Field transplantation of a freshwater bivalve, *Pyganodon grandis*, across a metal contamination gradient. II. Metallothionein response to Cd and Zn exposure, evidence for cytotoxicity, and links to effects at higher levels of biological organization

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Abstract: To examine links between the metallothionein (MT) status of an organism and its general health, we transplanted adult specimens of the freshwater bivalve *Pyganodon grandis* (formerly *Anodonta grandis*) from a less to a more contaminated lake in the mining area of Rouyn-Noranda, in northwestern Québec. The transplanted bivalves were maintained in open enclosures placed in the bottom sediments of the contaminated lake; in addition, indigenous specimens were maintained in control enclosures in their lake of origin. Up to 16 individuals were removed from pairs of enclosures at times $t = 0$ (June 1990), 5, 14, 30, 60, 90, and 400 d. Excised gill tissue was analyzed for metallothionein, Cd, Cu, Zn, Ca, and malondialdehyde (MDA), a product of lipid peroxidation. Metal partitioning in the gill cytosol, as determined on a subset of gill samples from transplanted molluscs, changed markedly during the experiment. After 400 d, Cd was present in the low molecular weight fraction of the gill cytosol, and symptoms of cellular toxicity were detected in the transplanted molluscs (elevated [MDA] and [Ca]). At the whole organism level, the marked transplanted bivalves grew more slowly over the 400-d experiment than did marked control bivalves in Lake Opasatica, and their condition index deteriorated over time.

Résumé : Des spécimens adultes du bivalve d'eau douce *Pyganodon grandis* (anciennement *Anodonta grandis*) ont été transplantés d'un lac relativement peu contaminé vers un lac très contaminé en Cd et en Zn, dans la région minière de Rouyn-Noranda (Québec). Les bivalves transplantés et témoins (gardés dans leur lac d'origine) furent confinés dans des enclos ouverts enfoncés dans les sédiments littoraux. Jusqu'à 16 individus furent retirés des enclos aux temps 0 (juin 1990), 5, 14, 30, 60, 90 et 400 jours. Les branchies, choisies comme organe cible, furent analysées pour la métallothionéine (MT), des métaux (Cd, Cu, Zn, Ca) et la malondialdéhyde (MDA; produit de la peroxydation lipidique des membranes). La répartition subcellulaire du Cd dans le cytosol des branchies, tel que déterminée pour certains mollusques transplantés, montrait des changements importants au cours de l'expérience. Après 400 jours, l'apparition de symptômes de toxicité cellulaire dans les branchies des mollusques transplantés ([MDA] et [Ca] élevées) coïncida avec l'association du Cd avec des ligands de faible poids moléculaire dans le cytosol branchial. La croissance moyenne sur 400 jours des bivalves transplantés, marqués à cet effet,

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était significativement ralentie par rapport à celle des bivalves témoins marqués maintenus dans le lac le moins contaminé. De plus, l'indice de condition de ces mollusques transplantés se dégrada dans le temps.

Introduction

Metallothionein (MT) is a low molecular weight, cysteine-rich metal-binding protein with high affinity for groups IB and IIB metal ions. Given its molecular properties and our present knowledge of its role in metal uptake, transport, storage, and excretion, MT offers considerable potential as a contaminant-specific biochemical indicator of metal exposure and (or) stress (Roesijadi 1992; Stegeman et al. 1992). Possible approaches include

(1) *Direct measurement of [MT] as an indicator of prior exposure to toxic metals.* In this case, it is assumed that basal levels of MT are low, and that any increase in concentration in an animal above these low levels is attributable to the induction of MT in response to an influx of toxic metals. Measurements of MT would in principle distinguish between the toxicologically significant intracellular fraction of metals and metals bound in unavailable form (Roesijadi 1981).

(2) *Examination of the distribution of toxic metals in cytosolic ligand pools as a means of evaluating metal stress at the biochemical level.* It has been suggested that the 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). In principle, this condition could be considered as symptomatic of metal stress and would be amenable to detection.

Our previous field work has shown that metallothionein concentrations in the freshwater bivalve *Pyganodon grandis* (formerly *Anodonta grandis*) respond in a concentration-dependent manner to ambient levels of Cd. This dependence was observed both spatially (interlake comparison along a contamination gradient; Couillard et al. 1993) and temporally (transplant experiment; Couillard et al. 1995). We concluded that MT is a promising biomarker of prior metal exposure (approach (1) above). Similar conclusions have been reached by other workers, based on laboratory investigations (Stegeman et al. 1992; NRCC 1985).

In contrast, field investigations of the validity of the second possible use of MT, i.e., as a biochemical indicator of metal-induced stress, are only slowly appearing in the literature (Klaverkamp et al. 1991; Brown et al. 1987; Johannson et al. 1986). Indeed, almost all the evidence for spillover in aquatic organisms has been derived from laboratory experiments (Jenkins and Mason 1988; Sanders et al. 1983) where the test organisms were exposed to (unrealistically) high metal concentrations.

In the present study, we carried out a field experiment designed to evaluate the potential of metallothionein as a biochemical indicator of metal-induced stress in *P. grandis*. Specimens were transplanted from a relatively unpolluted lake to a lake contaminated by Cd and Zn. To detect symptoms of cellular toxicity and test a hypothetical model of cytotoxicity, we monitored MT, cytosolic [Ca], and malondialdehyde (MDA; a product of lipid peroxidation) and determined the subcellular partitioning of cytosolic metals. Similarly, to investigate the relation between MT status and effects at higher levels of biological organization, we determined condition indices and shell growth rates of the specimens (organism response), as well as other characteristics of the indigenous bivalve populations involved in the transplant experiment.

The role of MT in the detoxification of excess trace metals and in the health maintenance of *P. grandis* has been evaluated within the framework of a hypothetical model of cytotoxicity (Viarengo 1989; Younes and Siegers 1984). In this model, [cytosolic metal]/[MT] ratios are taken to reflect the quantity of the metal available to express cytotoxicity (Fig. 1, step 1). The lipid peroxidation process (step 2) can be broadly defined as an oxidative deterioration of polyunsaturated lipids in the cellular membranes, initiated by reactive species of oxygen such as O^{2-}, $OH^{\cdot}$, $O_2^{\cdot}$ (Bus and Gibson 1979). Non-MT-bound nonessential metals, as well as non-MT-bound essential metals present in excess, would be expected to enhance the lipid peroxidation process, resulting in increased levels of malondialdehyde, a by-product of this peroxidation (Sunderman et al. 1985). It is well established that Cd and Cu, in particular, can stimulate this process, though by very different mechanisms (Hendry et al. 1992; Gill et al. 1990; Viarengo et al. 1990); Cu reacts with H_2O_2 directly, generating reactive oxygen species via Fenton-like reactions, whereas Cd, a redox-insensitive metal, acts by depleting the -SH antioxidant pools. 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 ATPase (Viarengo 1989; e.g., with Cd: Verbost et al. 1988; with Cu: Viarengo et al. 1988). 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).

Several criteria have been proposed for evaluating the adequacy of various biochemical indicators (Stegeman et al. 1992; Haux and Förlin 1989). Criteria are numbered below because we refer to them by their numbers in this text and in the accompanying article.

(1) The indicator should present an early warning capacity, i.e., the biochemical response should be predictive of effects at higher levels of biological organization (population, community) and should precede them.

(2) It should be specific to a particular contaminant or a class of contaminants.

(3) It should respond in a concentration-dependent manner to changes in ambient levels of the contaminant.

(4) Endogenous and exogenous factors that affect the

indicator should be known, so that sources of uncontrolled variation can be minimized.
(5) The indicator should be related to the health or fitness status of the organism.

The present study was designed to investigate criteria 1 and 5. However, we shall use all five criteria in our final evaluation of MT as a biomarker.

Materials and methods

Study area and experimental design

The transplant experiment was carried out in two lakes in the mining area of Rouyn-Noranda in northwestern Québec. This experiment was designed to follow changes associated with increased metal exposure as a function of time. The two lakes selected differed widely in their levels of contamination (Cd, Zn), and with respect to MT concentrations found in their indigenous mollusc populations, but they shared similar ecological characteristics (Couillard et al. 1995).

