AQUATIC EFFECTS TECHNOLOGY EVALUATION (AETE) PROGRAM

**Technical Evaluation
of Metallothionein
as a Biomarker for the Mining Industry**

AETE Project 2.2.1

AQUATIC EFFECTS TECHNOLOGY EVALUATION PROGRAM

Notice to Readers

Technical Evaluation of Metallothionein
as a Biomarker for the Mining Industry

The Aquatic Effects Technology Evaluation (AETE) program was established to review appropriate technologies for assessing the impacts of mine effluents on the aquatic environment. AETE is a cooperative program between the Canadian mining industry, several federal government departments and a number of provincial governments; it is coordinated by the Canadian Centre for Mineral and Energy Technology (CANMET). The program is designed to be of direct benefit to the industry, and to government. Through technical evaluations and field evaluations, it will identify cost-effective technologies to meet environmental monitoring requirements. The program includes three main areas: acute and sublethal toxicity testing, biological monitoring in receiving waters, and water and sediment monitoring.

The technical evaluations are conducted to document certain tools selected by AETE members, and to provide the rationale for doing a field evaluation of the tools or provide specific guidance on field application of a method. In some cases, the technical evaluations include a go/no go recommendation that AETE takes into consideration before a field evaluation of a given methods is conducted.

The technical evaluation are published although they do not necessarily reflect the views of the participants in the AETE Program. The technical evaluation should be considered as working documents rather than comprehensive literature reviews.

The purpose of the technical evaluations is to document specific monitoring tools. AETE committee members would like to note that no one single tool can provide all the information required for a full understanding of environmental effects in the aquatic environment.

Any comments concerning its content should be directed to:

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PROGRAMME D=ÉVALUATION DES TECHNIQUES DE MESURE D=IMPACTS EN MILIEU AQUATIQUE

Avis aux lecteurs

Une évaluation technique de la métallothionéine comme outil de surveillance biologique pouvant être utilisé par l=industrie minière

Le Programme d'évaluation des techniques de mesure d'impacts en milieu aquatique (ÉTIMA) vise à évaluer les différentes méthodes de surveillance des effets des effluents miniers sur les écosystèmes aquatiques. Il est le fruit d'une collaboration entre l'industrie minière du Canada, plusieurs ministères fédéraux et un certain nombre de ministères provinciaux. Sa coordination relève du Centre canadien de la technologie des minéraux et de l'énergie (CANMET). Le programme est conçu pour bénéficier directement aux entreprises minières ainsi qu'aux gouvernements. Par des évaluations techniques et des études de terrain, il permettra d'évaluer et de déterminer, dans une -efficacité, les techniques qui permettent de respecter les exigences en matière de surveillance de l'environnement. Le programme comporte les trois grands volets suivants : évaluation de la toxicité aiguë et sublétale, surveillance des effets biologiques des effluents miniers en eaux réceptrices, et surveillance de la qualité de

=ÉTIMA et de fournir une justification pour l=évaluation sur le terrain de ces outils ou de fournir des lignes directrices quant à leur application sur le terrain. Dans certains cas, les évaluations techniques pourraient inclure des recommandations relatives à la pertinence d =effectuer une évaluation de terrain que les membres de l =ÉTIMA prennent en considération.

Les évaluations techniques sont publiées bien qu =elles ne reflètent pas nécessairement toujours l'opinion des membres du l =ÉTIMA. Les évaluations techniques devraient être considérées comme des documents de travail plutôt que des revues de litérature

Les personnes intéressées à faire des commentaires concernant le contenu de ce rapport sont invitées à communiquer avec M ^me Diane E. Campbell à l'adresse suivante :

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EXECUTIVE SUMMARY

S.1 Introduction

The Aquatic Effects Technology Evaluation Program, AETE, has been established to assist the Canadian mining industry in meeting its environmental effects monitoring and related requirements, in as cost-effective a manner as possible. The program is coordinated by the Canadian Center for Mineral and Energy Technology (CANMET). The present report is a technical evaluation of metallothionein as a biomonitoring tool (biomarker) for the mining industry.

Metallothioneins (MT) are low molecular weight, cysteine-rich metal-binding proteins that show high affinity for Group IB and IIB metal ions. Studies involving aquatic animals have suggested a central role for these molecules in the regulation of the essential metals Zn and Cu; in the detoxification of these metals, when present in excess, and of nonessential metals such as Cd; and in the acquisition of metal tolerance for populations living in metal-contaminated environments.

S.2 Evaluation

The present evaluation of metallothionein is based principally on published field studies performed in mining regions. Peer-reviewed literature and reports of studies carried out by individual mining companies and by the AETE program were consulted. Criteria defined for biomarkers and in the terms of reference of the contract were used as guides for the evaluation process. Conclusions of this evaluation are provided point by point in the following.

Criteria: the indicator should respond in a dose-dependent manner to changes in ambient levels of the contaminant; the indicator should be specific to a particular contaminant or a class of contaminants.

Conclusion: strong field evidence (15 studies) supports the fact that metallothionein responds specifically in a dose-dependent manner to changes in ambient levels of a trace metal or of a group of trace metal (e.g. Cd, Cu, Zn, Ag).

Criterion: levels of the indicator should be related to the health or «fitness» status of the organism.

Conclusion: only 4 field studies examined this issue; results were in agreement with the criterion. In these studies, high metallothionein levels were associated with detrimental effects at the organism and population levels of biological organization. Hypothetical causes were an overwhelming of the detoxification mechanism including MT, or a metabolic cost, associated with MT synthesis, affecting directly the growth and/or reproduction of the host organism.

Criterion: the indicator should have an early warning capacity, *i.e.*, the biochemical response should be predictive of effects at higher levels of biological organization and should precede them.

Conclusion: one single study provided results consistent with an early warning capacity of the MT tool. MT decreases in a fish species and improvements in water quality preceded recoveries of phytoplanktonic, zooplanctonic, and benthic communities. This link appeared empirical.

Criterion: the basic biology/physiology of the biomonitor organism should be known so that sources of uncontrolled variation (growth and development, reproduction, food sources) can be minimized.

Conclusion: Peer-reviewed literature on the subject is scarce. The author concluded that non-toxicological factors influencing MT levels have not been adequately evaluated.

Criterion: applicability.

Conclusion: reliable analytical protocols for MT detection and quantification have been defined. MT is easy to quantify by metal-saturation methods, and metal-saturation methods would be easy to standardize on a countrywide basis.

Criterion: commercial availability.

Conclusion: metallothionein analyses are not available in the private sector, and specific cost estimates could not be made. MT certified reference materials are commercially available.

Criterion: practical limitations for carrying field work.

Conclusion: fresh samples have to be frozen quickly (<6 h after collection) and protected against long-term oxidation.

S.3 Recommendations

- Metallothionein can already be considered to be a useful biomarker of exposure to certain metals (e.g. Cd, Cu, Zn, and Ag).

- Metallothionein is not a «stand on its own» tool. As for any monitoring tool, the MT level in an organism has to be used in conjunction with other biotic and abiotic measurements to be interpreted unambiguously (e.g. section 6.9).

- Use of metallothionein as a means of evaluating metal effects on cells, organisms and populations is less well established. There is a need for fundamental, mechanistic research on understanding the role of metallothionein in metal toxicology.

- The early warning capacity of metallothionein is not established. Research efforts are to be directed notably to an increased understanding of fundamental ecological mechanisms, and the characteristics of tolerant organisms to metal exposure in nature.

- Some of the research needed on the use of metallothionein as a biomonitoring tool could be conducted under the auspices of the AETE program. This includes an investigation of the use of different species as sentinel organisms at selected sites, and their calibration with respect to the use of MT as a biomarker of exposure.

- Standardization of protocols of sample preparation, metallothionein extraction and quantification, and QA/QC checks would be required on a countrywide basis. The private sector could relatively rapidly develop the infrastructure required to offer MT services.

- Protocols of organism capture/collection and handling need to be standardized to minimize undesired variations in metallothionein concentrations.

Other recommendations are indicated in Chapter 7.

SOMMAIRE

S.4 Introduction

Le programme d'Évaluation des Techniques de Mesures d'impacts en Milieu Aquatique (ÉTIMA) a été mis sur pied afin d'assister l'industrie minière canadienne dans la sélection de techniques efficaces et peu coûteuses pour le biomonitoring des effets des métaux traces toxiques en milieu naturel. Le programme est géré par le Centre canadien de la technologie des minéraux et de l'énergie (CANMET). Le présent rapport constitue une évaluation technique de la métallothionéine en tant qu'outil de biomonitoring (biomarqueur) pour l'industrie minière.

Les métallothionéines (MT) ont attiré l'attention plus que toute autre molécule dans la catégorie des biomarqueurs biochimiques. Ce sont des protéines de faible poids moléculaire, riches en cystéines, largement répandues dans le règne animal, et qui possèdent une forte affinité pour les métaux de groupes IB et IIB. Les fonctions qu'on leur attribue sont la régulation des métaux essentiels Cu et Zn, et la détoxification de ces métaux, lorsque présents en excès, et des métaux non essentiels tel le Cd. Les MT sont également la base de l'acquisition de la tolérance aux métaux pour les populations animales vivant en régions

S.5 Évaluation

La présente évaluation de la métallothionéine comme biomarqueur se base principalement sur les études publiés réalisés en région minière. Des publications scientifiques corrigés par arbitrage, et les rapports d'études effectués par les compagnies minières et dans le cadre du programme ÉTIMA ont été consultés. Les critères énumés ici bas pour les biomarqueurs, et dans l'énoncé de travail du présent rapport, ont servi de guides dans cette évaluation. Les résultats de cette évaluation sont présentés point par point.

l'indicateur devrait répondre de façon dépendante (relation dose-réponse) à un changement dans la biodisponibilité du contaminant; l'indicateur devrait s'avérer spécifique à un contaminant particulier ou à une catégorie de contaminants.

Critère: applicabilité de l'outil métallothionéine.

des protocoles fiables et éprouvés sont disponibles pour la détection et la quantification de la MT. La MT est dosable facilement par les méthodes de saturation métallique, et ces méthodes pourraient être facilement standardisés à l'échelle nationale.

disponibilité au niveau commercial.

virtuellement aucune expertise analytique n'a été développé dans le secteur privé; des coûts spécifiques d'analyse n'ont donc pu être obtenus. Des échantillons certifiés de MT sont offerts par certaines firmes de produits scientifiques.

Critère: limitations au travail de terrain.

Résultat: les échantillons et spécimens fraîchement prélevés doivent être congelés relativement rapidement (<6 h après la récolte) et préservés contre l'oxydation à long terme.

S.6 <u>Recommandations</u>

- La métallothionéine peut déjà être considéré comme étant un excellent biomarqueur à certains métaux (e.g. Cd, Cu, Zn, Ag).

- La métallothionéine n'est pas un «outil-panacée» (stand on its own). Comme tout outil de monitoring, la concentration de MT dans un organisme doit être utilisé en conjonction avec d'autres mesures biotiques et abiotiques pour être interprété s approche hiérarchique, section 6.9).

- L'usage de la métallothionéine comme biomarqueur d'effet n'est pas aussi bien établi. Des efforts de recherche devraient être consentis pour l'élucidation du rôle de la

• Le caractère précoce du biomarqueur MT n'est pas établi. De la recherche devrait être
            niveau des mécanismes écologiques fondamentaux et des caractéristiques
                organismes ayant développé une tolé

naturel.

• Certaines recherches pourraient être accomplies sous les auspices du programme
                autres, cela inclut une étude du potentiel de différentes espèces comme
organismes-sentinelles, et   leur calibration quant à l'usage de la métallothionéine comme
biomarqueur d'exposition.

• Il serait impératif que les protocoles suivants soient standardisés sur une base nationale:
    préparation d'échantillons, extraction et quantification de la métallothionéine, protocoles
de contrôle de qualité Le secteur privé pourrait développer relativement rapidement les
    infrastructures requises à l'offre de services analytiques pour la MT.

• Les protocoles de capture/récolte et de manipulation d'organismes devraient être
                de minimiser la variabilité indésirable dans les concentrations de

D'autres recommandations apparaissent au Chapitre 7.

C H A P T E R   1

INTRODUCTION

1. <u>**INTRODUCTION**</u>

1.1 <u>Summary</u>

Metallothioneins (MT) are low molecular weight metal-binding proteins showing high affinity for Group IB and IIB metal ions. Metals usually associated with MT are Cd, Cu, Zn and, occasionally, Ag. Metals not reported to bind to MT are, notably, Pb, Ni, As, Al, Fe and Mn. Metallothioneins have been isolated in many animal phyla. However, capacity to synthesize MT may vary from one species to the other, and non-MT producer may be found. Studies involving aquatic animals have suggested a role for these proteins in the regulation of the essential metals Cu and Zn, in the detoxification of these metals, when present in excess, and of nonessential metals such as Cd, and in the acquisition of metal tolerance for populations living in metal-contaminated environments.

1.2 <u>Biochemical indicators of stress/biomarkers</u>

The Aquatic Effects Technology Evaluation program, AETE, has been established to review technologies that are offered for assessing the impacts of mine effluents on the aquatic environment; the program is coordinated by the Canadian Center for Mineral and Energy Technology (CANMET). The mandate of the program includes notably a field and technical evaluation of metallothionein as a biological monitoring tool for the Canadian mining industry. The present work represents the technical part of the evaluation.

Concern about pollutants derives from the effects that they cause, not from their mere presence in the environment. From an ecotoxicological view point, we need to know if organisms are exposed to doses that cannot be accomodated by natural processes such as elimination, metabolism and/or repair. If doses are beyond this limit, or are predicted to be beyond this limit, we need to know the effects this stress will have on target populations, and on the larger community/ecosystem.

Traditionally, attempts to assess the impacts of contaminants on aquatic ecosystems have involved measurements of chemicals in abiotic compartments and laboratory experiments performed under defined conditions (toxicity tests). To date, these approaches have met with only limited success (Cairns *et al*. 1993). On their own, direct chemical analyses on water and sediments are

unreliable predictors of ecological effects (Cairns *et al*. 1993; see also NRCC 1988). Extrapolation of laboratory-derived toxicological data to the field is fraught with difficulties (Cairns *et al*. 1993)[1]. An alternative and complementary approach to chemical impact assessments involves the use of biomarkers to monitor the response of individual organisms to toxic chemicals.

The biomarker concept is based on the assumption that contaminant-induced effects at the population, community, and ecosystem levels are preceded by biochemical reactions in individual organisms. According to a NRCC panel (1985), the detection and quantification of these chemical reactions could be developed as an early, sensitive and specific indicator of environmental stress. Many biomolecules and biochemical processes proposed over the years for this type of monitoring are still in an early stage of development. For metals, much of the attention in this area has focused on metallothioneins.

In the following sections, the author will provide an overview of metallothionein, describe a conceptual framework for the biomarker approach, provide a status of the utility of metallothionein as a biomarker, and identify research needs.

1.3 Overview of metallothionein (MT)

Several excellent reviews covering the chemistry, biochemistry, physiology, and molecular biology of metallothioneins (MTs) are available. For this reason, and also in the interest of conciseness, only a limited review of the subject will be presented here.

1.3.1 **Biochemistry**

Metallothioneins exhibit an unusual structure in the realm of metalloproteins; typical characteristics are (Stillman 1995, Roesijadi 1992):

- a low molecular mass of 6-7 kD, or 10 kD if estimated using size-exclusion liquid chromatography;

_____

[1] However, there is a general consensus on the fact that chemical analyses and bioassays are a necessary part of any comprehensive monitoring program (Cairns *et al*. 1993). The AETE program reflects this preoccupation; it has the task of evaluating acute and chronic toxicity testing methods, and water and sediment monitoring methods.

- a 61/62-amino-acid-sequence dominated by a 30% cysteine content, and a total absence of aromatic amino acids;

- a high metal content;

- an ultra-violet absorption spectrum characterized by an absence of absorbance at 280 nm, because of the absence of aromatic residues, and a peak of absorbance at 250 nm, because of the chemical bond metal-thiol;

- an absence of disulfide bonds means that the structure of the metal-free MT is that of a random chain;

- the three-dimensional structure of the protein has been investigated using nuclear magnetic resonance (NMR) and X-ray diffraction techniques for some categories of living organisms. Typically, metal ions are clustered into two distinct domains (Fig. 1), and are isolated from the external medium by the peptide chain with the exception of two deep crevices that provide direct access to the metal-thiolate structures in each domain.

Thus, each metallic ion is simultaneously chelated by several thiol-groups provided by cysteine. As a result, dissociation constants between metals and MT are very low. For example, for MT in horse kidney, the constant of the chelate Cd-MT is 5×10^{-20} M at pH 8.0; the chelate Cd-MT in the crab *Cancer pagurus* has a constant of $3.6 \times### 10^{-7}$ M at pH 8.0 and at a temperature of 20 °C. Binding constants for metal binding to MT thiolates determined *in vitro* follow the general order: Hg(II) >### Ag(I) ≈ Cu(I) >### Cd(II) >### Zn(II). In contrast to this firm binding, intramolecular and intermolecular metal exchange can be fast (Vallee and Maret 1993). Metal to protein stochiometries, elucidated by optical spectroscopy, indicate that each MT molecule is capable of binding 7 metal ions for Hg(II), Cd(II), Zn(II), 12 for Cu(I), and 12, 17 or 18 for Ag(I).

Metallothioneins occur naturally in a multiplicity of forms. All binding sites in the two domains may be occupied by Zn(II), Cu(I) or Cd(II), or each domain in a single MT molecule may be filled by different metal ions. Moreover, several protein isoforms are known to exist for many sources of MT and for different animal phyla.

Rabbit liver metallothionein 2a

M₇-MT

Cluster A

α domain

Cluster B
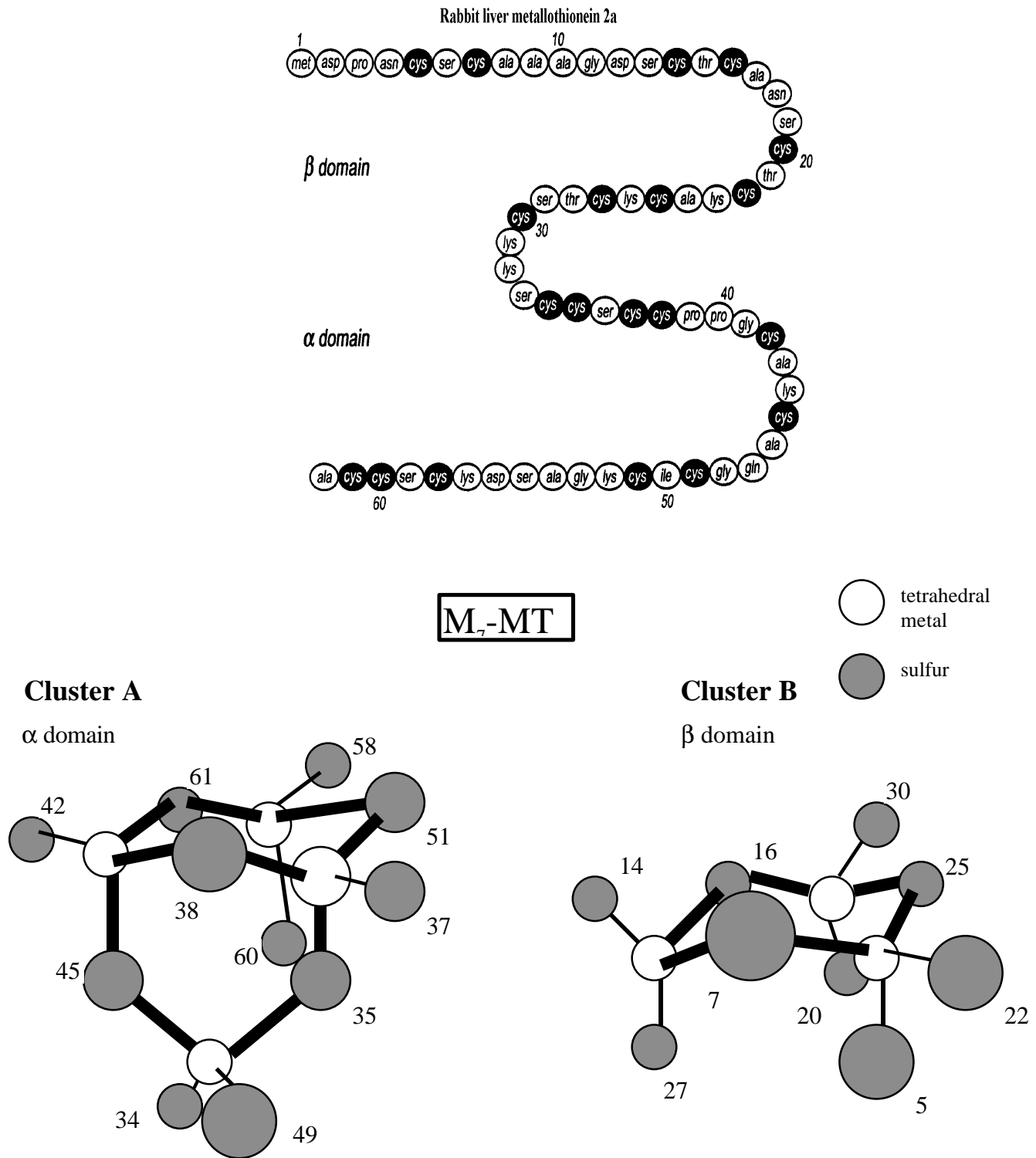
β domain

Figure 1:   Amino acid sequence, and three-dimensional structure of the two metal binding domains of rabbit liver metallothionein 2a. The numbering in the three-dimensional structure refers to the location of the cysteines in the 62-amino acid sequence of rabbit MT (adapted from Stillman 1995).

Table 1

Freshwater organism species for which metallothioneins have been reported .

CATEGORY	SPECIES	COMMON NAME	REFERENCE
AMPHIBIAN	*Rana catesbeiana*	bullfrog	Suzuki & Akitomi 1983
FISH	*Anguilla anguilla*	European eel	Roesijadi 1992
	Carassius auratus	goldfish	Roesijadi 1992
	Catostomus commersoni	white sucker	Roesijadi 1992
	Cyprinus carpio	common carp	Roesijadi 1992
	Esox lucius	northern pike	Norey *et al.* 1990
	Ictalurus punctatus	channel catfish	Roesijadi 1992
	Lepomis macrochirus	bluegill	Roesijadi 1992
	Morone saxatilis	striped bass	Roesijadi 1992
	Morone americana	white perch	Roesijadi 1992
	Onchorhynchus keta	chum salmon	Roesijadi 1992
	Onchorhynchus kisutch	coho salmon	Roesijadi 1992
	Onchorhynchus mykiss	rainbow trout	Roesijadi 1992
	Onchorhynchus tshawytscha	chinook salmon	Roesijadi 1992
	Perca fluviatilis	yellow perch	Roesijadi 1992
	Pimephales promelas	fathead minnow	Roesijadi 1992
	Salmo salar	Atlantic salmon	Wesson *et al.* 1991
	Salmo trutta	brown trout	Farag *et al.* 1995
	Salvelinus fontinalis	brook trout	Roesijadi 1992
	Salvelinus namaycush	lake trout	Palace & Klaverkamp 1993
	Semotilus margarita	pearl dace	Palace & Klaverkamp 1993
BIVALVE MOLLUSCS	*Anodonta anatina*	-	Streit & Winter 1993
	Anodonta cygnea	-	Roesijadi 1992
	Corbicula fluminea	Asiatic clam	Doherty *et al.* 1987
	Pyganodon grandis	floater mussel	Legrand *et al.* 1987
	Unio elongatus	-	Roesijadi 1992
INSECT	*Baetis thermicus*[a]	mayfly	Roesijadi 1992
	Eusthenia spectabilis[a]	stonefly	Everard & Swain 1983
CRUSTACEAN	*Austropotamobius pallipes*	crayfish	Roesijadi 1992
ANNELID OLIGOCHETE	*Limnodrilus hoffmeisteri*	freshwater worm	Roesijadi 1992

[a] Larvae are aquatic.

Metallothioneins have been isolated in many animal phyla. However, few proteins in animal species have been characterized to the extent that the structure and amino acid sequence have been determined. Metallothioneins or MT-like proteins have been reported in humans (Stillman 1995), in terrestrial and aquatic mammals, in amphibians (frogs and salamanders: Table 1), in reptiles (alligators), in birds, in marine and freshwater fishes (Table 1), and in protozoans (Piccinni and Albergoni 1996). Likewise, metallothioneins have been isolated in the following invertebrate groups: echinoderms, pogonophores, insects, crustaceans, molluscs, and annelids (see Table 1).

Metallothioneins have been grouped into three classes, on the basis of information on their structure and on their mode of synthesis (Fowler *et al*. 1987):

- Class I: polypeptides with locations of cysteine closely related to those in horse kidney metallothionein; also included are proteins similar to horse MT in several of their characteristics;

- Class II: polypeptides with locations of cysteine only distantly related to those in equine renal MT, such as yeast MT;

- Class III: nontranslationally synthesized metal-thiolate polypeptides such as cadystin, phytometallothionein, phytochelatin, or δ-glutamyl-cysteine-glycine. These polypeptides have been isolated in plants and fungi.

1.3.2 **Synthesis and degradation**

Table 2 (Cousins 1985) enumerates intrinsic and experimental factors inducing metallothionein synthesis in mammals. These factors, with the exception of metals, have not been adequately studied in aquatic organisms. Physiological and hormonal stimuli usually promote the production of zinc- or copper-thioneins (reported in Stillman 1995). However, the ability to induce metallothionein is generally much greater for metals (e.g. Klaassen 1981: Zn and Cd: 7- to 20-fold; see also Hamilton and Mehrle 1986), than for other inducers (e.g. Klaassen 1981: hormones hydrocortisone and dexamethasone: 1.4- to 1.8-fold).

Table 2

Physiological and experimental factors that result in metallothionein induction in mammals (adapted from Cousins 1985 and Onasaka *et al*. 1987).

PHYSIOLOGICAL FACTORS	EXPERIMENTAL FACTORS		
Development	Ag	Adjuvant arthritis	Glucagon
Dietary Zn	Cd	Alkylating agents	Interleukine 1
Infection	Cu	Diabetes	Isopropanol
Starvation	Hg	Endotoxin	Retinoic acid
Stress	Zn	Epinephrin	Turpentine
		Glucocorticoids	CCl4
		Ascorbic acid	

Cellular models of the synthesis and degradation of metallothionein have recently been proposed (see Roesijadi 1996, and Hogstrand 1991; Fig. 2). Their individual characteristics do not all have the same degree of certainty, and some of these characteristics remain to be validated in field situations. Induction of MT by metals would follow a pathway that results in increased levels of intracellular free zinc levels. Zinc would be displaced by intruding metals from Zn binding ligands in the cell. The displaced Zn would be available to bind to metal transcription inhibitors (MTI), releasing the transcription factors (MTF) from inhibition. Metallothionein expression would be initiated through binding of the MTF molecules to the corresponding metal regulatory element on the MT gene. Initially formed intracellular metal-ligand complexes would represent toxic interactions (e.g. turbot: George *et al*. 1996) and be repaired by metal exchange reactions with newly induced Zn-MT. Elimination of excess metal-protein complexes would proceed by exocytosis of their polymerized forms accumulated in lysosomes. This mechanism appears to be more efficient in eliminating cellular Cu and Zn than in excreting cellular Cd (reported in Hogstrand 1991: mammals and fish; George 1983: molluscs). These observations are consistent with the extremely long biological half-lives reported for Cd in many vertebrates (Hogstrand 1991, including man) and invertebrates (e.g. George 1983).
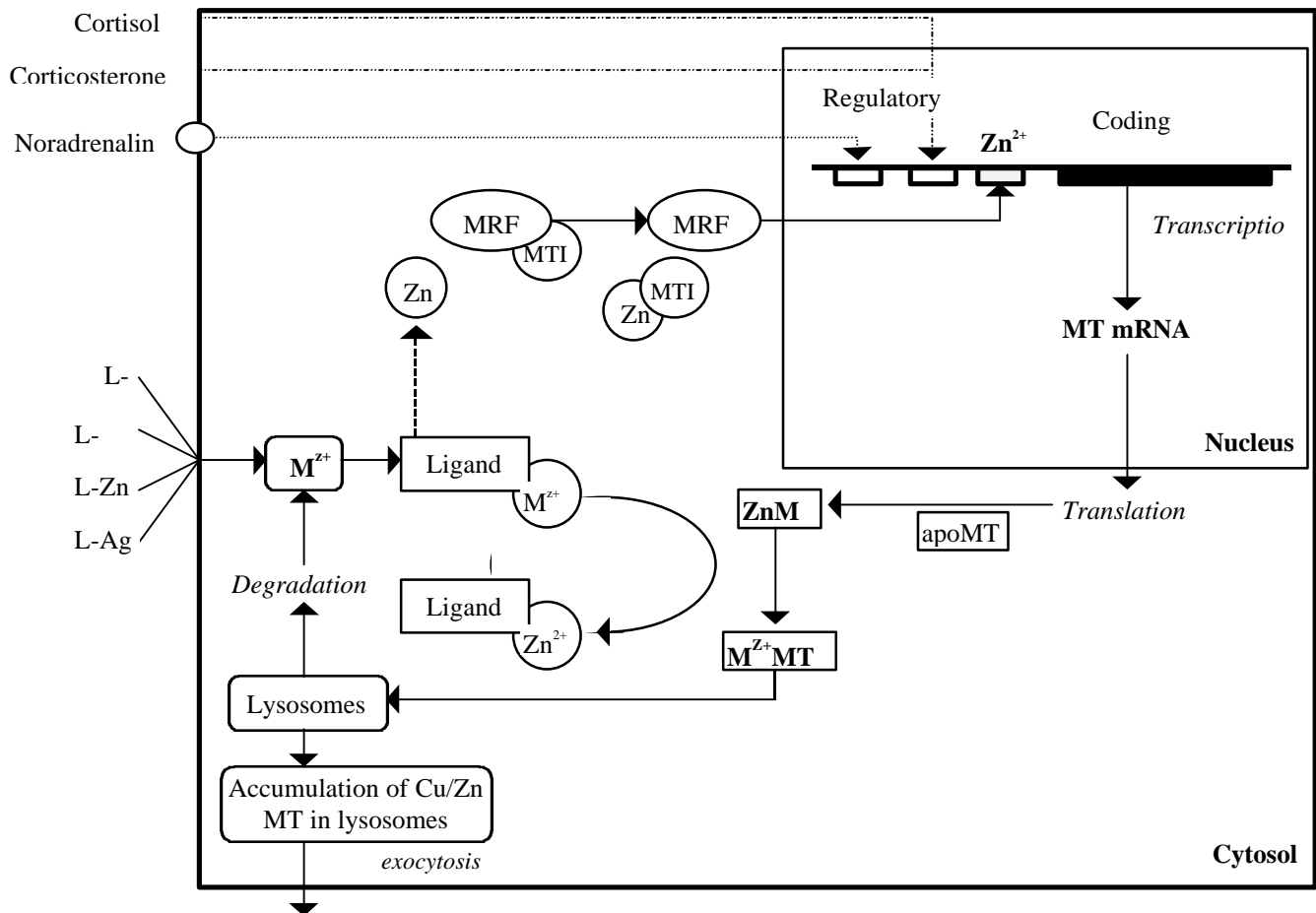
Figure 2: Model for the synthesis and degradation of metallothioneins at the cellular scale. Extracellular metal is assumed to be associated with a transport ligand. The synthesis of MT is induced by hormones and trace metals. Induction by the latter is mediated by metal-regulatory factors (MRF) and the appropriate regulatory elements. In higher organisms, the MRF would be under inhibition by a transcriptional inhibitor (MTI) that can only be released by Zn. Other metals would induce MT by displacing Zn from intracellular ligand binding sites, making additional Zn available for interacting with the inhibitor; transcription of the MTmRNA would ensue. Initially formed metal-ligand complexes can represent toxic interactions and be repaired by ZnMT through metal-metal exchange reactions. Excess Cu/Zn-thioneins can accumulate in lysosomes to be eventually excreted by exocytosis (adapted from Roesijadi 1996 and Hogstrand 1991).

