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METALLOTHIONEIN-LIKE METAL-BINDING PROTEIN IN THE BIOMONITOR *CHAOBORUS*: OCCURRENCE AND RELATIONSHIP TO AMBIENT METAL CONCENTRATIONS IN LAKES

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Abstract—Larvae of the insect *Chaoborus* are used to monitor cadmium (Cd) in lakes. We set out to determine if this animal possesses a metallothionein-like protein to which its Cd could be bound and if the concentrations of such a protein are correlated with those of Cd in the insect and in lakewater. To achieve our goals, we collected water and larvae of several *Chaoborus* species from 10 lakes situated along an environmental Cd gradient. We found that all of the *Chaoborus* species possess a metallothionein-like protein and that concentrations of the protein and of Cd differed among species. Concentrations of the metallothionein-like protein were directly related to those in *Chaoborus* and in lakewater. These direct relationships support the use of *Chaoborus* larvae as a Cd biomonitor.

Keywords—Metallothionein Cadmium *Chaoborus* Biomonitor Lake

INTRODUCTION

Larvae of the phantom midge *Chaoborus* have been proposed for use as biomonitors of the trace metal cadmium (Cd). They are well suited to this task because they are common in lakes world wide [1,2] and tolerate a wide range of pH (4–7.5) and Cd concentrations [3]. Most important, Cd concentrations in this putative biomonitor are consistently related to those in lakewater, even in highly metal-contaminated lakes [3]. It follows then that this insect is able to accumulate Cd without ill effect. To do so, this invertebrate likely possesses an effective Cd detoxification mechanism.

Metallothioneins are metal-binding proteins that are reported to play a key role in the binding and transport of Cd and other trace metals in animals [4]. These ubiquitous, low-molecular weight, cysteine-rich proteins have been found in several genera of aquatic insects, including *Chironomus* (Diptera; [5]), *Baetis* (Ephemeroptera; [6]), and *Eusthenia* (Plecoptera; [7]). There are, however, no published reports that metallothionein-like proteins exist in *Chaoborus* larvae.

In this article, we set out first to determine if *Chaoborus* larvae posses a metal-binding protein having the characteristics of metallothionein (MT). To achieve this, we collected *Chaoborus* larvae in large numbers; a minimum of several hundred individuals was required to provide enough biomass for a single sample. An ability to synthesize MT would explain, in part, the capacity of this insect to exist in metal-contaminated lakes. Second, because sympatric *Chaoborus* species are reported to vary in their Cd concentrations [8], we collected samples of more than one species in lakes where population densities allowed; we speculated that a lack of MT could lead to the restriction of some *Chaoborus* species to relatively uncontaminated lakes. Third, we measured Cd, Cu, and Zn concentrations in larvae to determine if larvae with high MT concentrations had correspondingly high concentrations of these trace metals. A strong correlation between the concentrations of MT and Cd but not those of Cu or Zn would suggest that Cd is the metal responsible for MT induction in larvae.

METHODS

Collection of field samples

Water samples and larvae of three species of *Chaoborus* were collected in September 1999 from 10 lakes located on the Canadian Shield (Table 1) in the Quebec City area (QC) and in the mining areas of Sudbury (ON) and Rouyn-Noranda (QC). One of the lakes in the Rouyn-Noranda region, Lake Turcotte, has been directly impacted by the dumping of mining residues and subsequent liming.

Insect larvae were collected after sunset by hauling a 250-μm plankton net horizontally in the water column of each lake until a sufficient number of *Chaoborus* was obtained. Insects were maintained at field temperatures and transported to the laboratory in plastic bags filled with lakewater. In the laboratory, *Chaoborus* larvae were sorted according to species [1]. Where numbers permitted, three replicate samples of 300 to 1,000 similar-sized fourth instar larvae of each species were sealed in polyethylene bags, frozen, and stored in liquid nitrogen. At the end of the sampling trip, frozen samples were sealed in plastic bags filled with nitrogen and were stored at −80°C for six months until homogenization.

Water samples were collected using in situ diffusion samplers (peepers) similar to those described by Carignan et al. [9]. These Plexiglas samplers comprise eight compartments of 4 ml each that were filled with ultrapure water (>18 MΩ cm) and separated from lakewater by a 0.2-μm polysulfone membrane (Gelman HT-200; Pall Life Sciences, Ann Arbor, MI, USA). After preparation, each sampler was sealed in a clean plastic bag prior to placement in the field. Two diffusion sam-

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Table 1. Location of the study lakes (codes in parentheses) with their total dissolved concentrations of the trace metals Cd, Cu, and Zn as well as their pH and the concentration of dissolved organic carbon (DOC) and calcium (Ca)

