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BIOCHEMICAL INDICATORS OF ENVIRONMENTAL STRESS CAUSED BY HEAVY METALS

Progress report No.1 to the Wildlife Toxicology Fund

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1. RESTATEMENT OF THE ORIGINAL PROJECT PURPOSE

1.1 Introduction

Ecological considerations. Freshwater sediments act as an important reservoir for metals of anthropogenic origin. Whether the fluxes of metals to sediments are gravitational or diffusive, the overall result is a marked enhancement of metal concentrations in the upper strata of lake sediments [13] - concentrations can attain levels 1000 - 5000 times higher (on a μg/g fresh weight basis) than those in the overlying water column. Given the presence at the sediment-water interface of a diverse and often abundant benthic community, this contamination of surficial sediments is of potential ecological importance.

Traditionally, attempts to define the impacts of contaminants on aquatic ecosystems have involved laboratory experiments under defined conditions (toxicity tests) and, to a lesser extent, field observations on impacted indigenous populations. To date these approaches have met with only limited success [14]: extrapolation of laboratory-derived toxicological data to the field is fraught with difficulties, as is the unambiguous interpretation of field observations (e.g.: changes in relative abundance, in species diversity, in community structure and biomass). An alternative and complementary approach involves the use of biochemical indicators to monitor the response of individual organisms to toxic chemicals and provides a measure of ecosystem health.

Biochemical indicators. The biochemical indicator concept is based on the principle that biological effects of toxic chemicals in the environment are initiated by the interaction of the toxic chemical with a receptor site in a living organism [14]. The assumption is made that effects at the ecosystem level are preceded by biochemical reactions in individual organisms, and that concentrations of the contaminant needed to initiate these reactions are lower than those required to provoke a life-threatening situation for the target organism or perceptible degradation of the ecosystem. The detection and quantification of these chemical reactions could then be developed as a sensitive, specific indicator of environmental stress.

For metals, much of the attention in the area of biochemical indicators has focused on metal-binding proteins, in particular on metallothionein (MT) and metallothionein-like compounds [8,14,17]. Most of the early research on the structure and function of MT was performed on mammals, but over the last 10-15 years marine invertebrates have been studied intensively and the role of MT in their metal metabolism has been clarified [8,17] - in distinct contrast, the role of MT in freshwater invertebrates has been little studied.

In marine invertebrates metallothionein has been implicated in the storage, transport and exchange of essential metals (Cu, Zn), and in

the detoxification of these and other non-essential metals (Ag, Cd, Hg). As a soluble protein present in the cytosol, with a demonstrably high in vitro affinity for such toxic metals as Ag, Cd and Hg, metallothionein is poised to sequester incoming metals and reduce their availability to critical biochemical sites within the cell [17]. In such a scheme, as applied to (marine) invertebrates, MT would play a role complementary to that of lysosomes and granules / concretions [20]. Additional support for the putative role of MT in metal detoxification is derived from the observation that exposure to elevated concentrations of Cd, Cu, Hg or Zn often induces the synthesis of MT; in the case of Cd and Hg, the toxic metals have been reported to displace the Cu and Zn normally associated with metallothionein. Laboratory exposure of several aquatic organisms to sublethal concentrations of toxic metals has been shown to produce both elevated levels of MT and acclimation to these metals, in a dose-dependent manner [14].

Metallothionein as a monitoring tool. Given the molecular properties of MT, and present knowledge of its role in metal uptake, transport, storage and excretion, how might it be used in the freshwater environment as a contaminant-specific biochemical indicator of metal exposure and/or stress? Possible approaches include:

(i) Direct measurement of [MT] as an indicator of prior exposure to toxic metals.

In this case, it is assumed that constitutive levels of MT are low, and that any increase in concentration above these low basal levels is attributable to the induction of MT in response to an influx of toxic metals. This approach would also furnish a measure of the toxicologically significant intracellular fraction of metals [15].

(ii) Examination of the relative distribution of toxic metals in cytosolic ligand pools as a means of evaluating metal stress at the biochemical level.

It has been suggested that excessive accumulation of metals beyond the binding capacity of available MT should result in their binding to other intracellular ligands (notably those of high molecular weight, HMW), a phenomenon termed "spillover" - metals bound to these other ligands are considered to be capable of exerting cellular toxicity [2]. In principle this condition could be considered as symptomatic of metal stress and would be amenable to detection.

1.2 Objectives and underlying hypotheses

The overall objectives of the proposed research are to elucidate the metal detoxification mechanisms that are operative in selected benthic invertebrates living in contaminated environments, and to evaluate the

potential use of molecules involved in metal detoxification (e.g., metallothioneins) as biochemical indicators of metal-induced stress.

The key hypotheses to be verified are:

(i) that the synthesis of metallothioneins can indeed be induced in representative freshwater invertebrates at contaminant levels typical of those encountered in polluted sediments;

(ii) that tissue concentrations of these proteins respond in a dose-dependent manner as a function of the bioavailability of metals (notably Cd) in the external environment;

(iii) that when the rate of metal bioaccumulation exceeds the net rate of biosynthesis of MT, "spill-over" of the metal occurs into other cellular binding sites; and

(iv) that this threshold corresponds to the onset of detectable adverse effects, as reflected in growth rates and physiological condition.

