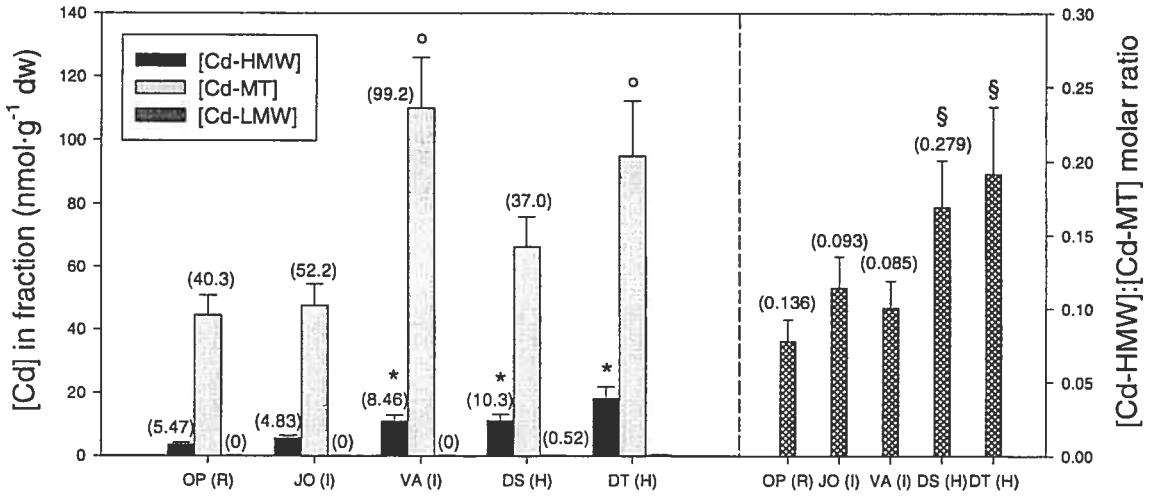

Figure 5.3. Sub-cellular distribution of Cd in the gill tissues of *P. grandis* specimens transferred from a clean lacustrine site to five lakes situated along a Cd concentration gradient. Values of the molar ratios of nonmetallothionein-bound cytosolic Cd (mainly Cd-HMW) to metallothionein-bound cytosolic Cd are also shown. Bars represent geometric means (+ 1 SE) calculated from least squares means and back-transformed; n = 3 replicate samples of three pooled individuals each for each site (except for DT for which n = 2 replicates). Values in parentheses show results of sub-cellular Cd partitioning measurements in non-caged bivalves (n = 1 composite sub-sample of 3 individuals). Bars for the HMW fraction marked with * are significantly different ($P < 0.05$; Dunnett's comparison method) from the control (OP); bars for the MT-like fraction marked with ° are significantly different ($P < 0.05$; Dunnett's comparison method) from the control (OP); bars for the Cd-HMW to Cd-MT ratio marked with § are significantly different ($P < 0.05$; Dunnett's comparison method) from the control (OP). There were no significant differences among the various sites for Cd concentrations in the LMW pool (one-way ANOVA: $F_{5,11}$ = 1.183; P = 0.3774). Sites were ranked as reference (R), moderately contaminated (I) or highly contaminated (H) according to the concentrations of Cd in water and sediments.

bound Cd (primarily Cd-HMW) to MT-bound Cd ratio in these specimens was significantly higher (Dunnett's test; $P < 0.05$) than that of bivalves from the control site. In the present study, the [Cd-HMW]:[Cd-MT] ratio represents the fraction of Cd available to express cellular toxicity and we assume that higher [Cd-HMW]:[Cd-MT] ratios correspond to less effective metal detoxification. Conversely, lower ratios suggest effective MT induction in an effort to protect more sensitive enzyme systems.

Although there is some experimental evidence that excessive Cd accumulation in aquatic invertebrates could result in increasing concentrations of this metal in the HMW ligand pool (e.g., Jenkins and Sanders, 1986; Wallace et al., 2000), our results contrast markedly with those from an earlier transplant experiment in which *P. grandis* was similarly transferred from L. Opasatica to L. Vaudray (Couillard et al., 1995). In this earlier experiment, Cd partitioning in the gill cytosol changed markedly during the exposure period, and after 400 days, the majority (i.e., 74 %) of cytosolic Cd was bound to LMW components, whereas only a small proportion of Cd was bound to the MT and the HMW fractions (12 and 14 %, respectively). It should be noted, however, that the ambient free Cd^{2+} ion concentrations reported in that study were somewhat higher (approximately ×2) than those observed at our most contaminated site. Moreover, in contrast to our study, the chromatographic profiles of Cd in gill cytosols of bivalves were obtained from a single composite sample, and the recoveries of Cd after HPLC fractionation were highly variable (e.g., 77–155% of total cytosolic Cd concentrations as judged from the mass balance calculations), therefore necessarily limiting the scope of the conclusions.