Specimens of the freshwater bivalve *P. grandis* of a nominal length of 8 cm (4–6 years old) were transferred from the relatively unpolluted Lake Opasatica to the highly contaminated Lake Vaudray (treatment T-VA). Indigenous mussels were used as control groups (Opasatica: C-OP, Vaudray: C-VA). Animals were held in open enclosures placed in the bottom sediments of each lake (eight animals per enclosure). The animals transferred to Lake Vaudray (T-VA) and the Lake Vaudray controls (C-VA) were sacrificed according to the sequence 0, 5, 14, 30, 60, 90, and 400 d (t = 0: 20 June 1990). Two enclosures, that is nominally 16 animals, were sampled at each date. The C-OP control group was sampled according to the sequence 0, 5, 90, and 400 d. Bivalves destined to be collected at 400 d were marked to follow their growth. In addition, 12 indigenous molluscs were collected outside the enclosures at each site at 400 days and are identified as OP-IND (Lake Opasatica) and VA-IND (Lake Vaudray).

In coding the various groups, we have used the following conventions: C, control; T, transferred; OP, Lake Opasatica; VA, Lake Vaudray; IND, indigenous, free living. The number of days of exposure completes the code, e.g., T-VA-90 d designates molluscs transferred to Lake Vaudray and collected after 90 days exposure. The location of the lakes, the rationale for site selection and the details of the experimental plan are summarized in the companion paper in this issue (Couillard et al. 1995).

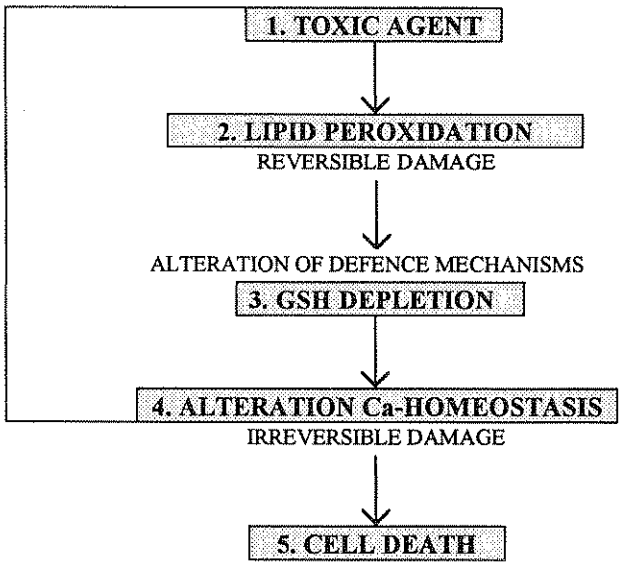
Bivalve analyses

The bivalves from all groups were dissected within 4 h of collection into four tissue groups: gills, mantle, digestive gland, and remainder. To obtain enough material for all the analyses, tissues from four bivalves were pooled yielding four replicate samples per treatment and per date, when no mortality had occurred. We computed, for each replicate, the following condition index (CI; Lucas and Beninger 1985):

$$[1] \quad CI = \frac{\text{Total flesh dry weight (g)}}{\text{Total shell weight (g)}}$$

Four pairs of demibranch gills from each replicate were retained at the time of dissection for determination of cytosolic Cd, Cu, Zn, and Ca concentrations ($[M]_{cyt}$), where M = metal. These samples were also used for determinations of malondialdehyde concentrations [MDA] as well as for the determination of the partioning of Cd, Cu, and Zn among cytoplasmic ligands. The tissues were preserved as described in Couillard et al. (1995), until the homogenization step. One-half gram of partially thawed tissue from each replicate was homogenized with a glass homogenizer and pestle in 5 mL of ice-cold 154 mM KCl solution; this extract was analyzed the same day for MDA. The remaining tissues were homogenized in three or nine weights of ice-cold 25 mM Tris solution, adjusted to pH 7.2, with a glass homogenizer and motor-driven Teflon pestle at 80 rpm; the homogenates were immediately frozen and stored at −80°C for eventual analysis of cytosolic metals. Dissection and preservation procedures and tissue metal determinations are fully described in Couillard et al. (1995). The occurrence of MT in *P. grandis* and the MT assay used here are discussed in Couillard et al. (1993).

Fig. 1. Model of trace metal cytotoxicity showing the sequence of events leading to cell death after lipid peroxidation. Variables used to evaluate the model are the ratios of cytosolic metal to MT concentrations, step 1; malondialdehyde concentrations, [MDA], step 2; and cytosol [Ca], step 4. The target organs are the gills. See text for explanations. Adapted from Viarengo (1989) and Younes and Siegers (1984).



For each sample of a given treatment obtained at 60 d (20 August 1990) and 90 d (17 September 1990), gravid females, identified by visual inspection of the gills, were counted. Larvae extracted from the marsupium of each female were weighed. Larval replicates, obtained by pooling four specimens, were analyzed later for MT and Cd, Cu, Zn, and Ca concentrations, and their dry weight (wt.)/wet wt. ratios were determined.

Malondialdehyde determinations in gills

MDA concentrations in the gills were measured on whole tissue homogenates by the colorimetric method of Sunderman et al. (1985), which is based on the reaction of thiobarbituric acid with MDA and related chromogens. Two

Table 1. Cadmium and Cu concentrations in HPLC gel permeation fractions of gill cytosols from specimens transplanted to Lake Vaudray (T-VA) and collected after 14, 90, or 400 d.

Molecular weight fraction	Elution volume (mL)	[M] in fractions for each treatment (μg/g dry wt.) T-VA-14 d [Cd]	T-VA-90 d [Cd]	T-VA-90 d [Cu]	T-VA-400 d [Cd]
High	4–6	0.74	0	1.32	0
	6–8	0.42	0	5.62	0
	8–10	0.59	0	1.41	1.09
MT[a]	10–12	0	0	0	0
	12–14	0	6.62	1.49	0.96
Low	14–16	0	0	0	5.73
	16–18	0	0	0	0
	18–20	0	0	0	0
	20–22	0	0	0	0
Σ fractions (μg/g dry wt.)		1.74 versus 2.28	6.62 versus 4.26	9.83 versus 10.87	7.79 versus 6.33
Recovery of total $[M]_{CYT}$[b] (%)		77	155	91	123

[a]10 kDa corresponds to an elution volume of 12.6 mL.

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

determinations of [MDA] were conducted on each homogenate and each determination was corrected for background absorption due to reagent blanks (about 0.01 mmol MDA equivalents/g wet wt.). Mean coefficients of variation (CV) for such determinations were ≤6%.

Metal concentrations in gill cytosols

Homogenized gill tissues were thawed and centrifuged at 170 000 × *g* for 1 h at 2°C. Subsamples (2 mL) from the supernatant were acidified with 0.5% ultrapure HNO_3 (Aristar; vol/vol). Concentrations of Cd, Cu, Zn, and Ca were measured by plasma atomic emission spectrometry.

Determination of metal partioning in gill cytosol preparations

For the transplanted molluscs collected at t = 14, 90, and 400 d, a composite sample representing one quarter of the gill tissue from four mussels was analyzed. A 100-μL subsample of the 170 000 × *g* gill cytosol supernatant obtained above (nonacidified) was fractionated by high performance liquid chromatography (HPLC: Waters 600 chromatograph) on a steric exclusion column (TSK gel SW 2000, 30 × 0.75 cm) and precolumn (Biorad, 7.5 × 0.75 cm). The column was eluted with a pH 7 solution containing 10 mM Tris-HCl, 100 mM NaCl, and 0.03% NaN_3 at a flow rate of 0.5 mL/min (Micallef et al. 1992). The eluant was degassed with helium before use and periodically degassed during HPLC operation.

The following molecular weight standards (Sigma Chemical Co., St. Louis, Mo.) were used to calibrate the column: dextran (2000 kDa, V_0 = 5.7–5.8 mL), bovine albumin (67 kDa), egg albumin (45 kDa), carbonic anhydrase (29 kDa), ribonuclease-*a* (13.7 kDa), cytochrome-*c* (12.4 kDa), aprotinin (6.5 kDa), vitamin B12 (1355 Da), and tryptophan (204 Da). Two-millilitre fractions were collected automatically (LKB 2211 SuperRac) up to 22 mL and analyzed for Cd and Cu by flameless atomic absorption spectrophotometry (AAS; Varian Techtron, model Spectra AA-30 equipped with a GTA-96 graphite tube atomizer). As an analytical control, a certified reference water sample (NIST, Trace elements in water, SRM No. 1643b) was diluted 1:4 in the eluant solution and analyzed by flameless AAS. After correction for the dilution factor, concentrations measured were within ±9% of the certified values for Cd and Cu. Metal concentrations in the chromatographic fractions were corrected for background metal concentrations in the eluant solution. Zn measurements were compromised by a high background in the eluant solution and, thus, are not reported. The chromatographic fractions were divided into three metal–ligand pools based on data from the molecular weight standards: high molecular weight pool (HMW), from the molecular weight cut-off of the column to 15 kDa; metallothionein pool (MT), from 15 to 3 kDa; and a low molecular weight pool (LMW), <3 kDa.