2. OUTLINE OF WORK DONE

To profit from the 1989 field season, we carried out the initial sampling phase of this project in June 1989 with the aid of complementary funds already available; confirmation of WTF funding was received in November 1989 and the initial instalment has been used to continue the work. The present report thus covers the 7-month period from last November to May 1990, but draws on the data generated from the samples collected earlier (June 1989).

2.1 Summary of proposed methodology [5]

The project involves field studies of representative benthic invertebrates at lacustrine sites located along a spatial geochemical gradient (pH, [Cd], [Zn]). Measured variables include the tissue concentrations of metals and metal-binding proteins, as well as the partitioning of metals among different cytoplasmic ligands; initially proposed target species included the filter-feeding bivalve Anodonta grandis and the detritivorous insect Hexagenia limbata.

Field transfer experiments between control areas with background metal levels and highly contaminated sites are planned in field year #2, to evaluate the time response of metals and metal-binding molecules to changes in environmental conditions. Complementary geochemical and biological parameters will be measured in order to define the contamination gradient, the physiological state of the organisms, and their growth rate.

2.2 Field work accomplished (1989-90)

To verify that tissue concentrations of MT respond in a dose-dependent manner as a function of the bioavailability of metals (Cd, Zn) in the external environment, surficial (oxic) sediment samples, bottom water samples and benthic invertebrates were collected by divers in June 1989 from lakes located along a previously identified contamination gradient in the Rouyn-Noranda area in northwestern Québec.

Bottom water samples were collected within 10 cm of the sediments and analyzed for dissolved Cd, Zn, Ca, dissolved organic and inorganic carbon, and pH. The **sediment samples** were subjected to a partial extraction procedure ($NH_2OH \cdot HCl$) designed to reduce and solubilize both the amorphous Fe/Mn oxyhydroxides present in the sediment and the metals associated with these solid phases; together with the pH, such measurements allow an estimate of the free-metal ion concentration to which the benthic invertebrates are exposed and hence can be used to define the metal contamination gradient [3,4].

After a depuration phase [9,18], the **benthic invertebrates** were dissected (A. grandis - gills, hepatopancreas, mantle + remainder; H. limbata - gills; gut; remainder) and the tissues analyzed. Measured variables included the tissue concentrations of metals (Cd, Cu, Zn) and metallothionein (determined by a ^{203}Hg-saturation method [1]).

For each type of material, appropriate NIST or NRCC certified standards were treated in the same manner to assure the analytical accuracy of the trace metal results.

3. BRIEF STATEMENT OF RESULTS; ASSESSMENT OF PROGRESS; EVALUATION OF LIKELIHOOD OF SUCCESS

The work performed to date has closely followed what was described in the research proposal. The only notable change has been to reduce the research effort devoted to Hexagenia limbata; this decision reflects the promising results obtained with the other benthic invertebrate, Anodonta grandis, as well as a shortened timetable (WTF funding for 2 years rather than 3) and a somewhat reduced budget.

The following progress report deals both with the method development work that was carried out from November 1989 - May 1990, and with the results of the 1989-90 field studies.

3.1 Research on analytical methods (1989-90)

Methodological research has been carried out in two areas: the ^{203}Hg saturation method for determining metallothionein concentrations, and the HPLC separation of metal ligand complexes present in the cytosolic fraction of various tissues.

^{203}Hg saturation method. The analytical performance of this method, developed by Anderson and Klaverkamp [1] for use on freshwater invertebrates, has been verified with tissues from Anodonta grandis:

- precision ± 10%;
- detection limit ≈ 1 nmole Hg/g dry wt;
- linear range 1 - 100 nmole Hg/mL tissue homogenate;
- recovery of spiked standard mammalian metallothionein 100 ± 5%.

In addition, the efficacy of our sample preservation technique has been checked. Metallothionein concentrations determined in tissues that had been stored for 15 or 60 d at -40 ^{o}C were indistinguishable from those determined on fresh samples.

HPLC separation. The apparent partitioning of metals among cytosolic ligands, as determined by size-exclusion chromatography followed by metal determinations on the various fractions eluting from the HPLC column, has been reported for many marine invertebrates (e.g.: [6,8,11,17]). If such distributions are to be considered representative of the subcellular metal speciation in the original organism (i.e., before dissection, homogenization, centrifugation and chromatographic separation), then it must be assumed that the metal-ligand complexes originally present in the cytosol are kinetically inert and thus do not dissociate or participate in ligand-exchange reactions in the time-frame imposed by the analytical procedure (several hours). As pointed out in our original proposal, this key point seems to have been ignored by virtually every researcher in the field.

Tissue samples from A. grandis are homogenized in ice-cold TRIS buffer (25 mm; pH 7.2) under N_2 and then centrifuged at 170,000 g for 60 min at 2^{o}C. Aliquots of the supernatant are separated on a HPLC gel permeation column (TSK gel SW2000) and the column effluent is monitored at 254 and 280 nm. Effluent fractions are collected and analyzed for metals. The optimal conditions (pH; ionic strength) for the chromatographic separation of the cytosolic ligands have been established; it proved essential to operate at a high ionic strength (> 100 nM NaCl) in order to minimize losses of model proteins on the column.

Using radio-isotopes (^{109}Cd; ^{203}Hg) we have labelled the cytosolic ligands and are presently verifying the stability of the ligand-metal complexes during passage down the HPLC column. If the complexes do not dissociate or participate in ligand-exchange reactions during this key separation step, we will be able to use the HPLC method to determine metal partitioning among different cytoplasmic ligands and to quantify metal "spillover".