1.3.3 **Functions**

The exact biological functions of metallothionein are still a subject of debate. Engel and Brouwer (1989) postulated that two interactive intracellular pools of MT exist in the cytosol. One is an *induced* pool (e.g. Cd-MT) responding to environmental fluctuations in trace metal levels. The other is a *constitutive* MT pool (e.g. Zn,Cu-MT) involved in normal metal regulatory processes. It is not known

if similar or different MT isoforms can fulfill both types of functions. The following sections summarize studies describing possible different functions of MT.

1.3.3.1 *Trace metal detoxification*

Given its remarkable affinity for and induction by trace metals, scientists have generally attributed to metallothionein a role in metal detoxification. Two classes of experiments have provided firm evidence for this protective function against essential and non-essential metal toxicity. Prior exposures of organisms in the laboratory to Cd, Cu, or Zn, at concentrations sufficient to induce MT-like proteins, conferred increased tolerance to subsequent metal exposures (Hogstrand 1991, Klaverkamp and Duncan 1987, Roesijadi and Fellingham 1987, NRCC 1985). The role of MT in metal tolerance was also demonstrated in natural populations of different mayfly species; larvae of the cadmium-sensitive species were not able to synthesize MT in response to Cd insult and, consequently, were not found in metal-contaminated environments (Roesijadi 1992).

The second class of experiments involved the genetic manipulation of cell lines, or of whole organisms (Liu *et al*. 1996), that either over- or under-produced MT. For example, some cell lines, disabled from synthesizing MT due to the hypermethylation of their MT genes, were especially sensitive to trace metals. In contrast, cultured cells, provided with an over-capacity to produce MT because of gene amplification (duplication), were much more resistant to trace metals (Roesijadi 1992: example with Cu and yeast; Hogstrand 1991).

1.3.3.2 *Role in essential metal regulation*

Early experiments suggested that metallothionein may act as a metal-transfer protein, but most of these studies were carried out *in vitro*, thus bypassing the complex cellular machinery involved in metal metabolism, notably specific molecules fulfilling a function in cellular metal distribution (Cu: ceruloplasmin and GSH, Vulpe and Packman 1995 and Cousins 1985; Zn: cysteine-rich intestinal protein [CRIP], Roesijadi and Robinson 1994). Recent studies tend to indicate that the degree of involvement of MT in essential metal metabolism is species- and organ-specific - examples follow.

Elegant work by Engel and Brouwer (1987) has provided evidence that metallothionein plays an active role in the metabolism and mobilization of metals during the molting process in decapod crustaceans (see Fig. 3). Notably, MT would scavenge Cu during the catabolism of the copper-containing respiratory protein hemocyanin in the pre-molt stages. Cu-MT would act as a copper donor for hemocyanin synthesis during rebuilding of tissues; glutathione is possibly an intermediate Cu(I) ligand in the process (Engel and Brouwer 1993). It is recognized that the molting process is under the control of ecdysteroid hormones. Torreblanca *et al*. (1996) injected the hormone 20-hydroxyecdysone to intermolt males of the crayfish *Procambarus clarkii*. The hormone induced loss of protein, Cu, and Zn in the hepatopancreas together with an important increase in MT. These observations are consistent with changes in blue crab metabolism during molt documented by Engel and Brouwer (1987) (cf. Fig. 3).

In contrast to the above, mouse strains for which MT genes were rendered non-functional did not exhibit differences in tissue Cu concentrations compared to control mice. Genetically-manipulated mice were able to grow and reproduce normally, provided that they were not subject to high Cu or Cd exposures (see Liu *et al*. 1996 and Vulpe and Packman 1995). These results suggest that MT does not play a major role in Cu homeostasis in these small mammals.

Unusually high levels of Zn and MT were found in livers of uncontaminated marine fish belonging to the family Holocentridae (Zn: up to 2000 μ g^{-1}, MT: up to 35% of liver protein content; Hogstrand and Haux 1996). Close relationships were found between liver [MT] and [Zn] ($0.84 < r < 0.99$), and MT appeared to be the most important ligand for total hepatic zinc. These results are intriguing and suggest that the high levels of MT and Zn in this fish family are linked to normal physiological processes.

A growth inhibitory factor from human brain tissue was recently found to be a metallothionein (termed human MT-III). Its expression did not appear to be regulated by metals or glucocorticoids. Alzheimer's disease is associated with a down-regulation of this MT (Vallee and Maret 1993).
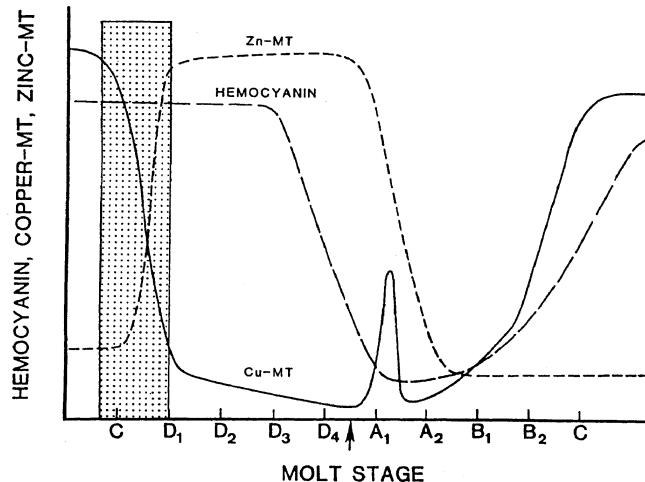
Figure 3:   Changes in hemocyanin, copper- and zinc-MT levels during the molt cycle of the blue crab *Callinectes sapidus*. The shaded area represents the period when the change from a predominantly Cu-MT to a Zn-MT occurs. The stages of the molt cycle are C, intermolt; D_1-D_4, premolt; A_1-A_2, softcrab; and B_1-B_2 paper shell crab; the arrow indicates time of ecdysis (adapted from Engel and Brouwer 1993).

1.3.3.3 *Protection against oxidative stress*

Several studies have suggested that metallothioneins are involved in the protection of the cells against oxidative stress (Bremner and Beattie 1990, Viarengo 1989). MT thiolate cluster would directly capture free hydroxyl (OH) and superoxide (O^{2-}) radicals and stabilize damaged membranes by an indirect release of MT-bound Zn (Viarengo 1989). Comparisons of antioxidant capabilities of dithiothreitol, apometallothionein, and Zn-thionein led Thomas *et al*. (1986) to conclude that the release of Zn ions from MT was far more important than the thiolate oxidation step in inhibiting oxidant-mediated membrane damage. Oxidative stress, a phenomenon of general occurrence in the animal kingdom, can be provoked by a variety of stresses: metals and compounds fostering the oxidative degradation of membranes, and food and water deprivation (Bremner and Beattie 1990).

A single study investigated the influence of capture stress on MT-like proteins in fish. In the liver of the striped mullet *Mugil cephalus*, the concentration of Zn associated with MT increased significantly from 100 to ~1200 nmol Zn g^{-1} wet wt in the seven days following field capture, handling, transportation to the laboratory and transfer to holding tanks (Baer and Thomas 1990).

The comprehension of the role of metallothionein in free-radical scavenging and in stress capture is incomplete and any descriptions of the above roles are still tentative at the moment (see Bremner and Beattie 1990).

1.3.3.4 *An apparent paradox*

An apparent paradox with metallothioneins is that the presence of these proteins would favor the bioaccumulation of toxic trace metals. This phenomenon is discussed in the following section, with a focus on Cd, a metal for which this trend is well documented.

Langston and Spence (1995) compared Cd uptake kinetics in several marine mollusc species. MT producers had net accumulation rates (μg Cd g^{-1} dry wt d^{-1}) 10-40 times more important than non-MT producers. Similarly, Cd concentrations in the natural populations of the species synthesizing MT were ~40 times higher than those of non-producing MT species. Langston and Spence (1995) explained that by sequestering intracellular Cd, MT may drive the process of further accumulation by maintaining the diffusion gradient responsible for the passive entry of Cd. In this context, Roesijadi and Robinson (1994) indicated that «one of the correlates of metallothionein induction is that an increased metal burden can be tolerated by an individual»(compared to a non-MT producer).

Why are toxic trace metals assimilated at all? One may speculate that, regarding essential element uptake, organisms have not evolved element-specific systems to minimize the possibility of adventious toxic trace metal uptake, or systems destined to pump out toxic elements taken up by accident. As a result, detoxification would have to be accomplished internally if an organism is to survive metal exposure. Simkiss and Taylor (1995) recently reviewed the literature addressing element transfer mechanisms across cellular membranes. They could not find any example, apart from bacteria, that organisms have developed systems to excrete toxic metals like Cd out of the cell. In addition, evidence suggests that Cd uptake occurs notably by Zn transporters and by leakage through poorly selective calcium channels. Examples of this have been given for various mammalian cell types, and for the gills of fishes and molluscs (Campbell PGC, pers. comm., Sept. 1996, address given in Chapter 5; reviewed in Roesijadi and Robinson 1994).

Similarities in essential element uptake systems across different phyla (see Simkiss and Taylor 1995), and recurrence of metallothionein-like proteins in these phyla (see previous section), suggest that metallothioneins have a long evolutionary history. These systems may not have been able to counter efficiently the internal build-up of certain non-essential elements because of constraints to evolutionary changes resulting from phylogenetic inheritance, embryogenesis and/or genetic architecture›(see ideas of Gould and Lewontin 1979). However, some evolutionary trajectories seem to have been favored. With regard to metallothionein, selection pressures have favored appearances of MT gene duplication (gene amplification: Maroni *et al*. 1987), and MT isoforms poised to sequester some trace metals specifically (Brouwer *et al*. 1992) to help natural animal populations develop increased tolerance to toxic metal insults (see also Beeby 1991 for terrestrial invertebrates).

A possible consequence of the increased capacity for metal sequestration in MT producers is an increase in the potential for trophic transfer of metals. Possible ecological or public health effects of this food-web transfer remain undefined at the moment (Roesijadi and Robinson 1994).

1.3.4 **Historical background**

Metallothionein was first isolated in 1957 by Vallee and co-workers in horse renal kidney (Vallee and Maret 1993). Since that time, research on the protein has extended to different animal groups and the diverse functions fullfilled by MT are still a subject of considerable interest. The number of publications per annum dealing with MT reached 300 in the early 1990s (Vallee and Maret 1993). A computer-assisted screening of the peer-reviewed literature covering a trimester of the 1995-1996 issues of Current Contents™ using the keyword metallothionein was performed. Two-hundred and thirty-two papers were detected in research areas as diverse as ecotoxicology and aquatic toxicology, mammalian and human toxicology, veterinary research and medecine and cancer research. Over the last 17 years, three international conferences were devoted entirely to metallothioneins; the proceedings of the communications have been published in supplement issues of Experientia (Kägi and *et al*. 1993). A book and a review article recently published deal exclusively with the induction, isolation, quantification and characterization of MTs (Stillman *et al*. 1992, Stillman 1995). Roesijadi (1992) extensively reviewed the literature on metallothioneins in aquatic animals.

CHAPTER 2

CONCEPTUAL FRAMEWORK FOR BIOMARKERS

2. <u>**CONCEPTUAL FRAMEWORK FOR BIOMARKERS**</u>

2.1 <u>Summary</u>

A **biomarker** is defined as a biological response to the exposure to an environmental chemical. This response, at the <u>below-individual level</u>, is not necessarily detected at the whole organism level. A successful biomarker should satisfy a number of criteria.

1. **Early warning capacity**: the biomarker response should be predictive of effects at higher levels of biological organization and should precede them.
2. **Specificity**: the indicator should be specific to a particular contaminant or for a class of contaminants.
3. **Dose-response relationship**: the indicator should respond in a concentration-dependent manner to changes in ambient levels of the contaminant.
4. **Sources of non-toxicological variability identified/understood**: the basic biology/physiology of the biomonitor organism should be known so that sources of uncontrolled variation (growth and development, reproduction, food sources) can be minimized.
5. **Direct relation with the health of the organism**: levels of the indicator should be related to the health or «fitness» status of the organism.

In this chapter, the conceptual framework underlying the biomarker approach is described in detail. To do this, the author has followed the mainstream in the evolution of the concept over the last 10-15 years. Munkittrick and McCarty (1995) highlighted the uses and abuses of the biomarker approach committed over the years. In the development of biomarkers, many studies failed to attempt to link biochemical changes to adverse effects at higher levels of biological organization, to mechanisms of responses, or to chemical exposure (Munkittrick and McCarty 1995). In addition, some of the criteria above defined for biomarkers are based on hypotheses that have rarely been validated in field situations. Thus, the present evaluation of metallothionein as a biomarker considers both its conformity with the above criteria and the solidity of the hypotheses underlying some of these criteria (no. 1 in particular). The bottom line is to statute on the usefulness of MT as a tool for the biomonitoring approach developed for the mining industry. The annotated bibliography on the subject (presented in the next chapters) will include mainly field studies realized in mining regions (peer-reviewed and unpublished literature).

2.2 Conceptual framework defining toxicity process in nature

A biomarker is defined as any biological response to an environmental chemical at the below-individual level, measured inside an organism or in its products (urine, faeces, hairs, feathers, etc...), indicating a departure from the normal status, that is not necessarily detected from the intact individual (see van Gestel and van Brummelen 1996). The biomarker approach has its roots in an ideological construct describing responses of individual organisms to stressors. Elaborated by Selye prior to the 1950s, this response can be decomposed into three main stages: alarm, compensation, and exhaustion (reported by Munkittrick and McCarty 1995, NRCC 1985). Early in the 1980s, Selye's theory was applied in aquatic toxicology to define an holistic response to chemical-induced toxicity. The resulting view, now widely accepted (Engel and Vaughan 1996; Luoma 1995, Munkittrick and McCarty 1995: Fig. 4, NRCC 1985), considers toxicity as a complex continuum of biochemical, physiological, individual, population, and community responses; within each level of biological organization, responses are in turn decomposed as primary, secondary and tertiary, and are the direct translations of alarm, compensation, and exhaustion. Figure 4 is an example of such a framework for toxic metal stress. Although many variants of this framework can be found in the open literature, some common principles emerge from these depictions.

Each level of biological organization includes a detoxification/compensation step. It follows that reactions to metal exposure at one level of biological organization are not necessarily translated to the next higher level. However, the greater the metal exposure, the more likely it is that responses will be detected further up. In addition, cause and effect will be more difficult to define as complexity increases from lower to higher levels of biological organization.

Organizational level	Secondary effect	Primary effect

Tertiary effect
Compensation overwhelmed
(complete degradation)
⇧

Community

Compensation ⟵ **Species is absent where it could be present**
- change in species dominance
- change in predator-prey relationship
▲

Tertiary effect
Compensation overwhelmed
⇧

Population

Compensation ⟵ **Individuals cannot survive or reproduce**
- genetically-based tolerance
- immigration
- age structure
▲

Tertiary effect
Compensation overwhelmed
⇧

Individual

Compensation ⟵ **Physiological stress**
- adult survival
- change in energy allocation devoted to growth and/or to reproduction

- weak individuals
- reproduction compromised
- vulnerability to stress
▲

Tertiary effect
Compensation overwhelmed
⇧

Physiological/ behavioural

Compensation ⟵ **Change or disruption of biochemical processes**
- behavioural adaptation
- acclimation
- adaptation in reproductive cycle
▲

Tertiary effect
Compensation overwhelmed
⇧

Cellular/ subcellular

Detoxification ⟵ **on functional components of cells**
- granules
- metallothioneins

Figure 4:   Examples of processes and effects that cascade from the cellular to the community levels of organization as metal toxicity is manifested (adapted from Luoma 1995 and NRCC 1985).

2.3 Criteria defined for biomarkers

Inferred from the cascade of events illustrated in Figure 4 is the principle that biological effects of toxic chemicals in the environment are initiated by the interaction of the toxic chemical with a biological receptor in a living organism (NRCC 1985). This principle is at the base of the biochemical indicator/biomarker concept. The assumption is made that effects at the ecosystem level are preceded by chemical reactions in individual organisms, and that concentrations of the contaminant needed to initiate these reactions are lower than those required to provoke a life-threatening situation for the target organism or perceptible degradation of the ecosystem. The detection and quantification of these chemical reactions could then be developed as a sensitive specific indicator of environmental stress. A number of criteria for biomarkers have recently been proposed (Stegeman *et al*. 1992, Haux and Förlin 1989; see also Cairns *et al*. 1993, Engel and Vaughan 1996 and van Gestel and van Brummelen 1996). Criteria are numbered below because we refer to them by their numbers in the following chapters.

1. The indicator should have an early warning capacity, *i.e*., the biochemical response should be predictive of effects at higher levels of biological organization and should precede them.

2. The indicator should be specific to a particular contaminant or for a class of contaminants.

3. The indicator should respond in a concentration-dependent manner to changes in ambient levels of the contaminant.

4. The basic biology/physiology of the biomonitor organism should be known so that sources of uncontrolled variation (growth and development, reproduction, food sources) can be minimized.

5. Levels of the indicator should be related to the health or «fitness» status of the organism.

Strict interpretation of MT induction is that aquatic organisms synthesize this protein to acquire tolerance and/or resistance to metal exposure (see Table 4). Thus, MT measurements would serve the purpose of an indicator of exposure and development of tolerance to metals since, theoretically, this

cannot be considered as a toxic effect *per se* (compensatory response: Fig. 4; see section 1.3.3). The appeal with such a biochemical reaction is that it gives early warning (NRCC 1985), inasmuch as this response can be related to individual-, population-, and higher levels effects (Cairns *et al*. 1993). Cairns *et al*. (1993) also indicate that the rationale for using early warning signals in biomonitoring is that it is easier and less costly to prevent impact than to restore after impact.

A corollary to the above is that metal-induced injury will occur at higher levels of biological organization only when detoxification mechanisms are swamped or overwhelmed. Note that higher level effects are the ones that are relevant from an ecological and management point of view (Cairns *et al*. 1993) and the detection of a detoxification failure would be significant in this regard. Hypothetical models have been suggested to describe failure of the detoxification mechanism involving metallothioneins.

2.4 The spillover hypothesis

The influence of metallothionein on intracellular metal distribution is expected to be dual. First, induction of MT would result in the interception and binding of metal ions that are taken up by the cell (Roesijadi and Robinson 1994; Fig. 2). Second, MT would remove metals from non-thionein ligands that include cellular targets of toxicity; this redistribution onto MT would represent a rescue function (e.g. Cd in oyster gill: Roesijadi and Klerks 1989). Cellular toxicity is expected when these functions are not carried out effectively.

It has been hypothesized that excessive accumulation of metals beyond the binding capacity of available MT should result in their binding to other intracellular ligands, a phenomenon termed «spillover». Metals bound to these other ligands are considered to be capable of exerting cellular toxicity (Brown and Parsons 1978; Fig. 5). In principle, the condition could be considered as symptomatic of metal stress and would be amenable to detection by an analysis of intracellular metal partitioning [2]

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[2] Because of the complex cellular interactions implied, total tissue concentrations of metals cannot be indicative of metal-induced cell injury. Note that for narcotic organic chemicals, whole body concentrations appear reasonable first approximations of the chemical levels present at cell sensitive sites (see McCarty and Mackay 1993). Toxicity of these non-reactive chemicals has been linked to their solubilities in lipids.

(Mason and Jenkins 1995, Stegeman *et al*. 1992). Thus, the degree of metal detoxification may represent a better indicator of metal-induced stress than the absolute measure of [MT].

Liquid phase of the cell (cytosol) {
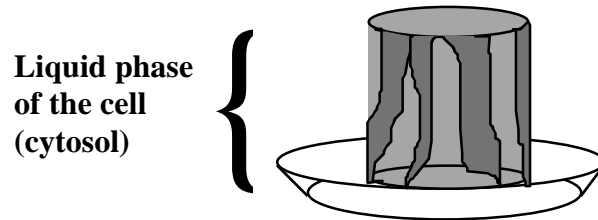
Cup: reservoir of MT available to sequester the metal M.

Saucer: cytosolic pool of enzymes (high-molecular-weight compounds) and small molecules constituting the cellular machinery.

Spillover: metal in excess of the binding capacity of the cellular MT pool.

Figure 5:   The spillover hypothesis is analog to a spillover of coffee in a coffee cup.

Sections 2.2, 2.3, and 2.4 describe the components of the mechanistically-based conceptual framework underlying the metallothionein biomarker approach. From here on, our evaluation of the utility of metallothionein as a biomarker will <u>not be limited exclusively</u> to judge of the compliance of MT with the criteria outlined in section 2.3. Because some of these criteria are based on hypotheses that have rarely been validated in field situations, our intention is to evaluate if the above framework helps to understand the mechanisms of metal action as manifested through different levels of biological organization. The biomarker paradigm has had a tendency to «deflate» in the last ten years. Because it appears extraordinarily difficult to predict effects of toxic metals at the community- and ecosystem-levels, theoreticians and practicians have tended to limit the predictive capabilities of biomarkers to the population-level. However, we will retain the original proposition outlined in NRCC (1985) in our willingness to undertake a thorough evaluation of MT as a biomarker.

2.5 <u>Suggested readings</u>

Mason, A.Z., and K.D. Jenkins. 1995. Metal detoxification in aquatic organisms. *In* A. Tessier and D.R. Turner (eds). <u>Metal speciation and bioavailability in aquatic systems.</u> John Wiley and Sons Ltd, Chichester. pp. 479-608.

Roesijadi, G. 1992. Metallothioneins in metal regulation and toxicity in aquatic animals. Aquat. Toxicol. <u>22</u>: 81-114.

Stegeman, J.J., M. Brouwer, R.T. Di Giulio, L. Förlin, B.A. Fowler, B.M. Sanders, and P.A. Van Veld. 1992. Molecular responses to environmental contamination - enzyme and protein systems as indicators of chemical exposure and effects. *In* R.J. Huggett, R.A. Kimerle, P.A. Mehrle and H.L. Bergman (eds). <u>Biomarkers - Biochemical, Physiological and Histological Markers of Anthropogenic Stress</u>. Lewis Publishers, Chelsea, MI. pp. 235-335.

A collection of papers dealing with the role of biomarkers in risk assessment <u>in</u>: Human and Ecological Risk Assessment, 1996, Vol. 2, No. 2, beginning at p. 243.

C H A P T E R   3

METALLOTHIONEIN INDUCTION

AND TOLERANCE TO METALS

3. **METALLOTHIONEIN INDUCTION AND TOLERANCE TO METALS**

One criticism that one may formulate on MT is that responses at the molecular level are far too removed, or not related to the health of individual organisms and populations (re: criterion 5, chap. 2). A pre-selection committee of the AETE program, the Biological Monitoring Technical Committee, examined many parameters that have been shown to be metal sensitive or to be related to the growth and reproduction of fish. Out of seventy molecular-level markers and molecules evaluated (Table 3), the committee retained metallothionein for a complete evaluation by the AETE program because it was the only molecular response which showed a direct relationship between elevated concentrations and resistance/tolerance at the whole animal level (Klaverkamp *et al.* 1996a). Table 4 gathers 6 laboratory and field studies supporting such a relationship for various animal groups. In addition, Klaverkamp *et al.* (1984) described 20 studies, realized between 1937 and 1983, that documented acclimation of fish species to metals but that did not measure concurrently MT concentrations.

Table 3

Molecular measurements on fish considered by the Biological Monitoring Group of the AETE program.

Metallothionein	MFO induction	Free radical damage
Cytochrome oxidase (CN)	Amino-levulinic acid dehydratase (Pb)	
	Succinic dehydrogenase and other mitochondrial enzymes (As)	
Xanthine oxidase	Superoxide dismutase	Allantoinase
Oxidative stress	Lysosomal degradation	Lipase
Sorbitol dehydrogenase	Glutamate dehydrogenase	Lactate dehydrogenase
Glutamate pyruvate transaminase	Glutamate oxaloacetic transaminase	
Amylase	Aspartate aminotransferase	Alanine aminotransferase
Leucine aminonaphthlamidase	Proximate analyses	RNA/DNA ratio
$O_2:N_2$ ratios	mRNA expression	Adenylate energy charge
Glycogen	Lipid	FFA
Triglycerides	Tryacylglycerol	Cholesterol
Glucose	Protein	Steroid hormones
Vitellogenin	Gonadotropic hormone	ATPase
Phosphorus	Calcium	Porphyria
Electrophoretic protein separations	Chromosomal aberrations	Sister chromatid exchanges
White blood cell ratios	Lactate	ACTH
ChEI	Cortisol	Corticosteroids
Neuroamines	Monoamine oxidase	Carbonic anhydrase
Alkaline/acid phosphatase	Lipofuschin	Creatinine
Bilirubin	β-Glucuronidase	

Table 4

Examples of studies showing that high MT concentrations confer increased tolerance/resistance to metals in fish and invertebrates.

Organism	Type of experiment	Estimation of [MT]			Estimation of resistance/tolerance to metals		
White sucker *Catostomus commersoni*	*In situ* toxicity tests in enclosures	[MT] in nmol g^{-1} (mean ± SD)			Mean time to death (min) Toxicity test Cd conc. (mg L^{-1})		
		Population	Liver	Kidney	Population	**10**	**30**
(Klaverkamp *et al*. 1991)		exposed	29.5±10.5	10.0±2.9	exposed	700	300
		reference	10.9± 9.8	4.9 ± 3.2	reference	305	160
Juvenile Coho salmon *Oncorhynchus kisutch*	Laboratory study	[MT] as µAmpere g^{-1} wet wt (mean ± SE) Liver			168-hr LC_{50} (µg L^{-1}; mean with 95% confidence limits) 168-hr LC_{50} = 2.423 [MT] + 48.6 (R^2=0.94)		
		acclimated 4 wks to 150 µg Cu L $^{-1}$		143±10	acclimated 4 wks to 150 µg Cu L $^{-1}$		470 (419-520)
(McCarter and Roch 1983)		100 µg Cu L $^{-1}$		140±10	100 µg Cu L $^{-1}$		394 (345-440)
		50 µg Cu L $^{-1}$		95±7	50 µg Cu L $^{-1}$		300 (255-343)
		$^{-1}$)		50	non-acclimated (1 µg Cu L $^{-1}$)		240 (219-263)
Carp *Cyprinus carpio*	Laboratory study	[MT] as µg metal mL $^{-1}$ eluant (analyse by liquid chromatography)			Time for 100% mortality (h)		
			liver	kidney	gill	Toxicity test Cd conc.: 15 mg L $^{-1}$	
Kito *et al*. (1982)		acclimated 14 d to 1 mg CdL $^{-1}$	0.3 Cd,0.3 Zn	1.0 Cd,0.5 Zn	0.1 Cd,0.2 Zn	acclimated to Cd	20
		5 mg Zn L $^{-1}$	0.1 Cd,1.2 Zn	0.1 Cd,0.7 Zn	ND Cd,0.7 Zn	acclimated to Zn	26
		non-acclimated	0.1 Cd,0.5 Zn	0.1 Cd,0.3 Zn	ND Cd,0.1 Zn	non-acclimated	15

Table 4 (continued)

Organism	Type of experiment	Estimation of [MT]		Estimation of resistance/tolerance to metals	
Juvenile rainbow trout *Oncorhynchus mykiss* (Dixon and Sprague 1981a,b)	Laboratory study	MT-like conc. (mg g $^{-1}$; mean $\pm$ SD) Liver Specimens exposed to 141 μg Cu L $^{-1}$ for 48 h control	48.8$\pm$10.9 32.7$\pm$4.3	Incipient lethal levels (ILL; μg Cu L $^{-1}$) (fiducial limits) Specimens acclimated to 131 μg Cu L $^{-1}$ for 7 d control	639 (585-699) 374 (309-453)
Marine blue mussel *Mytilus edulis* (Roesijadi and Fellingham 1987)	Laboratory study	[MT] as μg Cd on MT g $^{-1}$ gill (analyse by liquid chromatography) acclimated 28 d to 50 μg Cd L $^{-1}$ 10 μg Cd L $^{-1}$ non-acclimated	9.6 1.9 0.1	Cumulative % survival after 14 days of exposure Toxicity test Hg conc.: **75** μg L $^{-1}$ acclimated 28 d to 50 μg Cd L $^{-1}$ 10 μg Cd L $^{-1}$ non-acclimated	57 56 29

Natural populations of fruit fly *Drosophila melanogaster* (Maroni *et al.* 1987) — Laboratory study

Relative estimations

Fly strains with MT-gene duplications produced 1.7 to 2.1 times as much MT as wild type controls

% survival to the pupariation stage

Strains	[Cu] in food (mM)		
	2	**3.6**	**4.6**
MT gene duplications	94-106	84-96	80-95
Controls	68	42	35

Note: ND: non detectable

4.3 <u>Invertebrates</u>

4.3.1 **The Rouyn-Noranda mining area**

4.3.1.1 *History, pollutant sources and abiotic contamination*

Mining activities in the Rouyn-Noranda-Val D'Or mining area began in the early 1920s. At that time, it was a wilderness area accessible only by rivers and lakes. The area is in a complex geological zone characterized by rocks of Precambrian origin and glaciolacustrine deposits left behind by the post-glacial Lake Barlow-Ojibway at elevations of less than 300 m. Several geological faults cross the region delimiting mineral deposits rich in Au, Ag, Cu, and Zn (Surficial geology map No 1639A, 1987, and map No 900A, 1991; Geological Survey of Canada; Energy, Mines and Resources, Canada).