The subcellular distributions of metals in gills presented in this paper are operationally defined. Homogenization and fractionation procedures will normally cause organelle disruption and may lead to metal redistribution within cellular compartments. Gills homogenized as in our study, i.e., between a Teflon surface and a glass surface, should result in low organelle breakage (Julshamn and Andersen 1981). Competition experiments performed with commercial metallothionein (prelabelled with ^{109}Cd) and fresh bivalve cytosol extract demonstrated that no appreciable Cd exchange occurred during the 20 min preequilibration step or the subsequent chromatographic separation (Micallef et al. 1992). Thus, Cd–MT complexes in gill cytosols obtained after HPLC separation in this study can be

Table 2. Results of one-way analyses of variance applied to the temporal profiles of condition indices, [MDA(gill)], [Ca(gill cytosol)], and the [Cd(gill cytosol)]/[MT] ratio for each treatment series: T-VA, C-OP, and C-VA. Scheffe contrasts between group means at 0 and 400 d are indicated.

Variable	Treatment series	$F(df_1, df_2)$	P	Scheffe contrasts between means at 400 and 0 d
Condition index	T-VA	$F_{5,16} = 7.18$	≈0.001	Means different, $P < 0.05$
	C-OP	$F_{3,10} = 5.06$	>0.05	NS $P > 0.05$
	C-VA	$F_{4,15} = 1.33$	>0.05	
[MDA(gill)]	T-VA	$F_{6,19} = 2.52$	>0.05	
	C-OP	$F_{3,10} = 2.60$	>0.05	
	C-VA	$F_{5,18} = 0.28$	>0.05	
[Ca(gill cytosol)]	T-VA	$F_{6,19} = 13.57$	<<0.001	Means different, $P < 0.01$
	C-OP	$F_{3,10} = 3.14$	>0.05	
	C-VA	$F_{5,18} = 6.18$	<0.01	
[Cd(gill cytosol)]/[MT]	T-VA	$F_{6,23} = 2.79$	<0.05	Means different, $P \approx 0.05$
	C-OP	$F_{3,14} = 5.17$	<0.05	NS $P > 0.05$
	C-VA	$F_{5,18} = 8.52$	<0.001	

considered to represent the original distribution in the cytosol preparation.

Statistical analyses

Within each treatment series (T-VA, C-OP, C-VA), one-way analyses of variance were applied to test for significant differences over time of condition indices, [MDA], cytosolic [Ca] and [Cd]/[MT]. Similar analyses were used to compare measurements made in larvae from the T-VA, C-OP, and C-VA treatment groups at 90 d. Differences among group means were examined using Scheffe contrasts. The assumptions required for use of these parametric methods were generally met with the raw (nontransformed) data.

Results

Metal partitioning in gill cytosols of transplanted bivalves

The subcellular distribution of Cd in the gill cytosol of *P. grandis* was determined on three occasions during the course of the transplant experiment. Chromatographic profiles of Cd in gill cytosols of transplanted bivalves, T-VA, were obtained for a single composite sample at 14, 90, and 400 d (Table 1). For Cu, subcellular partitioning was determined only on day 90. Cadmium concentrations in several of the HPLC fractions (0.2–0.3 $\mu g \cdot L^{-1}$) were close to the analytical detection limit of 0.08 μg Cd·L^{-1}. The resulting analytical imprecision undoubtedly contributed to the variable recoveries of Cd after HPLC fractionation (77–155% of total $[Cd]_{cyt}$, as judged from mass balance calculations). Despite this variability, the HPLC profiles show important qualitative differences over the time course of the experiment.

Fourteen days after the initiation of the experiment, Cd was associated exclusively with HMW ligands in gill cytosols of transplanted bivalves. After 90 days, all the Cd had apparently shifted to fractions of moderate molecular size (15–3 kDa; Table 1) corresponding to the expected MT fraction. These results are consistent with the estimated response time of gill MT to the change in ambient Cd after transplantation (90 d; Table 4 in Couillard et al. 1995). In contrast to Cd, and in accordance with its status as an essential element, cytosolic Cu at 90 d was mainly bound (85%) to the HMW pool, which contains metalloproteins (Table 1). After 400 d, Cd appeared for the first time in fractions of low molecular weight (74% of cytosolic Cd; Table 1). The possible toxicological significance of this latter redistribution is considered in the Discussion.

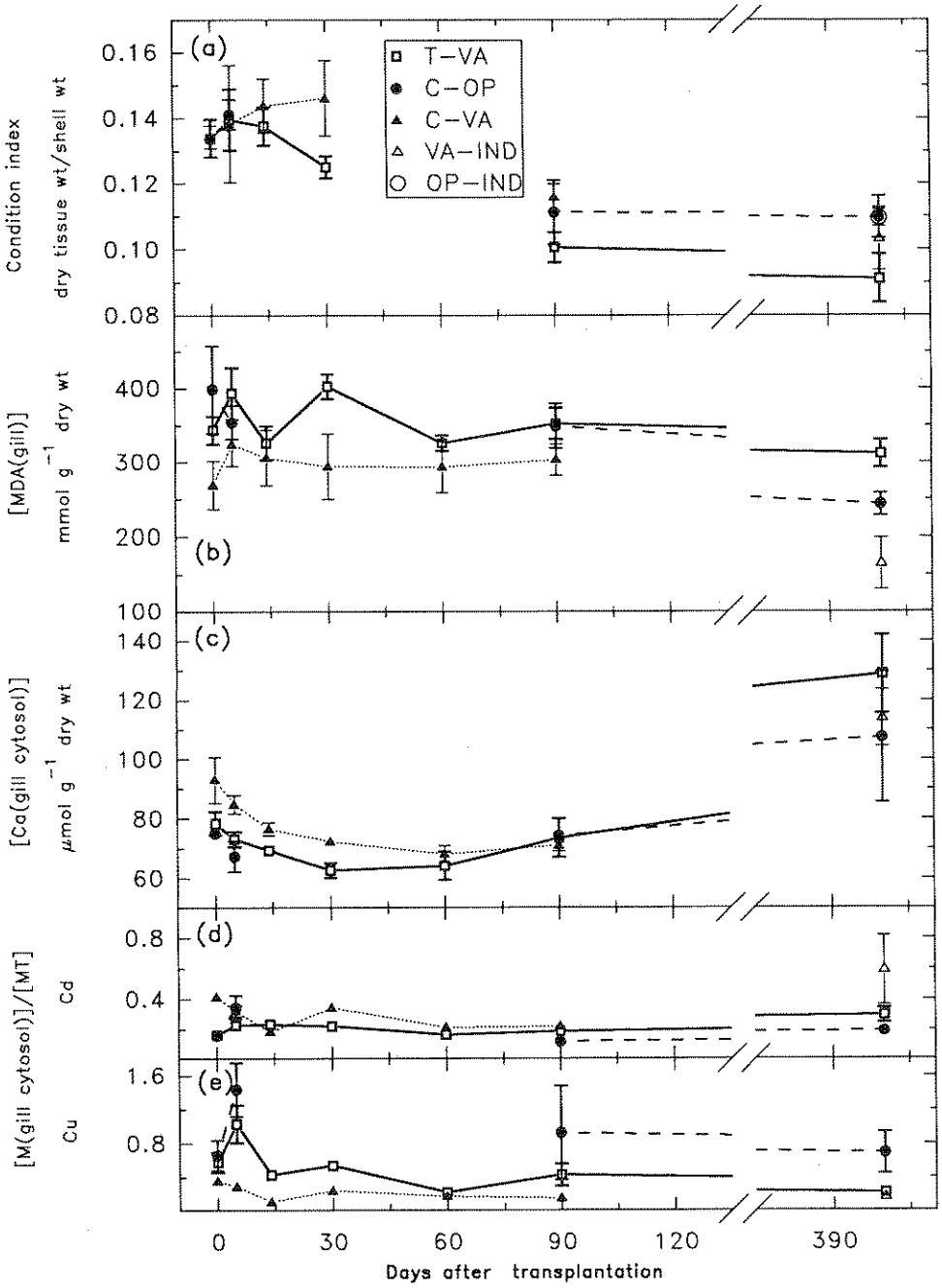
Trace metal cytotoxicity

To test the hypothetical model of trace metal cytotoxicity (Fig. 1), gills were chosen as a likely organ in which the postulated chain of cellular events might occur. Their exposure to ambient conditions is more direct than for internal organs; for example, gills accounted for 38–47% of Cd body burdens in control bivalves for this experiment, but contributed only about 21% to the organisms' total dry weight. Their contribution to MT burdens was even lower (only 11–14%). Moreover, gills of individual specimens from the indigenous population of Lake Vaudray were often deformed, whereas gills from Lake Opasatica specimens appeared more regular in shape.