3.3 Field work (1989-90)

The preliminary results of the multi-lake field study, as summarized below in point form, are promising - they strongly support a basic premise of the project, namely that metallothioneins are present in representative freshwater invertebrates exposed to contaminant levels typical of those encountered in polluted sediments, and that tissue concentrations of these proteins respond in a dose-dependent manner as a function of the bioavailability of metals (notably Cd) in the external environment.

Geochemistry

- The 11 lakes chosen for the 1989-90 sampling campaign clearly offer the desired metal contamination gradient (Table 1); e.g., for metals extracted from the surficial oxic sediments with a strong reducing agent ($NH_2OH \cdot HCl$), there is about a 25-fold concentration difference between the most and the least contaminated lakes. The contamination gradient is even more marked for the concentration of the free metal ions, $[M^{z+}]$, as estimated by the expression

$$[M^{z+}] = \frac{\{FeOM\}}{\{Fe\text{-}Ox\}} \cdot \frac{[H^+]^x}{n * K_a}$$

where FeOx = amorphous iron oxyhydroxide, and FeOM = metal M associated with this solid phase - see Campbell and Tessier [3].

	$[\ ]_{max}/[\ ]_{min}$		
	Cd	Cu	Zn
Extractable metal	25	22	28
Estimated free metal ion	180	5	300

- The more contaminated lakes include Duprat, Flavrian, Joannès, La Bruère, and Vaudry, whereas lakes Dufay, Héva and Opasatica are relatively uncontaminated.

Benthos

- Populations of the freshwater mollusc Anodonta grandis were found in each of the 11 lakes. To minimize biological variability, divers collected only actively filtering specimens of about 8 cm in length.

- From previous work [18,19] it is known that the reproductive status of freshwater molluscs may affect their metal levels. At the time of sampling (June 1989), none of the specimens were gravid.

- Average condition indices, as calculated according to the relation CI = 1000 x tissue dry weight (g) ÷ internal volume (mL), varied from a low of ≈25 g/mL (Joannès) to a high of ≈75 g/mL (Flavrian).

- The benthic detritivore Hexagenia limbata was present in significant numbers in some but not all of the 11 lakes (as evaluated by screening of numerous sediment samples collected

with an Ekman grab sampler). Small sample sizes and the resulting low tissue weights precluded accurate determination of metallothionein concentrations in Hexagenia - see below.

Metal bioaccumulation

- Concentrations of Cd and Cu in Anodonta grandis varied markedly from lake to lake; Zn levels were less variable.

- As expected from earlier work [3,4,18,19], the best predictor of variations in mollusc Cd concentrations among different lakes was $[Cd^{2+}]$, as estimated from the lake pH and the ratio of adsorbed Cd / amorphous Fe oxyhydroxides. The best predictor of mollusc Cu concentrations proved to be the concentration of filterable Cu measured in samples collected at the sediment-water interface (Table 2).

Metallothionein

- Earlier work in our laboratory [12] demonstrated the presence of a metallothionein-like protein in the gills and the hepatopancreas of A. grandis specimens that had previously been exposed to artificially high concentrations of Cd in the laboratory. This Cd-, Cu- and Zn-binding protein was subsequently detected in specimens collected from lakes in the Rouyn-Noranda mining area. The properties of this metal-binding protein (molecular weight; chromatographic behaviour; UV absorbance; stability after heat and acid treatment; polarographic behaviour, abundance of -SH groups) all are consistent with its designation as a metallothionein (MT).

- In the present study, concentrations of MT in Anodonta grandis varied from lake to lake (Table 3). Levels measured by the ^{203}Hg saturation method were less variable in the hepatopancreas than in the gills or the mantle + remainder; e.g., concentration ranges were narrower for the hepatopancreas (≈ 1.8-fold) than for the gills (≈ 4-fold) or the mantle + remainder (≈ 2.5-fold).

- Preliminary (bi-variate) statistical analysis showed that metallothionein levels in the gills and in the mantle + remaining tissues are significantly correlated ($p < 0.001$) with the free Cd^{2+} (and Zn^{2+}) concentrations in the ambient water from which the molluscs were collected (Table 4; Fig. 1).

- To our knowledge, this is the first demonstration that metallothionein levels in field populations of freshwater invertebrates vary along a metal contamination gradient in a consistent manner, i.e. as a function of the contamination gradient as defined by the free metal ion concentration, $[M^{Z+}]$.

- Metallothionein levels in the gills and in the mantle + remaining tissues are also significantly correlated ($p < 0.001$)

with Cd concentrations in these tissues (Table 5; Fig. 2); in contrast, correlations between [MT] and tissue levels of Cu or Zn are weak or non-existent. This would seem to indicate that $[Cd^{2+}]$ (and not $[Zn^{2+}]$) is the key environmental factor to which metallothionein levels in A. grandis are responding.

Toxicology

- As a preliminary evaluation of the spillover hypothesis, the ratio of tissue metallothionein (nmoles binding capacity/g dry wt) to tissue Cd (nmoles/g dry wt) has been calculated for various tissues; the implicit assumption is that higher ratios correspond to more effective Cd detoxification. Values of this ratio, as derived for each composite sample for each lake, were then compared to the condition indices for each lake. The condition index of the molluscs was positively correlated with the [MT]/[Cd] ratio in the gills and in the mantle + remaining tissues (Fig. 3). This result is particularly promising - the condition index of freshwater molluscs would be expected to be influenced by a variety of factors, notably the trophic status of the studied lakes and the availability and nutritional quality of the suspended matter; given this inherent variability, the observed relation between the condition index and the [MT]/[Cd] ratio is striking.