Also contrasting with the results of the study of Couillard et al. (1995), our experimental clams did not demonstrate a radical shift in the subcellular partitioning of Cd in the gill cytosol at the end of the experiment. Although the amount of Cd bound to the MT-like and HMW fractions increased along the Cd exposure gradient, there was, indeed, no apparent shift from one subcellular compartment to another (Fig. 5.4).

Relationships between Cd accumulation by bivalves and growth and mortality rates

Analysis of covariance indicated that mean shell growth varied significantly among all five transplant sites during the 400-day exposure period ($F_{4,363}$ = 37.3 ; $P < 0.0001$). The statistically significant site×density interaction ($F_{4,363}$ = 10.4; $P < 0.0001$) indicated that the effects of crowding on bivalve growth changed with site: increasing bivalve density in enclosures depressed growth for specimens transplanted in L. Joannès but

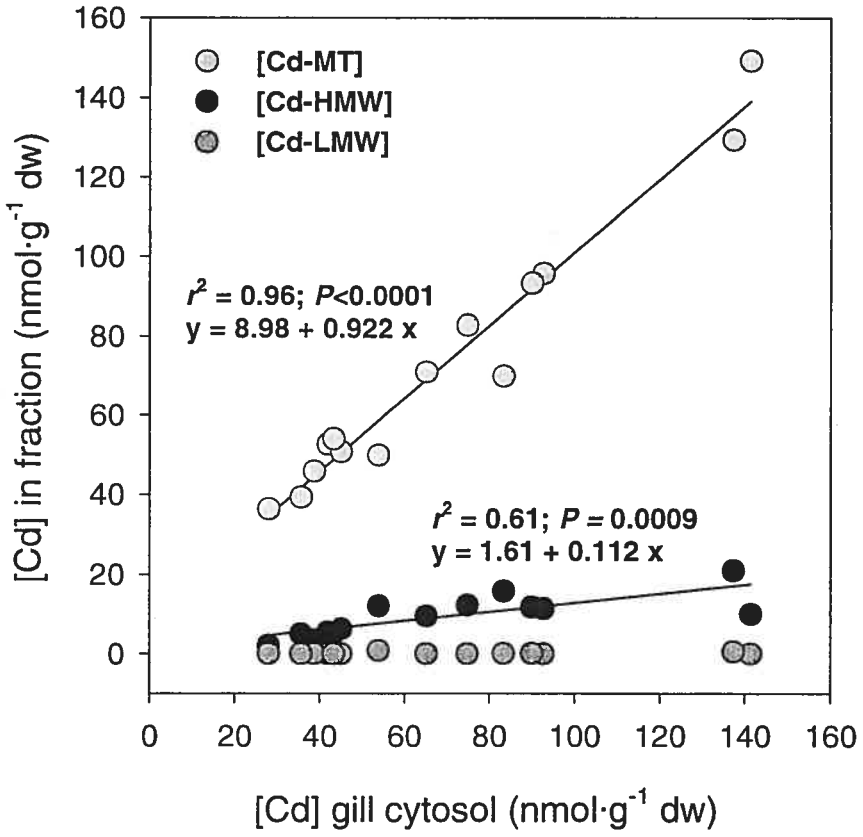

Figure 5.4. Relationships between gill cytosolic metal concentrations and Cd concentrations in three-metal-ligand pools identified as Cd-MT (the metallothionein-like pool), Cd-HMW (the high molecular weight pool) and Cd-LMW (low molecular weight pool) in transplanted bivalves. Each point represents data for a composite sub-sample of three individuals. Plotted lines were obtained by ordinary least squares regression.

enhanced growth for those in L. Dasserat (Fig. 5.5). Overall, there was a net shell increment for bivalves transplanted to the two intermediate-contaminated sites (Fig. 5.5). In these lakes, growth rates were comparable to those reported for the same species in oligotrophic pristine lakes (Downing and Downing, 1993). Surprisingly, we did not observe any significant change in length over the duration of the experiment for clams caged at the reference site, suggesting that bivalves experienced stressful conditions at this site. Although we have measured a number of environmental factors that have the potential to influence bivalve growth (such as food availability and water temperature) (Table 5-II),