Metal available to express cellular toxicity was represented by the ratio of metal concentration in the cytosol to MT concentration (see Discussion); we assume, for MT, that 1 mole of metal-binding sites as measured by the ^{203}Hg-saturation assay can bind 1 mole Cd, or 1.71 (12/7) moles Cu (Nielson et al. 1985). The implicit assumption is that higher $[M]_{cyt}$/[MT] ratios correspond to less effective metal detoxification. Lipid peroxidation was evaluated on the basis of the MDA concentration. Increases in $[Ca]_{cyt}$ were taken to indicate an increase in membrane permeability. To link these cellular events to metal toxicity at the organism level, we monitored mollusc condition indices (Eq. 1).

Temporal profiles of each of the above variables in the gills of control and transplanted bivalves appear in Fig. 2.

Fig. 2. Variations over time of condition indices, [MDA], [Ca], and [metal]/[MT] ratios in gill cytosols of transplanted and control bivalves. Each point is the mean ± SE of the replicate sample. T-VA, mussels transplanted from Lake Opasatica (OP) to Lake Vaudray (VA); C-OP and C-VA, specimens kept in enclosures in their source lakes, OP and VA respectively; OP-IND and VA-IND, specimens collected outside the enclosures in lakes OP and VA, respectively, at the end of the experiment (400 days). See text for discussion.



For the three treatments, mean [MDA(gill)] did not vary significantly with time (Fig. 2*b*; Table 2). Mean [Cu] in the gill cytosols of transplanted bivalves (T-VA) did not change significantly with time (results not shown). In addition, mean $[Cu]_{cyt}$/[MT] ratios for T-VA replicates were always <1, suggesting that transplanted specimens handled this essential metal efficiently for the duration of the transplant experiment (Fig. 2*e*).

Mean [Ca(gill cytosol)] and condition indices varied much more in the transplanted bivalves than in the controls (Figs. 2*a* and 2*c*, Table 2). Mean $[Cd]_{cyt}$/[MT] varied significantly with time in all three treatments (Fig. 2*d*, Table 2). Mean Cd concentrations in gill cytosols of transplanted bivalves were significantly higher at 90 and 400 d than the mean (±SE) value obtained for the T-VA replicate sample at 0 d ($[Cd]_{cyt}$(μg/g dry wt.) T-VA-0 d, 1.44 ± 0.19;

Table 3. 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). See text for discussion.)

Population characteristics	Lakes		
	Vaudray	Opasatica	Reference lake
Mean age (yr)	5.23	4.88	—
Growth rate	Similar[a]		—
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]See Fig. 3*a* in Couillard et al. (1995).

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

[c]Reference population of *Elliptio complanata* living in a relatively unpolluted lake in southern Québec (Downing et al. 1989).The number of gravid females per sample was computed from the percentage of females and hermaphrodites, and the relationship between the fraction of animals bearing eggs or glochidia and the allocation of male or female tissue in the gonad. Only the sample of bivalves in the size class 50–90 mm was considered for this calculation.

T-VA-90 d, 3.36 ± 0.51; T-VA-400 d, 7.06 ± 1.36; Scheffe contrasts, $P < 0.05$). For the final sampling date, the mean $[Cd]_{cyt}$/[MT] ratio and gill cytosol [Ca] for the transplanted molluscs (T-VA-400 d) were significantly higher than for the transplanted group at 0 d. The mean condition index of the group T-VA-400 d was significantly lower than that for the group T-VA-0 d. No such significant temporal differences for these three variables were detected between control bivalves in Lake Opasatica collected at 400 and 0 d (Table 2).

Mean gill MDA concentrations in transplanted specimens at 400 d differed significantly from those in control C-OP specimens (*t*-test for small samples; $t(5) = 2.67$, $P < 0.05$). At 400 d, mean [MDA] and $[Ca]_{cyt}$ of the T-VA-400 d group were the highest of the three groups, and the mean condition index of the T-VA-400 d specimens was the lowest (Fig. 2). These observations for the final sampling date are consistent with the model of trace metal cytotoxicity proposed earlier, though the sequential nature of the model is not revealed by our results (see Discussion).

Shell growth

Changes in shell growth, as well as in condition index, were assumed to be responses at the organism level to a change in metal exposure. Mean growth of marked transplanted bivalves over the 400-d experiment was significantly lower than mean growth of marked control bivalves from Lake Opasatica ([(mean length at 400 d − mean length at 0 d)/mean length at 0 d] × 100; T-VA, 1.70%, $N = 14$, mean shell increment = 1.7 mm; C-OP, 3.9%, $N = 9$, mean shell increment = 3.9 mm; two-sample *t*-test, $P < 0.001$). For each of the two groups, this effect of transplantation is superimposed on a general enclosure and marking effect on shell growth. As discussed in Couillard et al. (1995), we attribute this general effect to the fact that marked bivalves kept in enclosures were unable to migrate to microenvironments more favourable to growth. Shortage of food in the enclosures does not seem to be a plausible explanation for the enclosure and marking effect on shell growth because condition indices were similar for enclosed and indigenous species. As both the C-OP and T-VA molluscs originated from Lake Opasatica, and both treatment groups (C-OP-400 d and C-VA-400 d) were marked and confined, we can assume that the enclosure and marking effect was similar in both samples; thus, we conclude that the decrease in growth noted for the transplanted specimens is real and is correlated with the metal contamination in Lake Vaudray.

Responses at the population level

To evaluate the effects of metal stress on the bivalve populations involved in the transplant experiment, we have adapted the approach of Munkittrick and Dixon (1989) for in situ assessment of toxicant impacts on fish populations. 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. Characteristics of mussel populations from our study lakes are presented in Table 3. No detectable differences in mean age or mean growth rate were noted between the Lake Opasatica and Lake Vaudray populations. Moreover, mean condition indices of these populations were similar to that of the bivalve population in our reference lake, a pristine Precambrian Shield lake in northwestern Ontario (Huebner et al. 1990). In this latter study, measured dissolved [Cd] averaged 0.01 ± 0.001 nM and gill MT concentrations in indigenous *P. grandis* were lower than in Lake Opasatica (Couillard et al. 1995). These observations suggest that adults from our transplant lakes were not experiencing metal-related toxicity.

Fecundity data in Table 3 have been obtained from the control specimens C-OP and C-VA collected at 90 d. A population of the unionid *Elliptio complanata* in a relatively

Table 4. Metallothionein and metal concentrations, $[Cd]_{T(tissue)}$/[MT] ratios and total dry weight of larvae from the T-VA, C-OP, and C-VA treatment groups at 60 and 90 days (mean ± SE). For the statistical comparisons, the groups collected at 60 days are treated separately from the groups collected at 90 days.