3.4 Summary

As can be judged from the preceding sections, progress to date has been very satisfactory. A basic premise of the original proposal, namely that tissue concentrations of metallothionein in Anodonta grandis vary in a dose-dependent manner as a function of the bioavailability of metals (Cd) in the external environment, has been confirmed. The transfer experiments planned for the 1990-91 field season (see below) will enable us to evaluate the toxicological significance of MT, i.e. its role in the detoxification of metals. The likelihood of success appears high.

4. OUTLINE OF WORK PROPOSED

The work proposed for the upcoming funding period involves an intensive study of the seasonal variation of metallothionein levels in Anodonta grandis, coupled to transplant experiments from a relatively pristine lake to a contaminated lake. The work plan adheres closely to that described in the original proposal.

4.1 Seasonal variability.

The molecular binding patterns of metals in aquatic organisms have recently been reported to undergo marked seasonal changes, presumably in response to changes in water quality (temperature, pH) or to the metabolic perturbations associated with the reproductive or moulting cycles [7, 16]. To evaluate the importance of such seasonal changes for A. grandis, two sites (one highly contaminated - lake Vaudry; the other a control lake - lake Opasatica) have been selected for repetitive biological sampling over an annual cycle. Molluscs will be collected on four occasions during the ice-free season, at times chosen to bracket the reproductive cycle of A. grandis. The tissue concentrations of metals and metal-binding proteins will be determined.

4.2 Mollusc tranplant experiments.

Laboratory demonstrations of the induction of MT biosynthesis have almost invariably been carried out at very high metal concentrations relative to environmental levels, and for short-term exposures; as mentioned by Petering and Fowler [16], there is an urgent need for studies at environmentally realistic exposure levels. To demonstrate that the synthesis of metallothioneins can indeed be induced in representative freshwater invertebrates at contaminant levels typical of those encountered in the field, we shall carry out transfer experiments with A. grandis. Indigenous bivalves (≈ 150 individuals) will be collected, sized, weighed, aged and transferred from a pristine site to a highly contaminated site, where they will be maintained in enclosures placed in the bottom sediments; specimens from the contaminated site will be collected, characterized and maintained in nearby enclosures (control sample, to account for any enclosure effect and to reveal any seasonal variability). In a second control experiment, molluscs from the pristine site will be collected, manipulated, transported .... but returned to enclosures in their original (uncontaminated) lake. This second control sample will allow us to take into consideration any short-term stress imposed on the molluscs during the transfer step itself.

The transfers will be performed in late June 1990 and individuals will be removed from the enclosures (8 from each of two enclosures at a given time) at various times (t = 0, 5, 10, 30, 60, 90, 330 days). The tissue concentrations of metals and metal-binding molecules, as well as the partitioning of metals among different cytoplasmic ligands,

will be determined.

Having successfully carried out similar transfer experiments in the past, we fully appreciate the logistic and biological challenges involved. Our earlier studies showed that slow changes in whole-body and tissue metal burdens do occur after transfer ($t_{\frac{1}{2}}$ = 0.4 and 0.9 yr for Cd and Cu respectively), and that the condition of molluscs transferred from the control lake to the contaminated site deteriorated with time [19]. Building on this past experience, the present study aims to demonstrate the induction and accumulation of MT in a freshwater invertebrate, under field conditions, after imposition of metal stress.

To verify the applicability of the "spillover" phenomenon to A. grandis, the partitioning of metals among different cytoplasmic ligands will be determined (provided that the cytosolic metal-ligand complexes prove to be kinetically inert during chromatographic separation - see above). Such measurements have been reported for laboratory exposures of (marine) invertebrates, but to our knowledge no published data are available for field exposures of freshwater species. Finally, given the successful demonstration by Hinch et al. [10] of short-term effects of transplantation on the growth of the freshwater pelecypod Lampsilis radiata, we shall monitor mollusc growth and condition after 90 and 330 days and relate these variables to changes in the subcellular metal distribution.

5. STATEMENT OF EXPENDITURES OF WTF FUNDS FOR THE PRECEDING FUNDING PERIOD.

Expenditures of WTF funds for the period from November 1989 to May 1990 totaled $7 715$; an additional 3 220$ is already committed for the first month of year 2 (field work - travel advances).

	Nov.1989-May 1990
- Personnel service	$ 3692
- Travel	1835
- Supplies and material	1978
- Support services	210
Total:	$ 7715
- Funds committed for June 1990 field work	$ 3220
- Balance	$ 4065

The budget for 1989-90, including matching funds, is presented in Appendix A.

6. OUTLINE OF BUDGET FOR THE 1990-91 WTF GRANT

The $30,000 expected from WTF for 1990-91, together with balance of the first instalment, will be distributed as follows.

	June 1990 - May 1991
- Personnel service	$ 22750
- Field work	7950
- Supplies and material	6035
- Support services	550
Total:	$ 37285

The overall budget, including matching funds, can be found in Appendix A.

7. RESTATEMENT OF MATCHING FUNDING ARRANGEMENTS

Financial contributions to the present project are from the various sources described below.

