

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

Olivier Perceval<sup>a,\*</sup>, Yves Couillard<sup>b</sup>, Bernadette Pinel-Alloul<sup>a</sup>, Peter G.C. Campbell<sup>c</sup>

<sup>a</sup> Groupe de recherche interuniversitaire en limnologie (GRIL), Département de sciences biologiques, Université de Montréal, C.P. 6128, Succursale Centre-ville, Montréal, Québec, Canada H3C 3J7

<sup>b</sup> Division de l'évaluation des produits chimiques, Environnement Canada, Place Vincent Massey, 351 Bd Saint-Joseph, Hull, Québec, Canada K1A 0H3

<sup>c</sup> Institut National de la Recherche Scientifique – Eau, Terre et Environnement (INRS-ETE), Université du Québec, 490 de la Couronne, Québec, Canada G1K 9A9

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## Abstract

Relationships between Cd accumulation and subcellular distribution, and growth and mortality rates were examined in the freshwater bivalve *Pyganodon grandis* in a transplant experiment. Organisms were transferred from a clean lacustrine site to four lakes situated along a Cd concentration gradient in the mining region of Rouyn-Noranda. The bivalves were maintained in open enclosures placed in the bottom sediments of the littoral zone of all five lakes for 400 days. At the end of the experiment, metallothionein (MT) was measured in the bivalve gills with a Hg-saturation assay and Cd partitioning among the various cytosolic protein pools was determined by size-exclusion chromatography. Marked differences were observed among the five sites: the range in calculated free-cadmium ion concentrations in water overlying the sediments was 35-fold whereas Cd concentrations in the gill cytosol of the transplanted bivalves varied three-fold. In the transplanted bivalves, the distribution of gill Cd among the various cytosolic complexes also varied significantly among sites. For bivalves transplanted to the three most contaminated sites, Cd concentrations in the high molecular weight pool (HMW > 25 kDa) were significantly higher than the baseline levels determined from bivalves caged at the reference site; a similar trend was seen for Cd concentrations in the metallothionein pool (Cd–MT). For bivalves transferred to two of the high contamination sites, proportionately less of the gill cytosolic Cd was sequestered (i.e. detoxified) by MT-like proteins. Reductions in survival were also observed at these two sites, and these elevated mortalities, in turn, were consistent with the absence of indigenous bivalve populations at these sites. This result is compatible with our recent work on *P. grandis* populations living in lakes of the Rouyn-Noranda area, in which we demonstrated that excessive accumulation of Cd in the HMW pool of the gill cytosol of the individual mollusks could be related to the impairment of population health status.

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## 1. Introduction

Contaminants exert their toxicity at all levels of biological organization, from molecules to ecosystems. Undoubtedly, the ultimate levels of concern for ecotoxicologists are populations, communities and ecosystems. Indeed, measurements made on higher levels of organization generally provide the best indication of the ecological consequences of exposure to contaminants

(Underwood and Peterson, 1988). However, results of ecotoxicological studies on populations or communities are complex and highly variable, and therefore difficult to interpret. One common difficulty in these studies is to distinguish natural changes from those due to pollutants themselves. For example, it can be difficult from a field survey alone to establish the causes of observed changes in the number of species or their abundance, since these latter variables vary in time and space, as a result of density dependence acting through or together with changing levels of food supply, predation, parasitism, intra- or interspecific competition, migration and environmental variables (Sibly, 1999). Conversely, studies at the cellular level

\* Corresponding author. Tel.: +1 514 343 6111x3188; fax: +1 514 343 6216.  
E-mail address: olivier.perceval@umontreal.ca (O. Perceval).

can give insights into the mechanistic bases of toxicity, but lower level effects may not be necessarily translated into higher-level effects (see Luoma, 1995). In this context, some authors have recently emphasized the importance of studying organism-level responses, in combination with responses at different levels of organization, to monitor stress in natural environments (e.g. Maltby, 1999). Notably, the examination of the trade-offs between growth and mortality has been shown to be essential to understand how organisms cope with stress (Sibly and Calow, 1989).

Many aspects of metal accumulation in aquatic organisms, including toxicity, can be understood by examining the subcellular distribution of accumulated metal. In invertebrates, metal detoxification processes essentially involve the binding of metals with inducible metal-binding proteins (metallothioneins, or MTs), and their sequestration into mineralized concretions (Roesijadi, 1992; Langston et al., 1998; Marigomez et al., 2002; Wallace et al., 2003). With regard to the first detoxification pathway, it has been suggested that excessive accumulation of metals beyond the binding capacity of MT should result in their binding to other intracellular ligands (i.e. non-thionein ligands), and that this non-specific binding results in metal-induced toxicity at the cellular level (Brown and Parsons, 1978; Mason and Jenkins, 1995). These putative relationships between subcellular metal distribution and toxicity in aquatic invertebrates have been derived almost entirely from laboratory studies (e.g. Sanders et al., 1983; Sanders and Jenkins, 1984; Jenkins and Mason, 1988; Wallace et al., 2000). The extrapolation of these relationships to the field has been attempted only rarely (e.g. Campbell et al., 2005).

Cadmium is one of the most studied trace metals because of its toxicity, notably towards freshwater biota. For example, Cd was the most toxic of 63 metals and metalloids tested with the freshwater amphipod *Hyalella azteca*, during short-term exposures in the laboratory (Borgmann et al., 2005). In nature, Cd is significantly bioaccumulated by a wide variety of animals, and there is some evidence of Cd biomagnification along certain freshwater food chains (Croteau et al., 2005). Furthermore, for some freshwater invertebrates, the entry of this metal into the cell cytosol triggers the synthesis of MT (e.g. Couillard et al., 1993; Croteau et al., 2002).

In the field, we determined that the freshwater bivalve *Pyganodon grandis*, chronically exposed to Cd along an environmental Cd gradient, exhibited an increase in the concentration of Cd in the non-thionein ligand pool of the gill cytosol, and that this state was associated with symptoms of cellular toxicity (Giguère et al., 2003; Bonneris et al., 2005). For the same bivalve, we showed that population density, biomass, secondary production, turnover ratio and reproductive success decreased with increased Cd concentrations bound to high molecular weight ligands in gill cytosols of the individual mollusks (Perceval et al., 2004). However, it was not possible from this study alone to unequivocally assign to subcellular metal partitioning measurements any predictive role for population health because of the influence of environmental confounding factors (namely, the number of degree-days accumulated in the littoral zone of the lakes).