The Rouyn-Noranda copper smelter, built in 1927, has since been expanded many times. For the whole year of 1977, atmospheric emissions from the smelting complex included 485,000 tons (t) SO_2, 75 t Cd, 34 t Cu, 1540 t Pb, 610 t Zn, and 16 t Hg. Lesser emissions of Bi, Co, Fe, Ni, Sb, Se, and Te were also reported (BEST 1979a). Sediment samples obtained in the late 1970's indicated that surficial sediments in lakes located within 10 km of the smelter were severely contaminated by Cd, Cu, Zn, and Pb (BEST 1979b; Table 20). Air and water pollution controls have been introduced, but surficial sediments in lakes surrounding the smelter still reflect their history of contamination.

Twenty-four mine tailings parks, scattered over the area, present a potential for acid mine drainage. The tailings, of two types, are produced by the milling of base metal ores or by the treatment of gold ores (MENVIQ 1990).

Table 20

Mean trace metal concentrations (μ g g^{-1} dry wt) in the surface sediments of various lakes in the Rouyn-Noranda mining area in the late 1970's (adapted from BEST 1979b).

Lake	Distance from smelter	Cd	Cu	Zn	Pb	Hg (ng g^{-1} dry wt)
Osisko	<1	55	8900	10900	770	2000
Noranda	2.5	115	3400	3600	---	240
Rouyn	5	58	6200	14500	460	260
Dufault	6	17	1200	2200	380	200
Pelletier	6	16	1000	660	250	200
Beauchastel	13	5	100	270	68	110
Montbeillard	20	1	36	100	26	27
Caron	26	8	38	600	88	200
Regional back-ground level		<0.2	15-40	60-120	<1-10	20-90

4.3.1.2 *Experimental approach*

The present case study concerns the contamination of populations of the freshwater bivalve *Pyganodon grandis* inhabiting the Rouyn-Noranda mining area and an evaluation of metal-induced effects. The following key aspects were considered:

<div align="center">

Fate and distribution
(exposure)
↓
Bioavailability and uptake
↓
Effects

</div>
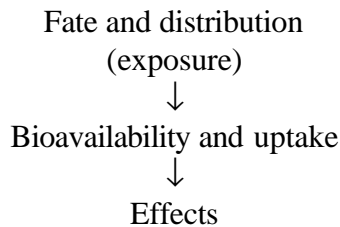
A geochemical modeling approach (see Box 15) was used to relate sedimentary variables to free-cadmium ion concentrations in the ambient water near the sediment-water interface, in order to define a lacustrine contamination gradient in terms of Cd^{2+}. Combining these surface complexation concepts with the Free-Ion model of trace metal-organism interactions (see Box 16)

Box No. 15

Geochemical partitioning model for estimating free metal ion concentrations in solution

Evidence for sorptive control of dissolved trace metal concentrations under oxic conditions is discussed by Campbell and Tessier (1996) (e.g. Cd: Tessier *et al.* 1993). Measured trace metal concentrations in oxic waters are consistently much lower than those calculated from solubility equilibria involving pure solid phases. Reactions other than metal precipitation must be involved in the geochemical control of dissolved [M] under such conditions. Given the presence in natural sediments of solid phases known to be important sorbents for trace metals, sorption reactions have generally been invoked to explain the observed undersaturation. The following is a schematic representation of metal coordination at the sorbent-water interface. The example is given for a hydrous metal oxide where 'X' can be an atom of Si, Ti, Al, Mn or Fe. Oxyhydroxides of Fe and sedimentary organic matter are known to be important sorbent phases.

$$\text{X-O-H} + M^{Z+} \Leftrightarrow \text{X-O-}M^{(Z-1)+} + H^+$$

or

$$\text{X-O-H}, \text{X-O-H} + M^{Z+} \Leftrightarrow \text{X-O}, \text{X-O} \diagdown M^{(Z-2)+} + 2H^+$$
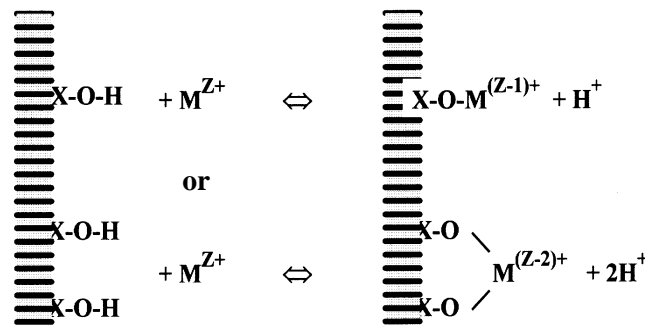
The complexation of a metal ion at the surface of the sorbent can be described by:

$$M^{Z+} + \equiv S\text{-}OH_x \overset{*K}{\Leftrightarrow} \equiv S\text{-}OM + x\,H^+$$
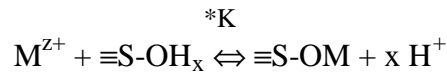
$$*K = \frac{\{\equiv S\text{-}OM\}\,[H^+]^x}{\{\equiv S\text{-}OH_x\}\,[M^{Z+}]}$$

where S=sorbent, $\{\equiv S\text{-}OH_x\}$=concentration of free binding sites on the sorbent, x=average apparent number of protons released when metal M is sorbed, $\{\equiv S\text{-}OM\}$= concentration of sites occupied by metal M, $[M^{Z+}]$=concentration of the free metal ion; $*K$=apparent overall equilibrium constant for sorption on the substrate. Concentrations of solid phases are indicated by {} parentheses, whereas concentrations of dissolved species are designated by brackets []; the notation '$\equiv$' refers to adsorption sites. Charges on the solid phases are omitted for simplicity.

Knowing the concentrations of sedimentary sorbent and sorbed metal, the ambient pH, and field-derived values of $*K$ and x, one can estimate the concentration of the free metal ion, M^{Z+}, in solution. Equilibrium conditions are assumed to prevail;

$$[M^{Z+}] = \frac{\{\equiv S\text{-}OM\}\,[H^+]^x}{\{\equiv S\text{-}OH_x\}\,*K}$$

Simplifying assumptions made for the use of this model, field-derived constant values, and examples of calculations are provided in Campbell and Tessier (1996) and Tessier *et al.* (1993).

Box No. 16

The Free-Ion Model

Biological responses to metals are determined not so much by the total concentration of the metal in an organism's environment, but by the concentration of the form(s) of the metal that can be taken up by the organism. The Free-Ion Model (FIM; equilibrium model; descriptions given by Campbell 1995, and Morel and Hering 1993) has been formulated to explain the importance of free-metal ion activities in the uptake, nutrition, and toxicity of cationic trace metals for organisms *obtaining their metal from water*. A simplified depiction of this model is shown below (adapted from Campbell 1995).

BULK SOLUTION   **PLASMA MEMBRANE**   **CELL**
   (mixed)

LABILE HYDROPHILIC COMPLEXES
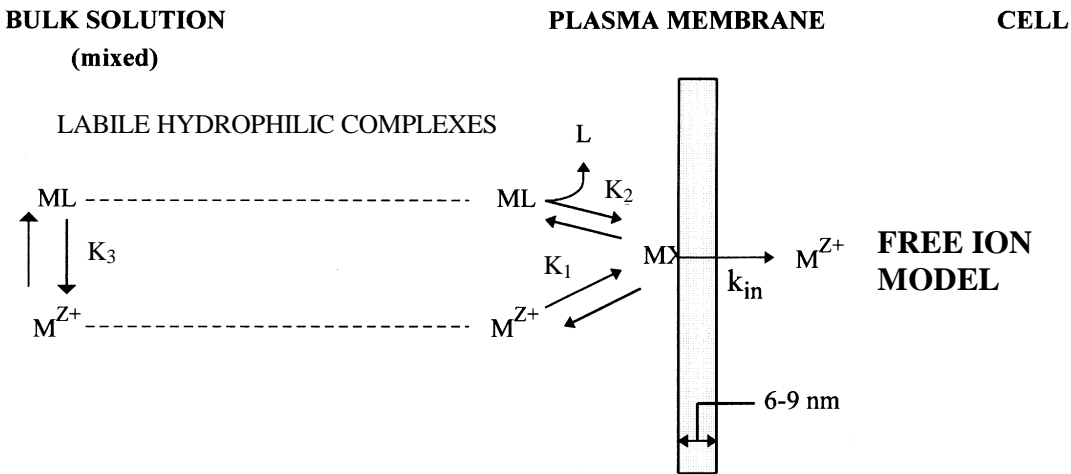
In the model, M^{Z+} = free metal ion; ML= metal complex in solution; M-X-cell = surface metal complex; K_1 and K_2 = equilibrium constants for formation of the surface complex; k_{in} = kinetic rate constant for internalization or transport of the metal across the biological membrane; K_3 represents the complexation reaction in solution (M+L $\leftrightarrow$ ML).

To elicit a biological response from an organism, whether it be metal uptake, nutrition, or toxicity, a metal must first interact with and/or traverse a cell membrane. The biological response is proportional to the concentration of the M-X-cell surface complex; in the range of metal concentrations of toxicological interest, variations of [M-X-cell] are assumed to follow those of $[M^{Z+}]$ in solution. In other words:

$$\text{Organism response} = f\,[\text{M-X-cell}]$$
$$= K_1\,[\text{X-cell}]\,[M^{Z+}]$$
$$\text{or } K_2\,[\text{X-cell}]\,[ML^{Z+}]/[L] = K_2 \cdot K_3[\text{X-cell}][M^{Z+}]$$

where f is a proportionality factor. Campbell (1995) describes the key assumptions underlying the Free-Ion Model, and critically reviews the numerous experimental studies undertaken to test the model.

it was demonstrated that the levels of Cd in the freshwater bivalve were controlled by the ambient free-ion activity. In turn, Cd bioaccumulation determined biological responses, that is detoxification of Cd by MT synthesis and adverse toxicological effects. In the following section, several elements will be discussed, extracted from the research papers, to review in detail the above approach.

4.3.1.3 *Dose-response relationships*

Study designs involved the collection of molluscs at lacustrine sites located along a metal-contamination gradient (Fig. 17). Measured biological variables included metal and metallothionein concentrations in indigenous organisms. Surficial sediments as well as pore and overlying waters were collected at each site to define metal contamination gradients. Highly significant relationships were obtained between metallothionein and Cd concentrations in the organisms, on one hand, and between MT and dissolved Cd^{2+} concentrations estimated from sediment-water sorptive equilibrium on the other (Fig. 18). In contrast, correlations with total metal concentrations in the sediments were statistically non-significant (Fig. 18; Couillard *et al*. 1993). These results may appear surprising for a benthic organism; however, *P. grandis* is a filter-feeder and the authors brought evidence supporting the idea that water is an important exposure vector for this bivalve (see Couillard *et al*. 1993, and Tessier *et al*. 1993).

In the study area, tissue MT concentrations were positively correlated with tissue Cd concentrations (Fig. 19; Couillard *et al*. 1993). In contrast, no such correlations were observed between [MT] and tissue levels of Cu or Zn (Fig. 19). These observations do not rule out the possible binding of metals other than Cd to MT in the freshwater mollusc (Couillard *et al*. 1993), nor do they mean that environmental concentrations of Cu and Zn do not vary along the metal contamination gradient. Indeed, there exists a strong relationship between body levels of Cu and dissolved Cu concentrations in the region (Fig. 20).

Figure 17: Locations of the sampling stations. Average wind direction at the Rouyn-Noranda smelting complex is also shown.
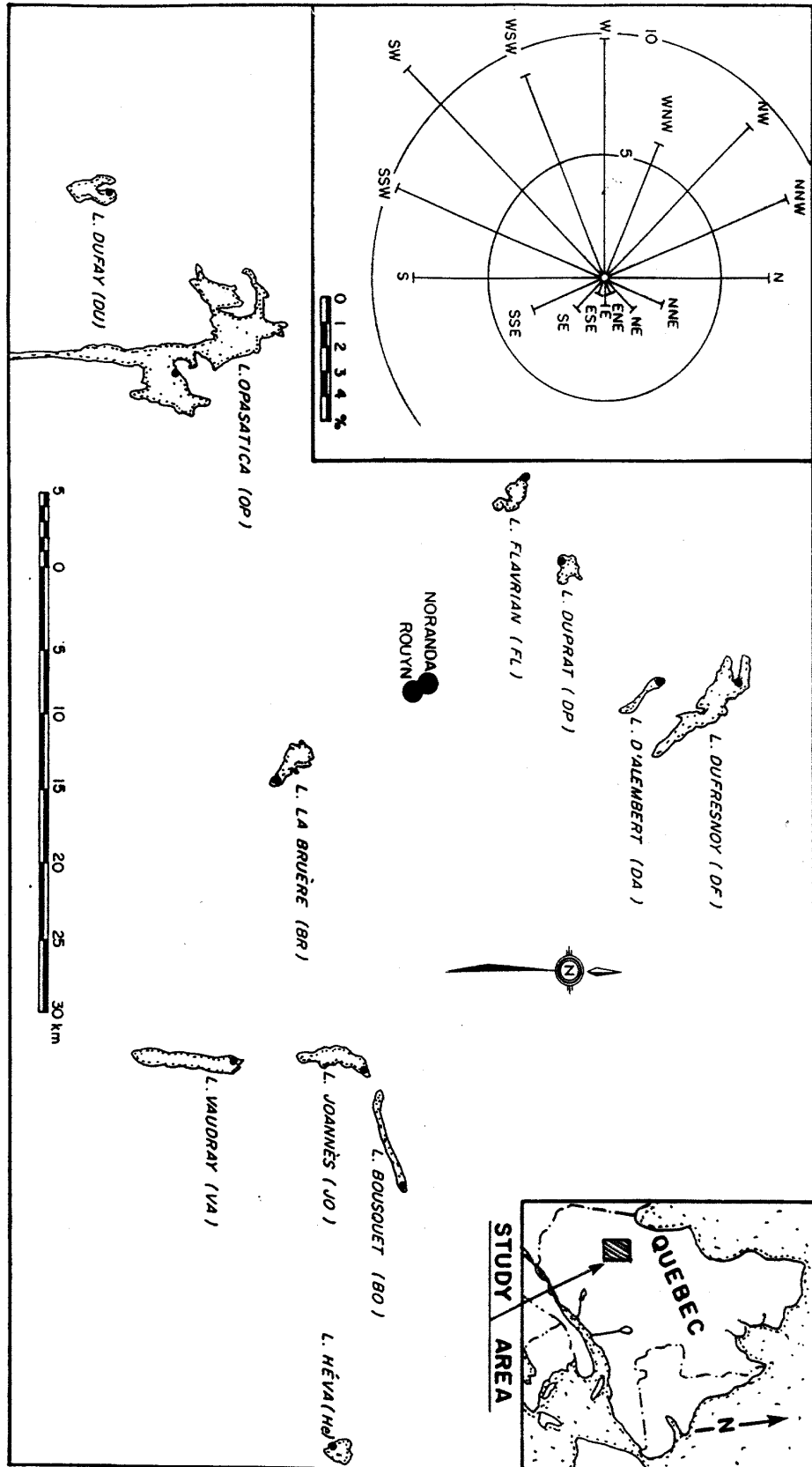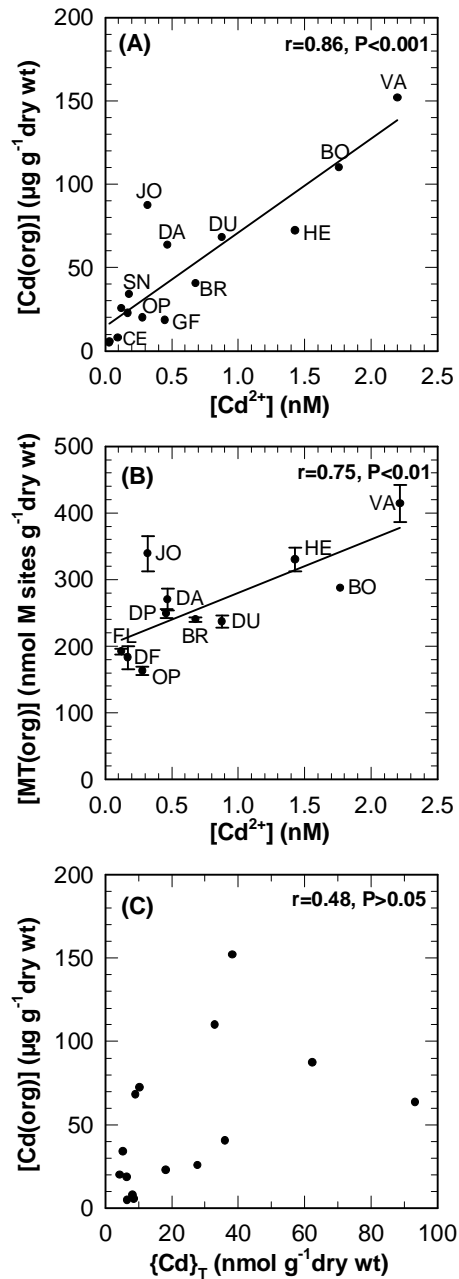
Figure 18: Scatter diagrams of (A) Cd, and (B) metallothionein concentrations in *Pyganodon grandis vs* [Cd^{2+}] calculated from sediment-water sorptive equilibria. (c) Relationships between mollusc and total sediment [Cd]. For correspondence between symbols and individual lakes, see map + SN: St. Nora (Ont.), GF: Gullfeather (Ont.), CE: Brompton (Qué) (adapted from Couillard *et al.* 1993, and Tessier *et al.* 1993).
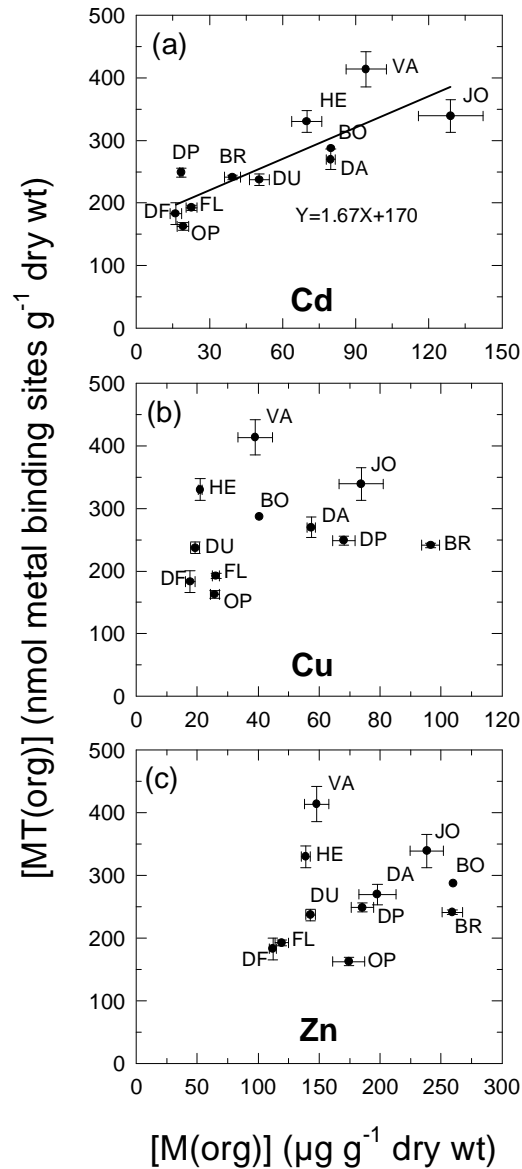
Figure 19: Scatter diagrams of MT concentrations *vs* concentrations of Cd, Cu, and Zn in the soft tissues of *P. grandis*. The molluscs were collected from 11 lakes chosen to represent a metal contamination gradient. Pearson correlation coefficients between whole organism [MT] and whole organism [Cd], [Cu], and [Zn] are, respectively, 0.83 ($P<0.01$), 0.22 ($P>0.05$), and 0.21 ($P>0.05$). For correspondence between symbols and individual lakes, see map (adapted from Couillard *et al.* 1993).
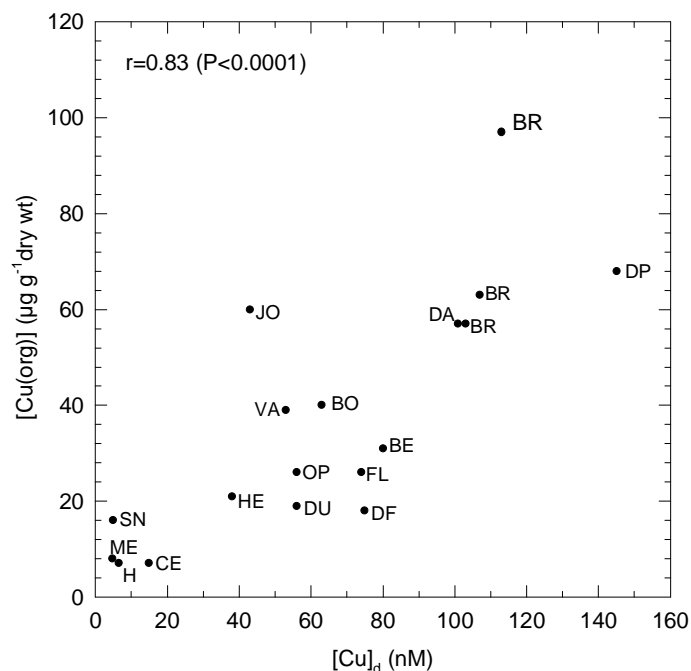
Figure 20: Scatter diagram of Cu concentrations in the soft tissues of *P. grandis vs* dissolved Cu concentrations (Cu_d) measured in lakes of the Rouyn-Noranda mining area, and in other lakes in Ontario and Québec. For correspondence between symbols and individual lakes, see map; and SN = St. Nora (Ont.), ME = Memphrémagog (Qué), CE = Brompton (Qué), H = Harp (Ont.) (adapted from Couillard 1993).

The above results are consistent with the reported potency of Cd in inducing MT biosynthesis in laboratory experiments (e.g. Roesijadi *et al*. 1988). Jones *et al*. (1988) showed that the relative ability of metals to induce metallothionein synthesis is inversely correlated with their softness parameter, σ_ρ. A «soft» electron acceptor is characterized by a high polarisability of its outer electronic shell and a tendency to form stable bonds with «soft» ligands, e.g. those containing free thiol groups, RS^-. Lower values of σ_ρ correspond to softer ions; ; σ_ρ values for Cd^{2+}, Cu^{2+}, and Zn^{2+} are, respectively, 0.081, 0.104, and 0.115 (Ahrland 1968).

In addition to the above spatial survey, the researchers selected two lakes from the 11 lakes sampled in the spatial study to demonstrate that bivalve MT concentrations also respond temporally to changes in the degree of environmental contamination (Fig. 21). Molluscs were transferred from Lake Opasatica to the highly contaminated Lake Vaudray (see Fig. 17), and kept in open plastic enclosures in contact with the bottom sediments for a duration of 400 days. Temporal increases in tissue [MT] in the transplanted bivalves were correlated with temporal increases in tissue [Cd] (see Couillard *et al*. 1995a). Only moderate seasonal variations in [MT] were observed for control mussels. At each site, these seasonal variations were insignificant (P>0.05) relative to the marked differences between MT concentrations in the bivalves from the two sites (Fig. 21).

Taken together, these results suggest that MT levels in *P. grandis* are particularly sensitive to increases in ambient [Cd] in the study area. Moreover, characteristics related to the basic biology/physiology of *P. grandis* appear less important than changes in metal bioavailability as sources of variation in [MT] (discussed in Couillard *et al*. 1995a).

4.3.1.4 *Toxic effects*

Transplant experiment

The above transplantation experiment was also designed to examine anticipated toxic effects on *P. grandis* caused by an abrupt increase in ambient metal levels. The selected lakes Opasatica and Vaudray differed widely in their levels of contamination and with respect to the MT concentrations found in their indigenous mollusc populations, but they shared similar ecological characteristics (Table 21). Comparisons of condition indices and shell growth rates for the two populations suggested that the sites were indeed of similar trophic status.
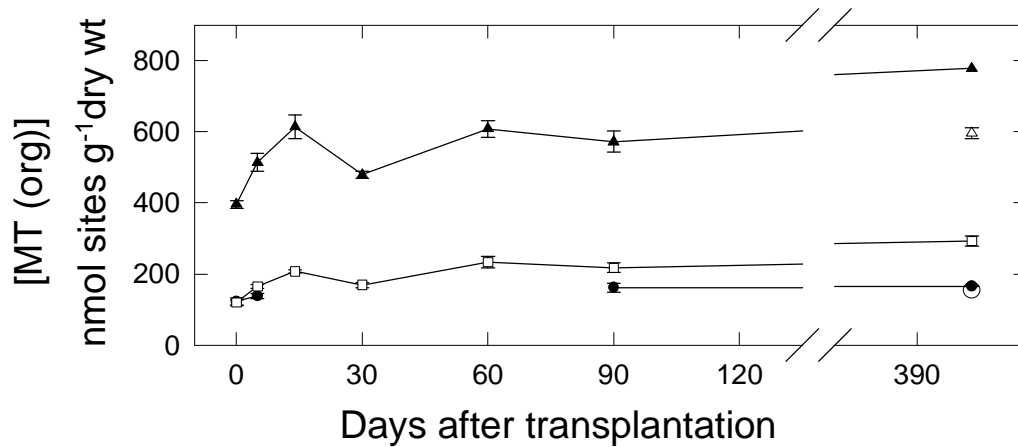
Figure 21: Variations over time of MT concentrations in whole bivalves transplan ted from a less (Lake Opasatica) to a more contaminated lake (Lake Vaudray) in the mining area of Rouyn-Noranda. Temporal variations in organism [MT] in the source and destination lakes are also indicated. Legend: : transplanted bivalves kept in enclosures; ●: control bivalves kept in enclosures in L. Opasatica; ▲: control bivalves kept in enclosures in L. Vaudray; ○: free indigenous bivalves in L. Opasatica; Δ: free indigenous bivalves in L. Vaudray (adapted from Couillard *et al.* 1995a).

A mechanism of cytotoxicity was investigated to assess if the transplanted bivalves suffered from metal-induced stress (Couillard *et al*. 1995b). In this model, non-metallothionein bound metal concentrations are taken to reflect the quantity of metal available to express cytotoxicity (Fig. 22, step 1). These metal pools would be expected to enhance the lipid peroxidation process (step 2; see Box 8), resulting in increased levels of malondialdehyde (MDA), a by-product of this peroxidation. It is well established that Cd, and Cu in particular (see Box 8), can stimulate this process.

Table 21

Geochemical and biological data for the two sites chosen for the mollusc transplantation experiment in the Rouyn-Noranda area. Metal and MT concentrations in sediments and biological tissues are expressed on a dry weight basis (adapted from Couillard *et al.* 1995a).

	Geochemical data			Biological data	
	L. Opasatica	L. Vaudray		L. Opasatica	L. Vaudray
Dissolved [M]			Whole organism		
[Cd] (nM)[a]	0.8	1.2	[MT] (nmol M sites g^{-1})	154 ± 10	596 ± 16
[Cu] (nM)[a]	56	53	[Cd] (nmol g^{-1})	178 ± 6	$1,560 \pm 60$
[Zn] (nM)[a]	40	174	[Cu] (nmol g^{-1})	680 ± 43	$1,260 \pm 60$
Calculated [Cd^{2+}] (nM)[b]	0.28	2.2	[Zn] (nmol g^{-1})	$3,130 \pm 150$	$4,880 \pm 280$
{M} in oxic sediments			Gills		
Extract.{Cu} (nmol g^{-1})[c]	54	88	[MT] (nmol M sites g^{-1})	109 ± 14	411 ± 20
Extract. {Zn} (nmol g^{-1})[c]	150	1200	[Cd] (nmol g^{-1})	279 ± 19	$2,560 \pm 150$
Total {Cd} (nmol g^{-1})	4.4	38	[Cu] (nmol g^{-1})	$1,435 \pm 155$	$3,060 \pm 200$
Total {Hg} (nmol g^{-1})	0.10	0.10	[Zn] (nmol g^{-1})	$6,660 \pm 260$	$10,140 \pm 830$
			Organism characteristics		
			Condition index	0.110±0.003	0.103±0.009
			Growth rate	Similar	

[a] Single measure at 10 cm above the sediments (June 1989).