1) CAMPBELL, P.G.C., R. CARIGNAN, A. TESSIER AND P.M. STOKES. NSERC Strategic research grant, 1988-1989, ≈61 000$/yr. Status = terminated 1989. Title: "Prédiction de la biodisponibilité de métaux traces présents dans les sédiments des eaux douces". An amount of 13 820$ was allocated in 1989 in support of the study of the spatial variability of tissue concentrations of MT as a function of the bioavailability of Cd and Zn.

2) TESSIER,A., P.G.C. CAMPBELL, R. CARIGNAN, J.C. AUCLAIR AND R.L. HARE. FCAR Team research grant, 1989-1992, ≈90 000/yr. Status = currently held. Title: "Biogéochimie de substances polluantes dans le milieu aquatique". The amount allocated for the first year (6 708$) corresponds to partial support of Dr. S. Micallef's postdoctoral stipend; the amount budgeted for year 2 (10 000$) corresponds to partial support of the SCUBA divers' and technicians' salaries.

3) TESSIER, A., P.G.C. CAMPBELL AND R.L. HARE. NSERC Strategic research grant, 1989-1992, ≈43 000$/yr. Status - currently held. Title: "Modélisation de l'accumulation de métaux traces chez des invertébrés benthiques". Amount allocated for year 2 (9 930$) corresponds to partial support for supplies and materials and for the field work.

4) CAMPBELL, P.G.C., A. TESSIER AND J. PELLERIN-MASICOTTE. Université du Québec, Fonds FODAR, 1990-91, 21 000$/yr. Status - currently held. Title: "Indicateurs biochimiques de stress causes par les metaux traces dans les ecosystemes aquatiques". Amount allocated for year 2 (13 800$) corresponds to partial support of the undergraduate student stipend and of the salaries of the laboratory technician and the laboratory manager.

5) One postdoctoral fellow (S. Micallef, biochemist) has worked on this project since October 1989. For the period October 1989 - March 1990 he held a postdoctoral fellowship from INRS (25 000$/yr). These fellowships are partially supported by INRS (50%), the remainder of the stipend being paid by the host professor. This corresponds to a contribution of 6 250$ from the INRS for fiscal 1989-90. In April 1990, Dr. Micallef was awarded a NSERC postdoctoral fellowship (27 000$/yr + 6 000$/yr supplement from INRS).

6) OTHER FUNDS. The graduate student working on this project (Yves Couillard) holds a NSERC postgraduate research award (13 500 - 15 000$/yr). In addition, our research centre contributes, from a FCAR infrastructure grant, to the general laboratory budget (partial salary of the lab manager; common laboratory supplies; instrument maintenance); funds from this source amount to ≈4 000$/yr.

These arrangements are virtually unchanged from those described in the original proposal. Note that contribution #3, originally listed as "applied for", is now confirmed. Contribution #4, obtained only recently, was not indicated in the original compilation of matching funds.

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Table 1: Geochemical data for the 11 lakes sampled during the 1989 field season.

Lake	sediment [M] extracted with $NH_2OH \cdot HCl$ (nmole/g)				filterable [M] (nmole/L)			
	Cd	Cu	Zn	Fe	Cd	Cu	Zn	pH[a]
Joannès (JO)	62	112	2260	98000	--	--	--	6.88
La Bruère (BR)	40	547	1600	77000	2.22	110	46	7.58
D'Alembert (DA)	80	102	2700	109000	2.13	100	108	6.80
Bousquet (BO)	24	28	1300	81000	2.76	62	204	5.66
Opasatica (OP)	3	54	150	33000	0.81	56	40	7.14
Héva (HE)	4	25	745	71000	2.21	37	142	5.82
Flavrian (FL)	49	70	2100	112000	2.65	74	245	6.94
Vaudry (VA)	30	88	1200	79000	1.15	52	174	6.44
Dufay (DU)	5	25	425	45000	2.13	55	53	6.36
Dufresnoy (DF)	19	67	800	102000	1.04	74	48	6.67
Duprat (DP)	54	114	4280	69000	1.15	145	161	7.08

[a]The pH was measured on water samples collected by divers at the sediment-water interface, on a single sampling date (June 1989). The value given for lake La Bruère was determined in July 1985.

Table 2: Correlations between metal levels in Anodonta grandis and those in its environment.

	Correlation coefficient (r)[a,b]								
	Gills			Hepato-pancreas			Mantle + remainder		
Predictor	Cd	Cu	Zn	Cd	Cu	Zn	Cd	Cu	Zn
$[M^{z+}]$	**0.60** ***	**0.51** ***	(-.2)	**0.59** ***	(0.2)	**0.66** ***	**0.66** ***	(0.2)	(0.2)
filterable [M]	(0.2)	**0.88** ***	(0.0)	(0.3)	**0.88** ***	(0.4)	(0.0)	**0.72** ***	(0.0)

[a] Pearson correlation between log-transformed variables ($\log_{10}$ (variable + 100)).

[b] N = 36-42; ***, $p < 0.001$; values in parentheses are not significant ($p > 0.05$).

Table 3: Metallothionein concentrations in specimens of the freshwater mollusc Anodonta grandis collected from the 11 lakes sampled during the 1989 field season.