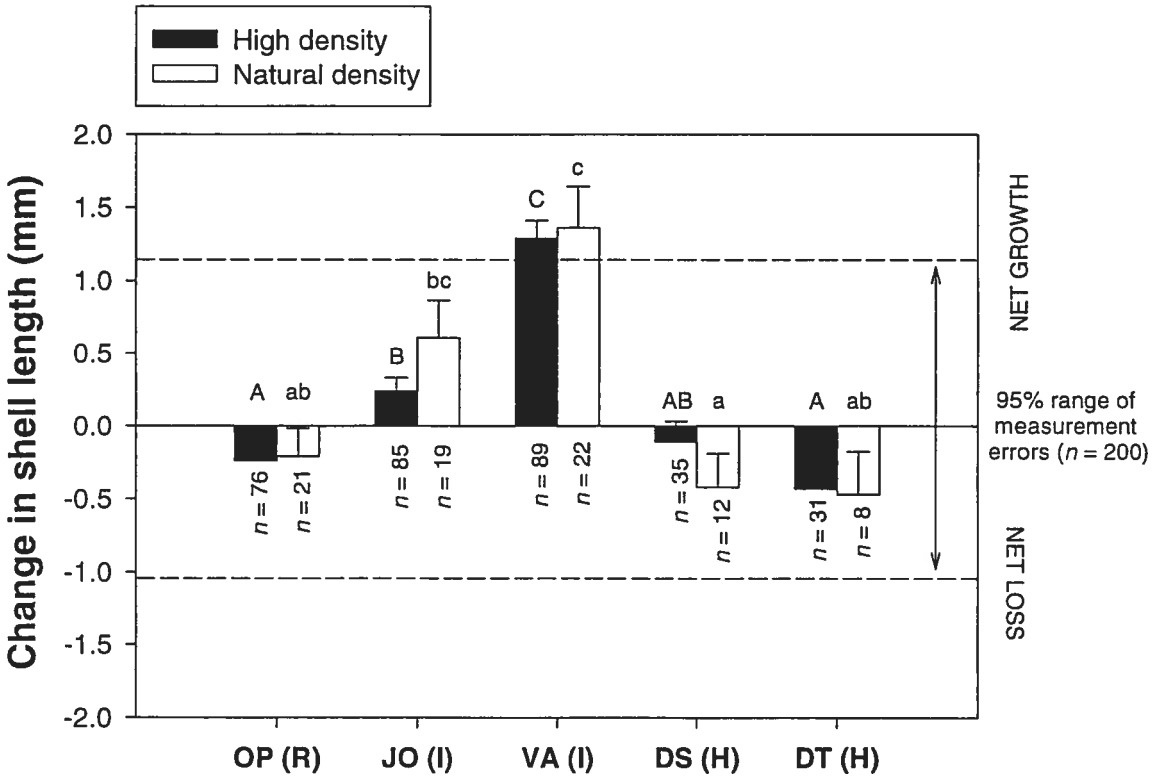

Figure 5.5. Shell growth of *P. grandis* (determined by direct measurement of changes in size of individuals) at the various transplant sites during the 400-day period of the experiment. Solid bars represent length-adjusted means (+ SE) for shell length variations in enclosures with high densities, open bars represent length-adjusted means for shell length variations in enclosures with natural densities. For high density treatments, means were adjusted to an initial shell length of 68.30 mm, whereas for natural density treatments, means were adjusted to an initial length of 68.94 mm. For each density treatment, the main effect of site was tested using the cage(site) mean square for the error term with 10 degrees of freedom; significant differences among sites are indicated by different letters ($P < 0.05$; Scheffé's multiple range test). Horizontal dashed lines show limits of 95 % of all measurement errors determined by re-measuring 10 *P. grandis* specimens of a range of shell sizes 20 times each, in random order. Sites were ranked as reference (R), moderately contaminated (I) or highly contaminated (H) according to the concentrations of Cd in water and sediments.

none of them could be positively related to growth inhibition in L. Opasatica (Pearson's correlation; $P > 0.05$ for all pairwise comparisons with environmental variables). Bivalves transplanted to the two highly contaminated sites did not grow significantly (Fig. 5.5).

Analysis of mortality rates using a two-way factorial ANOVA showed a strong effect of the destination site ($F_{4,20} = 28.6$; $P < 0.0001$). There was no statistically significant difference in mortality rates between density treatments ($F_{1,20} = 1.3$; $P = 0.2705$), indicating that maintaining the clams in enclosures at densities that are well above (i.e., more than 4×) the observed natural levels had no influence on their survival. Mortality of clams during the experiment was consistently low (i.e., ranging from 8 to 17 %) for bivalves transplanted to both the reference and intermediate-contaminated sites but increased significantly (Dunnett's comparison with the control; $P < 0.05$) at the two highly contaminated sites, where mean mortality rates exceeded 55% (Fig. 5.6).

Interestingly, of all the experimental clams, the individuals transplanted in L. Vaudray had the highest Cd-MT concentrations and exhibited the most successful growth and the lowest mortality rates. This strongly suggests that Cd binding to MT serves as a protective measure against Cd exposure. Conversely, we found that reductions in bivalve survival were positively correlated with increasing HMW-bound Cd to MT-bound Cd ratio (Fig. 5.7). This indicates that excessive accumulation of Cd in the heterogeneous high molecular weight pool, often referred as the metalloenzymes-containing pool, could be critical to *P. grandis*'s survival. Other studies have described relationships between the binding of metal to the various protein fractions and toxic effects. For example, reductions in prey capture efficiency in the grass shrimp *Palaemonetes pugio* were significantly related to Cd concentrations in high molecular weight proteins (Wallace et al., 2000). Similarly, reductions in the growth rate of fish (*Oncorhynchus keta*) and zooplankton coincided with increases of Hg in the HMW pool (Brown and Parsons, 1978). In those studies, the binding capacity of metallothionein was always surpassed, as evidenced by ratios of non-MT bound metal to MT-bound metal typically > 1. This was likely not the case in our experiment. The onset of toxicity in metal-exposed organisms, however, is not always associated with the saturation of MT. For example, Hamilton et al. (1987) observed mortality rates as high as 35% for brook trout exposed to water-borne Cd, while the amounts of Cd bound to MT were in general more than an order of magnitude below the total Cd-binding capacity of the MT. In that study, the authors found that mortality rates of

fish were well correlated with the amount of cadmium bound to non-thionein ligands in the tissues.