	Treatments at 60 days†		Treatments at 90 days†		
Variables	T-VA	C-VA	T-VA	C-OP	C-VA
MT (nmol M sites/g dry wt.)	61±5.7*a**	102±9.8*b**	26±8.5*a*	23±3.8*a*	26±2.6*a*
Cd (nmol/g dry wt.)	11±4*a*	22±4*a*	11±5*a*	5±3*a*	65±8*b***
Cu (nmol/g dry wt.)	260±20*a*	255±60*a*	620±145*a*	550±170*a*	1380±820*a*
Zn (nmol/g dry wt.)	520±30*a*	535±70*a*	470±8*a*	470±20*a*	660±30*b***
Ca (μmol/g dry wt.)	1800±140*a*	2160±130*a*	5400±430*a*	6070±200*a*	5200±14*a*
$[Cd]_{T(tissue)}$/[MT]	0.20±0.08*a*	0.22±0.06*a*	0.39±0.16*a*	0.21±0.13*a*	2.53±0.56*b***
Larval dry wt. per bivalve (g)	0.06±0.02*a*	0.04±0.01*a*	0.14±0.03*a*	0.25±0.05*a*	0.14±0.02*a*

Note: For each variable × time combination, significant differences among means are indicated by different lowercase letters, *a* and *b*. (Scheffe contrast or two-sample *t*-test: *, $P < 0.05$; **, $P < 0.01$).

†Replicate sample sizes: T-VA-60 d, 4; C-VA-60 d, 2; T-VA-90 d, 3; C-OP-90 d, 3; C-VA-90 d: 2.

uncontaminated lake in souththern Québec has been taken as our reference population (Downing et al. 1989). This bivalve has a reproduction strategy similar to *P. grandis* (ovoviviparity, Mackie 1984; common fish hosts, Clarke 1981) and the two species occur together in several lakes of the Rouyn-Noranda area (unpublished observations). Relative to Lake Opasatica and the reference lake, mussels from Lake Vaudray exhibited a lower incidence of gravid females per sample and a lower mean larval dry weight per individual, suggesting possible toxic effects on larval stages.

Further evidence for the existence of adverse effects of trace metals on the reproduction of *P. grandis* in Lake Vaudray is provided by an examination of MT and metal concentrations in larval stages. Larvae were removed from the parent demibranchs at 60 d (August 19; possibly embryonic stages) and 90 d. The high mean Ca concentrations of replicate samples at 90 d (Table 4) suggest that the larval shells were developing at that time (see Mackie 1984). At 90 d, for similar levels of MT, C-VA larvae had a ≈12-fold higher mean Cd concentration ($P < 0.01$) than C-OP larvae. C-VA-90 d larvae also showed a higher ratio, statistically highly significant, of tissue Cd to MT ($[Cd]_{T(tissue)}$/[MT]; Table 4). The situation was markedly different at 60 d, when C-VA larvae displayed, respectively, an approximately fourfold higher concentration of MT but approximately a threefold lower concentration of Cd in their tissues than at 90 d. This temporal trend was similar for larvae in the transplanted bivalves (T-VA). At 90 d, reproduction of the transplanted bivalves did not yet seem to be severely affected: the T-VA-90 d group exhibited an incidence of gravid females (5 gravid females of 11 specimens) similar to the C-OP-90 d group. For roughly equivalent MT levels at 90 d, larvae of the transplanted mussels had a higher mean Cd concentration and a lower larval dry weight per individual (not statistically different) than did larvae from control mussels in Lake Opasatica. With the exception of Zn levels in groups collected at 90 d, levels of Cu and Zn were similar for all larval groups on a given sampling date.

In principle, larval shells as well as soft tissues contributed to the Cd content of larvae samples obtained at 90 d. These shells probably did not yet possess a periostracum layer (Pennak 1989), known to have an enormous capacity to bioconcentrate trace metals in adults (e.g., bioconcentration factor for Cd in the zebra mussel (*Dreissena polymorpha*) periostracum ≈ 70 000; Bias and Karbe 1985). Lingard et al. (1992) measured Cd concentrations in isolated nacre layers of shells (periostracum removed), and in soft tissues of adult specimens of the freshwater bivalve *Elliptio complanata* from a softwater lake in Ontario. They obtained mean values of 3.3 nmol $Cd \cdot g^{-1}$, 0.5 nmol $Cd \cdot g^{-1}$, and 169 nmol $Cd \cdot g^{-1}$ dry wt. for the "nacre protein fraction," the "nacre crystal fraction," and the soft tissues, respectively.

Discussion

Metal partitioning in gill cytosols of transplanted bivalves

Roesijadi and Klerks (1989) exposed oysters, *Crassostrea virginica*, to Cd concentrations such that $[Cd^{2+}]$ fluctuated in the range of 35–40 nM for 24 d. At various times during exposure, they excised gills, exposed them to ^{109}Cd for 45 min, and studied the kinetics of Cd binding to MT and other intracellular ligands. They concluded that Cd binding in the gill is controlled by competition among available ligands displaying varying affinities for the metal, MT having the highest. Before induction of MT synthesis, estimated to have occurred at 4 d, Cd was bound predominantly to the particulate fraction and the basal MT pool, the size of which was not specified. After induction, MT became the major Cd-binding ligand and Cd initially bound to other fractions (particulate, HMW, or LMW) shifted onto MT.

The ambient Cd^{2+} concentrations and the exposure duration in the present field experiment are very different from the laboratory conditions of Roesijadi and Klerks (1989). Despite these differences, the behaviour of Cd in the gills of the transplanted bivalves, before and after induction of MT (Table 1), resembles the competitive binding of Cd described by these researchers and others (Bebianno et al. 1992; Brown et al. 1987; Hamilton et al. 1987).

Model of trace metal cytotoxicity

To detect symptoms of cellular toxicity and test a hypothetical model of cytotoxicity (Fig. 1), we monitored MT, cytosolic [Ca], and MDA and determined the subcellular

partitioning of cytosolic metals. Implicit in our testing of this model of metal cytotoxicity is the use of $[M]_{cyt}/[MT]_{cyt}$ ratios as biochemical indicators of intracellular metal availability. This hypothesis merits close scrutiny.

In the early literature on the role of MT in metal detoxification, excess cytosolic metal not bound to MT was deemed deleterious (Brown and Parsons 1978). Thus, a $[M_{cyt}]/[MT]$ ratio greater than 1 was associated with the occurrence of cytotoxicity (Hogstrand 1991). The laboratory studies at the origin of these ideas involved the exposure of organisms to single metals (e.g., Hg; Brown and Parsons 1978). However, in nature, animals are exposed to metal mixtures; hence, the $[M_{cyt}]/[MT]$ ratios expressed for an individual metal are no longer easily interpreted. In vitro studies suggest that the affinity of MT for different metals decreases in the order Hg(II) > Cu(I) > Cd(II) > Zn(II) (Roesijadi 1992). If this same sequence were applicable in vivo, then one might anticipate that Cd would be bound to MT only if the concentration of the latter exceeded the cytosolic Cu concentration (i.e., that Cu would outcompete Cd for binding sites on MT). This does not seem to be the case. For example, Brown et al. (1987) examined the cytosolic distributions of Cd, Cu, and Zn in livers of three fish species collected from highly contaminated and less contaminated southern California coastal sites. They observed for the three species at both stations that most of the cytosolic Cd (75–95%) was in the MT pool, even though significant proportions of Cu (7–50%) were bound to HMW cytosolic ligands other than MT. Similarly, in the present experiment, 90 d after the transfer of the molluscs to the contaminated lake 100% of the cytosolic Cd, but only 15% of cytosolic Cu, was associated with chromatograhic fractions including MT (Table 1; results discussed below).

Given these metal partitioning results, the ratios [Cd]/[MT], [Cu]/[MT], and [Cd + Cu]/[MT] might be of toxicological significance in relation to the physiology of the cell. We have tested the relevance of these ratios as indicators of metal availability in the cytosol in the model of trace metal cytotoxicity presented above. Ratios of [Zn]/[MT] were ignored in the statistical data treatment, because Zn on MT would presumably be easily displaced by Cu or Cd. Given its status as an essential element, the association of Zn with enzymes or with cytosolic biomolecules other than MT cannot be taken as an indication of Zn toxicity.

Links between cytosolic metal distribution and cytotoxicity

To test the model of trace metal cytotoxicity, we examined temporal profiles of key variables of the model in bivalves transplanted to a contaminated lake and in indigenous bivalves put in enclosures in the source and destination lakes (Fig. 2, Table 2).