In the present study, we have adopted a field manipulation approach to investigate metal exposure → bioaccumulation → effects relationships in *P. grandis*, specifically focusing on manifestations of toxicity at the organism level, as evidenced by alterations in growth and survival. The sub-lethal endpoint of growth is particularly relevant from an ecological perspective since it integrates all physiological processes that occur in an organism (Sheehan, 1984), and it has been linked to ecotoxicological impairment in bivalve populations (e.g. Bayne et al., 1985). In this context, we performed a transplant experiment with *P. grandis* in a series of lakes having similar habitat characteristics but differing markedly in their degree of environmental Cd concentration. Our approach was essentially based on the comparison of the chemical and biological properties of individuals that had been collected from a clean bivalve population and, after randomization and translocation, had subsequently been exposed to different environmental conditions at the transplant sites. The primary advantage of conducting transplant experiments rather than monitoring populations is the increased experimental control while maintaining a high level of environmental realism (de Kock and Kramer, 1994). Here, we used statistically similar groups of experimental animals with regard to population, size, and exposure history. We hypothesized that bivalves transplanted to sites along the Cd exposure gradient would exhibit increased concentrations of Cd in the non-thionein ligand pool, and that this condition would coincide with a decrease in organism growth and an increase in mortality rates.

## 2. Materials and methods

### 2.1. Experimental sites and treatments

Five sites were used for the transplant experiment with one site in each of five lakes (Lakes Opasatica (48°04'N; 79°17'W), Joannès (48°10'N; 78°41'W), Vaudray (48°05'N; 78°40'W), Dasserat (48°15'N; 79°24'W) and Dufault (48°18'N; 78°59'W)) located in the mining area of Rouyn-Noranda, northwestern Quebec, Canada. Although the sediments from these lakes contain elevated concentrations of several metals, including Cd, Cu, Zn and Pb, a recent study showed that the toxic effects observed in the indigenous benthic communities are probably caused primarily by Cd (Borgmann et al., 2004). Based on a preliminary study (Perceval et al., 2002), we selected five lakes having the widest Cd concentration gradient possible (calculated free Cd<sup>2+</sup> ion concentrations ranging from ~0.05 to 1 nM) from a set of lakes presenting water bodies with comparable trophic status. Lake Opasatica, which acted as the source of experimental animals, is a headwater lake with no point sources of metal pollution and is therefore classified as the reference lake. Lakes Joannès and Vaudray have been polluted by metals via atmospheric transport and are classified here as intermediate-contaminated lakes. Lakes Dasserat and Dufault are subject to point-source and atmospheric metal inputs and are classified as highly contaminated lakes. Table 1 shows Cd concentrations in water and sediment samples obtained from those lakes during recent sampling campaigns. The experimental sites were situated in the littoral zone

Table 1  
Cadmium concentrations in water (means  $\pm$  S.D.) and sediments of the study lakes

	Group				
	Reference (Lake Opasatica, OP)	Intermediate contamination		High contamination	
		Lake Joannès (JO)	Lake Vaudray (VA)	Lake Dasserat (DS)	Lake Dufault (DT)
Cd in the dissolved phase (nM)	0.05 (0.01)	0.35 (0.13)	0.68 (0.21)	1.04 (0.33)	3.40 (0.85)
Cd in oxic sediments (nmol g <sup>-1</sup> dry weight)	6.8	27.6	22.4	28.6	515
Calculated free Cd <sup>2+</sup> ion at the sediment–water interface (nM)	0.03	0.25	0.39	1.08	0.76

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

of the lakes, on the leeside, at a distance of  $\sim$ 40 m from the lakeshore. Water depth at all sites was approximately 3 m. To minimize the likelihood of human disturbance, sites were not marked by surface floats or buoys; instead, littoral flags were used to locate them. All sites were characterized by a gentle substrate slope (<5%) and homogenous sediments, and by the absence of dense beds of macrophytes. All sites contained a few resident *P. grandis*, except for the sites located in Lakes Dasserat and Dufault.

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

The experiment consisted of transferring *P. grandis* specimens from the reference site (Lake Opasatica) to the experimental sites situated along the Cd exposure gradient (Lakes Joannès, Vaudray, Dasserat and Dufault). In order to assess the baseline levels for accumulated Cd and metallothionein in transplanted *P. grandis*, a group of clams was caged in the source lake, Lake Opasatica. Previous experiments using freshwater bivalves showed that a post-transplant period of more than 1 year is usually necessary for the influence of the destination environment to surpass that of the source environment (e.g. Hinch and Green, 1989). Therefore, *P. grandis* specimens were exposed for a period of 400 days (from July 1999 to September 2000) at the various sites.

## 2.2. Bivalve collection, processing and deployment

In this experiment, it was impractical to standardize the transplanted bivalves by genotype. However, care was taken to collect all *P. grandis* specimens from the same site in the same lake and to randomly assign the collected clams to their transplant destinations. Thus, the genetic differences among the source

populations are likely reduced, and genetic variation will not be confounded with destination (site) effects.

On July 21, 1999, SCUBA divers collected  $\sim$ 900 actively filtering bivalves of a size range representative of the natural assemblage (i.e. shell lengths ranging from 51.2 to 92.9 mm) from a single littoral site in Lake Opasatica, in an area known to abound in *P. grandis*. In this lake, maximum densities of *P. grandis* exceeded six individuals m<sup>-2</sup>, with an average of one individual m<sup>-2</sup> at depths less than 6 m (Perceval et al., 2004). Each collected clam was marked in the boat by attaching a numbered plastic label (Dymo<sup>TM</sup>) to the posterior face of one valve with underwater glue (PC-Marine Putty Epoxy<sup>TM</sup>). Following marking, bivalves were measured along the axis of maximum growth (to the nearest 0.01 mm), using a digital vernier caliper (Mitutoyo<sup>TM</sup>). To minimize the stress associated with air exposure, marked animals were returned to the water within 5 min of collection. They were held inside temporary enclosures in the littoral zone of Lake Opasatica for 1 week, until their deployment at the various transplant sites. Twelve bivalves of similar size (70.1  $\pm$  1.0 mm; mean  $\pm$  95% confidence interval) were retained for the determinations of initial metallothionein and accumulated Cd concentrations and subcellular distribution of Cd before deployment; these individuals were transported to the field laboratory in coolers filled with lake water, and sacrificed for gill preparation within 12 h after collection.

A total of 760 bivalves were used in this experiment. Power analysis indicated that about 100 mussels per site were usually necessary to provide sufficient statistical power (i.e. >0.80) to detect significant differences in growth between two sites (at an  $\alpha$  level of 5%), when growth rates differed by 25% (Salazar and Salazar, 1995). Here, we randomly assigned a group of 152 bivalves to be transplanted to each of the five experimental sites: three enclosures received 32 animals each, yielding a density of 44 individuals m<sup>-2</sup> (referred to as high density treatments in the rest of the text), and the three others received eight animals each, yielding a density of 11 individuals m<sup>-2</sup> (referred to as natural density treatments) comparable with that of the indigenous populations from Lake Opasatica. We manipulated bivalve density in our enclosures primarily to assess the

effects of crowding on growth and mortality. Additionally, to test whether maintaining the clams within the enclosure had an influence on their metallothionein and Cd concentrations, and on shell growth and mortality rates, 32 marked *P. grandis* per site were “replanted” in their natural living position outside the experimental cages (i.e. non-caged bivalves). Following the procedures outlined in Salazar and Salazar (1995), size–frequency distributions of bivalves were kept similar across all sites and across all enclosures: a nested analysis of variance (ANOVA) confirmed that there were no significant differences in mean shell lengths among sites ( $F_{4, 725} = 0.047$ ;  $P = 0.996$ ) or among enclosures ( $F_{30, 725} = 0.030$ ;  $P = 1.000$ ). At the end of bivalve deployment, upper parts of enclosures were covered with a rigid plastic screen (mesh size 5.5 cm × 4 cm) to deter muskrat predation.