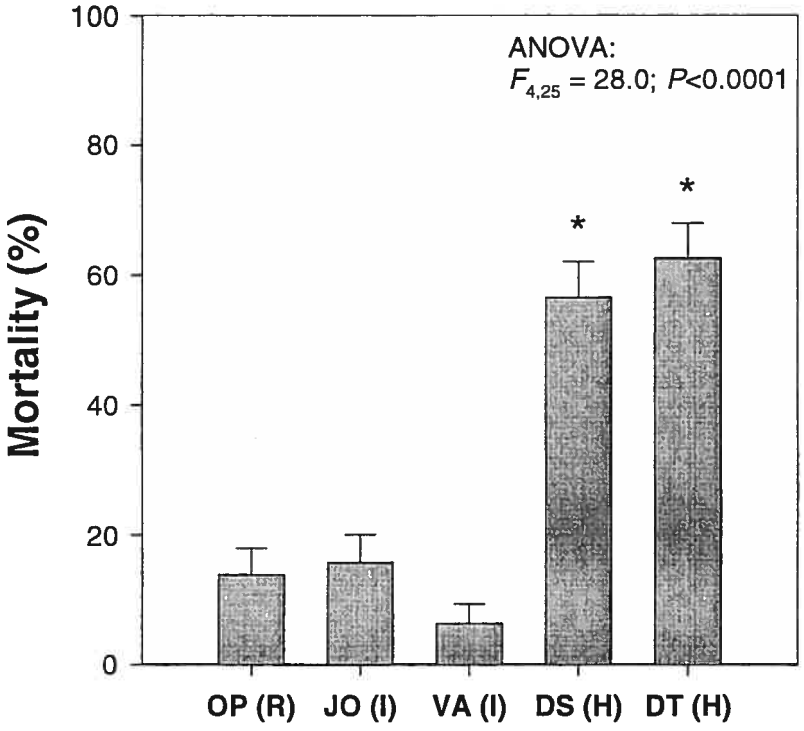

Figure 5.6. Proportion of *P. grandis* specimens that were found dead in enclosures at the end of the transplant experiment at each of the 5 lacustrine sites, both density treatments combined. Bars represent means (+ 1 SE) back-calculated from arcsine-transformed values; n = 6 independent experimental units (enclosures) for each site. Bars marked with * are significantly different (P < 0.05; Dunnett's comparison method) from the control. Sites were ranked as reference (R), moderately contaminated (I) or highly contaminated (H) according to the concentrations of Cd in water and sediments.