Region and lake	Location	pH	[Cd] (nM)	[Cu] (nM)	[Zn] (nM)	[Ca] (μM)	[DOC] (mg C/L)
Québec, Québec, Canada							
Bertrand (BE)	46°58′N, 72°01′W	5.2	0.3	2.6	100	8	5.6
Laflamme (LA)	47°19′N, 71°07′W	6.7	0.1	2.6	22	17	5.5
St. Joseph (SJ)	46°55′N, 71°40′W	6.7	0.03	5.2	10	19	3.3
Rouyn-Noranda, Québec, Canada							
Bousquet (BO)	48°14′N, 78°34′W	6.7	0.6	53	39	45	14.5
Caron (CA)	47°56′N, 78°58′W	7.2	0.7	72	46	114	10.9
Marlon (MA)	48°16′N, 79°04′W	7.4	0.9	147	35	59	10.2
Turcotte[a] (TU)	48°18′N, 79°04′W	5.2	8.7	134	2,427	30	4.6
Sudbury, Ontario, Canada							
Crooked (CK)	46°25′N, 81°02′W	6.2	1.5	317	81	23	5.7
Crowley (CW)	46°23′N, 80°59′W	6.7	0.7	127	48	22	2.8
Tilton (TI)	46°22′N, 81°04′W	6.4	0.9	106	63	29	2.7

[a] Manipulated lake.

plers were suspended in the epilimnion of each lake. After a 3-d equilibration period (3 d is considered to be sufficient for peeper equilibration; Tessier and De Vitre, unpublished results), the diffusion samplers were retrieved and water was collected immediately for the measurement of organic and inorganic carbon, trace metals, and major cations and anions.

Samples (4 ml) for organic carbon determination were removed from one compartment of each peeper using a pipette fitted with a persulfate-washed plastic tip and were injected into 4-ml glass bottles that had been previously heated for 6 h at 500°C and rinsed several times with ultrapure water. Samples (1 ml) for inorganic carbon determination were removed with a syringe from a second compartment and were injected through a septum into preevacuated and prewashed glass tubes. From the same compartment, a sample (1.5 ml) for dissolved SO_4 and Cl analyses was collected with a plastic-tipped pipette and was injected into prewashed microcentrifuge tubes (1.5 ml, high-density polyethylene). Samples for trace metals (Cd, Cu, and Zn; five compartments) and major cations (Ca, Mg, Na, and K; one compartment) were removed from the remaining compartments in each dialysis sampler by piercing the membrane with a pipette fitted with an acid-cleaned tip. These samples were injected into preacidified (53 μl of 1.35 N Anachemia HNO_3) high-density polyethylene bottles (4-ml capacity). On installation and retrieval dates, pH was measured with a portable pH meter (Microprocessor model HI9024/HI9025; Hanna Instruments, Woonsocket, RI, USA) in water samples collected at the depth of the dialysis samplers using a Van Dorn bottle.

Analyses

To minimize inadvertent trace metal contamination, labware, water-sampling materials, and vials were soaked in 15% nitric acid and rinsed in ultrapure water prior to use.

Metallothionein

Partially thawed *Chaoborus* larvae were gently homogenized, on ice, under a nitrogen atmosphere with a 50-ml manually operated glass tissue grinder. Homogenization was performed with four volumes of a phosphate buffer solution (0.02 M) adjusted to pH 7.5. A subsample (1 ml) was centrifuged at 30,000 *g* for 30 min at 4°C, and the supernatant was divided into four subsamples that were analyzed the same day for MT. Additional subsamples of the tissue homogenate were allocated for determination of the dry-weight:wet-weight ratio (1 ml) and Cd, Cu, and Zn concentrations (1 ml).

Metallothionein concentrations in *Chaoborus* larvae were measured with a mercury-saturation assay adapted slightly from Dutton et al. [10] and described in detail in Couillard et al. [11]. As a quality control, recovery of a standard (MT from rabbit liver; Sigma-Aldrich, St. Louis, MO, USA; stoichiometry of 7 mole metal/mole MT) was determined with every assay; the mean recovery for three determinations was 86 ± 8% (standard deviation). Metallothionein concentrations (i.e., [MT]) are mean values of both field and analytical replicates (± standard error) and are expressed as nanomoles of metal-binding sites per gram of dry tissue weight.