Lake	Metallothionein concentrations (nmole Hg/g dry wt) Gills	Hepatopancreas	Remainder
Joannès	199	387	421
La Bruère	156	307	259
D'Alembert	238	319	269
Bousquet	195	358	365
Opasatica	100	212	178
Héva	262	310	359
Flavrian	129	232	198
Vaudry	406	292	436
Dufay	220	232	244
Dufresnoy	154	242	173
Duprat	217	258	253

Table 4: Correlations between metallothionein levels in Anodonta grandis and extracellular metal concentrations.

Tissue [MT]	Correlation coefficient (r)[a,b] Aqueous metal $[Cd^{2+}]$	$[Cu^{2+}]$	$[Zn^{2+}]$
Gill (N=42)	**0.52** ***	(0.3)	**0.58** ***
Hepato-pancreas (N=40)	0.40 *	(0.3)	(0.3)
Mantle + Remainder (N=40)	**0.61** ***	0.38 *	**0.57** ***

[a]Pearson correlation between log-transformed variables ($\log_{10}$ {variable + 100}).

[b]For r values labelled ***, $p < 0.001$; *, $p < 0.05$; values in parentheses are not significant ($p > 0.05$).

Table 5: Correlations between metallothionein levels in Anodonta grandis and intracellular metal concentrations.

	Correlation coefficient (r)[a,b]								
	Tissue metal								
	Gill			Hepato-pancreas			Mantle + remainder		
Tissue [MT]	Cd	Cu	Zn	Cd	Cu	Zn	Cd	Cu	Zn
Gill (N=38)	**0.70** ***	(0.1)	(0.3)	--	--	--	--	--	--
Hepato-pancreas (N=40)	--	--	--	0.40 *	(0.0)	(0.1)	--	--	--
Mantle + Remainder (N=40)	--	--	--	--	--	--	**0.90** ***	(0.1)	0.42 **

[a]Pearson correlation between log-transformed variables ($\log_{10}$ {variable + 100}).

[b]For r values labelled ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; values in parentheses are not significant ($p > 0.05$).

Figure legends

Figure 1: Inter-lake variation of metallothionein levels in the gills of Anodonta grandis as related to the free Cd^{2+} concentration in the ambient lake water. The concentration of Cd^{2+} was estimated from the expression $\Gamma_{Cd} \cdot [H^+]^x / n \cdot K_a$, where Γ_{Cd} = {FeOCd}/{Fe-Ox}; see section 3.3 for details. The individual lakes are identified by a two-letter code, as indicated in Table 1.

Figure 2: Inter-lake variation of metallothionein levels in the gills of Anodonta grandis as related to the Cd concentrations in the same tissue. The individual lakes are identified by a two-letter code, as indicated in Table 1.

Figure 3: Relation between the condition index of Anodonta grandis (mg/mL) and the ratio of metallothionein : Cd in the mantle + remaining tissues (nmoles binding capacity/nmoles Cd bioaccumulated, expressed as %). The individual lakes are identified by a two-letter code, as indicated in Table 1.