Transplanted bivalves display the highest [MDA] and $[Ca]_{cyt}$ and the lowest condition index of the three treatments at 400 d (Figs. 2*a*, 2*b*, and 2*c*: group T-VA-400 d). It is tempting to relate these apparent symptoms of cellular toxicity to the detection of Cd in the low molecular weight cytosolic fraction (based on a single composite sample; Table 1). Similar shifts in cytosolic metal distribution have been reported in the literature. For example, in specimens of the marine bivalve *Macoma balthica* collected at monthly intervals in San Francisco Bay, Johannson et al. (1986) observed that [Cu], [Ag], and [Zn] in the LMW fractions increased steadily when the MT–metal pool appeared saturated at high cytosolic metal concentrations. However, they were not able to link such cytosolic metal profiles to indications of in situ metal stress. From laboratory experiments with the polychaete *Neanthes arenaceodentata*, Jenkins and Mason (1988) observed perturbations in reproduction at 10 nM Cd^{2+}; this coincided with increased accumulation of Cd in the very low molecular weight pool in the animal cytosol.

These studies, as well as the present investigation, suggest that shifts in cytosolic metal distribution do occur and that they may eventually prove to be useful in the diagnosis of metal-induced stress. However, the toxicological significance of metal (Cd) binding to ligands in the LMW cytosolic pool must first be elucidated.

Condition of indigenous molluscs

In contrast to the negative effects of the Lake Vaudray environment on the molluscs transplanted from Lake Opasatica (growth and condition index decrease), the indigenous adult bivalves in Lake Vaudray appear to be in reasonable health despite their higher body burdens of Cd, Zn, and Cu (cf. Table 1 in Couillard et al. 1995). In effect, bivalves from Lake Vaudray seem to have been able to counter the potential toxic effects of these high gill metal concentrations (*i*) by excluding the metal from the cytosol, e.g., Cu but not Cd, and storing it elsewhere in the gill tissue and (*ii*) by increasing the levels of MT in the gill cytosol.

Indigenous bivalves	Lake Vaudray		Lake Opasatica	
Gill tissue	Cd	Cu	Cd	Cu
Tissue metal ($nmol \cdot g^{-1}$ dry wt.)	2560	3060	280	1435
Cytosolic metal ($nmol \cdot g^{-1}$ dry wt.)	245	116	19	113
Tissue MT ($nmol\ sites \cdot g^{-1}$ dry wt.)	410		110	

Lake Vaudray free-living (VA-IND) as well as enclosed mussels (C-VA) exhibit lower levels of gill MDA than do bivalves from the other treatments for the duration of the experiment (Fig. 2*b*; cf. Pellerin-Massicotte 1994). A similar trend was reported by Rodriguez-Ariza et al. (1992), who collected specimens of the marine bivalves *Chamalea gallina* and *Crassostrea gigas* along the South Atlantic Spanish coast. Animals from the more contaminated areas (Cd, Cu, Hg, and Pb) had higher levels of antioxidant enzymes and lower levels of MDA. Presumably, the more contaminated organisms were better protected from oxidative stress. Similar tendencies were observed for fish populations living in environments contaminated by toxic metals (antioxidant compounds: Klaverkamp et al. 1991; Brown et al. 1987).

Critique of the observed results

The present experiment does not constitute an unambiguous field validation of the hypothetical model of trace metal

cytotoxicity presented in Fig. 1. Several inconsistencies are enumerated below.

(1) Results from the HPLC fractionation suggest that transplanted bivalves face a spillover of Cd into low molecular weight ligands in the gill cytosol at the end of the experiment (group T-VA-400 d). This spillover occurs even though the $[Cd + Cu]_{cyt}/[MT]$ ratio is less than 1 on day 400 in the T-VA group. Ratios <1 indicate that there should be enough MT to complex all the Cd in the gill cytosol. Moreover, the mean $[Cd]_{cyt}/[MT]$ ratio for the VA-IND group is greater than for the T-VA-400 d group, but the former group does not exhibit any obvious symptoms of Cd-induced toxicity: [MDA(gill)] and $[Ca]_{cyt}$ are low and condition index is high. A more thorough understanding of metal detoxification mechanisms will be necessary to help clarify the relationships between $[M]_{cyt}/[MT]$ ratios, representing nonthionein metal binding or expressing different metal availabilities in the cytosol, and cellular and organism responses observed in this transplant experiment. In particular, the dynamics of Cd, Cu, and Zn in gill cytosols and gill tissues during the course of exposure should be studied not only in transplanted molluscs but also in the control groups. The binding of Cd to MT may be the result of a competition for binding sites between Cd and constitutive metals such as Cu and Zn (Bebianno et al. 1992; Brown et al. 1987).

(2) Lipid peroxidation and cellular Ca regulation processes have rarely been studied in situ in bivalve molluscs. The kinetics of cellular oxidative processes and effects of natural factors on these processes are poorly understood (e.g., Ribera et al. 1989). Moreover, free Ca^{2+} rather than total cytosolic Ca is carefully regulated by cells, because of the role of the former as an intracellular messenger (Carafoli 1987). Consequently, the total concentration of cytosolic Ca is less indicative of disturbed Ca regulation than would be the Ca^{2+} concentration. In addition, the model presented in Fig. 1 is oversimplified as it does not take into account the dynamics of reactive oxygen species in the cell.

(3) The model implicitly assumes that toxic effects should occur first at the biochemical level. Then, higher level effects might be expressed if regulatory mechanisms cannot compensate for chemical exposure. With the set of variables that we have used to evaluate the model, we have not been successful in demonstrating such a sequence of events.

Clearly, the hypothetical model of cytotoxicity that we have tested is oversimplified; the physiological and toxicological links between Cd, Cu, Ca, and MDA in *P. grandis* may well be more tenuous and complicated than described in Fig. 1. Refinement of the suggested cause–effect relationships must await further experimentation.

Relationships between metallothionein status and metal effects at higher levels of biological organization

Organism responses: transplanted bivalves

Although we have not unambiguously validated the hypothetical model of trace metal cytotoxicity (Fig. 1) in our field experiment, results related to this hypothetical chain of (cellular) events have been noted. Thus, a link may be seen to exist among the following phenomena: the appearance of nonthionein-bound Cd in the gill cytosol (as measured by HPLC), the oxidative degradation of membranes and disturbed cellular Ca concentrations. These cellular events are associated with a deterioration of the condition index of the transplanted bivalves (Fig. 2*a*). In addition to these responses, transplanted mussels grew more slowly than control mussels over the 400-d experiment.

These results are encouraging if we are to relate the degree of metal detoxification in *P. grandis* to the health status of the organism in its natural environment (criterion 5). Critical steps are a proper analytical separation of nonthionein-bound metal in cytosol preparation, and the determination of the dynamics of the key metals (Cd, Cu, and Zn) at the organ level. Also, a better understanding of the links between nonthionein-bound Cd and the organism responses would be desirable.

Responses at the population level

The apparent well being of the indigenous bivalves from Lake Vaudray (VA-IND), despite their chronic exposure to Cd and Cu, as well as the anticipated oxidative stress, contrasts with the precarious state of health of the transplanted bivalves. It is tempting to relate this apparent tolerance to the presence of high MT concentrations in individuals from the Lake Vaudray population. Moreover, because mining and smelting operations in the Rouyn-Noranda area date from 1927, Lake Vaudray bivalves might well have evolved a genetically based tolerance to trace metals, given the rapidity with which the evolution of metal tolerance can occur under high selection pressure (Bradshaw et al. 1990). Because of its 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. It follows that the extent of tolerance to trace metals as a result of selection, in populations of *P. grandis* in the Rouyn-Noranda area, is an important issue; it has to be resolved if we wish to use MT as an early warning indicator of metal exposure and stress in this species, or in any animal species in general (criterion 1). In other words, the questions are as follows:

(1) Provided that we have correctly accounted for the seasonal factors affecting MT levels in a given species at a given site (discussed in Couillard et al. 1995), 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. Such limitations would restrict but not preclude the use of MT as a biomarker.

Table 5. Metallothionein status in the freshwater bivalve *Pyganodon grandis* as a potential biomarker of metal exposure and metal-induced stress. Summary of the results of the transplant experiment in relation to criteria for a biochemical indicator (see text for explanations).