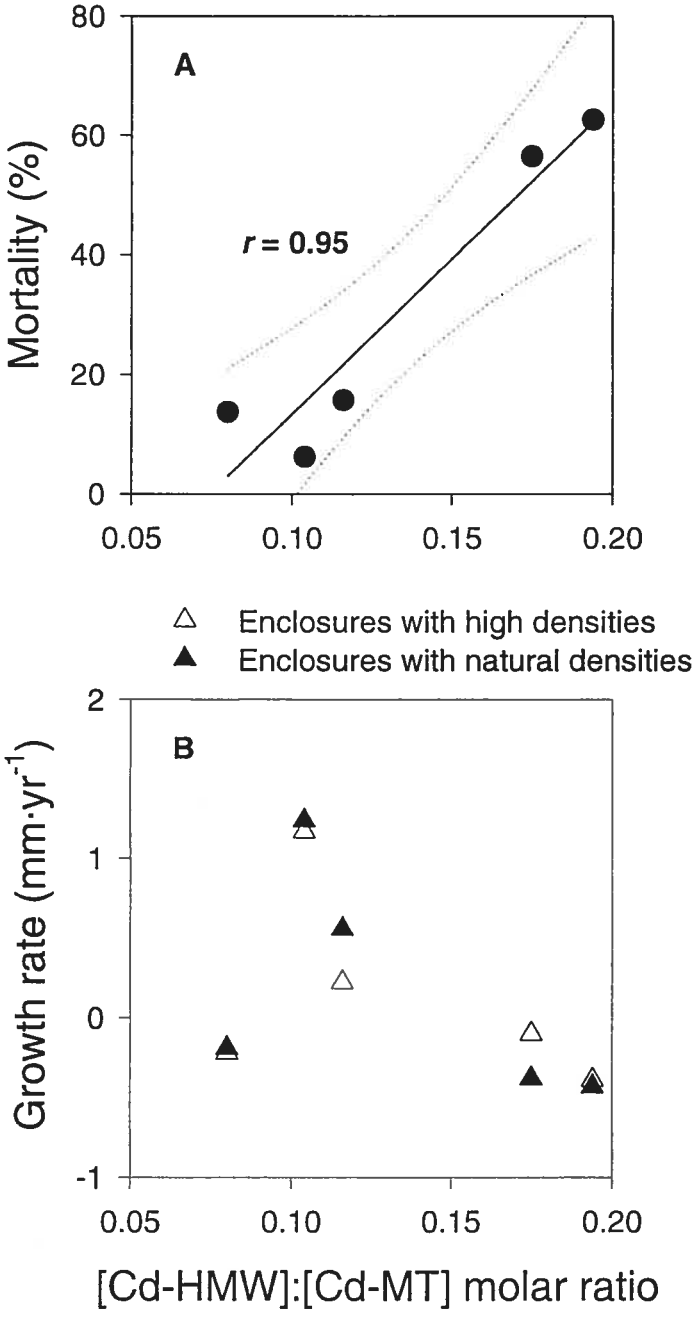

Figure 5.7. A) Mortality (full circles) and B) growth (open and full triangles) rates of transplanted bivalves in relation to the values of the ratio of [Cd] in the gill cytosolic HMW pool to [Cd] in the MT-like pool.

This work indicates that in response to excessive accumulation of Cd in the high molecular weight fraction of the gill cytosol, *P. grandis* exhibits lower survival rates. This result is consistent with those from our population study on *P. grandis* (Perceval et al., 2004). However, given some inconsistencies between our results and those obtained from a previous transplant experiment using the same species, the exact nature of Cd toxicity in *P. grandis* is still unclear and further investigations into the nature of the Cd-HMW fraction are needed. It is also possible that a clearer relationship between Cd accumulation and metal-induced toxicity emerges if the presence of other metals with a high affinity for MT (especially Cu) is also taken into account. It would then be possible to verify that non-essential metals such as Cd are actually replacing Cu in metalloenzymes.

5.5 Acknowledgements

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Chapitre 6

Conclusion générale

La recherche actuelle dans le domaine des biomarqueurs vise à définir leur utilité pour déterminer le niveau d'exposition d'organismes à des polluants chimiques et pour évaluer les effets toxiques de ces polluants en milieu naturel. Dans ce but, elle s'intéresse à la caractérisation des facteurs confondants susceptibles de modifier l'interprétation et l'évaluation des réponses du biomarqueur à un stress environnemental (Adams et al., 2001). Elle s'intéresse également à établir des relations statistiques ou causales entre la réponse du biomarqueur mesurée à l'intérieur des organismes et des effets biologiques à des niveaux élevés de l'organisation biologique (p. ex., populations, communautés) (McCarthy et Munkittrick, 1995; Adams et al., 2001). L'utilisation de la métallothionéine (MT) comme biomarqueur d'exposition à certains métaux traces a été validée à de nombreuses reprises en milieu naturel, chez une grande variété d'organismes aquatiques. Certains auteurs ont également suggéré d'utiliser la MT comme biomarqueur d'effets toxiques, en examinant la répartition intracellulaire du métal entre différents ligands cytosoliques, dont la MT (Stegeman et al., 1992). Des études menées la plupart du temps en laboratoire, dans des conditions expérimentales peu représentatives des conditions naturelles, ont en effet montré qu'il existait des relations entre la liaison non-spécifique du métal (le plus souvent le Cd ou le Cu) à des ligands autres que la MT et la manifestation d'effets toxiques au niveau cellulaire, et au niveau des organismes (détérioration de la reproduction, diminution de la croissance, altérations comportementales, etc.) (Sanders et al., 1983; Jenkins et Mason, 1988; Wallace et al., 2000). Le plus souvent, dans ces études, les conséquences sur l'état de santé des populations n'étaient qu'effleurées. Peu d'études en milieu naturel se sont intéressées au potentiel prédictif de la MT et de la répartition sub-cellulaire du métal comme indicateurs d'effets toxiques au niveau des populations. De plus, dans la majorité des études existantes (p. ex., Blaise et al., 2003; Farag et al., 2003), les caractéristiques écologiques des habitats, variables susceptibles d'influencer les réponses des populations, n'étaient pas considérées. Dans ce contexte, cette thèse représente une contribution significative à l'évaluation de la MT comme biomarqueur d'effets toxiques chez les invertébrés d'eau douce en milieu naturel, et à l'identification et à la compréhension des

variables pouvant modifier ces réponses toxiques à différents niveaux de l'organisation biologique.