[b] [Cd^{2+}] estimated from sediment/water sorptive equilibria; see Box 15 for details.

[c] [M] extracted for 6h at 96°C with 0.04 M $NH_2OH.HCl$ in 25% (vol/vol) HOAc.

In the case of sustained aggression by metals, several defence systems against oxidative damage would be impaired. A depletion of glutathione (GSH), the cell's main antioxidant, might be anticipated (step 3). Reactive oxygen species as well as intruding metals would alter the Ca-extruding systems of the plasma membrane, e.g. by interacting with -SH groups in the Ca-Mg
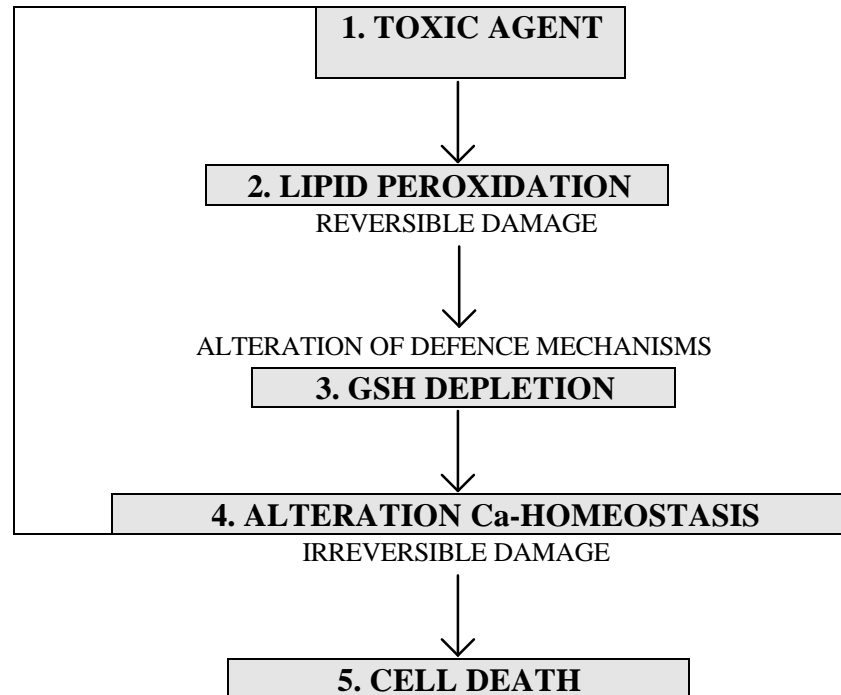
Figure 22: Model of trace metal cytotoxicity showing the sequence of events leading to cell death after lipid peroxidation. Variables used to evaluate the model are non-thionein-bound cadmium, step 1; malondialdehyde concentrations [MDA], step 2; and cytosol [Ca], step 4. The target organs are the gills (adapted from Couillard *et al.* 1995b).

ATP-ase. At this stage (step 4), an accumulation of Ca in the cytoplasm would occur; calcium-mediated functions of the cell would be impaired and eventually this would lead to cell death (step 5).

Gills were chosen as a likely organ in which the postulated chain of cellular events might occur; only steps 1, 2 and 4 were monitored over time. Important changes occurred in the course of the transplant experiment. Initially, in transplanted mussels, gill cytosolic Cd was mainly associated with high molecular weight (HMW) ligands (Table 22). After 90 d, Cd was associated with newly synthesized MT (see Couillard *et al*. 1995b). In contrast with Cd, and in accordance with its status as an essential element, cytosolic Cu was mainly bound to the HMW pool, which contains metalloproteins (Table 22). After 400 d, a large amount of cytosolic Cd was associated

Table 22

Cadmium and Cu concentrations in HPLC gel permeation fractions of gill cytosols from bivalves transplanted to Lake Vaudray and collected after 14, 90, or 400 d (adapted from Couillard *et al.* 1995b).

Molecular wt fraction (MW)	MW	[M] in fractions for each treatment ($\mu g \ g^{-1}$ dry wt)			
		14 d [Cd]	90 d [Cd]	90 d [Cu]	400 d [Cd]
High	> 15 kD	1.74	0	8.35	1.09
MT	15-3 kD	0	6.62	1.49	0.96
Low	< 3 kD	0	0	0	5.73
Σ fractions		1.74 *versus* 2.28[a]	6.62 *versus* 4.26[a]	9.83 *versus* 10.87[a]	7.79 *versus* 6.33[a]
Recovery of total [M]$_{cyt}$ (%)		77	155	91	123

[a] The total metal concentrations in gill cytosols were determined by plasma atomic emission spectrometry in digested subsamples of 170 000×g gill homogenates.

with low molecular weight (LMW) ligands, as if «spillover» had happened. This last shift in metal distribution coincided with increased oxidative degradation of membranes (MDA $\uparrow$, step 2, Fig. 22), and disturbed cellular Ca homeostasis (cytosolic Ca $\uparrow$, step 4, Fig. 22). In addition, condition indices and growth rates of the transplanted mussels declined over time (Couillard *et al*. 1995b).

Natural contamination gradient

Having detected abnormal intracellular Cd partitioning in the transplanted mussels at 400 d, the author verified if the same phenomenon occurred naturally along a contamination gradient in reaction to a chronic Cd exposure. The above series of lakes in the Rouyn-Noranda mining area (Fig. 17) was revisited and subcellular Cd distributions in mussel gills were determined along the contamination gradient.
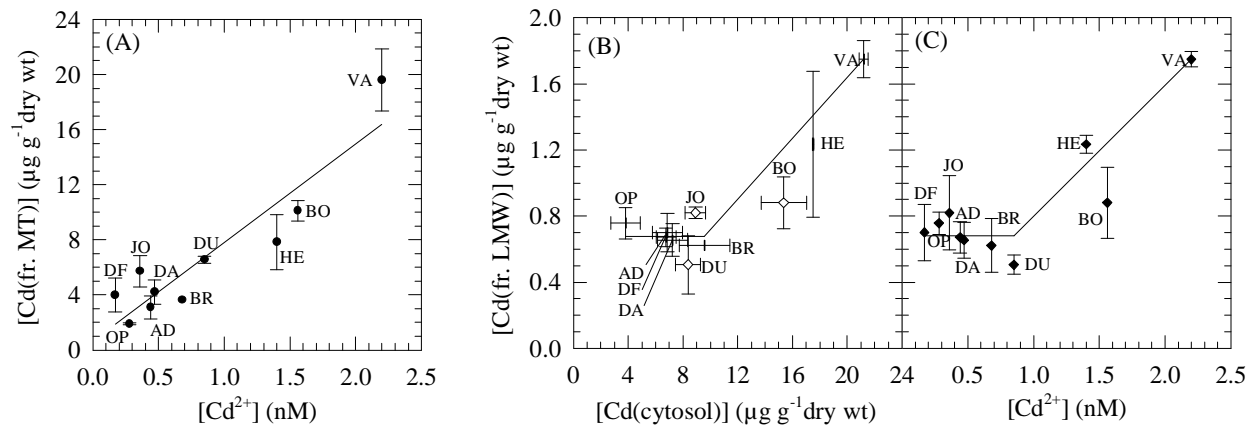
Figure 23: Inter-lake variation in the subcellular distribution of Cd among gill cytosol fractions from *P. grandis* collected in the Rouyn-Noranda area (Campbell *et al*., unpublished data). (A) Cd in the MT fraction and (C) Cd in the low molecular weight (LMW) fraction, as a function of the ambient Cd^{2+} concentration. (B) Cd in the LMW fraction as a function of [Cd(cytosol)]. Each point represents the mean $\pm$ SE for a particular site.

Results of chromatographic separations of gill cytosol extracts supported observations obtained earlier (Fig. 18B; Couillard *et al*. 1993) - amount of Cd associated with the MT peak» increased as a function of environmental $[Cd^{2+}]$ (Fig. 23A; unpublished results). Moreover, cytosolic Cd was not entirely chelated by MT - even in the least contaminated lakes, a part of this Cd was associated with LMW compounds (Fig. 23B). At environmental Cd^{2+} concentrations above a <u>threshold</u> of about 0.9 nM, the amount of cytosolic Cd bound to LMW cytosolic ligands increased markedly (Fig. 23C).

Does the above association of cytosolic Cd with low molecular weight ligands corresponded to the onset of deleterious effects at the organism or population levels? The authors had preliminary data to answer this question.
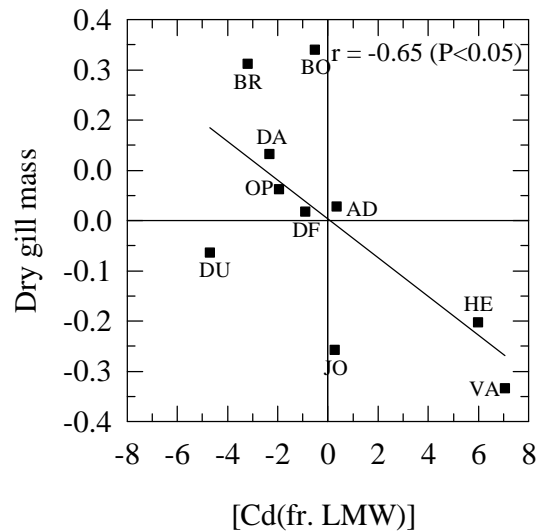
Figure 24: Scatterplot of the partial correlation between dry gill mass and Cd concentrations in the LMW fraction of indigenous bivalves, holding shell length constant (Campbell *et al*., unpublished data). Solid squares are the replicate samples. Vertical and horizontal lines indicate lack of departure of an observation from its estimation by shell length.

Dry gill masses decreased significantly with increases of Cd in LMW chromatographic fractions (Fig. 24). Lake Vaudray (VA) bivalves exhibited the highest Cd levels in these fractions, and the lowest gill masses of the 10 populations sampled in the spatial study. Moreover, parts of the gills of individual specimens from the Lake Vaudray population appeared necrotic, whereas gills from specimens belonging to the other populations appeared regular in shape (Couillard *et al*. 1995b). At the time of the transplantation experiment, individuals of the Lake Vaudray population tended to have a lower incidence of gravid females per sample and a lower mean larval dry weight per female relative to the least contaminated population (Table 23). In addition, larval stages exhibited a marked imbalance in tissue [Cd] to [MT] ratios, suggestive of a Cd spillover (Couillard *et al*. 1995b). Note that the formation of larval stages of *P. grandis* are carried in gill marsupia and

that these brood chambers are functionally isolated from the external medium during reproduction

(*i.e.* no direct exposure to water in the palleal cavity or to lakewater; Richard *et al*. 1991).

Table 23

Characteristics of the bivalve populations (*Pyganodon grandis*) from Lake Opasatica and Lake Vaudray, and from a reference lake, based on the approach of Munkittrick and Dixon (1989) [a] (adapted from Couillard *et al*. 1995b).

Population characteristics	Lakes		
	Vaudray	Opasatica	Reference lake
Mean age (yr)	5.23	4.88	---
Growth rate	Similar		---
Condition index	0.103±0.009	0.110±0.003	0.114[b]
Fecundity (% gravid female/sample)	29	50	38[c]
Mean dry wt larvae per bivalve (g)	0.14	0.25	---

[a] The principle of their approach is that a population of organisms found to be growing, reproducing, and surviving within the limits observed for a comparable reference population will be considered free from detrimental contaminant exposure effects.

[b] Reference population of *P. grandis* living in a pristine Precambrian Shield lake in northwestern Ontario (Huebner *et al*. 1990).

[c] Reference population of *Elliptio complanata* living in a relatively unpolluted lake in southern Québec (Downing *et al*. 1989).

4.3.1.5 *Summary*

The most important results from the described studies above are summarized below:

a. Tissue concentrations of metallothionein in *P. grandis* were strongly correlated with tissue Cd concentrations. In contrast, correlations between [MT] and the tissue concentrations of the essential metals Cu or Zn were non-existent.

b.   Spatial and temporal variations in MT levels in *P. grandis* closely reflected the changes in
the ambient free Cd concentration (from 0.15 to 2.5 nM Cd^{2+}), as estimated from
sediment/water sorptive equilibria. Together with the results in **a**, these observations
suggest that Cd^{2+} activity is the key environmental factor to which metallothionein levels
are responding in the studied lakes.

c.   Metallothionein concentrations in *P. grandis* collected from a given lake showed only
moderate seasonal variability (June-September), much less than the inter-lake variability
encountered along the contamination gradient.

d.   Shifts in cytosolic metal distributions were observed along the contamination gradient and
they appeared to be reproducible under severe metal stress (transplantation experiment).
These biochemical abnormalities were linked to deleterious effects at higher levels of
biological organization.

4.3.2 **The San Francisco Bay**

4.3.2.1 *History, pollutant sources and environmental contamination*

This case study was not performed in a mining region. However, it deals with the sub-cellular distribution of metals in relation to manifestations of toxicity in a natural population of an estuarine bivalve mollusc. San Francisco Bay is a structurally and temporally complex estuarine system (Fig. 25). Physical processes are influenced by seasonal cycles of wind, riverine inputs, salinity changes, and tidal regimes. As a result of this, distributions of trace metals in the water column, sediments and biota are necessarily heterogeneous in space and time (Luoma and Phillips 1988). Superimposed on this spatio-temporal variability in trace metal distributions is the diversity and number of anthropogenic inputs of contaminants to the bay. Inputs include 50 municipal waste water treatment plant discharges, 18 major industrial discharges including those of 6 petroleum refineries, inputs of untreated surface runoff, and trace element inputs occasioned by activities of 20 boat marinas, naval bases and coastal harbours. Metals are also remobilized during dredging of metal-enriched sediments, and from forty hazardous waste disposal sites disseminated on the shores of the bay (Luoma and Phillips 1988). On the basis of loadings from anthropogenic sources, and of the frequency and severity of metal contamination in water, sediments and biota, the trace metals of greatest concern are Ag, Cu, Cd, Se and Hg (Luoma and Phillips 1988). Table 24 provides ranges of sedimentary trace metal concentrations in San Francisco Bay, together with whole body metal levels in the deposit feeder mollusc *Macoma balthica* (the study organism) at the Palo Alto sand flat (South San Francisco Bay).
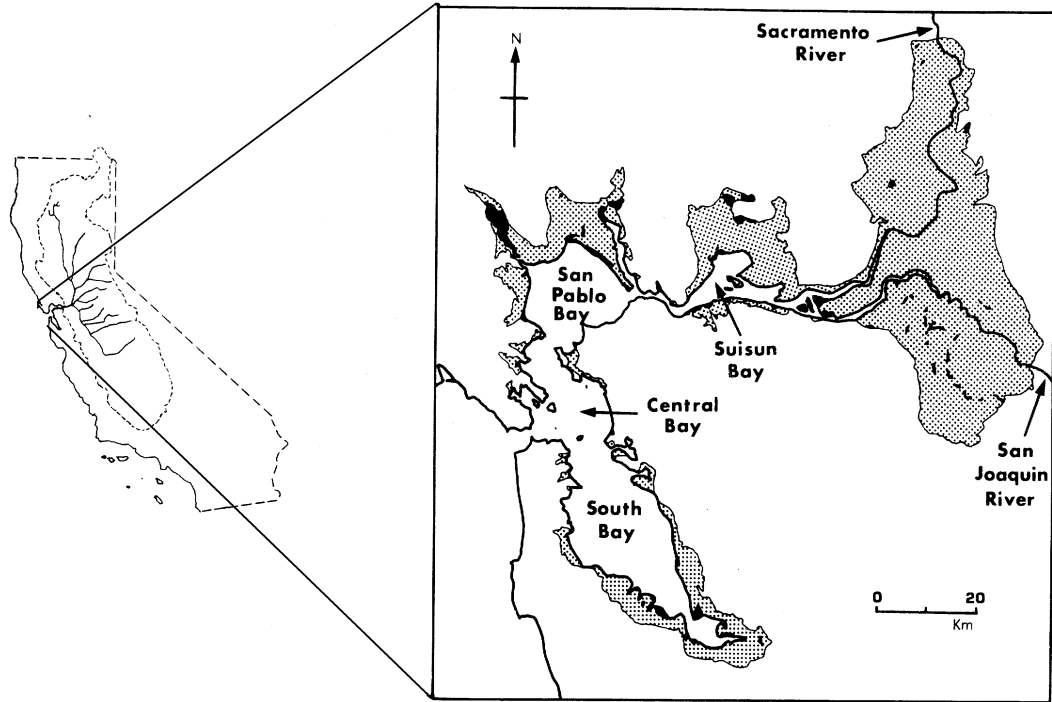
Figure 25: San Francisco Bay and its general location in California (adapted from Luoma and Phillips 1988).

Table 24

Ranges of sedimentary metal (M) concentrations in San Francisco Bay, and ranges of whole body metal levels in the bivalve *Macoma balthica* living on Palo Alto sand flat, South San Francisco Bay (adapted from Luoma and Phillips 1988, and Johannson *et al*. 1986).

Metal	Sediment M concentrations ($\mu g\ g^{-1}$ dry wt)	Metals in *Macoma balthica* at Palo Alto ($\mu g\ g^{-1}$ wet wt)
Ag	1.2 - 66.0	67[a]
Cd	2.0 - 15.6	~1
Cu	37 - 380	70 - 420
Hg	0.4 - 10.5	-
Ni	31 - 530	-
Pb	52 - 2900	-
Se	0.9 - 35	-
Zn	140 - 1890	200 - 520

[a] ($\mu g\ g^{-1}$ dry

4.3.2.2 *Cytosolic metal distributions and metal stress in Macoma balthica*

Specimens of the bivalve *M. balthica* were harvested on a monthly basis from January 1981 to June 1982 in South San Francisco Bay near Palo Alto. Cytosols were extracted from whole animals and submitted to gel filtration chromatography. Chromatographic fractions were analyzed for Ag, Cu and Zn, and were defined as a high molecular weight (HMW) metal ligand pool (>30 kD), a metallothionein (MT) pool (3 - 25 kD), and a low molecular weight (LMW) metal ligand pool (<3 kD; Johannson *et al*. 1986).

Results showed that concentrations of Cu and Ag in the HMW pool stayed relatively constant during the 2-year period (1 µg g^{-1} wet wt for Cu). Concentrations of Cu and Ag in the MT and LMW pool exhibited marked temporal variations. For Cu, levels ranged from 3 to 4.1 µg g^{-1} wet wt in the MT pool, and from ~0.1 to 4.1 µg g^{-1} wet wt in the LMW pool. When individual metal levels in fractions were considered altogether, independently of the time covariate, concentrations of Cu, Ag and Zn in the MT metal ligand pool were linearly correlated with their corresponding cytosolic levels; concentrations in the MT pool did however tend to plateau asymptotically at high cytosolic metal levels. Concentrations of Cu, Ag and Zn in the LMW metal ligand pool remained low at low cytosolic metal concentrations. However, above a threshold in cytosolic metal levels, the amount of metal bound to LMW cytosolic ligands increased markedly. Thresholds of cytosolic [Cu], [Ag] and [Zn] were, respectively, ~5 µg g^{-1} tissue wet wt, 200 ng g^{-1} tissue wet wt, and 15 µg g^{-1} tissue wet wt. The marked increase of these metals in the LMW pool coincided with the point where concentrations tended to plateau in the MT pool. These trends are graphically illustrated in Figure 26. Recall that a similar shift in cytosolic metal distribution was observed in the Rouyn-Noranda case study (section 4.3.1).

Johannson *et al*. (1986) did not perform *in situ* measurements to link metal «spillover» into the LMW metal ligand pool to indications of metal stress. However, converging lines of evidence

provided by a 17-year study of the Palo Alto sand flat suggested that the indigenous population of *M. balthica* was indeed metal-stressed (Luoma 1995). Reproductive anomalies were periodically observed. Production of biomass declined during years of increases in Ag and Cu enrichment. Moreover, the mollusc population temporarily disappeared when contamination by both metals was most severe. This population was six times more tolerant to Cu and Ag than other populations of *M. balthica* in San Francisco Bay. Luoma (1995) indicated that the large temporal variabilities in biomass production and metal adaptation were population-level attributes unique to *M. balthica* inhabiting the Palo Alto sand flat.

4.3.2.3 *Summary*

In specimens of the marine bivalve *Macoma balthica* collected at monthly intervals in San Francisco Bay, Johansson *et al*. (1986) showed that [Cu], [Ag] and [Zn] in the low molecular weight cytosolic ligand pool increased steadily during periods of high metal enrichment. They did not systematically link such cytosolic metal profiles with the onset of metal stress. Several lines of evidence, provided by a 17-year study of the mollusc population, did however suggest that there were links between the above biochemical measurements and adverse effects at organism- and population levels of organization.
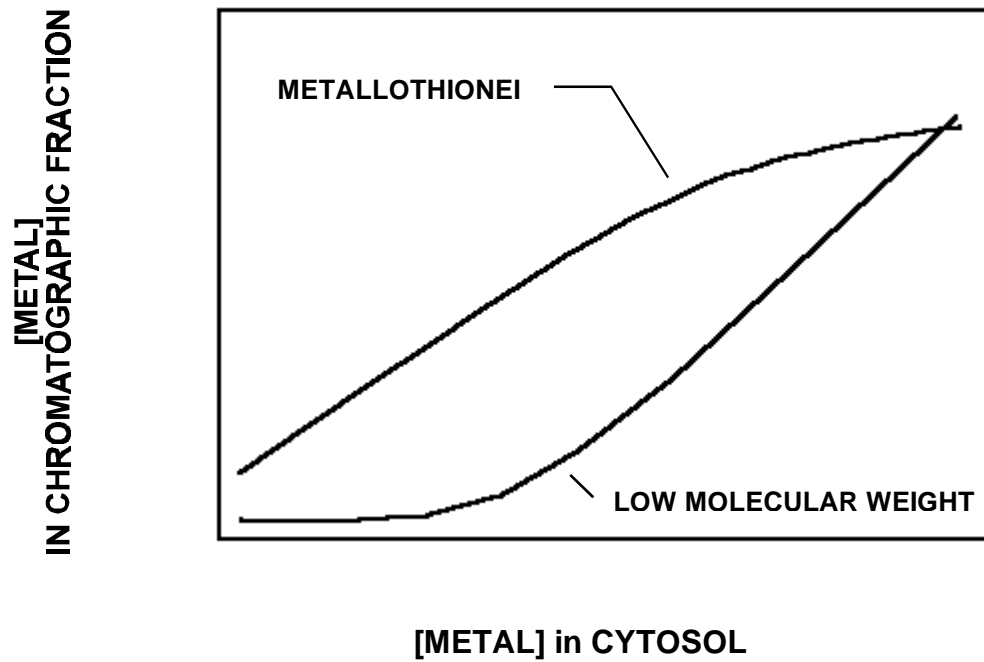
Figure 26: Schematic representation of variations in metal concentrations in the MT and low molecular weight chromatographic fractions as a function of total cytosolic metal levels in a temporal study of the bivalve population *Macoma balthica* living on the Palo Alto sand flat, San Francisco Bay, CA (adapted from Johannson *et al.* 1986).

4.3.3 **The ELA whole-lake cadmium experiment: studies with molluscs.**

4.3.3.1 *Reminder*

Lake 382, a small Precambrian Shield lake in the Experimental Lakes Area, received experimental additions of Cd over 6 consecutive ice-free seasons (1987-1992). Several animal species were monitored for metal uptake and metallothionein responses (see section 4.2.3.3); we report here the results obtained for the freshwater bivalve *Pyganodon grandis*. The rationale behind whole-lake experimental manipulation is discussed in section 4.2.3.1. The conduct of the present experiment and the fate of added Cd are described in section 4.2.3.2.

4.3.3.2 *Dose-response relationships*

Specimens of *P. grandis* were harvested in Lake 382 in the autumn 1989, *i.e.* after 3 experimental additions of Cd to the lake. Metallothionein levels responded temporally to the increase in the degree of metal contamination and several body parts produced MT in response to Cd exposure (Malley *et al.* 1993; Table 25). The gill MT response can be compared to MT levels measured in populations of *P. grandis* sampled in 1989 in the Rouyn-Noranda mining area (Couillard *et al.* 1993; section 4.3.1); values of gill [MT] and of [Cd^{2+}] were obtained for the two studies. As metallothioneins were measured by the same analytical method in both these investigations, a Hg-displacement assay, gill MT values in Malley *et al.* (1993) could easily be converted into units of nmoles metal binding sites g^{-1} dry tissue weight, assuming a molecular weight of 10 kDA for MT, a stochiometric ratio of 7 moles Hg mole^{-1} MT, and a dry wt to wet wt ratio similar to the Rouyn-Noranda gill samples. For the whole-lake Cd experiment, a mean epilimnetic [Cd^{2+}], averaged over the addition periods 1987, 1988 and 1989, was calculated using a speciation model for aqueous Cd developed by Wagemann *et al.* (1994) for L. 382. The model assumed that the total Cd concentration in water was distributed among 3 main pools: a free ion Cd^{2+} pool, a pool of Cd complexed to dissolved organic carbon (DOC), and a pool of Cd sorbed

Table 25

Metallothionein concentrations in body parts of *P. grandis* specimens collected from Lake 382, having received experimental additions of Cd, and from pristine Lake 377 (adapted from Malley *et al.* 1993).

Site	Date of collection	[MT] in µg g^{-1} wet weight (mean $\pm$ SE)				
		Mantle	**Gill**	**Foot**	**Kidney**	**Visceral mass**
Lake 377	21 Sept. 89	2.5 ± 0.5	13.7 ± 2.4	12.2 ± 1.5	33.7 ± 12.2	16.4 ± 3.5
Lake 382	20 Sept. 89	9.5 ± 3.4	35.0 ± 5.8	26.0 ± 4.3	118.3 ± 53.2	36.3 ± 5.2

Note: All values from Lake 382 indicated significantly different from those of Lake 377 in Malley *et al*. (1993).

to suspended particulate matter (SPM). Values of total [Cd] in water, [DOC], and SPM concentrations were obtained from Lawrence *et al.* (1996). Figure 27 shows the strong significant relationship obtained between gill metallothionein levels and Cd^{2+} concentrations for the Rouyn-Noranda mining area (see Couillard *et al.* 1993). The point for the ELA Lake 382 bivalve population is very close to its estimation provided by the Rouyn-Noranda regression. Several observations can be derived from these results.

a. Gill MT levels in the Rouyn-Noranda and in the Lake 382 bivalve populations responded similarly to Cd exposure as defined by the free metal ion Cd^{2+}. Note that these mollusc populations live in Precambrian Shield lakes and that a distance of ~ 1100 km separates ELA from the Rouyn-Noranda mining area.

b. The Cd exposure experienced by the L. 382 bivalve population over the first 3 years of Cd addition was in the low range of Cd exposures experienced by bivalves in the Rouyn-Noranda mining area.
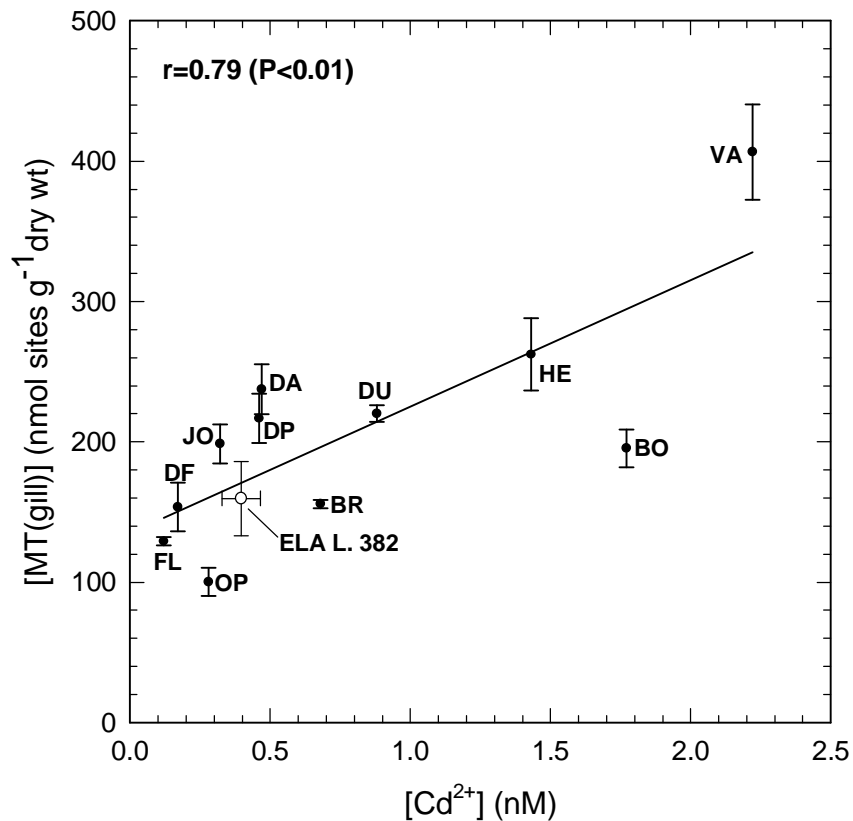
Figure 27: Relationships between metallothionein levels (mean ± SE) in gills of the bivalve *P. grandis* and Cd^{2+} levels in lakes of the Rouyn-Noranda mining area. Coordinates of the data point 'ELA L. 382' are (mean ± SE): MT: 160±26, Cd^{2+}: 0.40±0.07. Codes refer to the lake names indicated in the map, Figure 17. (Information sources: Lawrence *et al.* 1996, Wagemann *et al.* 1994, Couillard *et al.* 1993, and Malley *et al.* 1993).

c. Malley *et al.* (1993) did not document the state of health of the Cd-exposed bivalves in Lake 382. Some parallels can be drawn between this study and the Rouyn-Noranda study though. Gills of the Rouyn-Noranda populations in the low range of Cd exposure (*i. e.* Cd exposure similar to that in ELA L. 382; Fig. 27) appeared regular in shape. Gills of the Lake Vaudray bivalve population, exposed to very high Cd^{2+} levels, were deformed and damaged most

probably by the toxic action of Cd (Fig. 27; see also section 4.3.1.3 and Couillard *et al.* 1995b). Lake Vaudray gill dry weights were markedly lower than those recorded for less Cd-exposed bivalves (Fig. 24).