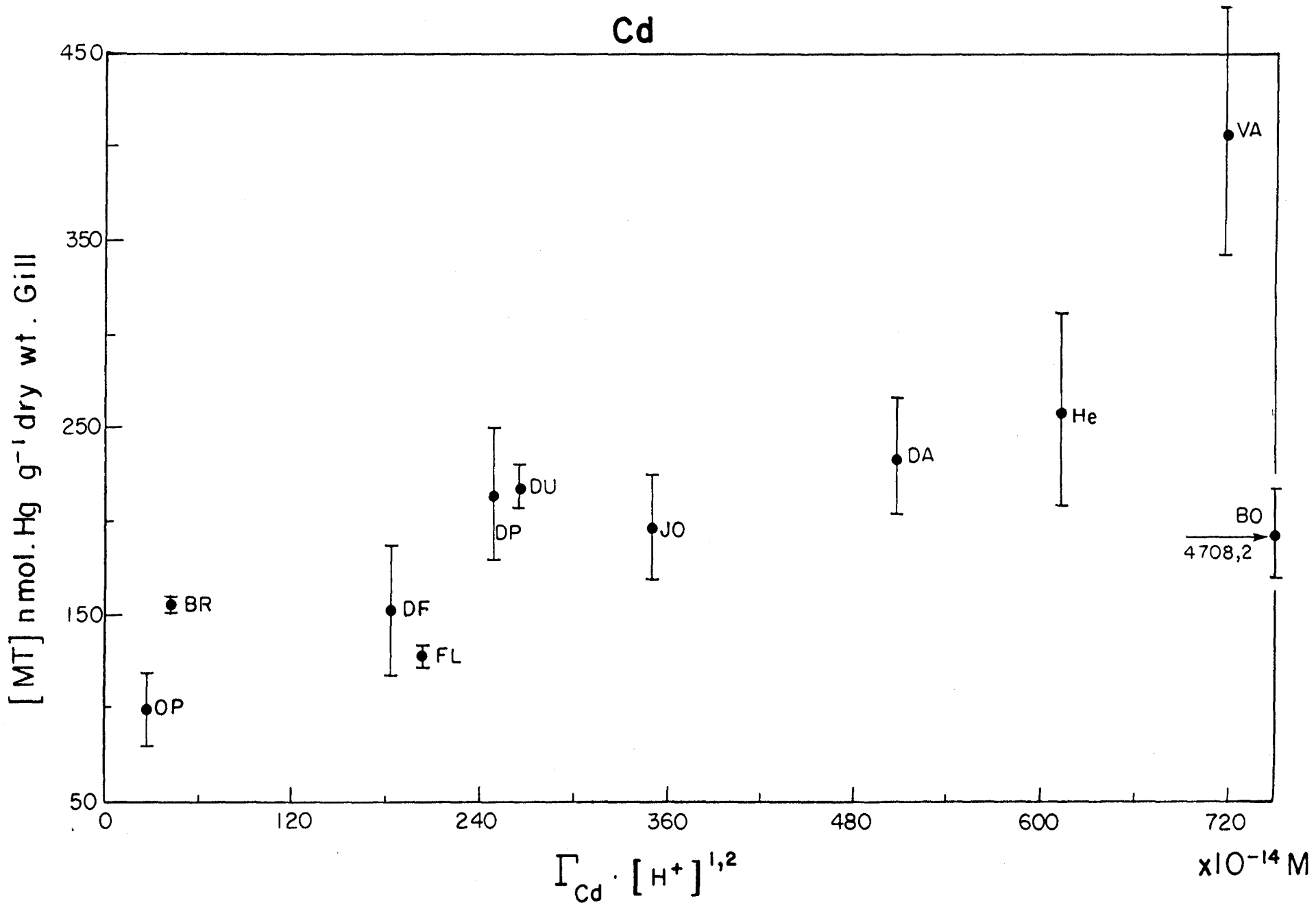

Figure 1: Inter-lake variation of metallothionein levels in the gills of Anodonta grandis as related to the free Cd^{2+} concentration in the ambient lake water. See section 3.3 for details.

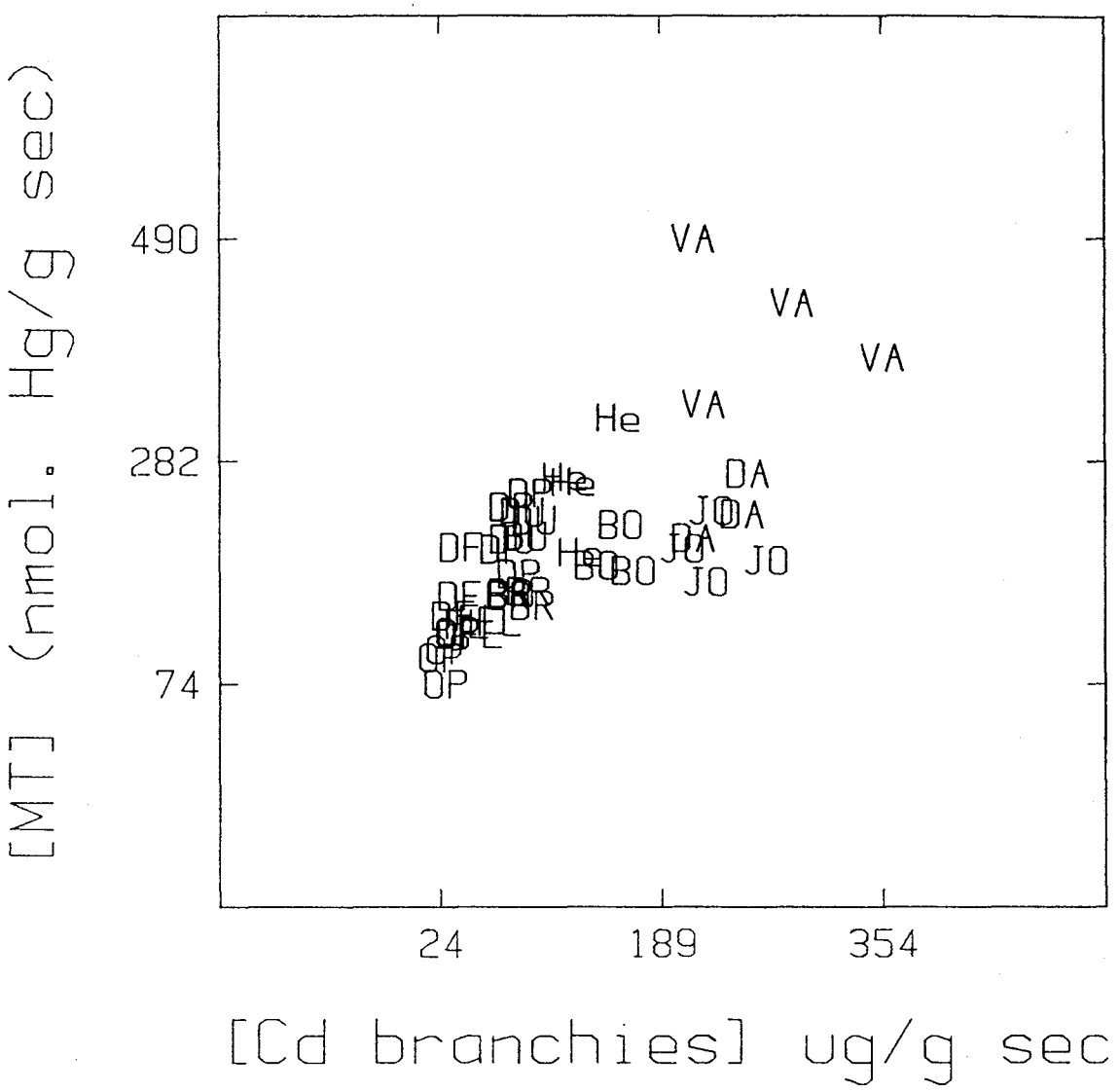

Figure 2: Inter-lake variation of metallothionein in the gills of Anodonta grandis as related to the Cd concentration in the same tissue.