Les concentrations de métallothionéine dans notre organisme modèle, le bivalve d'eau douce *Pyganodon grandis*, sont indirectement influencées par les caractéristiques physico-chimiques des lacs. Il existe peu d'études en milieu naturel ayant quantifié l'importance relative des variables environnementales confondantes, par rapport au gradient d'exposition au contaminant, sur les processus de bioaccumulation du métal et de synthèse de MT. Ce genre d'étude requiert l'utilisation de méthodes statistiques multivariables issues de l'écologie (partition de la variation, analyses en composantes principales), encore peu appliquées dans le domaine de l'écotoxicologie (Maund et al., 1999). Dans l'ensemble des 20 lacs étudiés au départ, près de 50% de la variation totale des concentrations de Cd dans les branchies du bivalve *P. grandis* était expliquée par des variables limnologiques confondantes (essentiellement le pH et la concentration en calcium dissous dans la colonne d'eau) (Perceval et al., 2002). Puisque la biosynthèse de MT est en partie contrôlée par l'entrée du Cd dans la cellule, la matrice des variables confondantes expliquait également plus de 40% de la variation totale des concentrations de MT chez cette espèce. Une procédure de sélection des lacs a été proposée pour réduire l'influence relative de ces facteurs confondants: les lacs ont été choisis de façon à maximiser le gradient de contamination en Cd, tout en minimisant la variation des facteurs écologiques pouvant masquer les réponses des bivalves indigènes au gradient de contamination. Les résultats de cette étude nous ont permis de démontrer que la fiabilité de la MT comme biomarqueur d'exposition au Cd était améliorée quand les sites d'échantillonnage étaient rigoureusement sélectionnés pour minimiser la variabilité d'ordre écologique.

Dans une étude connexe menée dans le groupe de lacs issus de cette procédure de sélection, Giguère et al. (2003) ont démontré que dans des populations de *P. grandis* exposées de façon chronique à des niveaux croissants de cadmium ambiant, les individus présentaient des concentrations croissantes de Cd lié non-spécifiquement à des ligands de faible et de haut poids moléculaires dans le cytosol des branchies. Cet état coïncidait avec l'apparition d'un stress oxydant au niveau des membranes cellulaires, manifesté par la

production de malondialdéhyde (MDA), un indicateur de peroxydation lipidique des membranes cellulaires. En nous basant sur le concept de continuum de toxicité des métaux (Luoma, 1995), nous avons émis l'hypothèse que ces effets toxiques observés à un niveau inférieur de l'organisation biologique (i.e., la cellule) pouvaient se propager à des niveaux supérieurs (i.e., la population). Dans les mêmes lacs, nous avons effectivement pu établir des corrélations significatives entre la dégradation de l'état de santé des populations de bivalves, se traduisant essentiellement par une diminution de la densité, de la biomasse totale, de la production, du taux de renouvellement et du succès de reproduction des populations, et l'augmentation des concentrations de Cd dans la fraction de ligands cytosoliques de haut poids moléculaire dans les branchies des organismes (Perceval et al., 2004a). Les résultats concernant la production secondaire nous intéressaient plus particulièrement, puisque cette variable constitue une mesure fonctionnelle du flux d'énergie à travers la population, et peut être considérée comme indicatrice de l'état de santé des écosystèmes. Ces résultats étaient en accord avec les observations faites au niveau des individus. Dans des organismes issus d'une même population de *P. grandis* transférés d'un milieu non-contaminé vers des sites lacustres présentant des niveaux élevés de contamination par le Cd, l'augmentation du rapport des concentrations de Cd associé à des ligands de haut poids moléculaire sur les concentrations de Cd lié à la MT dans le cytosol de branchies (ratio [Cd-HPM]:[Cd-MT]) à l'issue d'une période d'exposition de 400 jours, correspondait à une augmentation des taux de mortalité individuels (Perceval et al., 2004b). Dans cette expérience, le ratio Cd-HPM:Cd-MT témoignait du niveaux de détoxication du métal: ce ratio était plus faible lorsque le Cd était davantage lié à la MT, ce qui limitait la toxicité de ce métal, et il augmentait lorsque le Cd était plutôt associé à des protéines sensibles de haut poids moléculaire (p. ex., enzymes).

Pour évaluer la faisabilité d'utiliser un biomarqueur donné pour prédire des effets écotoxicologiques au niveau des populations ou des communautés, il est nécessaire de relier la réponse du biomarqueur à l'intérieur des organismes à la fois à des mesures d'exposition au contaminant, et à des mesures de leur toxicité à différents niveaux de l'organisation biologique (Adams et al., 2000). A priori, les résultats de cette thèse sembleraient indiquer que la répartition du Cd entre les ligands intracellulaires offre un