4.3.4 **Metallothionein in the burrowing larva of the mayfly*Hexagenia limbata***

A field study was carried out on populations of the burrowing larva of *Hexagenia limbata* (Ephemeroptera) living along a metal contamination gradient in the Rouyn-Noranda mining area (Couillard *et al.* 1995c). Tissue concentrations of MT in *H. limbata* were correlated with tissue Cd, but not with tissue Cu or Zn (Fig. 28). These results, together with the dose-response relationships obtained for the bivalve *P. grandis* (MT-tissue Cd; MT-Cd^{2+}; section 4.3.1.2), clearly suggest that Cd is the key environmental factor to which these organism metallothionein levels are responding in the studied lakes. Ranking of MT concentrations, by decreasing order, was made for insect larvae and mollusc specimens collected in the same lakes at similar periods (*H. limbata*: summer 1993, Fig. 28; *P. grandis*: summer 1989, Fig. 19). These rankings are:

[MT] *H. limbata*: BR>>JO>VA>DP>HE>DU~FL>OP>DA~BO

[MT] *P. grandis*: VA>JO>HE>BO>DA>DP>DU~BR>FL>OP

dissolved [Cd^{2+}]: VA>BO>HE>DU>BR>DA~DP>JO>OP>FL

Comparison of these rankings indicates that different animal species living in the same freshwater ecosystem can experience different toxic metal exposure regimes. An important determinant appears to be the relative importance of the different routes of metal uptake for each animal species. *H. limbata* larvae dig burrows in sediments and feed on organic-rich sedimentary particles; oxygen is supplied by gill ventilation of the burrow. *P. grandis* is a filter-feeding mollusc which is exposed to metals mainly through the dissolved phase (see Tessier *et al.* 1993).
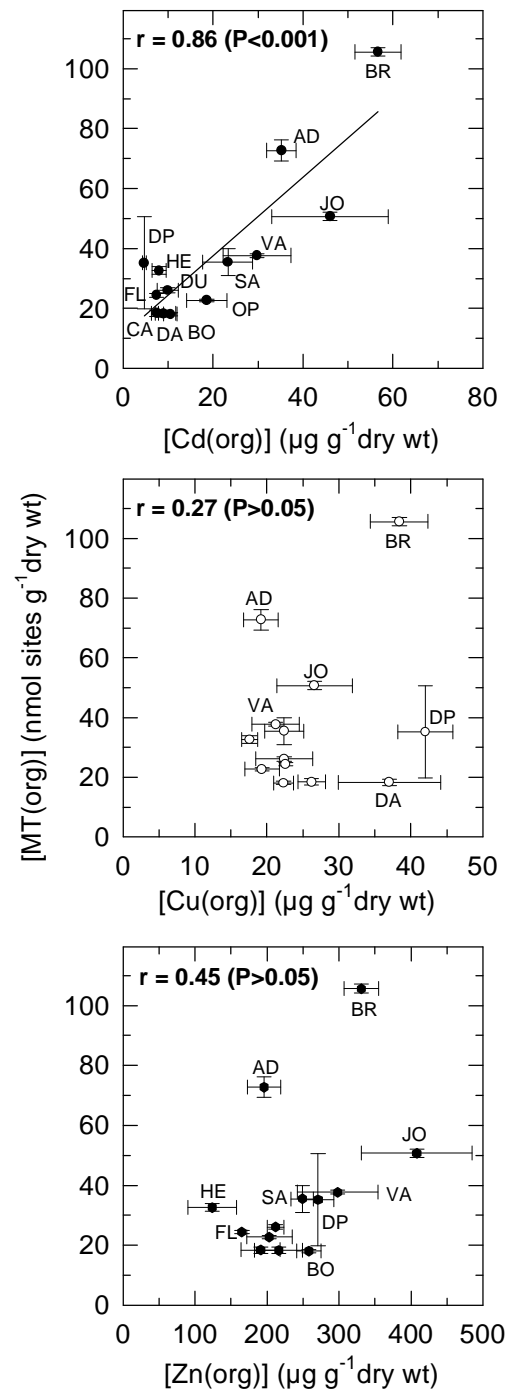
Figure 28: Scatter diagrams of metallothionein concentrations *vs* those of Cd, Cu, and Zn in the burrowing larva of the mayfly *Hexagenia limbata*. The larvae were collected from 13 lakes chosen to represent a metal contamination gradient in the Rouyn-Noranda

mining area. Codes refer to lake names given in the map, Figure 17 + AD: Adéline, SA: Savard, CA: Caron (adapted from Couillard *et al.* 1995c)

4.4 <u>Other organisms</u>

Apart from fish, aquatic insects and invertebrates, trace metal contamination of aquatic ecosystems will affect many terrestrial and semi-terrestrial organisms that rely on the aquatic environment for food sources, habitat or for reproduction. Direct effects may occur through bioaccumulation pathways (water and food). Indirect effects may be associated with changes in abundance and/or quality of food items (note that indirect effects act on aquatic organisms too). A considerable body of laboratory studies have investigated effects of trace metals on vertebrate organisms, including induction of metallothionein. In direct contrast, field studies examining acclimation and toxicological responses to trace metals for organisms such as aquatic birds and mammals, amphibians, and aquatic reptiles are rare. Studies reviewed in the following section were not performed in mining areas but are deemed worthy of interest for the present work.

4.4.1 **Tree swallows in the Experimental Lakes Area**

From 1986 to 1989, St. Louis *et al.* (1993) used nest boxes to attract tree swallows (*Tachycineta bicolor*, a green-backed swallow) to breed near the shores of seven lakes defining a pH gradient in the Experimental Lakes Area. Four of these lakes were used as references (114, 224, 303, 304); one lake was naturally acidified by organic acids (225); two lakes had been experimentally acidified in previous years (223 and 302). Lake 302 had been divided in two basins by a sea curtain; the south basin was acidified with H_2SO_4, and the north basin received additions of HNO_3 and H_2SO_4. Mean pHs of the lakes during this field study are indicated in Table 26. Nestlings were collected in nest boxes, and livers and kidneys were analyzed for tissue Cd, Cu, Zn and MT concentrations.

St. Louis *et al.* (1993) obtained a significant negative relationship between hepatic MT concentrations in nestlings and the mean pHs of the nest-site lakes (r=-0.69, P=0.0007, N=20; see Table 26). In addition, liver [MT] correlated with liver [Cu] and [Zn] (defined as a principal

Table 26

Lake pH, and hepatic metal and metallothionein concentrations (mean ± SE; from 1986 to 1989) in nestling tree swallows collected at ELA lakes along a pH gradient (adapted from St. Louis *et al.* 1993).

Lake	Lake pH	Liver metals ($\mu g\ g^{-1}$ dry wt)			Liver MT ($mg\ g^{-1}$ wet wt)
		Cd	Cu	Zn	
302 South	4.77	0.28 ± 0.05	42.6 ± 8.9	107.6 ± 9.4	0.155
225	5.11	0.25 ± 0.05	25.8 ± 5.2	85.0 ± 3.4	0.077
223	5.85	0.36 ± 0.10	12.1 ± 0.6	71.0 ± 2.5	0.034
302 North	5.99	0.20 ± 0.04	28.4 ± 5.5	95.3 ± 14.5	0.127
Reference (114,224, 303,304)	6.66	0.20 ± 0.02	17.3 ± 1.5	70.5 ± 1.8	0.037

component Cu + Zn factor), but not with [Cd]. The authors interpreted the above results as evidence that trace metals were transferred from acid lakes to birds, and that these bioaccumulated metals caused increases in hepatic MT production. Complex geochemical and biological processes underly these statistical relationships. Theoretically, more acidic waters favor a larger pool of bioavailable metals in the interstitial and superficial waters, as a direct result of decreased complexation of metal ions on important sorbent phases in sediments (see Box 15). Metals accumulated in nestling swallows may originate from contaminated dietary components, either by maternal transfer at the egg stage, or from food fed directly to nestlings (e.g. contaminated emerging chironomids [Diptera]: St. Louis *et al.* 1993).

4.4.2 **Studies with aquatic birds**

Some studies have reported strong dose-response relationships between metallothionein and tissular metal concentrations in the livers and kidneys of marine and freshwater birds. Metallothionein concentrations were often correlated with [Cd], [Cu] and [Zn], and rarely with [Hg]. These studies are summarized in the following table.

Table 27

Summary of studies examining relationships between metallothionein and bioaccumulated metals in aquatic birds. (***: P<0.001; **: P<0.01; *: P< 0.05).

Bird species	Field site	Correlations (r) between tissue [MT] and [Metal]		Reference
		Liver	Kidney	
Lesser black-backed gull *Larus fuscus* (N=56)	Marine littoral sites (N=2) in England and Scotland. Metal gradient undefined.	Cd: 0.46*** Cu: 0.34 ** Zn: 0.37 ** Hg: NS[a]	Cd: 0.83 *** Cu: 0.28 * Zn: 0.46*** Hg: NS	Stewart *et al.* (1996)
Cory's shearwater[b] *Calonectris diomedea* (N=33-34)	Azores islands. Metal gradient undefined.	Cd: NS Cu: NS Zn: 0.52 ** Hg: NA[a]	Cd: 0.41 * Cu: 0.55 *** Zn: 0.43* Hg: NA	Stewart *et al.* (1996)
Leach's storm-petrel[c] *Oceanodroma leucorhoa* (N=12)	Canadian Atlantic Coast Sites (N=2-3) more or less influenced by urban and industrial activities. Metal gradient undefined.		Cd: 0.69 **	Elliot *et al.* (1992)
Atlantic puffin[c] (N=12) *Fratercula arctica*			Cd: 0.85 *** Hg: 0.71**	Elliot *et al.* (1992)
Herring gull[c] (N=18) *Larus argentatus*			Cd: 0.87 ***	Elliot *et al.* (1992)
Greater flamingo[d] *Phaenicopterus ruber*	Camargue Rhône River delta, France.	Cd: NS Cu: 0.87 ** Zn: 0.85 ** Hg: NS	Cd: 0.72** Cu: 0.91 ** Zn: 0.82 ** Hg: 0.82 **	Cosson (1989)
Little egret *Egretta garzetta*		Cd: NS Cu: NS Zn: 0.66 * Hg: NS	Cd: NS Cu: NS Zn: NS Hg: NS	Cosson (1989)

[a] NS: non-significant; NA: non-available.

[b] Freshly dead fledglings were used.

[c] Tissues were also analyzed for Cu, Zn, Pb and major elements; relationships between [MT] and [Cu] and [Zn] were not provided in the paper.

[d] Birds were collected after they had starved and frozen to death in the ponds.

C H A P T E R   5

ANALYTICAL METHODS FOR DETECTION AND

QUANTIFICATION OF METALLOTHIONEIN

5. <u>**ANALYTICAL METHODS FOR DETECTION AND QUANTIFICATION OF METALLOTHIONEIN**</u>

This chapter describes the separation and detection of metallothioneins in biological tissues. Problems arising in initial treatment steps of the samples have been identified and precautions have been suggested. Quantification methods have been developed and improved over the last 20-25 years so that reliable analytical protocols can be suggested. Artifacts generated in analyses of metal composition of metal-binding ligands in cytosolic extracts have been recently identified, and research needs have been defined. The author assumes that the reader has a reasonable knowledge of liquid chromatography (LC) or high performance liquid chromatography (HPLC), and of techniques of metal quantification. The following expressions will be used: HMW and LMW: high- and low-molecular-weight cytosolic compounds; GF-AAS: graphite furnace atomic absorption spectrophotometry; AAS: flame atomic absorption spectrophotometry; ICP: inductively-coupled plasma; ICP-AES: ICP- atomic emission spectrometry; ICP-MS: ICP- mass spectroscopy; RIA: radioimmunoassay; ELISA: enzyme-linked immunosorbent assay.

5.1 <u>Summary</u>

Reliable methods for quantitative analysis of metallothionein are available. The author favors metal saturation assays for routine quantification of MT. Presently, MT measurements are not performed routinely by any private laboratory in Canada. Current evaluations of analytical costs suggest a commercial rate below 40$ for one determination of a MT concentration in a sample.

Reliable protocols of sample preparation for MT analyses have been described. In these protocols, precautions are taken notably to avoid the long-term oxidation of MT. Note that MT is not an enzyme and can be extracted and analyzed under conditions that would be highly unfavourable to the more labile enzymatic proteins. However, there is presently no standardized protocol, on a countrywide basis, for sample preparation and MT extraction and quantification.

Some institutional and governmental laboratories may offer highly specialized analyses such as determinations of primary, secondary, or tertiary structures of metallothioneins, and determinations of the intracellular partitioning of metals including their distributions on MT.

5.2 <u>Preparation of tissue samples</u>

Metallothioneins appear to be robust (e.g. Klaassen *et al*. 1993, see below). However, as for any protein, they are particularly susceptible to oxidation and to degradation by proteases during tissue homogenization, a critical step in this regard. Whole organisms, or specific organs can be used; a minimum of one gram of fresh tissue would be necessary to perform MT analysis, determination of a dry wt/wet wt ratio and, secondarily, a tissue metal analysis. Homogenizations should be done under conditions that perpetuate the cellular environment of the metal-binding proteins. Whenever possible, homogenizations are performed manually on fresh tissues using a glass homogenizer and pestle and a minimum number of strokes (disruption of subcellular organelles such as lysosomes is minimized). Homogenization buffers must be isotonic with the tissue. Osmotic pressures of internal fluids can vary appreciably from one organism to the other; values can be found in biology textbooks. In addition, tissue samples should be homogenized under an atmosphere of N_2 or argon, and on ice. The presence of antioxidant agents (DTT, mercaptoethanol) during isolation procedures may cause redistribution of metals among cellular components and dissociation of natural MT dimers. The first choice for MT preparation is to remove O_2 during homogenization procedures (Suzuki 1992; Marius Brouwer, USM Institute for Marine Sciences, Gulf Coast Research Laboratory, 703 E. Beach Drive, Ocean Springs, Mississippi, USA; pers. comm., June 1996).

Tissue homogenates, supernatant fractions, or fresh tissue samples (if homogenizations cannot be carried out immediately) should be stored at -20 °C or lower temperature until analysis. Reproducible results are not obtained when samples are repeatedly frozen and thawed. Adequate storage procedures minimize O_2 molecular diffusion through samples. For example, these can be sealed and stored in an atmosphere of nitrogen; homogenates and supernatants are purged carefully with nitrogen. Under good storage conditions, MT concentrations are normally stable for months to years (e.g. Couillard *et al*. 1993; Suzuki 1992). Prudhomme *et al*. (1993) evaluated the influence of sample preparation and duration of storage on MT levels of white sucker livers.

Recent experiments have demonstrated that metal-containing metallothioneins are very refractory to hydrolysis (Klaassen *et al*. 1993). Cd-MT and Zn-MT were completely resistant to

degradation for at least 16 h in the presence of lysosomal extracts (containing proteases) at pH 5.5. In *in vitro* incubations with purified proteases carried out at pH 5.5, rates of degradation of apo-MT, Cd-MT, and Zn-MT were 50200, 35, and 20.5 pmol mg^{-1} lysosomal protease min^{-1} respectively. Zn-MT degradation decreased as the protein underwent a transition from a metal-free form to a Zn-containing form. These results suggest that MT degradation by proteases will remain low to negligible, provided that sample preparations do not favor loss of metal from MT because of oxidative conditions (see Suzuki 1992).

5.3 Methods of quantification of metallothioneins

5.3.1 **Initial characterization**

The demonstration that an organism, or a tissue, contain metallothionein is normally the first step to do if MT presence and properties are not known for this organism or tissue. LC or HPLC seem to be the most convenient methods for this purpose (Suzuki 1992). From a chromatographic profile, one can determine the following properties of a metal-binding protein:

Property	**Detection by**
molecular weight	size-exclusion column
kinds and contents of metals (see section 5.4)	chromatographic fractions analyzed by GF-AAS, AAS, ICP-AES, etc...
absence of aromatic amino acids	low absorption at 280 nm
presence of a Cd-mercaptide bond in a Cd-MBP	high absorption at 254 nm
presence of protein isoforms	size-exclusion column and/or anion exchange column
high sulfur content indicative of -SH groups	chromatographic fractions analyzed for S by ICP-AES or ICP-MS. Sulfur must be measured under high vacuum conditions because the element emits at low wavelengths.

Complete characterization of a metallothionein requires ideally a determination of its amino-acid content and the amino acid sequence.

5.3.2 **Metal saturation methods**

A number of methods are available for quantitative analysis of MT (Fig. 29). Figure 30 is a typical flow diagram for metal saturation assays. The author considers them as the most convenient methods for routine quantification of MT. A supernatant fraction is obtained by centrifugation of homogenized tissues. An excess of metal M is then incorporated into the supernatant; the added metal must displace all the metal originally present in MT. Non-specifically-bound displacing metal M is removed by adding a massive dose of an exogenous protein (EP) for which the affinity for metal M follows preferentially the order $K_{MT-M} > K_{EP-M} > K_{GSH-M}$, etc... The complex EP-M is precipitated by heating or by lowering the pH of the mixture (MT is acid- and heat-resistant). After centrifugation of the preparation, the supernatant contains only the chelate MT-M. The metal concentration [M] of the supernatant is measured by AAS or by γ spectrometry if a radioactive γ emitter is employed. A molar binding capacity is calculated (nmol metal-binding sites g^{-1} tissue), and if the stochiometric ratio M:MT is known, [MT] can be expressed as a weight of protein g^{-1} tissue. Usually, M:MT ratios determined for mammalian MT are taken as reference values; these ratio are, however, not always known with certainty. Consequently, conversion of metal data to equivalent concentrations of MT and intercomparisons of MT levels reported in the literature must be done with caution. Metal-saturation assays are often used for quantification of MT in aquatic organisms (e.g. sections 4.2.2, 4.2.3, 4.2.5.2, 4.2.5.4, 4.3.1, 4.3.3, 4.3.4, 4.4.1, 4.4.2).

5.3.2.1 *Hg-saturation method*

In this assay, samples are incubated with an excess of ^{203}Hg in the presence of 10% trichloroacetic acid. In this acidic medium (pH<1), Hg has a higher affinity for thiol groups than any other metal constitutively bound to MT. Dutton *et al*. (1993) demonstrated successful displacement of Cu (96% release), Zn, and Cd by Hg in trials with rainbow trout hepatic MT, known to have high copper content. The characteristic overestimation of MT observed in the

Tissue samples

Pretreatment	homogenization centrifugation

Supernatant fraction (cytosol)

Purification	chromatography electrophoresis acid-treatment heat-treatment solvent-treatment

example of methods

Quantification	thiol groups (polarography) metals (metal saturation methods) protein epitopes (immunological methods)

example of methods

Metallothionein

Figure 29: Schematic progression of metallothionein assays.

original method (see Summer and Klein 1993), was overcome in the modified assay by adding an exogenous protein (e.g. chicken egg albumin or mammalian hemoglobin) to scavenge Hg bound to cytosolic ligands other than MT (Couillard *et al*. 1993; Dutton *et al*. 1993). Recovery of an internal standard of commercially available rabbit liver MT added to 53 liver, kidney, gill and intestine homogenates from white sucker and lake trout averaged 98 ±2%. A similar operation carried out on gill, digestive gland and remainder homogenates of bivalve specimens before ^{203}Hg addition yielded recoveries of 93 ±1%, 93 ±7%, and 96 ±1%, respectively (N=4: Couillard *et al*. 1993; Dutton *et al*. 1993). Thus, neither sample preparations nor the Hg saturation assay caused the loss or degradation of MT. With the modified assay, Dutton *et al*. (1993) indicated that they were able to detect 111 ng of MT; Couillard (1993) obtained a detection limit of 1 nmol Hg g^{-1} dry wt. Greater sensitivity may be achieved by increasing the specific activity of ^{203}Hg.

A benefit of acid conditions of the assay is that HMW proteins and exogenous added protein denature on contact and are separated from soluble Hg-MT by *one* centrifugation stage, in contrast with 3 cycles of heating-centrifugation required for the other metal saturation assays. The assay is miniaturized; Dutton *et al*. (1993) indicated that the method would appear to require   or

SUPERNATANT (MT-M$_1$)

← **1**. Ag, Hg, or Cd; added metal must displace
          all M$_1$ on MT

M M M	GSH	cysteine	P$_1$	P$_2$	P$_3$	P$_4$	
M − − M	M	M	M	M	M	M	M^{Z+}
M M							

← **2**. Exogenous protein
          $K_{MT-M} > K_{EP-M} > K_{GSH-M}$, $K_{cysteine-M}$ etc..

M M M	GSH	cysteine	P$_1$	P$_2$	P$_3$	P$_4$	M M
M − − M	|	|	|	|	|	|	EP
M M							M M

← **3**. Heating and/or acidification;
          centrifugation; MT is thermostable and
          acid-resistant.

SUPERNATANT

M   M   M
M −   − M
M   M

4. Measurement of M by AAS, or by γ
spectrometry if a γ emitter is used (specific
activity must be known).

5. Calculation of a molar binding
capacity (nmol metal-binding sites
g^{-1} tissue).

Figure 30: Flow diagram of a typical metal saturation assay for measuring metallothioneins. Critical steps of the assay are 1 and 2. Abbreviations: GSH: glutathione; P$_1$, P$_2$, P$_3$, P$_4$: supernatant labile proteins and enzymes; EP: exogenous protein (adapted from Couillard *et al.* 1993).

less of total preparatory time than the other metal saturation assays.

There is a controversy about the correct molar ratio of Hg:MT. A ratio of 7 has been routinely used (Summer and Klein 1993). Recently, Stillman (1995) titrated a rabbit liver apo-MT with aliquots of Hg(II) at room temperature and pH 2.0, *i.e.* in conditions similar to the Hg-saturation assay, and obtained the formation of a Hg_{18}-MT.

5.3.2.2 *Cd-saturation method*

This method is not recommended for the analysis of Cu-, Ag- or Hg-containing MT because Cd is unable to displace these metals from the protein. However, Summer *et al.* (1991) recently developed a Cd-saturation assay for Cu-containing metallothionein. The main features of the assay are:

- the precipitation of HMW Cd-binding proteins by acetonitrile;

- the removal of MT-bound Cu by ammonium tetrathiomolybdate;

- the removal of thiomolybdate in excess and its Cu-complexes by the anion exchanger DEAE-Sephacel;

- the saturation of apothionein with ^{109}Cd;

- the removal of excessive Cd by the cation exchanger Chelex-100.

Oxidation of apo-MT is avoided by using N_2-saturated solutions and by performing the test in an atmosphere of argon until Cd saturation. All reagents can be purchased to companies specialized in scientific products, and no chromatographic step is included. The method is miniaturized, and avoids inherent problems of conventional Cd-heme assays, such as the underestimation of MT because of the excessive stripping of Cd from MT by the added exogenous protein. The reported detection limit is 14 ng MT. Contrary to the Hg-saturation method, a well established metal:MT stochiometry of 7 is available for the assay (see Stillman 1995). The Cu content of MT can be determined by both uses of this assay and a conventional ^{109}Cd-saturation method which is incapable of removing Cu but can remove Zn and non-radioactive Cd. Note that such determinations can also be done by concurrent use of Hg- and conventional Cd-assays. This new

Cd-saturation assay was shown by Summer *et al*. (1991) to be specific to MT and to have the capacity of totally expel MT-bound Cu. However, its performance in analyses of environmental samples does not seem to have been evaluated; metals other than Cu are typically bound to MT in this type of sample.

5.3.2.3 *Ag-saturation method*

In this assay, metallothionein is incubated in the presence of an excess of silver at pH 8.5. Gagné(1991) presented a miniaturized version of this assay for quantifying MT in rainbow trout livers. However, complete copper displacement from MT by Ag has not been well documented (cf. Gagné1991; Gagné*et al*. 1990; Scheuhammer and Cherian 1986). In an early version of the method, Ag hardly displaced 50% of Cu in a Cd, Cu, Zn-MT extracted from rat liver, but the test material was not totally adequate to verify Cu displacement since MT copper content was low (Fig. 3 in Scheuhammer and Cherian 1986). The method is not recommended for analyses of tissues with high chloride content because silver precipitates with Cl (Summer and Klein 1993).

5.3.3 **Differential pulse polarography**

Detection of the change in current that occurs when a compound is either oxidized or reduced is the basis of this method. When MT is quantified by DPP, the reduction of hydrogen in the MT sulfydryl groups is measured. Different amounts of rabbit MT-I included as internal standards in polarographic cells were entirely recovered (Hogstrand and Haux 1992). Low amounts of tissues are required (100-200 mg for this assay). Hogstrand and Haux (1992) determined a detection limit of 7.2 μ MT g^{-1} liver of yellow perch. DPP has been used for metallothionein measurements in different fish species (e.g. sections 4.2.1., 4.2.5.1; Hogstrand and Haux 1992) and in invertebrates (e.g. Raspor and Pavičić 1996).

The major advantage of DPP is that the analysis is not influenced by the metal composition of MT (whereas metal saturation assays are). Specificity is highly dependent on sample preparation. Heating and centrifugation of the homogenates/cytosol extracts, and the use of an ammonium buffer containing Co (Brdicka buffer) make it possible to quantify the thiol groups of MT with negligible interference from other sulfydryl-containing compounds. A calibration curve is required,

preferably with the specific MT to be quantified. This might represent a disadvantage of the method if one chooses to purify a quantity of MT of the study organism for this purpose; isolation and purification procedures can be long.

5.3.4 **Immunological methods**

The principle of immunological methods is that metallothionein is an antigen and an antibody is raised against it by injecting MT molecules as foreign compounds in an animal species different from the study organism (Garvey 1991). The antibody specifically binds to a small region of a protein antigen (usually 5 to 7 amino acids), and serves as a probe for detection and determination of MT concentrations. In addition to the two techniques described below, western blotting uses antibodies raised against MT to detect it (Whitacre 1996; Garvey 1991).

5.3.4.1 *Radioimmunoassay*

A double-antibody radioimmunoassay (RIA) for the yellow perch *Perca fluviatilis* has been developed by Hogstrand and Haux (1990). Ingredients are a rabbit anti-perch MT globulin as the first antibody, a goat anti-rabbit immunoglobulin G as the second antibody, and labelled ^{125}I-MT of perch. In this assay, ^{125}I-labelled and native MTs compete against each other for a limited number of anti-perch MT antibodies. As the number of native MT molecules increases, a decreasing portion of the labelled MT will be bound to the antibodies. Thus, the larger is the amount of MT in the unknown sample, the smaller is the amount of ^{125}I bound to the anti-perch MT antibodies. Then, the antibody-antigen complex is precipitated with a second antibody and the precipitate is analyzed for ^{125}I. The antibody raised against liver perch MT cross-reacted with rainbow trout MT and was used to measure MT in brown trout in the Clark Fork River study (section 4.2.4).

The advantage of the method is its high sensitivity. MT can be detected in body fluids at concentrations as low as 3 ng mL^{-1} and in tissues at concentrations > 9 ng g^{-1} wet wt. A disadvantage is that the method is long and complicated. An appreciable amount of the metallothionein to be analyzed has to be purified to produce a ^{125}I-MT tracer, and a calibration curve is needed. The assay includes periods of incubation lasting 72 h in total. Use of an internal MT standard for quality control is hardly possible with this assay.

5.3.4.2 *Enzyme-linked immunosorbent assay*

A double-antibody ELISA was developed for the marine mussel *Mytilus edulis* by Roesijadi *et al*. (1988). The procedure utilized a goat anti-mussel MT IgG as the first antibody, a rabbit anti-goat IgG conjugated to horse radish peroxidase as the second antibody, and purified mussel MT as an antigen. The functioning of this assay is very similar to the preceding RIA. The reference antigen is immobilized on wells of microtiter plates. This and the competing antigen (native MT) compete against each other for a limited number of anti-mussel MT antibodies. As the number of native MT molecules increases, a declining portion of reference antigen is bound to the antibodies. Plates are washed after the first incubation. The primary antibody bound to the immobilized reference antigen is put in presence of the second antibody linked to an enzyme. Finally, the substrate, a chromophore, is incorporated and the disappearance of the substrate is monitored by colorimetry.

This is a very sensitive assay with a working range of 1 - 21 ng MT. Like the RIA, the method is long and complex. Mussel metallothionein has to be purified to synthesize a mussel MT antibody; a calibration curve for the assay is required. The assay includes periods of incubation lasting ~ 40 h in total.

5.4 Intracellular metal partitioning

Many investigators have studied the distribution of metals among different cytosolic ligands to gain understanding of the MT induction process and to detect metal detoxification failure as exemplified by metal «spillover» Ideally, this type of quantitative analysis would be undertaken *in situ* within the cell without disturbing the cellular metal compartmentation. There is presently no technique which allows for this form of analysis although secondary ion mass spectrometry of hydrated cryosections might be useful in this matter (Mason AZ, California State University Long Beach, Department of Biological Sciences, CA, USA, pers. comm., August 1996; Roesijadi G, University of Maryland, Center for Environmental and Estuarine Studies, Chesapeake Biological Laboratory, Solomons, MD, USA, pers. comm., July 1996). The study of changes in the subcellular distribution of metals involves first the isolation of the different metal-binding ligands,

and second the quantification of the different metals associated with each of the ligand pools. These distributions are operationally defined as homogenization and fractionation procedures will normally cause organelle disruption and may lead to metal redistribution within cellular compartments. The following shows how different researchers have minimized artifacts generated by the above operations. Copper seems to be the ‹enfant terrible› in this area.