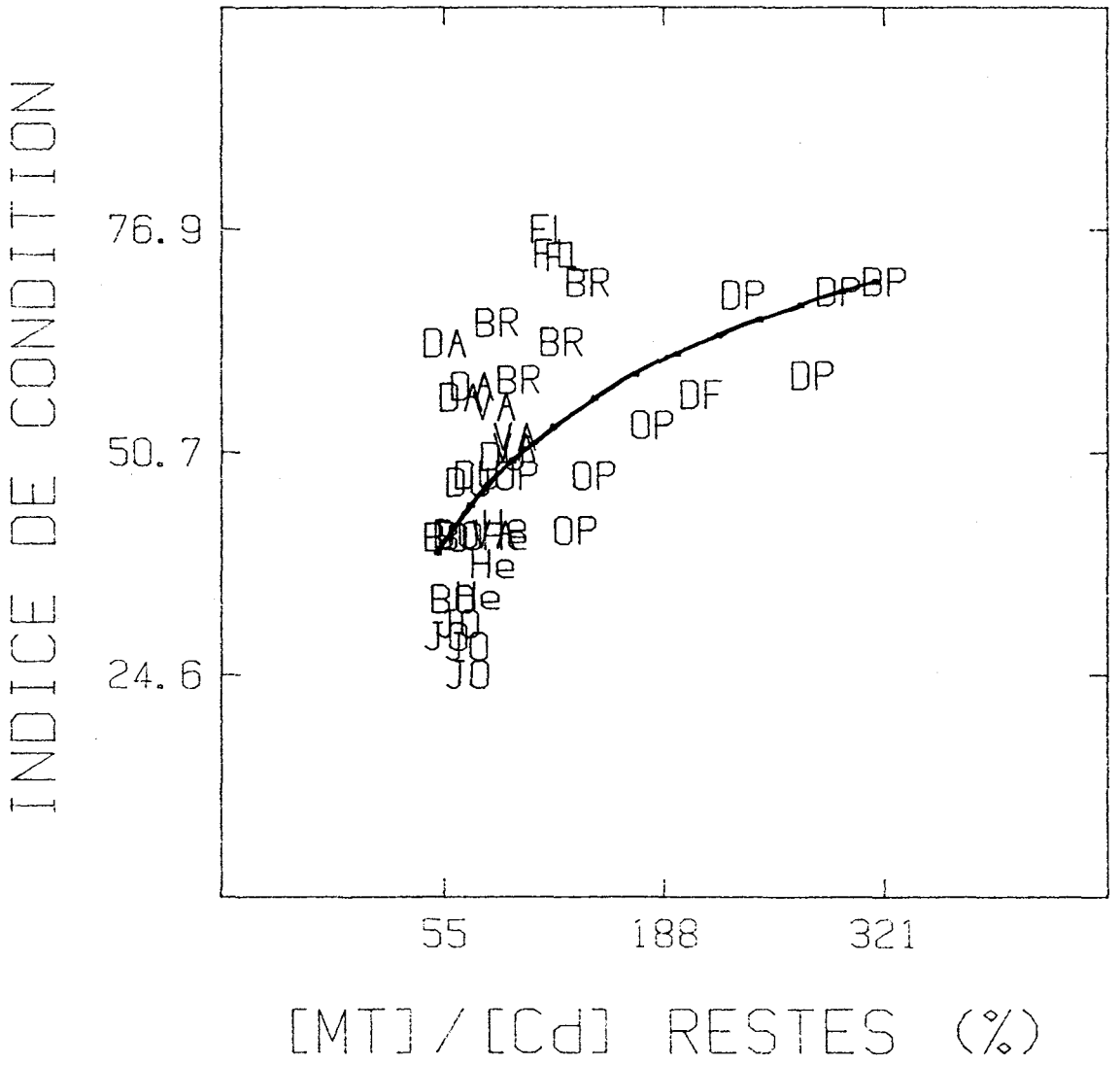

Figure 3: Relation between the condition index of Anodonta grandis (mg/mL) and the ratio of metallothionein : Cd in the mantle - remaining tissues (nmoles binding capacity / nmoles Cd bioaccumulated, expressed as %).

APPENDIX A

BUDGET (1 June 1989 to 31 May 1991)

	1989-90			1990-91		
	Project Budget	WTF	Matching Grants	Project Budget	WTF	Matching Grants
ERSONNEL SERVICE	$	$	$	$	$	$
Postdoctoral fellow	13416	--	13416	33000	--	33000
Graduate student	13500	--	13500	18000	3000	15000
Undergraduate student	--	--	--	10500	3500	7000
Technicians	9863	3477	6386	30000	15000	15000
Professional (lab manager)	215	215	--	2500	1250	1250
Employer contribution to fringe benefits (11%)	1108	--	1108	3575	--	3575
RAVEL						
Field work + conference	6459	1835	4625	13850	7950	5900
UPPLIES AND MATERIAL						
Field and lab supplies	4609	1978	2631	10065	6035	4030
UPPORT SERVICES						
Photocopying, typing, etc.	389	210	179	1100	550	550
TOTAL	49559	7715	41845	122590	37285	85305