Criteria No.	Results	Remarks
1. MT should have an early warning capacity; predictive of effects at higher levels of biological organization.	Our study: Possible link between [MT] and perturbations in reproduction in metal-impacted populations (preliminary results). See also Klaverkamp et al. (1991) and Johansson et al. (1986).	Long-term, field study is needed. Such a relation should also be sought in environments variously contaminated with trace metals. Relation between the MT response and genetic characteristics of the bivalve population should be addressed. Study of effects of trace metals on *P. grandis* larvae would be helpful.
2. MT should be specific to a particular trace metal or for a group of trace metals.	Our study: MT in *P. grandis* seems to respond strongly to Cd (Couillard et al. 1993, 1995).	Laboratory studies suggest that Cd is a good inducer of MT.
3. [MT] should respond in a concentration-dependent manner.	Our study: Strongly suggests such a response in an in situ situation whether on a temporal (Couillard et al. 1995) or spatial basis (Couillard et al. 1993).	Should be verified in a broader array of environments with varied physico-chemical conditions and with other trace metals.
4. All factors, endogenous as well as exogenous, affecting the MT should be known.	Our study: MT levels in our indigenous populations showed only modest seasonal fluctuations (encouraging) (Couillard et al. 1995).	Long-term, field study is needed. Seasonal fluctuations of [MT] in other environments with more complex hydrologic and geochemical processes should be studied.
5. Degree of metal detoxification should be related to the health status of the organism in its natural environment.	Our study: In gill cytosols of transplanted bivalves at 400 days, there is an apparent link among nonthionein-bound Cd, oxidative degradation of membranes, and disturbed cellular Ca concentration. The deterioration of condition indices suggests an occurrence of toxicity at the organism level (Preliminary results).	More thorough understanding of metal detoxification mechanisms in *P. grandis* is necessary, e.g., dynamics of Cd, Cu, and Zn in gill cytosols during long-term exposures.

Relationships between MT variations in response to metal exposure and population-level responses have also been examined in terms of the ability of the Lake Opasatica and Lake Vaudray bivalve populations to cope with their more or less metal-contaminated environments (notion of fitness). From the analysis of Tables 3 and 4, it would appear that developing larvae present in the gill marsupia of indigenous parent bivalves from Lake Vaudray, paradoxically a metal-tolerant population, face a Cd-induced stress. This stress might in turn result in reduced larval recruitment at the population level. Our analysis relies on similar molluscan stocks in similar environments using consistent sampling procedures. Nevertheless, unsuspected differences may exist. The impaired reproduction hypothesis should be tested concurrently in the field and in the laboratory under more controlled conditions.

Concluding remarks

The present field transplant experiment was designed to evaluate the potential of metallothionein as a biochemical indicator of prior metal exposure and metal-induced stress. Using as a framework the criteria recently proposed for an ideal biomarker (Stegeman et al. 1992; Haux and Förlin 1989), we have summarized the results of our experiment in Table 5 and identified areas where research is needed to refine this particular analysis. In the development of a validated biomarker for toxic metals, one of the crucial issues to be addressed is the relationship between the biomarker levels in a given population and the present or future state of the aquatic community.

The transplant experiment was designed to avoid ecological differences between sites, such that any changes in MT status and organism health could be attributed to a change in metal exposure. To minimize the influence of factors other than metal contamination on the biochemical and physiological responses considered, we selected sites that shared similar ecological characteristics. Similarities in the availability of resources for our test species were assessed by comparing the condition indices and growth rates of the respective indigenous bivalve populations. Such approaches have been used by others (McCuaig and Green 1983; Haukioja and Hakala 1978). The rigorous verification of this assumption of ecological similarity would ultimately require intensive temporal sampling, simultaneously at the two sites, of target variables reflecting the availability of resources and habitat quality, e.g., the suspended food particle size spectrum, calorific values for

these food particles, degree-days, water turbulence, wind exposure, etc.

Although not yet at an application stage, MT monitoring in *P. grandis* would benefit from the qualities of molluscs as bioindicators of trace metal pollution (Phillips 1980). In particular, *P. grandis* is able to tolerate very high contaminant burdens and survive without undue adverse effects. As a sedentary organism it is representative of the area of collection. It is long lived, of reasonable size, and is widely disseminated in North America (Clarke 1981). It is also easy to transplant; transplantation of specimens with similar contamination histories and MT genetic characteristics could provide strong evidence for site-specific impacts (Clements 1991). Such an approach would be well suited for the assessment of environmental benefits gained by pollution control measures.

One of the main problems impeding the field use of biochemical indicators of contaminant effects is that research attention has focused on developing new methods, rather than on improving and validating known biochemical responses for field use (Veersteg et al. 1988). In the development of biomonitors, determinations of no observable effect concentrations (NOEC) are often subject to regulatory considerations (the question being whether metals at measured environmental levels have a demonstrable biological effect). Changing the focus to a scientific goal of understanding how metals affect biological responses might be more profitable (Luoma and Carter 1991).

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References

Bebianno, M.J., Langston, W.J., and Simkiss, K. 1992. Metallothionein induction in *Littorina littorea* (Mollusca: Prosobranchia) on exposure to cadmium. J. Mar. Biol. Assoc. U.K. **72**: 329–342.

Bias, R., and Karbe, L. 1985. Bioaccumulation and partitioning of cadmium within the freshwater mussel *Dreissena polymorpha* Pallas. Int. Rev. Gesamten Hydrobiol. **70**: 113–125.

Bradshaw, A.D., McNeilly, T., and Putwain, P.D. 1990. The essential qualities. *In* Heavy metal tolerance in plants: evolutionary aspects. *Edited by* A.J. Shaw. CRC Press, Boca Raton, Fla. pp. 323–334.

Brown, D.A., and Parsons, T.R. 1978. Relationship between cytoplasmic distribution of mercury and toxic effects to zooplankton and chum salmon (*Oncorhynchus keta*) exposed to mercury in a controlled ecosystem. J. Fish. Res. Board Can. **35**: 880–884.

Brown, D.A., Bay, S.M., Greenstein, D.J., Slazay, P., Hershelman, G.P., Ward, C.F., Westcott, A.M., and Cross, J.N. 1987. Municipal wastewater contamination in the Southern California Bight. II. Cytosolic distribution of contaminants and biochemical effects in fish livers. Mar. Environ. Res. **21**: 135–161.

Bus, J.S., and Gibson, J.E. 1979. Lipid peroxidation and its role in toxicology. Rev. Biochem. Toxicol. **1**: 125–149.

Carafoli, E. 1987. Intracellular calcium homeostasis. Annu. Rev. Biochem. **56**: 395–433.

Clarke, A.H. 1981. The freshwater molluscs of Canada. National Museum of Natural Sciences, National Museum of Canada, Ottawa, Ont.

Clements, W.H. 1991. Community responses of stream organisms to heavy metals: a review of observational and experimental approaches. *In* Metal ecotoxicology—concepts and applications. *Edited by* M.C. Newman and A.W. McIntosh. Lewis Publishers, Chelsea, Mich. pp. 363–391.

Couillard, Y., Campbell, P.G.C., and Tessier, A. 1993. Response of metallothionein concentrations in a freshwater bivalve (*Anodonta grandis*) along an environmental cadmium gradient. Limnol. Oceanogr. **38**: 299–313.

Couillard, Y., Campbell, P.G.C., Tessier, A., Pellerin-Massicotte, J., and Auclair, J.C. 1995. Field transplantation of a freshwater bivalve, *Pyganodon grandis*, across a metal contamination gradient. I. Temporal changes in metallothionein and metal (Cd, Cu, and Zn) concentrations in soft tissues. Can. J. Fish. Aquat. Sci. **52**: 690–702.

Downing, J.A., Amyot, J.-P., Pérusse, M., and Rochon, Y. 1989. Visceral sex, hermaphroditism, and protandry in a population of the freshwater bivalve *Elliptio complanata*. J. North Am. Benthol. Soc. **8**: 92–99.

Gill, K.D., Sandhir, R., Sharma, G., Pal, R., and Nath, R. 1990. Perturbations in lipid peroxidation and antioxidant enzymes on cadmium exposure to growing rats. J. Trace Elem. Exp. Med. **3**: 79–89.

Hamilton, S.J., Mehrle, P.M., and Jones, J.R. 1987. Evaluation of metallothionein measurement as a biological indicator of stress from cadmium in brook trout. Trans. Am. Fish. Soc. **116**: 551–560.

Haukioja, E., and Hakala, T. 1978. Life-history evolution in *Anodonta piscinalis* (Mollusca, Pelecypoda). Oecologia, **35**: 253–266.