potentiel de diagnostic des effets néfastes au niveau des populations. Cependant, à cause des effets confondants des facteurs écologiques sur les réponses des populations, il a été difficile d'assigner à la MT ou à d'autres biomarqueurs potentiels (Cd-HPM) un rôle prédictif d'effets écotoxicologiques sur l'état des populations de bivalves, et ce malgré la sélection initiale de lacs présentant des caractéristiques d'habitat comparables. Des analyses de corrélations partielles nous ont en effet permis de mettre en évidence un changement dans les facteurs structurants des populations de bivalve dans les lacs étudiés. À l'heure actuelle, la quantité de chaleur accumulée dans la zone littorale des lacs est la variable expliquant le mieux les variations inter-lacs des réponses des populations. Cette variable dépend essentiellement des caractéristiques morphologiques des lacs (volume, profondeur moyenne, etc.), dont nous n'avions pas tenu compte dans le choix des lacs. En raison des efforts de mitigation au début des années 80 de la part de la fonderie Noranda, la principale source polluante de la région, le stress anthropique exercé par le Cd sur les populations a diminué au cours du temps, et il est probable que le Cd ait été remplacé par des facteurs naturels comme agents structurant des populations de *P. grandis* dans les lacs de la région. Un suivi à long terme des niveaux de contamination par les métaux traces dans les lacs étudiés indique, en effet, une diminution de près de 60 % des concentrations ambiantes de Cd, sous la forme de l'ion libre Cd^{2+}, entre 1989 et 1998 (Perceval et al., 2004c). Parallèlement à cette diminution, les concentrations de Cd accumulé et de MT dans les organismes indigènes de ces mêmes lacs ont baissé de plus de 40 %.

Il est également possible que les populations de *P. grandis* aient développé des mécanismes compensatoires permettant aux individus de contrer la toxicité du métal dans des conditions d'exposition chronique, expliquant ainsi l'absence de relations significatives entre les variables des populations et la variable d'exposition au Cd, après avoir contrôlé l'influence des facteurs écologiques confondants. L'acquisition d'une tolérance génétique par des populations soumises à de faibles concentrations environnementales de métaux a été démontrée chez de nombreux organismes aquatiques (Klerks et Weis, 1987).

Dans cette thèse, nous n'avons considéré qu'une seule voie possible de détoxication du métal dans notre organisme modèle, c.-à-d. la liaison du métal avec des protéines

inductibles s'apparentant aux MTs. Notre compréhension des mécanismes de toxicité des métaux traces au niveau cellulaire s'en trouve nécessairement limitée, en comparaison d'études récentes utilisant des techniques de centrifugation différentielle (Wallace et al., 1998). L'utilisation de cette technique nous aurait permis d'élargir notre « fenêtre analytique », et d'avoir une image plus complète de la distribution du métal à l'intérieur de la cellule. L'application récente de la centrifugation différentielle à notre organisme modèle *P. grandis* (Bonneris et al., 2003), nous a d'ailleurs permis de constater que dans les branchies d'individus exposés de façon chronique au cadmium, au cuivre et au zinc, les concrétions minérales riches en calcium (granules) étaient le site préférentiel de séquestration (et donc de détoxication) de ces trois métaux.

Dans ce travail, nous avons considéré le Cd comme étant l'unique source de contamination dans les lacs étudiés. Ceci constitue évidemment une vue simplifiée du milieu environnant, puisque dans les lacs de la région de Rouyn-Noranda, le cadmium se retrouve le plus souvent associé au cuivre et au zinc (voir chapitre 4). Ce choix était justifié par les résultats d'études antérieures sur *P. grandis*, et qui désignaient le Cd comme le seul métal inducteur de la MT chez cette espèce (p. ex., Couillard et al., 1993; Wang et al., 1999). Cependant, l'étude des trois métaux combinés et plus spécifiquement des interactions compétitives entre le Cd et le Cu pour les sites de fixation de la MT nous aurait permis de lever certains doutes sur la nature exacte de la toxicité du Cd chez *P. grandis* (chapitre 5).

Les résultats de cette thèse mettent en évidence la nécessité de considérer, dans le futur, les caractéristiques de l'habitat, composantes intrinsèques des écosystèmes, en même temps que les composantes reliées aux activités anthropiques dans les protocoles d'échantillonnage des études environnementales utilisant les biomarqueurs. L'idée de comparer les effets toxiques des polluants en interaction avec les autres facteurs écologiques est encore très rarement abordée en écotoxicologie. À ce sujet, Chapman (2002) recommandait récemment une meilleure intégration des études écologiques (i.e., approches holistiques) et toxicologiques (i.e., approches réductionnistes) afin de mieux évaluer les risques environnementaux de la présence des contaminants dans les milieux