### 2.3. Water and sediment sampling

To evaluate the habitat quality and monitor food availability for transplanted bivalves, experimental sites were sampled on four occasions in the summers of 1999 and 2000. Water samples were collected in polyethylene bottles at ~30 cm above the sediments by SCUBA divers and were analyzed using the methods described in Perceval et al. (2002). Measured properties included Secchi depth, dissolved oxygen at the bottom ( $O_2$ ), pH, specific conductivity (Cond), dissolved calcium (Ca), dissolved organic carbon (DOC), total phosphorus (TP), chlorophyll a (Chl a), total suspended solids (TSS) and carbon and nitrogen content in seston (Seston C and Seston N). Before filtration, water samples for chlorophyll a and sestonic C and N analyses were run through a Nitex sieve to remove the >80  $\mu\text{m}$  fraction; analysis of gut contents of another unionid bivalve, *Elliptio complanata*, showed that 90% of the ingested particles were smaller than 80  $\mu\text{m}$  (Tessier et al., 1984). Water temperature was recorded at each site at 5 h intervals for the duration of the experiment using *in situ* computerized data loggers placed in waterproof containers and fixed to a plastic rod approximately 30 cm from the sediment surface. Degree-days available for bivalves between July 1999 and September 2000 were calculated by the rectangular method as described in Young and Young (1998).

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

### 2.4. Bivalve retrieval and analyses

On day 400 (September 2000), bivalves were retrieved from the experimental sites by SCUBA divers, placed in coolers filled

with lake water, and transported to the field laboratory where they were processed within 12 h after collection. Of the 760 bivalves used in the experiment, 218 died and 57 were not recovered. The majority (i.e. 53) of missing clams were those initially transplanted outside the experimental cages. Upon return to the laboratory, all surviving specimens were measured for total shell length (0.01 mm accuracy). For each site, a sub-sample of nine *P. grandis* of similar size (nominal shell length 70 mm, actual mean shell lengths: 69.8 ± 1.0, 69.2 ± 1.2, 69.9 ± 1.0, 71.8 ± 3.0 and 68.2 ± 7.9 mm for OP, JO, VA, DS and DT, respectively) was randomly selected from enclosures with high densities (when-ever possible, we selected three animals from each of the three enclosures with high densities). In addition, three other animals were chosen among the non-caged bivalves for chemical analyses. Gills from each selected clam were manually isolated with a scalpel. We choose gills as our target organ based on a previous study, in which we had demonstrated that gills contained the greatest proportion of the total metal burden (40 ± 13% for Cd) in *P. grandis* (Tessier et al., 1993). Assuming that metal uptake and metal partitioning are similar in each of the demibranchs that composed the gills of *P. grandis*, we used one part of these large lamellae for metallothionein (MT) quantification and the other part for determining bioaccumulated Cd concentrations and sub-cellular Cd partitioning. Gill tissues from three individuals were pooled, frozen in liquid nitrogen, sealed in plastic bags filled with nitrogen, and stored at –80 °C until the homogenization step. This procedure provided three replicate samples for each site as well as one control for caging effects for all measurements. MT analyses were carried out 8 months after collection whereas measurements of sub-cellular partitioning were done within 16 months of collection.

Partially thawed gill tissues were gently homogenized under a nitrogen atmosphere with a motor-driven glass tissue grinder (Dual Co.), and the homogenized tissues were kept on ice to inhibit protease activity. To limit organelle disruption, homogenization was limited to 80 rev/min. Homogenization was performed with ice-cold 25 mM Tris buffer (OmniPur) adjusted to pH 7.2; the tissue-to-buffer ratio was adjusted to 1:19 (wet weight tissue:weight of buffer) for MT measurements and to 1:3 for the determinations of accumulated Cd concentrations and subcellular Cd partitioning. A sub-sample (~1 mL) of each gill homogenate was dried in an oven at 65 °C for 48 h to determine the dry to wet weight ratio. In this calculation, the dry weight values were corrected for the contribution of the Tris buffer (i.e. a mass of 0.00762 g of salts was subtracted from the total dry mass for each mL of buffer used during homogenization).

#### 2.4.1. MT concentration in gills

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

of both field and analytical replicates and are expressed as nanomoles of Hg-binding sites per gram of dry tissue weight.

#### 2.4.2. Cd concentrations in gill cytosols

Homogenate sub-samples (1 mL) were ultracentrifuged at  $160,000 \times g$  for 1 h at  $2^\circ\text{C}$  and the supernatants filtered through Acrodisk filters (0.45  $\mu\text{m}$  porosity filters, Gelman, usually used as prefilters before ion chromatography). Filtrate supernatants were then acidified with  $\text{HNO}_3$  (final acid concentration 0.5%, v/v) and analyzed for Cd concentrations by atomic emission spectrometry (ICP-AES: Varian Vista). The recovery of Cd in spiked samples was within 95% of the amount added.

#### 2.4.3. Determination of Cd partitioning in gill cytosols

Sub-samples (155  $\mu\text{L}$ ) of the  $160,000 \times g$  gill cytosol supernatants obtained above (non-acidified) were fractionated by high-performance liquid chromatography (HPLC, Waters Action Analyzer Chromatograph, equipped with a Waters 996 photodiode array detector) on a steric exclusion column (BIOSEP-SEC-S 2000; 30 cm  $\times$  0.75 cm). The column was eluted with a mobile phase of 10 mM Tris, 100 mM NaCl, and 0.03%  $\text{NaN}_3$ , adjusted to pH 7 at a flow rate of 0.5 mL  $\text{min}^{-1}$ . The eluant was degassed with helium before each use and the HPLC column was washed periodically with an injection of 1 mM EDTA in the same mobile phase. All reagents were of HPLC grade.