Haux, C., and Förlin, L. 1989. Nine selected assays for health status in natural fish populations. *In* Chemicals in the aquatic environment: advanced hazard assessment. *Edited by* L. Lander. Springer-Verlag, Berlin. pp. 197–215.

Hendry, G.A.F., Baker, A.J.M., and Ewart, C.F. 1992. Cadmium tolerance and toxicity, oxygen radical processes and molecular damage in cadmium-tolerant and cadmium-sensitive clones of *Holcus lanatus* L. Acta Bot. Neerl. **41**: 271–281.

Hogstrand, C. 1991. Regulation of metallothionein in teleost fish during metal exposure. Ph.D. thesis, University of Göteborg, Göteberg, Sweden.

Huebner, J.D., Malley, D.F., and Donkersloot, K. 1990. Population ecology of the freshwater mussel *Anodonta grandis grandis* in a Precambrian Shield lake. Can. J. Zool. **68**: 1931–1941.

Jenkins, K.D., and Mason, A.Z. 1988. Relationships between subcellular distributions of cadmium and perturbations in reproduction in the polychaete *Neanthes arenaceodentata*. Aquat. Toxicol. **12**: 229–244.

Johannson, C., Cain, D.J., and Luoma, S.N. 1986. Variability in the fractionation of Cu, Ag, and Zn among cytosolic proteins

in the bivalve *Macoma balthica*. Mar. Ecol. Prog. Ser. **28**: 87–97.

Julshamn, K., and Andersen, K.-J. 1981. Studies on major and minor elements in molluscs in western Norway. V. Protein binding of zinc, cadmium and copper in tissues of oyster (*Ostrea edulis*), common mussel (*Mytilus edulis*) and horse mussel (*Modiolus modiolus*), taken from unpolluted waters. Fiskeri dir. Skr. Ser. Ernaer. 1. pp. 235–245.

Klaverkamp, J.F., Dutton, M.D., Majewski, H.S., Hunt, R.V., and Wesson, L.J. 1991. Evaluating the effectiveness of metal pollution controls in a smelter by using metallothionein and other biochemical responses in fish. *In* Metal ecotoxicology—concepts and applications. *Edited by* M.C. Newman and A.W. McIntosh. Lewis Publishers, Chelsea, Mich. pp. 33–64.

Lingard, S.M., Evans, R.D., and Bourgoin, B.P. 1992. Method for the estimation of organic-bound and crystal-bound metal concentrations in bivalve shells. Bull. Environ. Contam. Toxicol. **48**: 179–184.

Lucas, A., and Beninger, P.G. 1985. The use of physiological condition indices in marine bivalve aquaculture. Aquaculture, **44**: 187–200.

Luoma, S.N., and Carter, J.L. 1991. Effects of trace metals on aquatic benthos. *In* Metal ecotoxicology—concepts and applications. *Edited by* M.C. Newman and A.W. McIntosh. Lewis Publishers, Chelsea, Mich. pp. 261–300.

Mackie, G.L. 1984. Bivalves. *In* The Mollusca. Vol. 7. *Edited by* K.L. Wilbur. Academic Press, New York. pp. 351–417.

McCuaig, J.M., and Green, R.H. 1983. Unionid growth curves derived from annual rings: a baseline model for Long Point Bay, Lake Erie. Can. J. Fish. Aquat. Sci. **40**: 436–442.

Micallef, S., Couillard, Y., Campbell, P.G.C., and Tessier, A. 1992. An evaluation of the HPLC – gel chromatographic method for analyzing metallothioneins in aquatic organisms. Talanta, **39**: 1073–1079.

Munkittrick, K.R., and Dixon, D.G. 1989. Use of white sucker (*Catostomus commersoni*) populations to assess the health of aquatic ecosystems exposed to low-level contaminant stress. Can. J. Fish. Aquat. Sci. **46**: 1455–1462.

Nielson, K.B., Atkin, C.L., and Winge, D.R. 1985. Distinct metal-binding configurations in metallothionein. J. Biol. Chem. **260**: 5342–5350.

NRCC. 1985. The use of biochemical indicators in the assessment of ecosystem health—their development and validation. Associate Committee on Scientific Criteria for Environmental Quality, National Research Council of Canada, Ottawa, Ont. NRCC Rep. 24371.

Pellerin-Massicotte, J. 1994. Oxidative processes as indicators of chemical stress in marine bivalves. J. Aquat. Ecosyst. Health, **3**: 101–111.

Pennak, R.W. 1989. Fresh-water invertebrates of the United States. Protozoa to Mollusca. 3rd ed. Wiley, New York.

Phillips, D.J.H. 1980. Quantitative aquatic biological indicators. Applied Science Publishers, London.

Ribera, D., Narbonne, J.F., Daubeze, M., and Michel, X. 1989. Characterization, tissue distribution and sexual differences of some parameters related to lipid peroxidation in mussels. Mar. Environ. Res. **28**: 279–283.

Rodriguez-Ariza, A., Abril, N., Navas, J.I., Dorado, G., López-Barea, J., and Pueyo, C. 1992. Metal, mutagenicity, and biochemical studies on bivalve molluscs from Spanish Coasts. Environ. Mol. Mutagen. **19**: 112–124.

Roesijadi, G. 1981. The significance of low molecular weight metallothionein-like proteins in marine invertebrates—current status. Mar. Environ. Res. **4**: 167–179.

Roesijadi, G. 1992. Metallothioneins in metal regulation and toxicity in aquatic animals. Aquat. Toxicol. **22**: 81–114.

Roesijadi, G., and Klerks, P.L. 1989. Kinetic analysis of cadmium binding to metallothionein and other intracellular ligands in oyster gills. J. Exp. Zool. **251**: 1–12.

Sanders, B., Jenkins, K.D., Sunda, W.G., and Costlow, J.D. 1983. Free cupric ion activity in seawater: effects on metallothionein and growth in crab larvae. Science (Washington, D.C.), **222**: 53–55.

Stegeman, J.J., Brouwer, M., Di Giulio, R.T., Förlin, L., Fowler, B.A., Sanders, B.M., and Van Veld, P.A. 1992. Enzyme and protein synthesis as indicators of contaminant exposure and effect. *In* Biomarkers—biochemical, physiological, and histological markers of anthropogenic stress. *Edited by* R.J. Hugget, R.A. Kimerle, P.M. Mehrle Jr., and H.L. Bergman. Lewis Publishers, Chelsea, Mich. pp. 235–335.

Sunderman, F.W., Jr., Marzouk, A., Hopfer, S.M., Zaharia, O., and Reid, M.C. 1985. Increased lipid peroxidation in tissues of nickel chloride-treated rats. Ann. Clin. Lab. Sci. **15**: 229–236.

Veersteg, D.J., Graney, R.L., and Giesy, J.P. 1988. Field utilization of clinical measures for the assessment of xenobiotic stress in aquatic organisms. *In* Aquatic toxicology and hazard assessment. Vol. 10. *Edited by* W.J. Adams, G.A. Chapman, and W.G. Landis. ASTM STP 971. American Society for Testing and Materials, Philadelphia, Pa. pp. 289–306.

Verbost, P.M., Flik, G., Lock, R.A.C., and Wendelaar Bonga, S.E. 1988. Cadmium inhibits plasma membrane calcium transport. J. Membrane Biol. **102**: 97–104.

Viarengo, A. 1989. Heavy metals in marine invertebrates: mechanisms of regulation and toxicity at the cellular level. CRC Crit. Rev. Aquat. Sci. **1**: 295–317.

Viarengo, A., Canesi, L., Pertica, M., Poli, G., Moore, M.N., and Orunesu, M. 1990. Heavy metal effects on lipid peroxidation in the tissues of *Mytilus galloprovincialis* Lam. Comp. Biochem. Physiol. **97C**: 37–42.

Viarengo, A., Mancinelli, G., Orunesu, M., Martino, G., Faranda, F., and Mazzucotelli, A. 1988. Effects of sublethal copper concentrations, temperature, salinity, and oxygen levels on calcium content and on cellular distribution of copper in the gills of *Mytilus galloprovincialis* Lam.: a multifactorial experiment. Mar. Environ. Res. **24**: 227–231.

Younes, M., and Siegers, C.-P. 1984. Interrelation between lipid peroxidation and other hepatotoxic events. Biochem. Pharmacol. **33**: 2001–2003.