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Annexe 1

Programme FORTRAN 77 permettant de sélectionner un sous-ensemble de lacs ayant des caractéristiques limnologiques semblables et des indices de contamination très différents. Ce programme d'optimisation détermine la combinaison de 10 lacs parmi x lacs pour laquelle le rapport R est maximum (R = inertie matrice toxicologique/inertie matrice limnologique).

```
C Ce programme maximise le rapport de la variance du tableau 1 sur celle
du tableau 2.
C
      Integer p1,p2,pmax,lac(10),indlac(10)
      Parameter (nmax=30),pmax=50)
      Real*8 Mat1(nmax,pmax),Mat2(nmax,pmax),var1,var2,rapport,rapref
      Character tt*8
      String namea
C Initialisation
      rapref=0.0
      rapmin=100000.0
C BBEdit is the 'creator' for output files
      call F_Creator('R*ch')
C Fichier de sortie
      write(*,*)
      write(*,*) 'Nom du fichier de sortie'
      open (3, file=*'Output file: ',Status='new')
      inquire(unit=3,name=namea)
      write(*,*) 'Fichier de sortie: ',namea
C Fichier d'entrée
      write(*,*) 'Nom du premier fichier d''entrée (variance à
maximiser)?'
      open (1,file=*,Status='old')
      inquire(unit=1,name=namea)
      write(*,*) 'Fichier 1: ',namea
      write(*,*) 'Nombre de lacs, nombre de variables?'
      read(*,*) n,p1
      do 6 i=1,n
      6 read(1,*) (Mat1(i,j), j=1,p1)
      write(*,*) 'Centrer et réduire ces variables? -- 0 = non, 1 = oui'
      read(*,*) noui
      if(noui.eq.1) then
          Call Stand(n,p1,nmax,pmax,Mat1)
          write(3,*) 'Premier fichier d''entrée, centré-réduit: '
          write(*,*)
          do 8 i=1,n
```

```
      8       write(3, 100) (Mat1(i,j), j=1,p1)
            write(3,*)
            endif
C Second fichier d'entrée
      write(*,*) 'Nom du second fichier d''entrée (variance à
minimiser)?'
      open (2,file=*,Status='old')
      inquire(unit=2,name=namea)
      write(*,*) 'Fichier 2: ',namea
      write(*,*) 'Nombre de variables?'
      read (*,*) p2
      do 10 i=1,n
     10 read(2,*) (Mat2(i,j), j=1,p2)
      write(*,*) 'Centrer et réduire ces variables? -- 0 = non, 1 = oui'
      read (*,*) noui
      if(noui.eq.1) then
            Call Stand (n,p2,nmax,pmax,Mat2)
            write(3,*) 'Second fichier d''entrée, centré-réduit: '
            write(*,*)
            do 12 i=1,n
     12       write(3,100) (Mat2(i,j), j=1,p2)
            write(3,*)
            endif
C Démarrer l'horloge
      write(*,*)
      call time(tt)
      write(*,*) tt
      debut=secnds(0.0)
C Début des 184756 combinaisons de 10 lacs parmi 20
      nn=0
      do 50 i1 =   1,n-9
      do 50 i2 =i1+1,n-8
      do 50 i3 =i2+1,n-7
      do 50 i4 =i3+1,n-6
      do 50 i5 =i4+1,n-5
      do 50 i6 =i5+1,n-4
      do 50 i7 =i6+1,n-3
      do 50 i8 =i7+1,n-2
      do 50 i9 =i8+1,n-1
      do 50 i10=i9+1,n
      indlac(1)=i1
      indlac(2)=i2
      indlac(3)=i3
      indlac(4)=i4
      indlac(5)=i5
      indlac(6)=i6
      indlac(7)=i7
      indlac(8)=i8
      indlac(9)=i9
      indlac(10)=i10
      nn=nn+1
C Variance de la première matrice pour les lacs sélectionnés
      var1=0.0
      do 22 i=1,10
      do 22 j=1,p1
     22 var1=var1+Mat1(indlac(i),j)**2
```

```
C Variance de la seconde matrice pour les lacs sélectionnées
      var2=0.0
      do 26 i=1,10
      do 26 j=1,p2
     26 var2=var2+Mat2(indlac(i),j)**2
C Rapport des variances
      rapport=var1/var2
      if(rapport.lt.rapmin) rapmin=rapport
      if(rapport.gt.rapref) then
          rapref=rapport
          do 28 i=1,10
     28     lac(i)=indlac(i)
            endif
     50 continue
      write(*,101) nn
      write(*,102) rapref
      write(*,103) rapmin
      write(*,104) lac
C Lire l'horloge
      write(*,*)
      call time(tt)
      write(*,*) tt
      fin=secnds(debut)
      write(*,109) fin
      stop
    100 format(8f10.5)
    101 format( 'Nombre de combinaisons =',i8)
    102 format( 'Rapport de variance pour la sélection =',f10.5)
    103 format( 'Rapport minimum des variances =',f10.5)
    104 format( 'Lacs sélectionnés:',10i3)
    109 format( 'Durée du calcul:',f10.2,' sec.'/)
      end

      Subroutine Stand(n,p,nmax,pmax,Mat)
      integer p,pmax
      Real*8 sx,sx2,xbar,ety,dfln
      Real*8 Mat(nmax,pmax)
C Centrage et réduction des données
      dfln=dfloat(n)
      do 20 j=1,p
      sx=0.0
      sx2=0.0
      do 10 i=1,n
      sx=sx+Mat(i,j)
     10 sx2=sx2+Mat(i,j)*Mat(i,j)
      xbar=sx/dfln
      ety=(sx2-((sx**2)/dfln))/dfloat(n-1)
      ety=dsqrt(ety)
      do 12 i=1,n
     12 Mat(i,j)=(Mat(i,j)-xbar)/ety
     20 continue
      return
      end
```