The column was calibrated for molecular weight estimations using dextran (2000 kDa,  $V_0 = 5.7$  mL), bovine albumin (66 kDa), egg albumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa), myoglobin (17 kDa), ribonuclease A (13.7 kDa), vitamin B<sub>12</sub> (1355 Da) and tryptophan (204 Da) as standard markers. Eluting fractions (1 mL) were collected automatically up to a volume of 20 mL and physically combined into three metal–ligand pools: a high molecular weight (HMW) pool (25–25 kDa), eluting from 10 to 16 min after injection, a MT-like pool (25–1.3 kDa), eluting from 16 to 24 min after injection and a low molecular weight (LMW) pool (<1.3 kDa), eluting from 24 to 40 min after injection. Pooled fractions were then analyzed for Cd by graphite furnace atomic absorption spectrophotometry (GFAAS: Perkin-Elmer model Simaa 6000 with Zeeman background correction and a Perkin-Elmer model AS-72 automatic sampler). A matrix modifier (0.1% Pd and 0.06%  $\text{Mg}(\text{NO}_3)_2$ ) was added to the samples before measurements. Analytical procedural blanks and certified reference water samples (NIST 1643d and 1640d, National Institute of Standards and Technology, Gaithersburg, MD, USA) were analyzed during each analytical run. Procedural blanks indicated no appreciable contamination ( $N = 52$ : below the detection limit) and certified samples were quantitatively recovered ( $N = 36$ :  $108.1 \pm 10.3\%$  and  $107.3 \pm 7.2\%$  for NIST 1643d and 1640d, respectively).

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

#### 2.5. Data analyses

One-way analyses of variance (ANOVA) were performed to test the differences among transplant sites for MT and bioaccumulated Cd concentrations and for Cd concentrations in each of the three metal–ligand pools in bivalve gills. Following ANOVA, we used the Tukey's multiple comparison procedure to identify, which sites differed from each other. This test is designed to make all pairwise comparisons between means, while maintaining a type I experimentwise error rate constant for each comparison (Day and Quinn, 1989). We used the  $\log_{10}$ -transformed values of the response variables for all analyses.

Bivalve growth was calculated from direct measurement of changes in shell lengths during the 400-day experimental period. To evaluate the precision of shell measurements, we determined the 95% range of all measurement errors by re-measuring 10 *P. grandis* of a range of shell sizes (58–89 mm) 20 times each, in random order: the measurement error found in blind repeated trials was  $\sim 1$  mm in 95% of the 200 trials. An analysis of covariance (ANCOVA) was used to test for differences among transplant sites, density treatments and enclosures in growth of *P. grandis*. Because the growth rates of unionids have been shown to be dependent upon individual size (Hinch et al., 1986) and because all individuals were not the same size at the beginning of the experiment, the initial shell length was used here as the covariate. Following ANCOVA, we used the Tukey's test to identify, which sites differed from each other. Both the response variable and covariate were  $\log_{10}$ -transformed to meet the requirements of normally distributed and homoscedastic residuals.

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

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

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

### 3. Results and discussion

Results of physico-chemical measurements made on water and sediment samples from the various sites are shown in

Table 2  
Physical and chemical properties of water and sediments at the five lacustrine sites chosen for the transplant experiment

	Group				
	Reference (Lake Opasatica)	Intermediate contamination		High contamination	
		Lake Joannès	Lake Vaudray	Lake Dasserat	Lake Dufault
<b>Water quality variables</b>					
Secchi depth (m)	1.6	1.4	2.1	2.4	2.4
Bottom O <sub>2</sub> (% saturation)	99.2 (6.8)	99.9 (10.3)	93.4 (1.7)	95.3 (1.7)	96.0 (2.4)
Degree-days	1376	1272	1266	1389	1344
pH <sup>a</sup>	7.25–7.86	7.24–7.71	7.10–7.49	6.90–7.72	7.19–7.43
Conductivity (μS cm <sup>-1</sup> )	97 (14)	63 (10)	36 (7)	83 (15)	159 (27)
Ca (mg L <sup>-1</sup> )	9.2 (0.7)	7.7 (0.7)	3.5 (0.4)	9.3 (0.4)	19.3 (0.3)
DOC (mg L <sup>-1</sup> ) <sup>b</sup>	7.0 (0.5)	10.5 (0.6)	8.0 (0.1)	5.5 (0.4)	5.1 (0.4)
TP (μg L <sup>-1</sup> ) <sup>b</sup>	3.0 (0.9)	2.2 (0.4)	1.4 (0.1)	2.8 (0.04)	1.9 (0.4)
Chl a (μg L <sup>-1</sup> )	0.68 (0.16)	1.22 (0.89)	0.72 (0.19)	1.13 (0.36)	0.88 (0.29)
TSS (mg L <sup>-1</sup> )	4.79 (1.09)	3.23 (1.65)	1.52 (0.39)	2.76 (0.38)	2.36 (0.51)
Seston C (mg C L <sup>-1</sup> )	0.35 (0.05)	0.41 (0.11)	0.36 (0.13)	0.43 (0.05)	0.44 (0.03)
Seston N (mg N L <sup>-1</sup> )	0.055 (0.006)	0.057 (0.013)	0.052 (0.027)	0.057 (0.005)	0.058 (0.009)
<b>Sedimentary variables</b>					
% Sand (>53 μm)	34.9 (8.8)	39.8 (2.3)	79.5 (4.1)	62.3 (1.2)	60.7 (5.5)
% Silt (>2 and <53 μm)	52.9 (7.5)	58.0 (3.0)	19.0 (3.0)	17.5 (0.6)	39.3 (5.5)
% Clay (<2 μm)	12.2 (1.4)	2.1 (1.0)	1.5 (1.2)	20.1 (1.1)	0.0 (0.0)

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

<sup>a</sup> Range.

<sup>b</sup> DOC and TP were measured only once during each summer.

**Table 2.** All the lakes selected for transplant destinations have circumneutral pH and low to moderate conductivity. They are all oligotrophic, with mean total phosphorus (TP) concentrations <5 μg L<sup>-1</sup> and chlorophyll a biomass ~1 μg L<sup>-1</sup>. Water overlying the sediments was well oxygenated at all sites. Chlorophyll a, and sestonic C and N analyses revealed little difference among the five sites with regard to the quantity and quality of food available for bivalves (the ranges in Chl a, Sest N and Sest C concentrations were 1.8-, 1.3- and 1.1-fold, respectively). Similarly, water temperatures recorded at the sediment–water interface during the experiment were comparable for all transplantation sites (the range in degree-days was 1.1-fold). In contrast, dissolved Ca concentrations exhibited appreciable variability among the sites (the range in Ca concentrations was nearly six-fold). Sediment grain size also varied among the sites (Table 2). Although attempts were made to locate areas with similar substrate type, sites in Lakes Opasatica and Joannès had muddy sediments (>60% silt and clay) whereas those located in Lakes Vaudray, Dasserat and Dufault had sandy sediments (60–80% sand).