Brouwer and collaborators (pers. comm., July 1996) found that marine crabs exposed to high levels of dietary copper contained large amounts of LMW Cu-containing compounds in their lysosomes. Disruption of the cell by tissue homogenization caused a large reduction of cytosolic catalase activity, the activity of which is inhibited by lysosomal copper. They noted that tissue homogenization may result in bringing cytosolic Zn-MT and copper complexes sequestered in lysosomes (*i.e.* Cu(I)-GSH, and Cu-MT degradation products) together resulting in Zn displacement from MT. Brouwer strongly recommended the use of a ‹soft» homogenization procedure which results in low organelle breakage. Such an approach of sample preparation for MT and cytosolic metal partitioning is provided with much details in section 5.2.

Additional artifacts may arise during chromatographic cytosol fractionations. Molecular weight markers are used to calibrate chromatographic columns that discriminate cytosolic compounds on the basis of their size/mol. wt. (e.g. HPLC size-exclusion column). It was shown that only the use of high-ionic-strength mobile phases would result in the successful elution of all protein markers. Presumably, high electrolyte concentrations in eluants (NaCl) mask silanol groups in the column packing, thereby suppressing protein column interactions (Micallef *et al*. 1992; Mason *et al*. 1990). The use of high-ionic-strength elution buffers is not without drawback though. Corrosion of stainless steel HPLC components has been illustrated by quantifying changes in the composition of the mobile phase during elution. Appreciable increases were recorded in the concentrations of Fe, Cr, Mn, Ni, Sn, and, to a lesser extent, in levels of Cu and Cd (Mason 1989). Thus, non-metallic hardware appeared desirable for the above type of HPLC operation. Mason (1989) provided examples of this type of material: columns with external glass supports; fluoropolymer, or plastic ferrules and couplings; pump modules with heads composed entirely of inert ceramics or titanium alloys.

The kinetic stability of pure metal-MT complexes in HPLC was shown to be strongly dependent on the quantity of protein taken for elution (Mason *et al*. 1990). Total recovery of Cd was observed for sample size larger than 1 g. Poor recovery of Zn was obtained up to 250 g, whereas the recovery of Cu was constantly higher than predicted from the original composition of MT (Table 28). The authors interpreted the results as follows. Reactions in the size exclusion column favored the sequestration of Zn from MT to the column and its isomorphous replacement by Cu scavenged from the mobile phase or column packing. If one assumes that the total number of binding sites for Zn on the column is fixed and limited, as is the total mass of Cu available in the mobile phase or column, then the relative degree of Zn exchange will be most pronounced at low protein loadings. This explanation should apply to any metal binding complex. Work of Micallef *et al*. (1992) was in agreement with the above findings. Pure MT solutions labelled with [109]Cd or [203]Hg, and injected in an HPLC size exclusion column (quantity injected: 100 g) similar to that of Mason *et al*. (1990), were entirely recovered; MT labelled with [65]Zn was not entirely recovered. In addition, competition experiments performed with commercial MT pre-labelled with [109]Cd and fresh bivalve cytosol extract demonstrated that no appreciable Cd exchange occurred during the 20 min pre-equilibration step or the subsequent chromatographic separation. Poor recoveries of [65]Zn-MT were obtained after the HPLC fractionation of a mixture of this protein and cytosol (Table 29).

To summarize the above, intracellular metal partitioning protocols that minimize methodological artifacts should include the following:

1. a ‹soft› sample homogenization and preparation procedure (section 5.2);
2. a chromatographic setup that minimizes metal contamination from all sources:
   - non-metallic/inert physical components (columns, HPLC pumps, etc...),
   - the use of ultrapure salts for the preparation of elution buffers;
3. the use of high-ionic-strength elution buffers to suppress adsorption problems
   (e.g. 10 mM Tris pH 7.2, 100 mM NaCl);

Table 28

Kinetic stability of Zn-, Cu-, and Cd-MT bound during HPLC chromatography. Comparison of observed and expected recovery of Cd, Zn, and Cu from injecting different quantities of protein (adapted from Mason *et al*. 1990).

	Recovery of metals (%)		
MT injected (ng)	Cd	Cu	Zn
500	82	338	--
2500	94	152	31
5000	100	140	48
25000	102	116	80
50000	101	110	89
250000	100	100	96

Note: An HPLC size-exclusion column (TSK SW2000, 7.5 mm × 60 cm) was eluted with a low-ionic-strength buffer, 60 mM Tris-HCl, pH 7.5.

Table 29

Competition experiments between radiolabelled MT and mussel cytosol extract: recovery (%) relative to total quantity loaded to the column of ^{203}Hg, ^{109}Cd, and ^{65}Zn in eluate fractions after chromatographic separation (adapted from Micallef *et al.* 1992).

Fractions	Hg		Cd		Zn	
	MT-Hg only	MT-Hg + cytosol	MT-Cd only	MT-Cd + cytosol	MT-Zn only	MT-Zn + cytosol
HMW	0	1.2-3.6	0	0.1-0.3	0.1	0.3-0.9
MT	93-110	47-89	96.5-98	102-106.6	52-58	50-60
LMW	0	0.5-1.1	0.2	0.6-1.0	0.1-0.2	0.4-0.5
Range of total recovery (%)	93-110	49-93	97-98	103-107	52-58	50-58

Note: An HPLC size-exclusion column (TSK SW2000, 30 ×0.75 cm) was eluted with a high-ionic-strength elution buffer (10mM Tris-HCl, 100mM NaCl, pH 7).

4. a high protein loading (e.g. 250 μg) to the chromatographic column during each elution cycle;

5. a careful washing of the column (e.g. by EDTA) between each elution to eliminate

memory effects.

Protocols   for subcellular distribution of Cd among gill cytosol fractions from *P. grandis*
included these characteristics (see Table 22, and Fig. 23).

5.5 Analytical costs and expertise in Canada

Metallothionein is relatively easy to quantify by metal saturation methods. Klaverkamp *et al.*
(1996a) indicated that, in the laboratory of the Freshwater Institute at Winnipeg, the cost per
replicate sample using a ^{203}Hg-saturation assay was 10 dollars which covered the technicians
salary, the analytical materials and supplies. Approximatively 60 assays could be conducted in a
week. The above rate would have to be multiplied by a factor of 3 or 4 to obtain an approximation
of the commercial rate. In comparison, the cost for a tissue metal analysis, including several
metals, may reach 70 dollars per biological sample in a private laboratory (Beak 1996a).

A minimum number of replicate samples would have to be analyzed to be able to detect
significant differences between MT concentrations at exposure and reference sites. With this
consideration in mind, Klaverkamp *et al.* (1996a) evaluated MT data for fish from two relatively
uncontaminated sites, one in Great Slave Lake, N.W.T., and the other in a small lake in ELA.
Using statistical power analyses, with α set at 0.05, 1-β at 0.95, and δ (the magnitude of change)
at 100%, the authors determined the following. Six, 9 and 17 lake whitefish would be required to
detect a doubling of MT in kidney, liver and gill, respectively. Three, 10, and 11 northern pike
would have to be analyzed to detect a doubling of MT in kidney, liver and gill, respectively. For
white sucker, 5, 12 or 14 specimens would be necessary to detect a doubling in liver, kidney and
gill, respectively. The authors did not mention if they controlled for size and/or age in their
analyses of the data.

The above information provides a rough idea of the analytical costs associated with
determinations of MT levels in fish samples. Additional costs are associated with activities of
specimen collection, and sample preparation. Examples of such costs are given by Beak (1996a)
for the 1995 AETE field study (section 4.2.5.3).

The author is not aware of any private laboratory performing MT measurements on a routine basis. Rather, these would be done on a custom basis in institutional or governmental laboratories and under the supervision of a scientific authority. Some addresses are provided below.

Analytical expertise in Canada

(A) Metal saturation methods and subcellular metal partitioning:

(i)   Dr Jack Klaverkamp
      Department of Fisheries and Oceans
      Freshwater Institute
      501 University Crescent
      Winnipeg, Manitoba, R3T 2N6
      Tel: (204) 983-5003
      FAX: (204) 984-6587
      Specialities: MT in freshwater fish; gel filtration liquid chromatography.

(ii) Dr PGC Campbell
      Université du Québec
      Institut national de la recherche scientifique, INRS-Eau
      2800 rue Einstein, suite 105
      Sainte-Foy, Québec, G1V 4C7
      Tel: (418) 654-2538/3777
      FAX: (418) 654-2600
      e-mail: Campbell@UQuebec.CA
      Specialities: MT and subcellular metal partitioning in freshwater bivalves and insects; size-exclusion HPLC.

(iii) Dr MG Cherian
      Department of Pathology
      Health Science Centre
      University of Western Ontario
      London, Ontario, N6A 5C1
      Speciality: Silver saturation method.

(iv) M. Michael D. Dutton
      Dutton Analytical Services
      32 Fairview Avenue
      Kitchener, Ontario, N2H 3E8
      Tel: (519) 579-2947
      e-mail: MDDUTTON@biology.watstar.uwaterloo.ca
      Speciality: MT in freshwater fish.

    (v) Canadian Wildlife Service, Ottawa, Ontario: MT in birds
          Environment Canada, Centre Saint-Laurent, Montréal, Québec: MT in fish
          McGill University, McDonald Campus, Ste-Anne de Bellevue,   MT in ducks
          Québec.

(B) Polarography and radioimmunoassay
    (No expertise known in Canada).

    (i) Dr Christer Hogstrand
         University of Kentucky
         TH Morgan School of Biological Sciences
         Thomas Hunt Morgan Building
         101 Morgan Blvd
         Lexington, Kentucky, USA
         Tel: (606) 257-7751
         FAX: (606) 257-1717
         Speciality: MT in marine and freshwater fish.

5.6 <u>Quality assurance/quality control associated with analytical protocols</u>

QA/QC checks for metal saturation assays and chromatographic fractionations are similar to those performed for quantitative analyses of metal residues in tissues. External and/or internal standards and procedural blanks are routinely run along with sample analyses. Purified mammal metallothionein is commercially available (e.g. Sigma Co.) for these purposes. Specifications on the metal content of each synthesized MT batch are indicated on the bottle. However, as a precautionary measure, the metal content may be measured by AAS on a pure aqueous solution of MT (acidified by HNO_3 0.5%). Klaverkamp *et al*. (1996a) obtained a very high degree of correspondance between concentrations of MT standard samples measured by a mercury saturation method and MT concentrations expected from specifications (N = 345, R^2 = 0.996). Note that internal MT standards are not possible for immunological quantification methods. If MT measurements were to be included in biological monitoring programs for the mining industry, standardization of protocols for sample preparation, MT extraction and quantification would be required on a countrywide basis. Round-robin exercices would be also desirable.

C H A P T E R   6

DISCUSSION

AND

RESEARCH NEEDS

6. **DISCUSSION AND RESEARCH NEEDS**

6.1 <u>Summary on research needs</u>

Table 30

Hypotheses and issues that need to be addressed for the use of metallothionein in biomonitoring programs

Theme	Hypothesis/issue	Remark
	Fundamental research	
Metallothionein as a biomarker of effect	Successful demonstration, in nature, that the over-whelming of the detoxification mechanism including MT is associated with deleterious effects on the host organism.	See section 6.3.
	Successful demonstration, in nature, that there exists a metabolic cost (associated with MT induction) to tolerance to metal exposure, that decreases the performance of the host organism.	See section 6.3.
Early warning capacity of the MT biomarker	Anticipation of effects at community- and ecosystem-levels of biological organization: we need to understand the functioning of complex whole ecosystems.	See section 6.4.1.
	Anticipation of effects at the population-level of biological organization: relation between the MT response and genetic characteristics of the animal population should be investigated.	See section 6.4.2.
	Development of active quantitative biomonitoring in order to fully exploit the early warning capacity of MT.	See section 6.4.3.

Table 30 (continued)

Theme	Hypothesis/issue	Remark
<td colspan="3">Research to increase the efficiency of MT as a cost-effective tool for biomonitoring programs</td>		
Minimization of the effects of non-toxicological factors on MT levels	Factors to consider: age/size, sex differences, influence of reproduction/spawning, seasonality (temperature, food sources, etc...), stress caused by capture/sampling.	See section 6.6.
Choice of sentinel species for MT measurements	What are good sentinel species? Freshwater bivalves; Aquatic insect larvae; Small forage fish species? Young-of-the-year for large species? Higher vertebrates? Critical target organ?	See sections 6.8 and 6.9.3. This research could be done within the AETE program. Note that it can be legitimate to select a non-sentinel species because of its economic and social value (e.g. adult Atlantic salmon).
Reference sites in mining regions	Determination of these sites may be facilitated by an initial analysis of size-fractionated surface and profundal sediments. Several of these sites, presenting a variety of habitat and trophic conditions, could be selected.	See sections 6.5 and 6.9.2.
Establishment of an overall monitoring strategy for the mining industry	Possible elements to consider: Tier-testing strategy, hierarchical approach, determination of indirect and non-direct effects, retrospective analysis.	See section 6.9. This research/ reflexion process is done within the AETE program.

Note: For this second part of the table, the 1997 field campaign of the AETE program will provide data relative to some of the above aspects (Re: Technical Committee Meeting, November 13-14 1996, North Vancouver, BC).

6.2 Metal inducers of metallothioneins

To address the question of metallothionein induction in indigenous aquatic organisms, the author has considered results and data from the case studies presented in Chapter 4. In these studies, the principal metal inducer was determined on the basis of the links and correlations reported between metal and MT concentrations, and on the metallic composition of the metal-binding proteins.

Cadmium and copper appeared to be responsible for most of the *in situ* MT biosyntheses, followed by Zn. Silver (Ag) and mercury (Hg) were only occasionally linked to the MT induction process (Table 31). The preponderance of a particular metal or of a group of metals in MT induction in an animal species at a metal-contaminated site appears to be function of a multiplicity of factors. Metal induction potencies, physiological characteristics of the organism, metal enrichment in food and water, and metal bioavailability may all exert a determining influence on the above induction process (e.g. George and Olsson 1994; Roesijadi *et al*. 1988).

Mercury is reported to have the highest *in vitro* affinity for metallothionein, greater than Cd, Zn, Cu or Ag (see section 1.3.1). Despite this affinity, relationships are rarely reported between Hg and MT in field surveys. The environmental speciation of Hg (organic and inorganic forms), and Hg-Se antagonism in organisms may be responsible for this apparent inconsistency (Stewart *et al*. 1996).

Metallothionein concentrations increased in a dose-dependent manner in the case studies in Chapter 4, carried out along metal contamination gradients. These dose-response relationships are consistent with the understanding that constitutive levels of MT were «low» and increases in concentration noted in organisms above these low levels were attributable to the induction of MT in response to an influx of inducing metals. Results obtained in these field studies suggest that MT conforms to criteria 2 and 3 for biomarkers mentioned in section 2.3, namely that the biomarker should respond in a concentration-dependent manner to changes in ambient levels of a particular contaminant or class of contaminants.

Table 31

Individual metals thought to be responsible for the induction of metallothioneins in field populations of organisms examined in the case studies of Chapter 4. The metal inducer is deduced on the basis of links/correlations between metals and MT levels reported in these studies.

Study organism(s)	Tissue	Other metals present	Study sites
Inducing metal: Cu			
Rainbow trout *Oncorhynchus mykiss*	Liver	Cd, Zn	Campbell River Drainage British Columbia Section 4.2.1
Brown trout *Salmo trutta*	Liver	Ag, Cd, Zn, (Pb)	Clark Fork River Montana Section 4.2.4
Yellow perch *Perca fluviatilis*	Liver	Zn (**co-inducer**)	River contaminated by Zn and Cu in Sweden Section 4.2.5.1
Marine bivalve *Macoma balthica*	Whole organism	Ag (**co-inducer**), Zn	San Francisco Bay California Section 4.3.2
Tree swallow *Tachycineta bicolor*	Liver	Zn (**co-inducer**)	ELA lakes defining an acidification gradient Section 4.4.1
Greater flamingo *Phaenicopterus ruber*	Liver, kidney	Cd, Zn, Hg (**co-inducers**)	Rhone River, France Section 4.4.2
Inducing metal: Cd			
White sucker *Catostomus commersoni*	Liver	Cu, Zn (**co-inducers**), (Hg)	Flin Flon region Manitoba Section 4.2.2
White sucker, Lake trout *Salvelinus namaycush*	Liver, kidney	- - -	ELA, Ontario Section 4.2.3
Yellow perch *Perca fluviatilis*	Liver	Cu, Zn	Emå River, Sweden Section 4.2.5.1
Bivalve mollusc *Pyganodon grandis*	Gills, whole organism	Cu, Zn	Rouyn-Noranda region Québec Section 4.3.1
	Gills, mantle, foot, kidney, visceral mass	- - -	ELA, Ontario Section 4.3.3
Insect larvae *Hexagenia limbata*	Whole organism	Cu, Zn	Rouyn-Noranda region Section 4.3.4
Seabirds: Atlantic puffin Leach's storm petrel Herring gull	Kidney	Cu, Zn Hg = **co-inducer** in Atlantic puffin	Canadian Atlantic Coast Section 4.4.2

Seabird:	Liver, kidney	Cu, Zn (**co-inducers**),	Littoral sites in England
Lesser black-backed		(Hg)	and Scotland
gull			Section 4.4.2

6.3 Detection of toxic effects caused by trace metals in nature

6.3.1 **Metal spillover**

In principle, toxicity occurs when compensatory mechanisms are overwhelmed, saturated, or damaged by metal influx (see Fig. 4). Cytotoxicity would appear when metallothionein biosynthesis cannot kinetically prevent metal from accumulating in other protein fractions, a phenomenon termed spillover (Mason and Jenkins 1995; Luoma and Carter 1991). Laboratory experiments with fish and invertebrates indicated that the appearance of metals in very low-molecular-weight complexes heralded the onset of metal-induced stress (reported in Mason and Jenkins 1995). Similar shifts in intracellular metal partitioning have been observed in areas contaminated by trace metals, indicating that the above cytosolic signal of metal stress was reproducible in field situations (see sections 4.3.1 and 4.3.2). The biochemical signal was concomitant with deleterious effects at higher levels of biological organization (organ, organism: section 4.3.1; population: section 4.3.2). These results do not strictly conform to the «spillover» hypothesis as originally formulated by Brown and Parsons in 1978 for MT. Indeed, failure to detoxify excess cytosolic metals need not reflect complete saturation of metal-binding sites on MT followed by an overflow of excess metal to sensitive cellular sites. Binding of the metal in excess in the cell to MT is to be seen as the result of a competition for binding sites between this metal in excess and constitutive metals on MT, such as, for example, essential metals (see Couillard *et al*. 1995b; Roesijadi 1992; Roesijadi and Klerks 1989; Hamilton *et al*. 1987; see Fig. 31).

The above results are certainly noteworthy if one is to relate the degree of metal detoxification to the health status of an organism in its natural environment (Criterion 5 for a biomarker: section 2.3). However, at the same time, it must be recognized that most field studies have not been able to convincingly demonstrate a mechanistic linkage between biochemical responses and adverse effects at higher levels of biological organization (see Mason and Jenkins 1995; Roesijadi 1992; Luoma and Carter 1991); further research is needed.
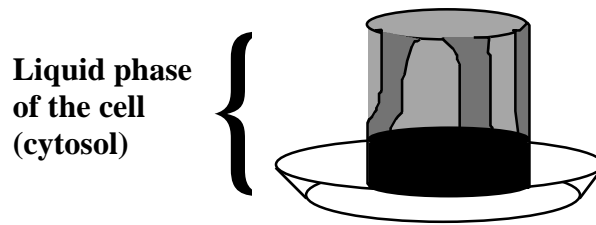
Cup: reservoir of MT available to sequester the metal M.

Black area in the cup: part of the reservoir of metal-binding sites on MT unavailable for sequestering the metal in excess in the cell.

Saucer: cytosolic pool of enzymes (high-molecular-weight compounds) and small molecules constituting the cellular machinery.

Spillover: metal in excess of the binding capacity of the cellular MT pool.

Figure 31: Modification of the spillover hypothesis: the overflow of excess metal to sensitive cellular sites results from a competition between this metal in excess and constitutive metals on MT (see text for explanation).

Of particular importance is the identity of low-molecular-weight metal-ligand complexes. Glutathione (GSH) is one of the candidate ligands binding metals in this pool (Mason and Jenkins 1995). Metal ions forming complexes with GSH would reduce the amount of GSH available for removing reactive oxygen species, predisposing the organism to oxidative stress (see Box 8; Christie and Costa 1984).

6.3.2 **Metabolic costs of adaptation to metal exposure**

It is often postulated that high levels of metallothionein in an organism at a metal-contaminated site reflect acclimation or adaptation of this organism to the chronic metal contamination of its habitat (Stegeman *et al*. 1992). In two comprehensive field studies described in Chapter 4, elevated MT concentrations were associated with cytotoxicity and adverse effects at the organism- and population-level of organization (Flin Flon study: section 4.2.2; Clark Fork

River study: section 4.2.4). Farag *et al.* (1995) evokated the concept of the metabolic cost to adaptation to help explain the poor state of health of brown trout living in the Clark Fork River.

This notion of cost associated with acclimation has interested scientists for the last 15-20 years (see Hoffman and Parsons 1991, Chapter 7; Luoma and Carter 1991; Weis and Weis 1989). The establishment of a mechanism enabling an organism to tolerate high metal exposures takes a toll on energy reserves normally directed to growth and reproduction. Thus, a metabolic cost of acclimation/tolerance can reduce an organism's scope for growth (Calow 1991), and its reproduction potential. Note that this metabolic cost does not represent <u>a toxic effect *per se*</u>; rather, it expresses a detoxification activity, the absence of which would preclude an organism from inhabiting a contaminated environment. These ideas are synthesized as follows.

<div align="center">

detoxification activity $\uparrow$

resistance and tolerance to metal exposure $\uparrow$

energy devoted to detoxification activity $\uparrow$

energy devoted to growth and reproduction $\downarrow$

(deleterious effect)

</div>

If costs in energy allocated for adaptation to metal stressors are so excessive that they deeply perturb the birth - growth - death cycle of organisms, the population will not persist. Such sublethal effects may only postpone population extinction (there is a theory of population dynamics to support this: Sibly 1996; Mulvey and Diamond 1991). A parallel can be drawn with the Lake Hamell white sucker population in the Flin Flon region (section 4.2.2.4). This population was compared to one living in a physically and chemically similar uncontaminated lake in the area (Klaverkamp *et al*. 1991; Franzin 1984). Briefly, Lake Hamell fish had more MT in their tissue, and were more tolerant of Cd toxicity than control fish (Chapter 3). Yet, the Lake Hamell population experienced an important recruitment failure of young-of-the-year fish, and population size was decreased (Franzin 1984); no individuals could be caught in the lake in 1986 (Klaverkamp *et al*. 1991).

It is appropriate at this point to review some studies examining the links between metabolic cost and tolerance mechanisms involving metallothionein. At stake here is the ecotoxicological

significance of measurements of MT levels and MT induction. Can increased MT concentrations represent both *compensatory and adverse responses to metal exposure* (because of reductions of growth and reproductive potentials caused by metabolic costs of acclimation)?

Four laboratory studies were performed on different fish species to demonstrate that increased tolerance to metal exposure incurred metabolic costs (Hobson and Birge 1989; Roch and McCarter 1986; McCarter and Roch 1983; Buckley *et al*. 1982; Dixon and Sprague 1981a,b). In these experiments, groups of fish were put in ‹acclimation› metal solutions for varying lengths of time. Groups were then ‹challenged› by high metal concentrations, and tolerance and/or resistance responses were determined. Metallothionein concentrations, fish weights and/or lengths were measured concurrently. Significantly increased tolerance/resistance, and increased MT levels relative to controls, together with significant reductions in growth were taken as evidence of metabolic costs associated with acclimation to metals. In the study of Dixon and Sprague (1981a,b) acclimated specimens were returned to control media to verify if tolerance was lost, and if growth resumed at normal rates.

Results of the above studies are given in Table 32. The experiment of Dixon and Sprague (1981a,b) showed that elevated [MT] were associated with increased tolerance to Cu, and reduced salmonid growth. Depressions of growth were greatest early during the exposures. In addition, the authors demonstrated that a threshold level of Cu was required to activate the tolerance mechanism. Trout pre-exposed to [Cu] below the threshold (30 μ L^{-1}; see Table 32) proved to be more sensitive than control trout (not pre-exposed) in acute toxicity tests. The authors suggested that ‹some deleterious effects of acclimation [for the former trout] was carried over and

$^{-1}$ were returned to uncontaminated media and growths of these trout resumed.

Hobson and Birge (1989) denoted an apparent relationship between MT induction in fathead minnows and their acclimation-induced tolerance and resistance to Zn. After 7 and 14 days of

Table 32

Characteristics of various fish species acclimated to different metals in laboratory. Values in parentheses are those of controls, and asterisks indicate significant differences between treatments and controls (where tests were conducted; *: $P<0.05$, **: $P<0.01$).

Fish species	Acclimation level of metal	Estimation of resistance/tolerance to acute metal exposure	Estimation of fish performance			MT concentrations
Juvenile rainbow trout *Oncorhynchus mykiss* (Dixon and Sprague 1981a,b)	Cu (μg L^{-1}) duration: 21 d	Incipient lethal level (μg Cu L^{-1})	Mean dry weight of fish (g)			Mean MT-like concentrations (mg g^{-1} liver)
	30	266 (329)	0.57 (0.57)			--
	58	349 (333)	0.83 * (0.75)			--
	94	515 * (311)	0.68 * (0.77)			--
	131	564 * (274)	0.52 * (0.57)			48.8 (32.7) [a]
	194	708 * (371)	0.83 * (0.98)			--
Developing rainbow trout from alevin to fry stage (Roch and McCarter 1986)	Mixture of Zn:Cu:Cd ratio 400:20:1 duration: 300 degree-days Zn (μg L^{-1})	96 hr-LC$_{50}$ (μg Zn L^{-1})	Mean length of fish (cm)	Mean wet weight of fish (g)		Mean MT concentrations (nmol g^{-1} liver)
	65	370 * (225)	5.0 (5.2)	1.4 (1.3)		90.6 ** (48.4)
	120	480 * (225)	4.6 (5.2)	1.1 (1.3)		123.6 ** (48.4)
	215	--	4.5 * (5.2)	1.1 * (1.3)		201.7 ** (48.4)
Juvenile Coho salmon *Oncorhynchus kisutch* (Buckley *et al.* 1982, and McCarter and Roch 1983)	Cu (μg L^{-1}) duration:10 wks	168 hr-LC$_{50}$ (μg Cu L^{-1}) (after 16 wks of acclimation)	Mean wet weight of fish (g)	Mean condition factor of fish		Mean MT conc. (μAmpere g^{-1} liver wet wt)
	70	310 * (220)	13.7 (14.8)	1.11 (1.16) [b]		81 (64) [c]
	140	550 * (220)	10.2 (14.8)	1.06 (1.16) [b]		136 (64) [d]
Subadult fathead minnow *Pimephales promelas* (Hobson and Birge 1989)	Acclimation level of Zn: 1.8 mg L^{-1} duration in d	Resistance (LC$_{50}$) expressed as experimental value/control value	Tolerance (LT$_{50}$) the ratio	Mean standard length of fish (mm)		MT-like conc. (μg Zn in MT fr./g tissue wet wt) [e]
	7	0.63 * **sensitization**	0.63 *	24 (24)		4.5 (1.3)
	14	0.76 * **sensitization**	0.63 *	--		26.1 (1.3)
	21	0.95	0.88	27 (29)		31.6 (1.3)
	35	0.98	0.98	30 * (33)		30.1 (1.3)

[a] Treatment fish were exposed to 141 **g** Cu L^{-1} for 2 d, prior to sacrifice, and were not pre-acclimated to Cu.

[b] The fish lost appetite immediately upon Cu exposure but gradually recovered appetite and resumed growth. [c] Fish were acclimated to 50 **g** Cu L^{-1} for a period of 10 wks. [d] Fish were acclimated to 150 **g** Cu L^{-1} for a period of 10

wks. [e] Metal content on MT was determined by metal analyses of cytosolic chromatographic fractions obtained by liquid chromatography.

exposure, LC_{50} and LT_{50} values were significantly lower than those of controls. These values returned to control values after 21 d of exposure and remained constant through 35 d. MT-like concentrations followed a similar pattern, with a steady increase to 21 d and a plateau from 21 to 35 d of exposure. Growth was significantly depressed only after 35 d of exposure. The short-term sensitization of treated fish to acute Zn exposure do not fit the response pattern expected for such a study destined to highlight a metabolic cost caused by MT induction (see above and Table 32). However, it is not known with certainty if MT induction occurred. First, MT concentrations were measured by an indirect method *i.e.* by evaluation of metal content in chromatographic fractions corresponding to MT. Second, Zn diffusing in the fish may have simply displaced metals bound to the constitutive MT pool without provoking the synthesis of new MT molecules. Consistent with this is the observation that the MT pool was 75% saturated with Zn after 7 and 14 d of acclimation to Zn, but was saturated at 97% with Zn after 21 d of exposure (Hobson and Birge 1989). It is known that Zn exhibit a low potency to induce MT. Moreover, Hobson and Birge (1989) reported that, in contrast with salmonids, cyprinid fish do not show acclimation-induced tolerance to Zn.

In the current author's opinion, the above studies do not constitute a demonstration of metabolic cost associated with activation of tolerance mechanisms to metal exposure including MT. Some inconsistencies are enumerated below.