### 3.1. Cd accumulation and subcellular distribution in transplanted bivalves

Transplanting bivalves from one place to another implies that they are disturbed (handled, marked and caged) and moved. Therefore, treatments to evaluate the effect of moving and disturbing the animals need to be included in transplant experiments. In the present study, effects of caging were evaluated by

transplanting a group of clams outside the enclosures at each site. With the exception of clams transplanted to Lake Dasserat, gill Cd and MT concentrations in non-caged bivalves were generally comparable with those measured in caged animals (Fig. 1), suggesting that caging procedures had only a minor influence on metal accumulation and MT synthesis in *P. grandis* in our experiment. Effects of moving were estimated (indirectly) by collecting bivalves from the reference site in Lake Opasatica and then caging them at the same site. Prior to deployment, bivalves from Lake Opasatica had mean (±S.D.) gill cytosolic Cd and MT concentrations of 39 ± 2 nmol g<sup>-1</sup> dry weight and 74 ± 4 nmol Hg-binding sites g<sup>-1</sup> dry weight, respectively. These concentrations did not differ significantly (*t*-test; *P* > 0.05) from those measured in gill samples of bivalves after a 400-day exposure at the same site (mean cytosolic Cd and MT concentrations were 36 ± 7 nmol g<sup>-1</sup> dry weight and 66 ± 19 nmol Hg-binding sites g<sup>-1</sup> dry weight, respectively). Assuming that no major changes in ambient metal concentrations occurred in Lake Opasatica during the experiment, these results indicate that moving and disturbing (i.e. caging) the clams had no significant effects on their MT or bioaccumulated Cd concentrations. Similarly, there were no significant effects of moving the animals on their subcellular Cd distribution (*t*-test; *P* > 0.05 for each gill cytosolic fraction).

Accumulation of Cd by clams depended upon transplant destination ( $F_{4,9} = 15.1$ ;  $P = 0.0005$ ) and generally increased with water and sediment Cd exposure (Fig. 1A and Table 1). Gill cytosolic Cd concentrations for bivalves transplanted to Lakes Vaudray, Dasserat and Dufault were, respectively, 3-, 1.8- and

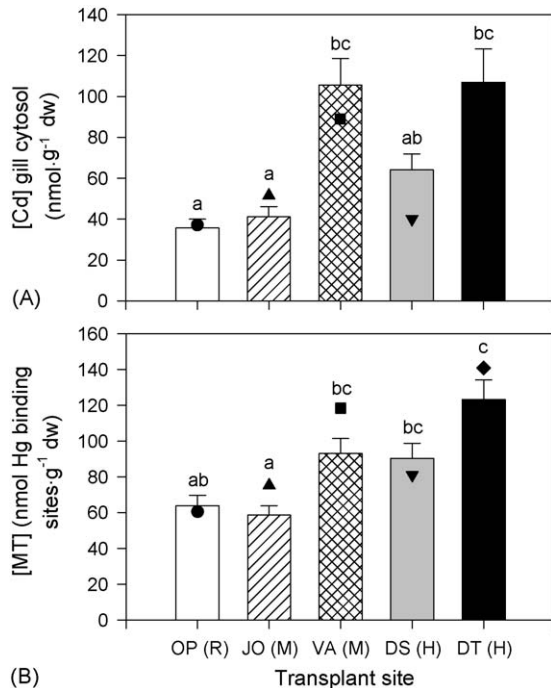


Fig. 1. (A) Cd and (B) metallothionein (MT) concentrations in the gill cytosol of *P. grandis* specimens transferred from a clean lacustrine site to five lakes situated along a Cd exposure gradient. Bars represent geometric means (+1 S.E.) calculated from least squares means and back-transformed;  $n = 3$  replicate samples of three pooled individuals each for each site. Bars with different letters represent groups with significantly different means ( $\alpha = 5\%$ , Tukey's test for multiple comparison). Black symbols above the bars represent the values for accumulated Cd and MT concentrations for bivalves transplanted outside the experimental cages ( $n = 1$  composite sub-sample of three individuals). The value of gill cytosolic Cd concentration for non-caged bivalves from Lake Dufault could not be determined, due to an unfortunate loss of the sample during ultracentrifugation procedure. Sites were ranked as reference (R), moderately contaminated (M) or highly contaminated (H) according to the concentrations of Cd in water and sediments.

3-fold higher than the baseline levels of Cd determined from clams caged at the reference site. Surprisingly, bivalves from Lake Vaudray exhibited levels of Cd that were comparable with those observed in bivalves from two most contaminated sites despite lower Cd exposure. This result may be partly explained by the remarkably low dissolved Ca concentrations measured in Lake Vaudray during the experiment (Table 2). Indeed, we know from previous work conducted in lakes of the Rouyn-Noranda region that Cd accumulation in *P. grandis* generally decreases with increasing Ca concentrations in lake water (Perceval et al., 2002), presumably due to the competitive interaction between  $\text{Ca}^{2+}$  and  $\text{Cd}^{2+}$  at gill binding sites. Total gill MT concentrations in bivalves after a 400-day exposure were also significantly different among transplant sites ( $F_{4, 10} = 10.7$ ;  $P = 0.0012$ ) (Fig. 1B), and they were directly related to accumulated Cd concentrations (Fig. 2). Several previous studies have reported a linear relationship between concentrations of MT and Cd in *P. grandis* (e.g. Wang et al., 1999; Giguère et al., 2003) and other aquatic invertebrates (Mouneyrac et al., 2000; Croteau et al., 2002; Bebianno and Serafim, 2003).

The comparison of our results with those from a recent study by Giguère et al. (2003) indicated that *P. grandis* spec-

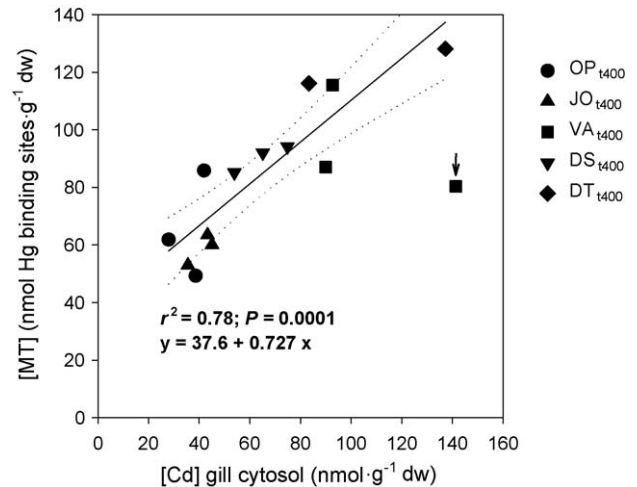


Fig. 2. Relationships between metallothionein (MT) concentrations and cytosolic Cd in samples of gill tissues of transplanted bivalves. Each point represents data for a composite sub-sample of three individuals. The ordinary least squares regression line is shown with the 95% confidence interval; the data point indicated by an arrow was excluded from the analysis.

imens transplanted to L. Joannès and Vaudray did not reach the Cd and MT concentrations of the indigenous populations even after 400 days, suggesting that steady-state between internal and external media was not reached. This result demonstrates that transplanted bivalves are an imperfect surrogate for natural populations.