1. The complex cellular machinery involved in metallothionein induction, and in the acquisition of tolerance for metal-exposed individuals is poorly understood - much research effort is channelized towards this research area though. The notion of a metabolic cost associated with metal tolerance is consequently vague. In term of ATP equivalents, a molecule of MT is expected to be ‹cheap›to produce because of the protein's small size.

2. A rigourous demonstration of the notion of metabolic cost associated with acclimation would
    require that the compensatory/detoxification mechanism not be overwhelmed at the exposure
    levels used to acclimate the organisms. Results obtained in the above laboratory studies and   in
the field study of Farag *et al.* (1995) do not rule out the alternative hypothesis that non-
    metallothionein bound metal causes adverse effects like reductions in fish growth; - this
    would represent the manifestation of toxic effects caused by a «spillover»of metal from MT,
    <u>and not</u> the expression of a metabolic cost of metallothionein synthesis. Increases in lipid
    peroxidation in some fish from the Clark Fork River (Farag *et al*. 1995) are best understood
    as the promotion of oxidative stress by Cu ions non-specifically bound to cellular membranes
(see Box 8).

3. The occurrence of metabolic costs associated with MT induction caused by t race metals may
    be metal- and species-specific. MT may not be induced and/or tolerance of the organism to
    the metal may not be acquired (re: Hobson and Birge 1989).

4. Growth declines of treated fish observed in the above experiments may be caused by an
    initial retardation of growth and these growth rates may resume to control levels later during
    exposure (see note b in Table 32). Such short-term effects would rarely be observed in nature
    and one would have to define a new endpoint to detect if such a metabolic cost to MT
    induction exist for field populations that have been exposed to metals for years.

6.3.3 **Research needs**

More research efforts are needed to shed light on the problem of detecting cytotoxicity, metal
spillover, and costs of adaptation in nature (sections 6.3.1 and 6.3.2). Thus, careful *in situ*
evaluation of the protection provided by the metal detoxification mechanisms involving MT would
be desirable. Capacities of detoxification mechanisms to counter effects of toxic substances,
defined as «counteractive capacity»(see Roesijadi 1992), are considered to have finite limits and can
be compromised when upper limits are approached. This would lead to toxic effects definable in

terms of organism performance (growth, fecundity, survival; Fig. 32A). These ideas are embodied in the spillover hypothesis associated with MT induction (section 2.4).
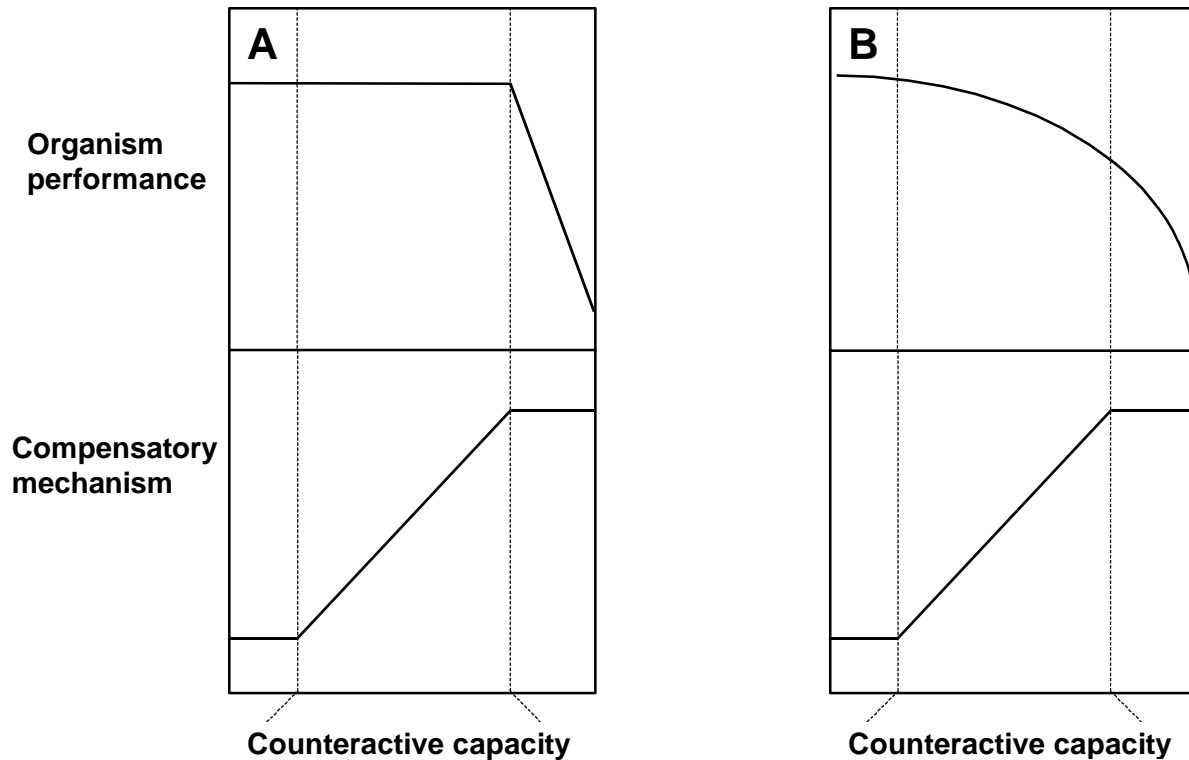
Figure 32: Hypothetical relationships between the performance of an organism (growth, fecundity, survival) and the behaviour of a compensatory mechanism. Exposure concentrations over which the compensatory mechanism provides protection is defined as the ʻcounteractive capacityʼ. Curve shapes do not necessarily reflect a real situation. See text for explanations (adapted from Roesijadi 1992).

Conversely, degradation of an organism performance may occur simultaneously with mobilization of the compensatory mechanism if the «counteraction» is less than perfect (Fig. 32B). In Figure 32B, an association is assumed to exist between the expression of the detoxification activity and the degrading state of the organism; such a relationship is also embodied in the concept of the cost to adaptation to contaminant exposure. Note that the latter concept remains elusive at this time. Information is scarce in the published literature on the nature of adaptive costs to contaminant exposure. Can they be defined as the consequence of an initial toxic biochemical interaction, or do they represent a true diversion of energy towards defense mechanisms quantifiable in terms of

ATP equivalents? In line with the former thesis, Roesijadi (1992) indicates that several examples exist in which the ability of metallothionein to intercept and sequester incoming metals has been less than perfect and metals have bound to other structures concomitantly with binding to MT, even if the nominal counteractive capacity was not exceeded as in Fig. 32B.

To determine which of the two types of relationships depicted in Figures 32A and 32B prevail for an organism in its natural environment, organism performance and the functioning of the compensatory mechanism have to be analyzed simultaneously (Roesijadi 1992). For metallothionein, the degree of metal detoxification could be evaluated in terms of MT concentrations, and intracellular metal partitioning would indicate if counteractive capacity is exceeded (metal spillover; Fig. 32). Organism performance may be characterized by individual life-history measures (mortality, birth rates), growth and reproduction (Sibly 1996). Incidences on the population may be assessed by determinations of population growth rate (Sibly 1996), age structure and recruitment (Luoma and Carter 1991). Populations of organisms should be studied at a number of metal-contaminated localities and control sites. Food and habitat quality and quantity should be carefully examined at each site since these variables will also influence individual- and populations-level performances. The question of control sites is treated in section 6.5. To summarize, if for a given organism, relationships such as those in Fig. 32A are likely to reflect well its biochemical and physiological responses to toxic metal aggression, then direct measurement of [MT] in this organism can only be considered as a simple indicator of prior exposure to toxic metals. Only when the detoxification system is overwhelmed would toxic effects occur; a biochemical signal corresponding to this status would be a diagnostic shift in intracellular metal partitioning (metal spillover). On the other hand, if relationships such as those shown in Fig. 32B prevail, then MT concentrations significantly higher than those of control populations would be taken as the manifestation of both a compensation response and a deleterious effect reflected by a general decline in the fitness/performance of the organism.

6.4 Early warning capacity and the biomarker concept

Early warning indicators would be particularly useful in evaluations of new mining operations and of efficiency of mitigation measures at existing mine sites. Such indicators would allow for management actions to be implemented before conditions have deteriorated to the point where exosystem organization is affected (Cairns *et al*. 1993). In principle, metallothionein possesses this early warning capacity because its induction precedes higher-level responses, and concentration of the metal needed to trigger MT induction is lower than those required to elicit higher-level responses (see section 2.3). The discussion that follows indicates if or how the potential early warning capacity of MT could be exploited in nature.

6.4.1 **Community and ecosystem integrity**

Results obtained in two studies suggested the potential early warning capacity of metallothionein (re: criterion 1 for biomarker, section 2.3) as a biomarker of effect. Following water quality improvements in the Campbell River system, rainbow trout MT levels declined before sensitive phytoplanktonic and zooplanktonic species reappeared at metal-impacted sites (section 4.2.1). Subsequent to experimental additions of Cd to the Experimental Lakes Area Lake 382, elevated MT levels were recorded in many organisms well before any symptoms of metal stress were detected in populations, communities, or ecosystems (sections 4.2.3 and 4.3.3; see also Schindler 1996). It is acknowledged that use of microcosms, mesocosms, and field experiments including whole ecosystem manipulations have improved the predictability of toxicant-induced responses of aquatic communities. Detoxification mechanisms have been studied; metal-sensitive species and taxonomic groups have been identified, and indirect effects, such as loss of prey species, have been documented (Schindler 1996; Luoma 1995; Clements and Kiffney 1994; Luoma and Carter 1991). However, linkages between metal doses and biochemical responses on one hand, and community- and ecosystem-level responses on the other, have not emerged (e.g. section 4.2.1.3), probably because a multitude of factors are involved in determining the latter responses. In the present state of knowledge, it appears rather illusory to think that any single biochemical biomarker can be developed to provide early warning of an anticipated toxicant-stress <u>at the community- and ecosystem-level</u>.

A major impediment to this type of research is that there is <u>no unifying ecological theory</u> explaining the functioning of ecosystems. For example, a model developed to predict effects of environmental stress on the structure and function of a type of aquatic biological community may not work for an other type of aquatic community (e.g. Locke 1996). Pratt and Cairns (1996) suggest that there are some myths in ecotoxicology to «slay»

(i)   Communities appear to lack appreciable redundancy. Impacts that result in local extinction of species have functional consequences even though these changes may be difficult to measure.

(ii) Biological communities are not in delicate equilibrium. Rather, communities are dynamic and species turnover is the rule.

(iii) All communities are not equal in their resilience following dist urbance by anthropogenic stressers. Some communities can recover to predisturbance levels, some others cannot.

(iv) Communities do not exist. However, they are conveniently defined as subsets of ecosystems that share properties such as species richness, succession, nutrient cycling and productivity.

The reader will find in appendix a new theoretical framework describing ecosystem function and integrity proposed by Kay and Schneider (1994).

6.4.2 **Acquisition of metal tolerance in nature.**

The comprehensive literature review accomplished in this report has provided several examples suggesting that exposure to metals in nature could result in physiological acclimation within the life-span of an exposed animal. Genetically-based tolerance to metals might also develop provided that exposed populations exhibit broad genetic variation (example with MT: Klerks and Levinton 1993). Because of this genetic component, Luoma and Carter (1991) consider that elevated tolerance to a metal in one population, relative to other populations of the

same species, might constitute a metal-specific response at the population level of organization. This adds additional complexity in the interpretation of the meaning of elevated MT levels (Stegeman *et al*. 1992); this issue has to be resolved if one wishes to use MT as an early warning indicator of metal exposure and stress <u>at the population-level</u> (re: criterion 1 for biomarker, section 2.3). In other words, the questions are as follows (Couillard *et al*. 1995b):

(1) Provided that the seasonal factors affecting MT levels in a given species at a given site have been correctly accounted for, can a high MT concentration under these conditions be considered an early signal of metal exposure and stress in this species? Or do high MT concentrations already constitute a response at the population level (a negation of the idea that [MT] could serve in an early warning capacity)?

(2) Will two populations of the same species, which differ in their MT genes, produce different MT levels under the same exposure conditions? If the answer is yes, then [MT] in a given species at a given site cannot be interpreted in its absolute sense; both genetic differences and differences in bioavailable metal concentrations will have to be considered to interpret the results correctly.

6.4.3 **Active quantitative biomonitoring**

The proactive capacity of metallothionein may, perhaps, be best exploited by the use of the active biomonitoring technique, given the potential pitfalls of using natural components of ecosystems for that purpose (sections 6.4.1 and 6.4.2). This technique (ABM: de Kock and Kramer 1994), also known as active quantitative biomonitoring, is based on transplantation of organisms at sites of interest - it is described below.

Active quantitative biomonitoring is currently used in Europe for the determination of trace metal concentrations in biota and in external media (de Kock and Kramer 1994; Kraak *et al*. 1991). However, the technique has infrequently been used to monitor biological effects (reviewed in de Kock and Kramer 1994); further development of this approach necessitates research. Issues

to consider include cage effects, selection of reference sites ecologically similar to exposure sites (see section 6.5 for a discussion of this point), the selection of the appropriate sentinel species (see section 6.7), and the demonstration that biological effects observed in caged organisms reflect accurately the anticipated site-specific impacts on their indigenous congeners and their population. In principle, active quantitative biomonitoring does not allow the possibility of predicting effects beyond the population-level of biological organization. However, trends and empirical links between ecological components are easier to establish when ecological communities have been extensively studied before and during mining operations (see section 4.2.1 and 4.2.3).

6.4.3.1 *Brief description of the technique*

Advantages of active quantitative biomonitoring through transplantation experiments (de Kock and Kramer 1994) are as follows:

- resolution power is optimized by the use of similar groups of organisms with regard to population, size, age, contamination history, and genetic characteristics, for comparing chemical stress at different locations;

- sites may be selected independently of the natural (non) occurrence of the biomonitor species;

- **the exposure period is known** Toxicokinetic information can be profitably used to provide discrete estimates of bioavailable chemical levels under natural condition s. Use of the biomonitor organism as a probe requires that the species be calibrated (see Box 33). Furthermore, the possibility is offered to follow chronologically biochemical responses such as metallothionein induction, and possible stress effects at physiological- and organism-levels of organization (see sections 4.2.1.2 and 4.3.1.4);

- transplantation experiments allow solid and accurate site-specific evaluations. Sampling stations can be precisely localized upstream and downstream of effluent disc harges, all other conditions being the same; spatial and temporal controls are possible (Green 1982). This type of biomonitoring is proactive, *i.e.* it provides early warning.

Active quantitative biomonitoring has also inherent disadvantages:

- it is logistically complicated;

- success of transplantation is not warranted; equipment can be stolen or vandalized;

- deployment in a foreign environment may adversely affect the biomonitor; cage/enclosure effects are possible. This problem is less likely to occu r if the species is naturally present at the site or if its ecological amplitude is large.

Box No. 33

Toxicokinetic model formalism

The rate of uptake and excretion of a chemical in an aquatic organism can be described as (de Kock and Kramer 1994; Landrum *et al.* 1992; Gobas *et al.* 1988):

$$d\,C_{org}/d\,t = k_1 C_W - k_2 C_{org} + k_F C_F - k_E C_{org} - g\,C_{org}$$

where C_W, C_{org} and C_F are chemical concentrations in water, in the organism, and its food respectively; k_1, k_2, k_F, and k_E are rate coefficients of chemical uptake from water, elimination to water, uptake from food, and elimination by egestion to feces, respectively; g is a first-order growth rate constant that accounts for the effect of growth on elimination as new tissue mass dilutes toxicant body burden. The assumption is made that uptake and excretion by the organism exhibit first-order kinetics.

A short exposure duration can be selected to minimize organism growth; it follows that the expression $g \times C_{org}$ can be solved from the model. If bioaccumulation of a trace metal by an organism occurs largely through chemical uptake from water, the toxicokinetic model can be further simplified. Organism chemical concentration can be used to calculate expected bioavailable trace metal level in water as defined, for example, by the free metal ion concentration. In relocalization/deployment schemes, this concentration can be determined using:

$$C_W = C_{org}/([k_1/k_2] \times [1 - e^{-k_2 t}])$$

where t is the time elapsed since the initial deployment. If steady state is achieved (*i.e.* $d\,C_{org}/d\,t = 0$ and $t_{95} = -\ln 0.05/k_2$), the above relationship simplifies to:

$$C_W = C_{org}/BCF$$

where BCF is the bioconcentration factor defined as k_1/k_2.

Rates of uptake and depuration of metals derived from solution and food can be determined using a combination of laboratory experiments (to discriminate efficiently routes of uptake from food and water in environmentally-realistic conditions), and field transplantation (to calibrate the biomonitor species in natural conditions).

6.5 Reference sites in mining regions

Comparisons between metal-impacted and reference ecosystems are essential in the biological effects monitoring for the mining industry. The reference ecosystems have a role in defining the characteristics of minimally-impacted populations and communities (Schindler 1996). To test whether an effluent discharge generates adverse effects in a biological community, an appropriate sampling design would be to sample an area unaffected by the effluent but otherwise similar to the area just below the effluent discharge. Temporal controls would be obtained by sampling both control and impacted areas before and after the beginning of the discharge (see Green 1982, p. 30). Such designs have been used in field evaluations performed for the mining industry (e.g. sections 4.2.5.3 and 4.2.5.4). However, the above sampling strategy appears to be inefficient in large-scale metal extraction regions because the extent and complexity of contamination is great (whether it be due to past mining practices or to the presence of natural surface mineralization), and zones of influence of past and present mining «activities»overlap on each other (Moore and Luoma 1990). Under these conditions, reference sites for a given mining operation may be difficult to locate; sources of contaminants are often numerous and may subtly affect areas which appear unimpacted at first sight (e.g. section 4.2.5.3). Therefore, in large mining regions, a cost-effective biomonitoring approach might be based on two elements of a sampling strategy that follows. First, the nature, extent and types of contamination must be understood at the *scale* of these regions (Moore and Luoma 1990). Second, biomonitoring for individual mining operations must be carried out using sampling designs recognized to be effective for these kinds of study (e.g. Green 1982; see above); knowledge (obtained from step one above) of the presence/absence of «true» reference sites in these studies will be of great help in interpreting results.

Detection of minimally-impacted sites may be facilitated by initial analysis of size-fractionated surface and profundal sediments, which normally represent main contaminant reservoirs in aquatic systems (see section 4.2.3.2). Sediment enrichments by anthropic sources may be evaluated by comparing metal concentrations in surface sediments to those measured in pre-industrial sediment strata. Low to minimal contamination would be indicated by a sediment enrichment factor ~ 1. Several precautions have to be taken to get interpretable and unbiased results and are described below (see Stephenson *et al*. 1996b, and Carignan *et al*. 1994).

- Sediments should be obtained by coring in lacustrine or riverine depositional zones;

- the coring device must not generate artifacts; core compaction and loss of superficial sediments must be minimal during the coring operation;

- post-depositional alterations in contaminant profiles should be minimal. Diagenetic processes and bioturbation may result in contaminant mobil ity within the sediment column;

- composition of profundal and modern sediment deposits should be similar, particularly for organic matter content and granulometry;

- to obtain a good correspondance between sediment depth and its age, rates of sediment deposition, and rates of supply of chronological markers (e.g. ^{210}Pb, ^{137}Cs) must have remained constant with time, or must have varied in a known manner.

In addition to the above, drainage basins including reference sites should ideally be unaltered. Agricultural and urban activities should be minimal, and pollutant point sources should be virtually absent. There should be no loss of natural littoral zones or spawning areas, and no overfishing. If very few or no control sites are available, historical records obtained from sediment analyses may be the best reference points available in the region. Paleoecological studies provide historical records of pre-settlement biological conditions (Cairns *et al.* 1993; Smol 1992).

The Experimental Lakes Area in northwestern Ontario includes a number of true reference ecosystems typical of the Canadian Precambrian Shield. The ecosystems are in an area of low atmospheric pollutant deposition; fishing, hunting, logging and mining activities have not affected their immediate watersheds (Schindler 1996; Malley and Mills 1992). Natural variability in characteristics such as water chemistry, phytoplankton photosynthesis and biomass has been studied for ~ 20 years in ten ELA lakes - data bases are available (Malley and Mills 1992). Populations and communities could be considered free from deleterious metal exposure effects if most of their characteristics or parameters fall within the limits of comparable reference sites in the ELA or within the mining region (Malley and Mills 1992; Munkittrick and Dixon 1989).

6.6 <u>Factors, other than metal contamination, influencing metallothionein</u>

    <u>concentrations</u>.

Relatively few endeavours have been devoted to research dealing with intrinsic and extrinsic factors, other than metal contamination, that influence metallothionein concentrations in aquatic organisms. Intrinsic factors may include cellular metal requirements, reproductive cycle, growth and development, genetic characteristics, size, age, sex (Metcalfe-Smith *et al*. 1996; Engel 1988) and stress caused by capture and sampling (Baer and Thomas 1990). Extrinsic factors include notably seasonal cycles of temperature (Engel 1988). Engel and Brouwer (1987) demonstrated that the molting process had a dramatic influence on the concentration and metal composition of MT in the blue crab *Callinectes sapidus* (see section 1.3.3.2). During an annual cycle, concentrations of MT varied between 4.4 and 17.7 nmol metal-binding sites g $^{-1}$ wet wt (*i.e.* a 4-fold variation in [MT]) in specimens of an indigenous population of the bivalve *Corbicula fluminea* (2 replicate samples obtained for each of 21 time points; Baudrimont M., unpublished results). Conversely, field studies with the freshwater bivalve *Pyganodon grandis* suggested that characteristics related to the basic biology and physiology of this mollusc were less important than changes in metal bioavailability as sources of variation in [MT] (Couillard *et al*. 1995a, Fig. 21; Kalhok and Cyr 1997).

Reproduction appears to have a marked influence on MT levels in fish (George and Olsson 1994). Olsson *et al*. (1987) studied variations in hepatic MT and Zn concentrations during an annual reproductive cycle in female rainbow trout held in the laboratory. Metallothionein levels increased concomitantly with those of cytosolic Zn; the ratio of the highest MT level to the lowest MT level reached 6. Mobilization of Zn was mediated by the reproduction hormone 17 β-estradiol. To minimize the effects of reproduction and other extrinsic factors on fish [MT], George and Olsson (1994) recommended that fish sampling not be carried out during periods of rapid

changes in water temperature, or during sexual maturation, and that collection of juvenile individuals was preferable.

Some groups of teleost fish maintain high constitutive concentrations of metallothionein because basal levels of essential metals are high (George and Olsson 1994; section 1.3.3.2). This is the case of salmonids for which hepatic levels of Cu (150 - 350 μg g^{-1}) and MT (100 - 240 μg MT g^{-1}) are normal (George and Olsson 1994; *O. mykiss* and *S. salar*).

The question of endobiotic and exogenous influences on MT concentrations awaits further research. If metallothionein is to be used as a monitoring tool, it is imperative that sources of uncontrolled variation in its concentration be minimized (re: criterion 4 for a biomarker, section 2.3). Indeed, increases in the precision and resolution of biomonitoring programs can be achieved by reducing within-site variation relative to among-site variation (Metcalfe-Smith *et al*. 1996, Engel 1988).

6.7 Sampling in a biological conservation perspective

6.7.1 **Tissue biopsy and catheterization**

Non-destructive sampling techniques are desirable in studies involving threatened or endangered species or sensitive populations (Depledge and Fossi 1994, p. 168). Tissue biopsy and use of catheters offer this possibility but few techniques have been described in the scientific literature. Ross (1984) employed catheterization to obtain *small* samples of gonads from reproductively inactive as well as ripe fish. The catheter was a tubule with an outside diameter of 1 mm, an inside diameter of ~ 0.7 mm, and a length of 20 cm. The tubule was gently inserted in the cloaca and, after penetration of the ovary, was removed while simultaneously applying mouth suction. The technique could be performed underwater (coupling a syringe to the catheter). Negligible mortality resulted from these operations. Harvey *et al*. (1984) carried out biopsies on 64 adult largemouth bass to obtain liver samples. Each specimen was anesthetized prior to surgery, and a 2.5-3.0 cm long incision was made below the pectoral fin. Approximately 1 g of liver tissue was excised. Then a 0.2% nitrofurazone solution (bactericide) was applied into the wound area and tissues were sutured. The operation lasted approximately 5 min as it was

performed in an open, non-sterile atmosphere. Two months after the biopsies, survival was 81%, all the incisions were completely healed, and no histologic abnormalities were observed. During his doctoral studies, a colleague developed a biopsy method to obtain samples of ovaries from muskellunge and pike (Bernard Lebeau, B.A.R. Environmental Inc., Nicholas Beaver Park, R.R.3, Guelph, Ontario, N1H 6H9; pers. comm., August 1996). Each specimen was anesthetized with MS-222, and the head and gills were maintained underwater by imparting a 30 ° inclination to the body. A 4-cm incision was made in the flanks and a 1-cm^3 portion of ovary was obtained. Peritoneal and skin tissues were then sutured using standard procedures. The operator gently manipulated all the internal organs and wore gloves disinfected with a bactericide solution prior to any surgery. Thirty-seven muskellunge and 3 pike were submitted to the above surgery, and returned to their lake. Monitoring fish displacements in the lake by telemetry indicated that none of the above specimens were lost. Berg *et al*. (1995) developed a biopsy method for freshwater bivalves. A wooden wedge was inserted between the two valves of an animal, and a 1-cm^2 portion of mantle tissue was cut with forceps and fine scissors. Comparisons of groups submitted to biopsies and control groups indicated that their respective survivals were not significantly different from each other one year after the mantle biopsies.

The feasability of obtaining biopsies of target tissues for trace metal bioaccumulation and toxicity should be investigated. Important organs are gills for freshwater bivalves, and liver and kidney for fish and higher vertebrates.

6.7.2 Transplantation of organisms

Transplantation of organisms constitutes an avenue to evaluate trace metal contamination and induced-stress at a given location while sparing potentially metal-stressed populations at this location. Relocated organisms may originate from an abundant population nearby, or may have been raised in an aquaculture facility[3]. Transplantation experiments are usually carried out within an active biomonitoring strategy (described in section 6.4.3).

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[3] In this regard, note that aquaculture has been useful for reintroducing/reestablishing fish populations in areas previously restored or for which pollutant inputs have declined (e.g. Sudbury area). Dr. Richard Neves of the U.S.

6.7.3 **Other measures of biological conservation**

Conservation principles should guide investigators if destructive sampling is required. A minimum number of organisms or composite samples should be collected to obtain the desired precision on estimates. A preliminary sampling is of great help to define these numbers, and to provide a basis for evaluation of sampling design (Green 1982). Organisms do not need to be sacrificed for individual growth measurements and for studies of population dynamics (Depledge and Fossi 1994).

6.8 <u>Selection of organism species for biomonitoring</u>

Monitoring biochemical-, organism-, population-, or community-level responses to trace metal exposures require the selection of particular animal species. Two motivations exist in this selection process. First, a particular species can be chosen because of <u>its economic and social value</u>; thus monitoring the recovery of its populations is relevant to the broad ecosystem objectives of maintaining indigenous organism populations (Renner 1996; Cairns *et al*. 1993; examples with Atlantic salmon: see sections 4.2.5.4 and 4.2.5.5). Second, <u>as expected in any biomonitoring program</u>, the candidate organism has to reflect chemical site-specific impacts; criteria have been defined for this purpose (Langston and Spence 1995; Crawford and Luoma 1993; Table 33). Crawford and Luoma (1993) indicate that any reasonable monitoring program should include the analyses of several resident taxa to assess different types of metal stress and to determine the possibility of trophic contaminant transfer. For example, two species found at the same metal contaminated site may experience widely different levels of metal exposure and stress (e.g. a mollusc *vs*. a burrowing mayfly in lakes of the Rouyn-Noranda area: sections 4.3.1 and 4.3.4.), or may experience metal stress originating from different metal elements (e.g. trout *vs*. plankton in the Campbell River basin: section 4.2.1). In addition, compensation mechanisms such

National Biological Survey, Blacksburg, Virginia, is exploring the possibility of raising populations of a variety of native bivalves in hatchery ponds (pers. comm., March 1995).

as MT induction may result in enhanced uptake of toxic trace metals, with the resulting danger that these metals may be transferred through food webs to terminal predators (Stegeman *et al*. 1992). Table 34 is a list of organisms appearing suitable for the present objectives of biomonitoring. The reader should consult this table in conjunction with Table 33 describing

Table 33

Criteria by which organisms are selected as being suitable for biomonitoring purposes (Langston and Spence 1995).

A.	Organisms should be relatively sedentary in order to be representative of the environment under study, or their mobility should be restricted by habitat barriers.
B.	There should be a simple correlation between metal concentrations in the tissues of the selected organism and the average ambient bioavailable metal concentrations.
C.	Organisms should be of widespread geographical distribution, abundant, easy to identify and sample, and should provide sufficient tissue for analysis.
D.	Organisms must be reasonably hardy and tolerant to environmental conditions and be amenable to laboratory experimentation or transplantation in order to investigate metal kinetics.

specific criteria for the selection of biomonitor organisms. All the species suggested are in intimate contact with aquatic habitats for dietary sources, shelter, reproduction, or life-cycle requirements. Since toxic trace metal transfer through aquatic food webs do not stop at the water line, top predators other than fish are included. Aquatic insects have not been used to a large extent in biomonitoring studies (Hare 1992). Yet, they represent an attractive group because they are found in freshwater of all types, are abundant and easy to collect (technicians, at INRS-EAU, developed a simple technique which allows rapid collection of mayfly larvae in sediments), are sedentary and thus representative of local conditions, and their internal metal concentrations appear to be related to those of their environment (Crawford and Luoma 1993; Hare 1992).

In designing biomonitoring programs, George and Olsson (1994) stressed that species selected should contain low «natural» levels of metals and/or metallothionein. These levels were high

in the Atlantic salmon *S. salar* eating a crustacean diet, and in decapod crustaceans. Protocols of

organism collection and handling need to be standardized to minimize undesired variations in metal

and metallothionein concentrations (see section 6.6) and in various parameters (see Metcalfe-

Smith *et al.* 1996; Crawford and Luoma 1993).