Results from subcellular Cd partitioning showed that the majority of Cd in the gill cytosol of transplanted bivalves was always associated with MT-like proteins (Fig. 3). Conversely, Cd was never detected in the low molecular weight (LMW) ligand pool. The concentration of Cd found associated with high molecular weight (HMW) fraction varied significantly among sites ( $F_{4, 9} = 18.5$ ;  $P = 0.0002$ ), and systematically increased as environmental Cd concentration increased (Fig. 3 and Table 1): Cd–HMW concentrations in bivalves from Lakes Joannès, Vaudray, Dasserat and Dufault were, respectively, 1.5, 3, 3.1 and 5× higher than those measured in clams from the reference site. In contrast, bivalves from the two most contaminated sites did not necessarily exhibit the highest concentrations of Cd–MT complex (Fig. 3); maximum concentrations of MT-bound cytosolic Cd were instead found in clams transferred to Lake Vaudray. This result is somewhat different from that obtained for total gill MT, for which maximum concentrations were measured in the specimens transplanted to Lake Dufault (Fig. 1B). The discrepancy between these two results could be partly explained by the fact that the HPLC method probably overestimated the true concentrations of Cd–MT complex for one of the three replicates obtained from the experimental clams of Lake Vaudray (Fig. 4).

Although there is some experimental evidence that excessive Cd accumulation in aquatic invertebrates could result in increasing concentrations of this metal in the HMW ligand pool (e.g. Jenkins and Sanders, 1986; Wallace et al., 2000), our results contrast markedly with those from an earlier transplant experiment in which *P. grandis* was similarly transferred from Lake Opasatica to Lake Vaudray (Couillard et al., 1995). In this earlier experiment, Cd partitioning in the gill cytosol changed markedly

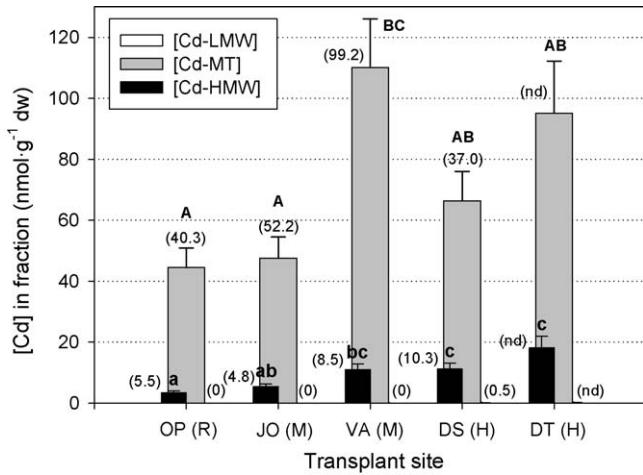


Fig. 3. Subcellular distribution of Cd in the gill tissues of *P. grandis* specimens transferred from a clean lacustrine site to five lakes situated along a Cd concentration gradient. Bars represent geometric means (+1 S.E.) calculated from least squares means and back-transformed;  $n=3$  replicate samples of three pooled individuals each for each site (except for DT for which  $n=2$  replicates). Values in parentheses show results of subcellular Cd partitioning measurements in non-caged bivalves ( $n=1$  composite sub-sample of three individuals). Subcellular Cd distribution for non-caged bivalves from Lake Dufault could not be determined, due to an unfortunate loss of the samples during ultracentrifugation procedure. Bars with different letters represent groups with significantly different means ( $\alpha=5\%$ , Tukey's test for multiple comparison); statistical comparisons using lower case letters are for Cd concentrations in the HMW pool and upper case letters are for Cd concentrations in the MT-like pool. There were no significant differences among the various sites for Cd concentrations in the LMW pool (one-way ANOVA:  $F_{4,9}=0.884$ ;  $P=0.51$ ). Sites were ranked as reference (R), moderately contaminated (M) or highly contaminated (H) according to the concentrations of Cd in water and sediments.

during the exposure period, and after 400 days, the majority (i.e. 74%) of cytosolic Cd was bound to LMW components, whereas only a small proportion of Cd was bound to the MT and the HMW fractions (12% and 14%, respectively). It should be noted, how-

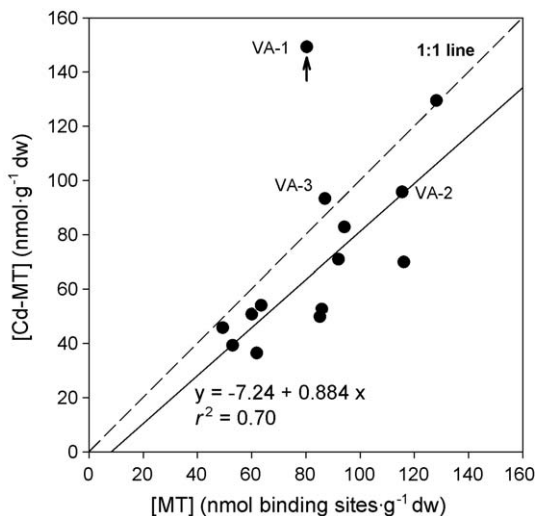


Fig. 4. Relationship between cadmium bound to the MT-like pool, as measured by HPLC, and metallothionein (MT) concentrations, as measured by the Hg-saturation assay. Each point represents data for a composite sub-sample of three individuals. The ordinary least squares regression line is also shown; the data point indicated by an arrow was excluded from the analysis.

ever, that the level of contamination of Lake Vaudray declined markedly between 1993 and 2000 (Perceval et al., in press); the ambient free  $\text{Cd}^{2+}$  ion concentration reported for Lake Vaudray in the earlier study was two times higher than those observed in the present study. Nevertheless, total dissolved Cd concentrations measured at our high contamination sites were still 7–22 times higher than the water quality criteria set by the Canadian water quality guidelines for the protection of aquatic life in freshwaters (CCME, 1999).

The results of these two transplantation experiments using *P. grandis* are not totally irreconcilable in the light of the results of recent simulations of the binding behavior of metals with sensitive biological sites (such as gill membranes and intracellular binding sites) under different metal exposure conditions. Simulations for Cd showed that at low aqueous metal concentrations, high-affinity binding sites will be easily (i.e. linearly) filled. However, as the number of these high-affinity sites is finite, high aqueous Cd concentrations will recruit an increasing number of low-affinity binding sites (Playle, 2004). Analogously, our experimental bivalves exposed to moderate environmental Cd levels (compared to those reported in the study of Couillard et al. (1995)) exhibited a monotonic increase of Cd bound to the MT-like and HMW fractions along the Cd exposure gradient (i.e. similar to a titration of high-affinity gill binding sites) without any concomitant increase of Cd in the LMW fraction (Fig. 5).