Table 34
Candidate organism species suggested for metallothionein field surveys and monitoring use for the mining industry. See text for discussion.

Organism(s)	MT producer	Advantage(s)	Disadvantage(s)	Notes
Freshwater bivalves e.g. *Pyganodon grandis*	Y	Conform to criteria A,B,C,D Excellent biomonitors. No licences required for collections. Extended knowledge on biology and ecology of *P. grandis.*	Measures of conservation have to be taken to sample some populations.	*P. grandis* is widely disseminated in North America. (see Couillard *et al.* 1995b).
Freshwater insects e.g. burrowing mayfly *Hexagenia limbata*	Y	Conform to criteria A,C,D; B? Populations are often nume-rous; there is no ethical problem to sample them. No licences required for collections. Extended knowledge on biology and ecology of *H. limbata*.	Low social value. Individuals are small: composite samples are necessary for most analyses. Isolation of specific organs requires much work.	*H. limbata* is abundant and widely distributed in North America. Source: Hare (1992)
Fish brown bullhead *Ameiurus nebulosus* white sucker *Catostomus commersoni* Yellow perch *Perca flavescens*	? Y Y	Conform to criteria A,C,D; B? Populations of *C. commersoni* and *P. flavescens* are often numerous.	Measures of conservation have to be taken to sample some populations. Licences required for collection.	*C. commersoni* and *P. flavescens* are widespread in Canada. Source: Scott and Crossman (1974).
Fish rainbow trout *Oncorhynchus mykiss*	Y	Conforms to criteria A,B,C,D High economic value. Biology well known. Home range size is small to moderate in lakes and rivers.	Measures of conservation have to be taken to sample some populations. Licences required for collection.	*O. mykiss* is widespread in western Canada. Source: Scott and Crossman (1974).
Predator fish lake trout *Salvelinus namaycush* pike *Esox lucius* Atlantic salmon	Y Y Y	High economic value. Widespread geographical distribution; however, *S. salar* is restricted to eastern Canada. Extended knowledge on	High mobilities make these species poor repre-sentants of site-specific impacts. Measures of conservation are desirable to sample these species	Lake trout is a sentinel species in the Great Lakes. See remarks on *S. salar* in text. Sources: Scott and

Organism(s)	MT producer	Advantage(s)	Disadvantage(s)	Notes
Salmo salar brown trout *Salmo trutta*	Y	biology, ecology and toxicology.	e.g. tissue biopsy. Licences required for collection.	Crossman (1974); Cairns *et al.* (1993)
Fish Young-of-the-year	species-dependent	Within-site variabilities in MT and metal concentrations are reduced because of sedentarity and the absence of endobiotic influences by sexual maturation and reproduction.	Individuals are small. Composite samples may be necessary for some analyses. Licences are required for collection.	

Table 34 (continued)

Organism(s)	MT producer	Advantage(s)	Disadvantage(s)	Notes
Fish Small forage species	species-dependent	Several species conform to criteria A and C. Economic value. Populations are often numerous. Large toxicological data base for *Pimephales promelas*.	Licences required for collection. Composite samples may be necessary for some species.	Widespread species in Canada: *P. promelas, Couesius plumbeus, Notropis atherinoides N. hudsonius, Rhinichthys cataractae, Semotilus margarita.* Source: Scott and Crossman (1974).
Amphibian bullfrog *Rana catesbeiana* adults and tadpoles	Y	Conforms to criteria A and C Economic value.	Measures of conservation have to be taken to sample some populations. Toxicological data base is small.	Home range size of adults is <3m radius. Population densities can be locally very high ~1000/ha adults ~10^4-10^5/ha tadpoles Source: U.S. EPA (1993).
Mammal muskrat *Ondatra zibethicus*	?	Conforms to criteria A and C Economic importance (fur animal).	Measures of conservation have to be taken to sample some populations. Toxicological data base is small. Licences required for collection.	Home range size of adults is 0.05-0.17 ha. Population densities in open water riverine areas may vary from 3-9 animals/ha. Source: U.S. EPA (1993).
Bird Top predator herring gull *Larus argentatus*	Y	Widespread and abundant throughout the Great Lakes; smaller colonies or isolated pairs may be seen along lakes and rivers in inland areas (*i.e.* discontinuous distribution). Biology well known. Large toxicological data base.	Low social value. High mobility makes this species poor biomonitor of site-specific impact. Licences required for collection.	Several toxicological studies including this bird in the Great Lakes. Mean foraging radius: 5-15 km. Source: U.S. EPA (1993).

Bird Top predator great blue heron *Ardia herodias*	?	Social value. Biology well known. May reflect local impacts because foraging radius is relatively small.	Discontinuous distribution. Measures of conservation have to be taken to sample some populations. Licences required for collection.	Source: U.S. EPA (1993).

6.9 Determination of an overall monitoring strategy for the mining industry

The statement of work for the present technical evaluation encompassed the making of recommandations on the best use of the metallothionein tool within an overall monitoring strategy for the mining industry»The author participated to a recent AETE Technical Committee Meeting (13-14 November 1996, Vancouver). During the meeting, much of the tasks given to the participants consisted of analyzing and evaluating the different tools and technologies available for mine biomonitoring, and defining the elements of a good environmental monitoring strategy. As a matter of fact, scientists have paid recently much attention in defining the elements of efficient environmental effects monitoring programs (EEM; Hodson *et al.* 1996; Suter II and Loar 1992). These discussions have demonstrated the need for additional understanding and research in **all** aspects of biomonitoring, *including the establishment of an overall biomonitoring strategy*. It is not the intention of the author here to propose what should be the best biomonitoring approach, but rather to provide elements that could be considered in defining such an approach.

A good environmental effects monitoring program would provide answers (quantitative) to clear, well defined questions (e.g. is there an environmental effect?; Hodson *et al.* 1996). One of the main goals of the AETE program is to evaluate and identify biotools and technologies that will help to answer the above questions at the lowest cost possible. Chapman illustrated that there were no simple answers to such questions because of the complex processes that underlie those, and suggested to include in the notion of cost, the cost of using inappropriate information to make erroneous decisions (AETE Technical Committee Meeting, Vancouver; point 5.4.2 Pre-survey studies; see also Chapman 1995). In the definition of a cost-effective biomonitoring strategy, the author thinks that there is no «stand on its own»tool; some tools are more useful and scientifically defensible than others though (the MT tool is promising in this regard).

6.9.1 Hierarchical approach

As discussed in section 6.4, our comprehension of the functioning of ecosystems is limited to the extent that it is not possible to obtain all the dose-response linkages between levels of

biological organization that are necessary to help protect aquatic habitats from metal toxicity (Luoma and Carter 1991; see also appendix). A **hierarchical approach** could be useful in this assessment process since knowledge of responses at one level of biological organization can be critical in anticipating, predicting, or understanding responses at the other levels (Luoma and Carter 1991). For example, the absence of obvious metal impacts on an animal population does not guarantee that it will be capable of tolerating subsequent stressors. Information on the general state of this population and on the individuals that make it up is necessary for a proper analysis. Conversely, the absence of a taxon at a given site is not necessarily caused by an extreme metal contamination. Information would be desirable on chemical concentrations in other abiotic compartments and in other biota, together with a general appraisal of the habitat quality of the site (see non-direct effects below). The hierarchical approach to investigations of ecotoxicological problems is gaining consensus among researchers (Engel and Vaughan 1996; Newman and Jagoe 1996; Munkittrick and McCarty 1995; Depledge and Fossi 1994; Cairns *et al.* 1993; Roesijadi 1992; Luoma and Carter 1991). Schneider and Kay (1994) indicate that «.neither a reductionist nor an holistic approach is sufficient...»in the study of ecosystems. Munkittrick and McCarty (1995) talk of an «effects-driven mechanistic understanding»Additional benefits of the evaluation of ecological impacts from a hierarchical perspective are that the approach fosters advancement of knowledge for predictive purposes (and help ecotoxicology transit to a mature science; see preface of Newman and Jagoe 1996) and that it provides multiple lines of evidence for the above assessment. Measurements are suggested at levels of biological organization «that draw mechanistic interpretation from the next lower level and attempts to predicts effects at the next higher level» Examples are (Newman and Jagoe 1996):

Theory/model/mechanism	Help in predicting/ understanding	At the level	Reference
Geochemical speciation models Free-Ion Model	Trace metal bioaccumulation	Cellular/organism	See Boxes 15 and 16
Mechanism of detoxification including metallothionein	Metal-induced deleterious effects	Cellular/physiological/ organism	Chapters 2 and 4
Theory of population dynamics (based on individual life histories)	Density/ viability of a population	Population	Calow (1994)

6.9.2 Indirect and non-direct effects

Two types of effects are not properly taken into account by the hierarchical approach: indirect and non-direct effects.

An **indirect effect** of a chemical on an organism refers to a chemical-induced impact on its food base or habitat. Decline of the organism's performance may ensue, and not being related at all to direct chemical exposure (Munkittrick and McCarty 1995). A **non-direct effect** results from changes in physico-chemical or ecological conditions which are not caused by contaminant exposure. Examples given by Munkittrick and McCarty (1995) include changes in benthic communities associated with sediment texture and quality, or loss of habitat not related to chemical pollution. Careful attention has to be brought in the selection of reference sites in order to avoid introducing artefacts caused by non-direct effects.

The work of Adams and Ryon (1994) represents an example of a hierarchical approach, assorted with an evaluation of indirect effects, in the study of the response of redbreast sunfish to Hg and PCBs in a stream in Tennessee. The authors concluded that the use of appropriate biochemical biomarkers, conventional population- and community-level responses, nutritional indices indicative of indirect effects, and the use of proper statistical techniques provided the best strategy for evaluating fish health.

6.9.3. Other elements

The EEM program can be defined as an ongoing and iterative examination of environmental quality in relation to industrial activity and environmental regulations (Hodson *et al*. 1996). The numerous measurements suggested by the use of a hierarchical approach and by the evaluation of indirect effects could be reduced in number in subsequent cycles of EEM when site-specific impacts are well understood.

In addition to the above measures, a preliminary step of gathering of background information would be useful (see Crawford and Luoma 1993). The **retrospective** should involve a thorough search for and interpretation of previous studies conducted in the study region and dealing with

distributions of contaminants in aquatic systems and/or with their effects on resident biota. The search should help to define which species to target for monitoring in a region, and then the relevant information on the biology of the target species could be obtained through a literature review.

In the following, the author has compiled the recent recommendations of two researchers, made in the context of increasing our understanding of ecotoxicological problems.

Recommendations of Cairns (1994):

(i)   He advocates an increased level of coordination and integration between disciplines (e.g. ecologists, toxicologists, ecotoxicologists, geochemists) to facilitate interactions and increase the speed at which information is transferred between these disciplines.

(ii)   He recommends that the spatial scale of assessments be consistent with the spatial scale of the stress. He notes that satellite imaging and aerial photography can collect relevant ecological information on large scales. Thermal infrared remote sensing has been useful to show that more developed forest ecosystems degrade more energy (see appendix). Schneider and Kay (1994) suggested the use of this type of information to help evaluate ecosystem integrity. Thermal infrared remote sensing does not appear to have been used to measure energy budgets of aquatic systems.

(iii)   Cairns recommends that the temporal scale of assessment be consistent with the temporal scale of stress (information on long term processes is needed).

Recommendations of Renner (1996).

Renner identified frequent problems in the selection of endpoints for ecological risk assessment:

(i)   Endpoint is too vague.

(ii)   Public does not value the endpoint (e.g. fishery makes a better endpoint than midges).

(iii)   Endpoint is not exposed to the stressor (e.g. better to use resident taxa).

(iv)   Endpoint does not reflect the complexity of the ecosystem. (e.g. to preserve native freshwater mussels, information is required on both the occurrence of fish hosts and on the habitat quality).

C H A P T E R   7

CONCLUSIONS

AND

RECOMMENDATIONS

7. **CONCLUSIONS AND RECOMMENDATIONS**

A. Metallothionein can already be considered to be a useful biomarker of exposure to certain metals (notably Cd, Zn, Cu and Ag).

B. The use of metallothionein as a biomarker of exposure has certain similarities to the use of tissue metal concentrations to monitor trace metal contamination. However, the two measures could be considered not ‹redundant› with respect to each other because:

- MT responds to the toxicologically significant intracellular fraction of metals (metal in cell soluble fraction) whereas a proportion of tissular metals can be found in biologically unavailable form (e.g. granules);

- organisms synthesize MT as a defense against metals in excess, and there is convincing evidence of a relationship between the MT response and the acquisition of tolerance to metal at the whole animal level.

C. Metallothionein is not a «stand on its own» tool. As for any monitoring tool, the MT level in an organism has to be used in conjunction with other biotic and abiotic measurements to be interpreted unambiguously (e.g. section 6.9).

D. The use of metallothionein as an effects biomarker is less well established. Significant progress in this direction depends on the successful demonstration, in nature, that there are metabolic costs to tolerance to metals and/or that overwhelming of detoxification mechanisms, including MT, are associated with deleterious effects on the host organism.

E.   The early warning capacity of metallothionein is not established; anticipation of effects at the population-, community- and ecosystem-level of biological organization is subordinate   on an adequate understanding of the functioning of complex whole ecosystems. Development   of active biomonitoring techniques is desirable if MT is to serve eventually an early warning   function.

F.   Some of the research needed on the use of metallothionein as a biomonitoring tool could be conducted under the auspices of the AETE program. This includes an investigation of the use of different species as sentinel organisms at selected sites, and their calibration with respect to the use of MT as a biomarker of exposure.

G. Metallothionein does not appear to be a cost-effective tool to monitor responses of highly mobile fish species to metal exposure as it may be the case with, perhaps, Atlantic salmon. Dose-dependent MT responses might be obtained by placing these fish in enclosures; however, cage effects are largely unknown for this species.

H.   Metallothionein analyses are not available commercially, but the private sector could readily develop the capabilities to do so. MT could be easy and reasonably inexpensive to analyze, but the costs of the analytical techniques remains to be   established.

I.   Standardization of protocols of sample preparation, metallothionein extraction and quantification, and QA/QC checks are required on a countrywide basis before any implementation of the MT tool is suggested.

J.   Protocols of organism capture/collection and handling need to be standardized to minimize undesired variations in metallothionein concentrations.

Table 35

Evaluation of metallothionein against a set of criteria defined for a biomarker, and provided in the statement of work for the present report. Ranking: 1: excellent; 2: very good; 3: good; 4: fair; 5: bad; N.A.: Information non available because field evidence is scarce.

Criterion	Ranking	Remark
Relative sensitivity (dose-response) Re: Criterion No. 3 defined for a biomarker.	1	Peer-reviewed field validation
Ecological relevance (predictive capability of effects, warning systems) Re: Criterion No. 1 defined for a biomarker.	N.A.	Issue not adequately investigated in field situations. More research is needed.
Relationship with the health of the organism Re: Criterion No. 5 defined for a biomarker	N.A.	Issue not adequately investigated in field situations. More research is needed.
Validation (peer-reviewed, field and laboratory)	Relative sensitivity: 1 Ecological relevance: 5 Relationship with the health of the organism: 4	See evaluations for the 3 criteria above.
Variability (temporal, spatial representativeness, sampling effort and variance) Re: Criterion No. 4 defined for a biomarker	N.A.	Non-toxicological factors influencing MT concentrations have not been adequately evaluated in nature. More research is needed.
Chemical specificity Re: Criterion No. 2 defined for a biomarker	2	Peer-reviewed field and laboratory validation.
Site-specificity	1	Sentinel (sedentary) organisms must be used.

Table 35 (continued)

Criterion	Ranking	Remark
Applicability	2	Reliable analytical protocols for MT detection and quantification have been defined. MT is easy to quantify by metal-saturation methods.
Repeatability	2	Laboratory and field-validated
Practical limitations for carrying field work	3	Fresh samples should be frozen and protected against long-term oxidation. See Chapter 5.
Commercial availability	MT analyses: 4 MT certified samples: 1	MT services are not now widely provided within the private sector.
Response time (exposure, rapidity)	3	Largely dependent on kinetics of metal uptake and excretion of the biomonitor organism.
Interpretability (statistical evaluation, confidence, clarity)	Exposure: 1 Effect: 4	In the present state of knowledge, very high MT concentrations might be associated with adverse effects, provided that non-toxicological factors influencing [MT] have been taken into account. More research on this aspect is needed as indicated above.

C H A P T E R　　8

REFERENCES

C H A P T E R　　8

7. **<u>REFERENCES</u>**

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C H A P T E R   9

GLOSSARY

9. **GLOSSARY**

Acclimation: adaptative changes of an organism in response to the manipulation of a single environmental parameter (e.g. metal concentration).

Bioaccumulation: a net retention of a contaminant by an organism over time, that is the influx of a contaminant exceeds its efflux. Bioaccumulation may not be associated with an increase in an organim's contaminant concentration, which may be caused by a loss in body weight.

Bioavailability: the portion of a contaminant in the environment whose presence in or mobilization from water, sediment, or diet gives rise to measurable accumulation by an organism.

Biomarker: a biological response to the exposure to an environmental chemical. This response, at the below-individual level, is not necessarily detected at the whole organism level.

Cytosol: soluble phase of the cell (operationally defined).

Detoxification: any strategy, process or mechanism that prevents, minimizes or reverses the potential for metals to chemically interact, via non-specific binding, in a detrimental fashion with essential molecules.

Dose: a dose can be expressed in one of the three ways:
   1. the amount of the substance actually in an organism;
   2. the amount of the substance entering the organism (usually in food, water, sediment, or air that is breathed);
   3. the concentration of the substance in the environment.

Ecological risk assessment: procedure which evaluates the likelihood that adverse ecological effects may occur, or are occurring, as a result of one or more stressors/contaminants. Ecological risk assessment may examine many ecological components.

Ecosystem integrity: ability of an ecosystem to maintain its organization (structure and function) in the presence of changing environmental conditions. There is a range of organizational states for which the ecosystem is considered to have integrity.

Induction: in the biological sense, activation of a phenomenon which occurs with a certain lag relatively to the trigger of this phenomenon. Metallothionein induction by a metal expresses an increased synthesis of the protein (relative to a basal level) in response to an internal injection of the metal or to an increased exposure of the organism to this metal. A delay in the production of MT is anticipated because of the complexity of its biosynthesis mechanism at the cellular scale.

Metalloprotein: protein which has metal incorporated in its structure; it includes MT, metalloenzymes, and other non-enzymatic proteins such as hemoglobin (Hb) and hemocyanin.

Metallothionein (MT): cysteine-rich, heat-stable, low-molecular weight protein which chelates metals such as Cd, Cu and Zn. Functions attributed to MT include detoxification, and regulation of these metals.

Oxidative stress: group of biological perturbations in an organism, including lipid peroxidation (see Box 8), provoked by an over-exposure to O_2 and its radicals (intra- or extra-cellularly).

Resistance: capacity of an organism to survive for a limited period in an environment which will generates eventually a lethal effect.

Spillover: refers directly to the spillover hypothesis; see Fig. 5 for a graphical depiction of this hypothesis.

Steady-state: when used in the context of toxicokinetic studies, refers to the condition in which organism uptake and excretion rates are balanced and further net bioaccumulation does not occur.

Tolerance/adaptation: capacity of an organism to survive indefinitely under an array of environmental conditions. This capacity may involve a genetic basis.

Toxicity: capacity of a chemical to cause injury to a living organism. This is usually defined with reference to:
(i) the species and its life stage,
(ii) the dose of the chemical,
(iii) distribution of dose in time (acute dose, chronic dose),
(iv) type and severity of injury (lethal response, sub-lethal response e.g. immunotoxicity, genotoxicity, cytotoxicity, avoidance),
(v) time needed to produce injury (acute, chronic).

APPENDIX

ECOLOGICAL FRAMEWORK

OF KAY AND SCHNEIDER

Ecological framework of Kay and Schneider

Kay and Schneider (1994) have recently proposed a theoretical framework for discussing ecosystem function and integrity. Discussion of this framework is relevant because some principles described in their approach appear useful in the context of the environmental effects monitoring for the mining industry.

The Kay and Schneider framework is based on the thermodynamic principle which states that as systems are moved away from equilibrium, they will utilize all avenues available to counter the applied gradients. As these gradients increase, so does the system's ability to oppose further movement from equilibrium. Incoming solar energy is a source of disequilibrium; to dissipate this energy, ecosystems organize themselves as individual chemical factories (species), doubled by a chain of energy degraders (grazing chain) and matter simplifiers, or in other words the detrital cycle, to insure a continuing supply of material for energy-degrading processes (Schneider and Kay 1994). The thesis of the authors that a more developed ecosystem degrades more energy relies on recent studies on the energetics of terrestrial ecosystems. In one of these studies, Luvall and Holbo (1989) used a thermal infrared multispectral scanner to demonstrate that thermal radiation varied with the structure of forest stands. Degradation of incoming solar energy increased with the more mature or less perturbed ecosystem (Table 36). These results and other studies presented in Schneider and Kay (1994) led the authors to suggest that impacted ecosystems are smaller, have fewer trophic levels, recycle less, and leak nutrient and energy. All of these are signs of disorganization and a step backward in development.

The above theoretical framework depicts ecosystems as complex self-organizing systems that are dynamic and not deterministic in nature (cf. Pratt and Cairns 1996, see section 6.4.1). Superimposed on these characteristics, the process of self-organization would have, in essence, a degree of unpredictability and would exhibit phases of rapid changes or catastrophes [4] (see also Schindler 1996: The element of surprise, p. 380).

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[4] The authors use the recent theories of catastrophe and chaos to describe complex system development.

Table 36

Radiative estimates for different forest ecosystem types in western Oregon. The data is presented from least (quarry) to most developed (400 year old forest with 3 tiered plant canopy) (adapted from Schneider and Kay 1994, and Luvall and Holbo 1989).

	Quarry	Clearcut	30 year old Douglas Fir Plantation	30 year old regenerating forest	400 year old Douglas Fir forest
K^* (W m^{-2})	718	799	854	895	924
R_n (W m^{-2})	445	517	730	771	830
R_n/K^* (%)	62	65	85	86	90
Surface T (°C)	50.7	51.8	29.9	29.4	24.7

Note: K^* = incoming net solar energy; it is inversely proportional to the albedo of the site.

R_n = net radiation transformed into nonradiative processes at the surface.

R_n/K^* = percent of net incoming solar radiation degraded into non radiative processes.

Kay and Schneider (1994) indicate that ecosystems can respond to changes/perturbations in five qualitative different ways (see Fig. 34):

1. The system operates as before after an initial destabilisation.

2. The system can operate at a different level using the same structures it originally had (e.g. reduction or increase in species numbers).

3. New structures emerge in the system that replace or augment existing structures (e.g. new paths in food web).

4. A new system made up of quite different structures can emerge (e.g. Clearwater Lake, Sudbury area, following the disappearance of its fish populations; see below).

5. The ecosystem collapses completely, and no regeneration occurs (e.g. desertification).

The above classification suggests that if environmental conditions were to return to a pre-impacted state, there is no guarantee that a given ecosystem would return to its original state. An important implication of this is that, from a practical point of view, considerable efforts can be engaged to reduce point source loadings, and to reintroduce historically lost animal populations following mitigation measures. Yet, expected results at the ecosystem level may be very disappointing (e.g. Stokes 1984). This discussion is substantiated by the biological recovery
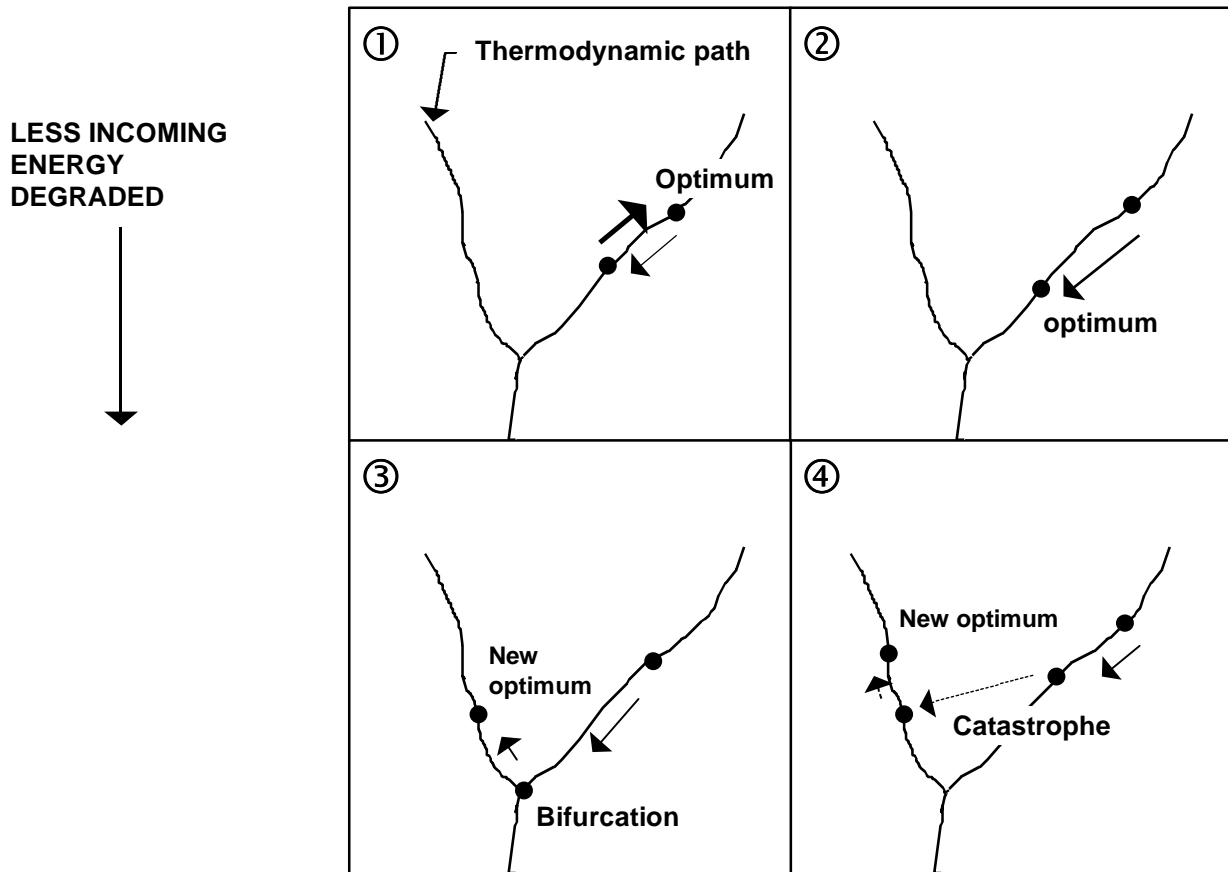
Figure 34: Schematic representations of the possible qualitative ways an ecosystem can respond to a perturbation. Numbers correspond to those of the above classification of Kay and Schneider (1994): 1.- operation as before; 2.-operation at an earlier successional stage; 3.- new optimum operating point; 4.- the system reaches a new optimum point through a catastrophic event (adapted from Kay 1991) .

studies undertaken in lakes of the Sudbury area, Ontario, following water quality improvements (Keller *et al*. 1992; Keller and Yan 1991). Continued reductions of industrial emissions of contaminants have resulted in partial recovery of many biological communities. Many recovering lakes are moving toward communities typical of natural Precambrian Shield lakes in the area (Table 37). Keller and Yan (1991) predicted that the greatest increases in species richness would occur in the larger, deeper lakes with abundant inflows, reflecting the overall potential for both internal and external recolonization sources (Schneider and Kay 1994 mention an abundant biodiversity pool) and with substantial increases in water quality. The least increases in species

Table 37

Summary of physico-chemical and biological changes in some lakes of the Sudbury area which have occurred solely through emission abatements at the Sudbury smelters in the early 80˙s. pH and Cu levels are indicated together with the year of sampling in parentheses (adapted from Keller *et al*. 1992 and Keller and Yan 1991).

Lake	Distance from Sudbury (km)	pH Before	pH After	Cu (μg L^{-1}) Before	Cu (μg L^{-1}) After	Phyto-plankton	Benthic algae	Zoo-plankton	Zoo-benthos	Fish
Clearwater	13	4.2 (73)	4.7 (84)	98	49	-	-	N	-	fishless
Swan	15	4.0 (77)	5.1 (84)	64	9	Y	decline	N	-	fishless
Wavy	21	4.4 (74)	4.7 (86)	33	21	-	-	Y	-	-
Joe	28	5.7 (75)	6.3 (86)	20	4	-	-	Y	-	-
SansChambre	29	5.4 (81)	5.9 (85)	4	3	-	-	Y	-	-
Laundrie	80	4.9 (75)	5.6 (86)	3	2	-	-	Y	-	-
Whitepine	89	5.5 (80)	5.9 (86)	1	1	-	-	Y	Y	Y

Note: Most potential for recovery: Wavy, Joe, Laundrie.
      Least potential for recovery: Clearwater, Swan.

richness would occur in lakes with fewer colonization sources and with minimal changes in metal exposure. Lake Clearwater was predicted to have a very low potential to recover (Table 37). The lake did not collapse according to Stokes (1984). As a system, the lake appeared dynamic and functional, with viable communities, but appeared simplified in terms of species richness, and in the number of trophic levels (no fish). Lake Clearwater has flipped from one thermodynamic branch to another (Kay 1991; Fig. 34, case 4; Stokes 1984).

To conclude, Schneider and Kay (1994) stated that ecosystems are so complex in space and time, that it is not *a priori* possible to make precise and deterministic predictions about the future development of self-organizing systems; such systems could only be understood from a hierarchical perspective that is the simultaneous study of several levels of biological organization.