Also contrasting with the results of the study of Couillard et al. (1995), our experimental clams did not demonstrate a radical shift in the subcellular partitioning of Cd in the gill cytosol at the end of the experiment. Although the amount of Cd bound to the MT-like and HMW fractions increased along the Cd exposure gradient, there was, indeed, no apparent shift from one subcellular compartment to another (Fig. 5). Similar results were reported in the spatial study by Giguère et al. (2003) for *P.*

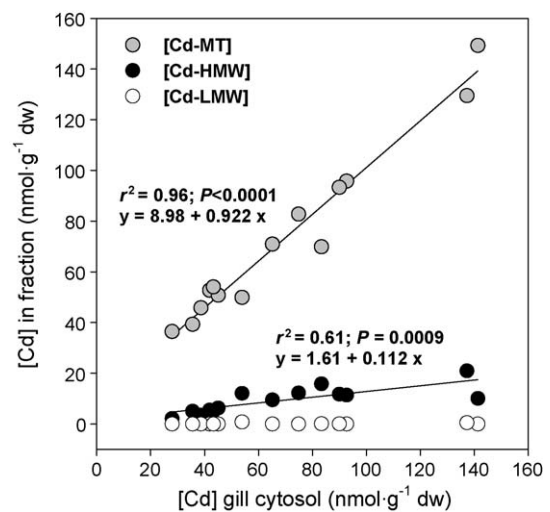


Fig. 5. Relationships between gill cytosolic Cd and Cd concentrations in three-metal-ligand pools identified as Cd-MT (the metallothionein-like pool), Cd-HMW (the high molecular weight pool) and Cd-LMW (low molecular weight pool) in transplanted bivalves. Each point represents data for a composite sub-sample of three individuals. Plotted lines were obtained by ordinary least squares regression.

*grandis* individuals that had been chronically exposed to metals since the egg stage.

### 3.2. Relationships between Cd accumulation by bivalves and growth and mortality rates

Analysis of covariance indicated that mean shell growth varied significantly among all five transplant sites during the 400-day exposure ( $F_{4, 363} = 37.3$ ;  $P < 0.0001$ ) but not between density treatments ( $F_{1, 363} = 0.002$ ;  $P = 0.965$ ). However, the site  $\times$  density interaction was highly significant ( $F_{4, 363} = 10.4$ ;  $P < 0.0001$ ), suggesting that the effect of transplant destination on bivalve growth was different for the two density levels. Growth rates of experimental clams have then been subsequently analyzed for each density treatment separately. Retrospective power analyses indicated that the power that was actually achieved to detect the differences in mean growth rates among the sites was consistently high in our experiment (i.e.  $>0.99$  for each density level). Overall, there was a net shell increment for bivalves transplanted to the two intermediate-contaminated sites for both density treatments (Fig. 6). In these lakes, growth rates were comparable to those reported for the same species in oligotrophic pristine lakes (Downing and Downing, 1993). Surprisingly, we did not observe any significant change in length over the duration of the experiment for clams caged at the reference site. We noticed that these bivalves were covered with a

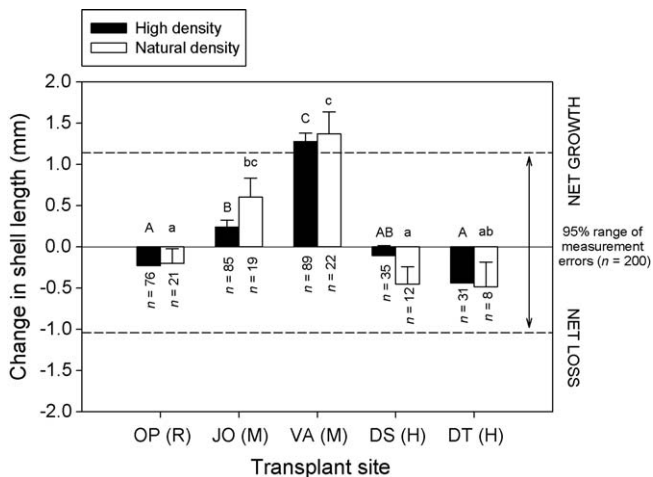


Fig. 6. Shell growth of *P. grandis* (determined by direct measurement of changes in size of individuals) at the various transplant sites during the 400-day period of the experiment. Solid bars represent length-adjusted means (+S.E.) for shell length variations in enclosures with high densities, open bars represent length-adjusted means for shell length variations in enclosures with natural densities. For high-density treatments, means were adjusted to an initial shell length of 68.30 mm, whereas for natural density treatments, means were adjusted to an initial length of 68.94 mm. For each density treatment, the main effect of site was tested using the cage (site) mean square for the error term with 10 degrees of freedom. Significant differences among sites are indicated by different letters ( $P < 0.05$ ; Tukey's multiple comparison procedure); statistical comparisons using lower case letters are for natural density treatments and upper case letters are for high-density treatments. Horizontal dashed lines show limits of 95% of all measurement errors determined by re-measuring 10 *P. grandis* specimens of a range of shell sizes 20 times each, in random order. Sites were ranked as reference (R), moderately contaminated (M) or highly contaminated (H) according to the concentrations of Cd in water and sediments.

thin layer of mud when we retrieved them and that the particulate matter content of the water column was elevated at that site (Table 2). We hypothesize that these specimens must have experienced stressful conditions probably brought about by sediment smothering. Consistent with this explanation, none of the environmental factors known to influence bivalve growth (i.e. food availability and water temperature) were related to growth inhibition in our experiment (Pearson's correlation,  $P > 0.05$  for all pairwise comparisons with environmental variables). Bivalves transplanted to the two highly contaminated sites did not grow significantly (Fig. 6).

Analysis of mortality rates using a two-way factorial ANOVA showed a strong effect of the destination site ( $F_{4, 20} = 28.6$ ;  $P < 0.0001$ ). There was no statistically significant difference in mortality rates between density treatments ( $F_{1, 20} = 1.3$ ;  $P = 0.2705$ ), indicating that maintaining the clams in enclosures at densities that are well above (i.e. more than  $4\times$ ) the observed natural levels had no influence on their survival. Mortality of clams during the experiment was consistently low (i.e. ranging from 8% to 17%) for bivalves transplanted to both the reference and intermediate-contaminated sites but increased significantly (Tukey's test;  $P < 0.05$ ) at the two highly contaminated sites, where mean mortality rates exceeded 55% (Fig. 7).

Interestingly, of all the experimental clams, the individuals transplanted in Lake Vaudray had the highest Cd–MT concentrations (even when the replicate sample with the highest value is ignored, see Fig. 4) and exhibited the most successful growth and the lowest mortality rates. This would suggest that Cd binding to MT may serve as a protective measure against Cd exposure. On the other hand, one can legitimately ask how can these bivalves do so well despite a significant accumulation of Cd in the HMW ligand pool of their gill cytosols? The heterogeneous

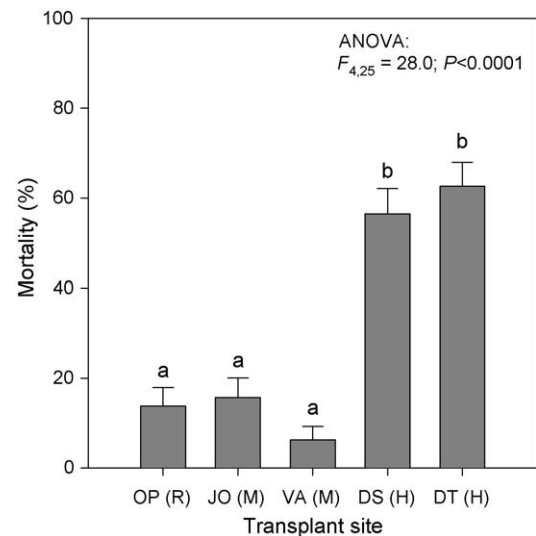


Fig. 7. Proportion of *P. grandis* specimens that were found dead in enclosures at the end of the transplant experiment at each of the five lacustrine sites, both density treatments combined. Bars represent means (+1 S.E.) back-calculated from arcsine-transformed values;  $n = 6$  independent experimental units (enclosures) for each site. Bars with different letters represent groups with significantly different means ( $\alpha = 5\%$ , Tukey's test for multiple comparison). Sites were ranked as reference (R), moderately contaminated (M) or highly contaminated (H) according to the concentrations of Cd in water and sediments.

high molecular weight pool is defined here as the fraction of metal ligands between 255 and 25 kDa, which includes metalloenzymes. Given that these enzymes are often identified as potential targets for Cd (Mason and Jenkins, 1995), and that they are involved in the majority of important cellular processes, we would have expected that the excessive accumulation of Cd in this metal ligand pool would coincide with a decrease in organism growth and an increase in mortality rates (as was observed in the bivalves from Lakes Dasserat and Dufault). The subcellular Cd distribution in the clams from Lake Vaudray differed from that observed in the bivalves from the two most contaminated sites in the sense that proportionately more metal accumulated in the gill cytosol was sequestered (i.e. detoxified) by MT-like proteins (Fig. 3). However, it is not obvious to us why the increased relative importance of Cd–MT should result in more effective Cd detoxification. Parallel studies on juvenile yellow perch (*Perca flavescens*) from Lake Vaudray also suggested abnormally high Cd accumulation (Kraemer et al., 2006). Since these two species do not share the same food resources, we are led to speculate that some geochemical factor may be favoring Cd bioaccumulation in this lake.

To explore relationships between the subcellular distribution of Cd and the growth or mortality of the transplanted bivalves, we only determined the distribution of Cd at the end of the metal exposure period. The interpretation of the results is thus limited by this lack of information about metal distribution changes that might have occurred during the experiment. Also, since we did not evaluate the subcellular partitioning of Cd in the particulate fraction of bivalve gills, we cannot rule out the possibility of a failure, for the clams transplanted in the two most contaminated sites, of the detoxifying mechanisms that involve the sequestration of metals into calcium concretions. This hypothesis is unlikely, however, because accumulated Cd concentrations in chronically exposed *P. grandis* were shown to increase proportionally in both the granules and the MT pool along the Cd concentration gradient, indicating a constant partitioning between these two compartments (Bonneris et al., 2005). Finally, our analyses have inevitably been performed on the specimens that were found alive at the experiment's end (i.e. on all individuals that kept their valves tightly closed). For that reason, subcellular metal partitioning measurements made on bivalves from the high contamination sites are not necessarily representa-

tive of animals that suffered from a complete breakdown of their detoxification mechanisms. By definition, organisms in this latter category have died during the course of the experiment, and it is more likely that we actually detected the early stages of this breakdown in many of the surviving clams from Lakes Dasserat and Dufault.

Other studies have described relationships between the binding of metal to various intracellular ligands and toxic effects. For example, reductions in prey capture efficiency in the grass shrimp *Palaemonetes pugio* were significantly related to Cd concentrations in high molecular weight proteins (Wallace et al., 2000). Similarly, reductions in the growth rate of fish (*Oncorhynchus keta*) and zooplankton coincided with increases of Hg in the HMW pool (Brown and Parsons, 1978). In those studies, the binding capacity of metallothionein was always surpassed, as evidenced by ratios of non-MT bound metal to MT-bound metal typically >1. This was likely not the case in our experiment, in which the proportion of non-thionein bound Cd never exceeded 30% of the total metal accumulated in the bivalve gill cytosol. The onset of toxicity in metal-exposed organisms, however, is not always associated with the saturation of MT. For example, Hamilton et al. (1987) observed mortality rates as high as 35% for brook trout exposed to water-borne Cd, even though the amounts of Cd bound to MT were in general more than an order of magnitude below the total Cd-binding capacity of the MT. In that study, the authors found that mortality rates of fish were well correlated with the amount of cadmium bound to non-thionein ligands in the tissues.

According to our results, the exact nature of Cd toxicity in *P. grandis* is still unclear and further investigations into the nature of the Cd–HMW fraction are therefore needed. Nevertheless, some of the elements presented here are compatible with our recent work on *P. grandis* populations living in lakes in the Rouyn-Noranda area (Perceval et al., 2004), and notably with the fact that bivalve densities are in general negatively correlated with Cd concentrations in the gill cytosolic HMW pool of the individual mollusks. For example, clams transferred to the high contamination sites (i.e. Lakes Dasserat and Dufault) exhibited high levels of Cd in the HMW pool of their gill cytosol and high mortality rates; in turn, these elevated mortalities were consistent with the absence of indigenous *P. grandis* populations in these sites (Table 3). Bivalves were formerly present in Lake Dufault,

Table 3  
Concentrations of Cd associated with the high-molecular weight fraction of gill cytosols and mortality rates (this study) and individual densities (Perceval et al., 2004) of *P. grandis* in the lakes selected for the transplant experiment

	Group				
	Reference (Lake Opasatica)	Intermediate contamination		High contamination	
		Lake Joannès	Lake Vaudray	Lake Dasserat	Lake Dufault
Cd–HMW (nmol g <sup>-1</sup> dry weight)	3.5	5.5	11.1	11.2	18.2
Mortality (%)	13.8	15.7	6.2	56.5	62.6
Bivalve density (number of individuals m <sup>-2</sup> ) (mean ± 95% CI)	1.12 (0.66–1.58)	0.77 (0.31–1.22)	0.18 (0.10–0.26)	0 <sup>a</sup>	0

<sup>a</sup> In the impacted section of Lake Dasserat; see the text for explanations.

as indicated by the discovery of empty shells in the littoral zone of the lake during a field survey done by our research team in the summer of 1999. The contemporary status of Lake Dasserat, in conjunction with the results of our experiment, also provide convincing evidence that an important pollution by metals (especially by Cd) has swept most resident species of bivalves out of this lake. Abandoned mine tailings located in the lake's watershed largely contribute to the total metal loading to the lake. All the lake is affected by this metal influx, except for a large basin situated in the northern section of the lake; this non-impacted section currently supports sustainable populations of *P. grandis*, whereas, as was mentioned above, we were unable to find a single individual in the contaminated part of the lake.

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