Metal analyses

Tissue homogenates were freeze dried (FTS Systems™, Mountain View, CA, USA), weighed, and digested at room temperature in 4-ml high-density polyethylene vials with concentrated nitric acid (Aristar grade, 100 μl/mg dry wt sample) for 7 d. Hydrogen peroxide (40 μl/mg dry wt sample) was added 24 h prior to final dilution with ultrapure water (760 μl/mg dry wt sample). Samples of similar weight of a certified reference material (lobster hepatopancreas, TORT-1, National Research Council of Canada) were submitted to the same digestion procedures during each analytical run. Trace metal concentrations measured in this reference material were consistently within the certified range. Cadmium concentrations in animals were analyzed by flameless atomic-absorption spectrophotometry (AAS, Spectra AA-30; Varian, Palo Alto, CA, USA), whereas Cu and Zn concentrations were measured by inductively coupled plasma atomic-emission spectroscopy (Vista; Varian).

Total dissolved Cd, Cu, and Zn concentrations in the peeper samples were measured by flameless AAS (SIMAA 6000; Perkin-Elmer, Norwalk, CT, USA). Certified reference water samples (riverine water reference material, National Research Council of Canada) were also analyzed for Cd, Cu, and Zn during each analytical run, and measured trace metal concentrations were consistently within the certified range. Major cation concentrations were measured by flame AAS (Spectra AA-20; Varian). Concentrations of SO_4 and Cl were measured by ion chromatography (DX300; Dionex AutoIon, Sunnyvale,

Table 2. Metal concentrations (Cd, Cu, and Zn, ±95% confidence interval) and metallothionein-like protein concentrations (MT ± standard error) in final instar *Chaoborus* larvae as well as the ratio of tissue [Cd] to [MT]; sample numbers are given in parentheses

Region and lake	Species	[Cd] (nmol/g)	[Cu] (nmol/g)	[Zn] (μmol/g)	[MT] (nmole binding sites/g dry wt)	Cd/MT
Quebec, Québec, Canada						
Bertrand	*americanus*	6.0 ± 0.59 (*n* = 3)	64 ± 9 (*n* = 3)	2.0 ± 0.63 (*n* = 3)	133 ± 15 (*n* = 3)	0.04
Laflamme	*flavicans*	4.3 ± 0.81 (*n* = 2)	97 ± 8 (*n* = 2)	1.2 ± 0.32 (*n* = 2)	100 ± 9 (*n* = 3)	0.04
St. Joseph	*punctipennis*	7.1 ± 1.4 (*n* = 3)	72 ± 2 (*n* = 3)	1.4 ± 0.29 (*n* = 3)	120 ± 11 (*n* = 3)	0.06
Rouyn-Noranda, Québec, Canada						
Bousquet	*flavicans*	36 ± 1.6 (*n* = 3)	102 ± 5 (*n* = 3)	1.4 ± 0.10 (*n* = 3)	187 ± 1 (*n* = 2)	0.19
	punctipennis	38 (*n* = 1)	97 (*n* = 1)	1.5 (*n* = 1)	241 (*n* = 1)	0.16
Caron	*flavicans*	44 ± 1.3 (*n* = 3)	107 ± 2 (*n* = 3)	1.3 ± 0.001 (*n* = 3)	163 ± 4 (*n* = 3)	0.27
Marlon	*punctipennis*	54 ± 3.5 (*n* = 3)	107 ± 31 (*n* = 3)	1.2 ± 0.07 (*n* = 3)	183 ± 2 (*n* = 3)	0.29
Turcotte	*americanus*	43 ± 5 (*n* = 3)	100 ± 8 (*n* = 3)	1.6 ± 0.03 (*n* = 3)	179 ± 7 (*n* = 3)	0.24
	punctipennis	15 ± 0.9 (*n* = 2)	122 ± 1 (*n* = 2)	1.2 ± 0.001 (*n* = 2)	193 ± 11 (*n* = 2)	0.08
Sudbury, Ontario, Canada						
Crooked	*punctipennis*	72 ± 2 (*n* = 3)	145 ± 12 (*n* = 3)	1.2 ± 0.06 (*n* = 3)	199 ± 5 (*n* = 3)	0.36
Crowley	*punctipennis*	130 ± 5 (*n* = 3)	164 ± 13 (*n* = 3)	1.0 ± 0.05 (*n* = 3)	196 ± 14 (*n* = 3)	0.66
Tilton	*flavicans*	125 (*n* = 1)	171 (*n* = 1)	1.7 (*n* = 1)	230 (*n* = 1)	0.54
	punctipennis	141 (*n* = 1)	138 (*n* = 1)	1.3 (*n* = 3)	268 (*n* = 1)	0.53

CA, USA). Dissolved inorganic carbon concentrations were obtained by gas chromatography (GC 5890 Series 2: Hewlett-Packard, Palo Alto, CA, USA), and dissolved organic carbon concentrations were obtained with a total organic carbon analyzer (TOC-5000A; Shimadzu, Kyoto, Japan) using the combustion-infrared method. The Windermere humic aqueous model ([12]; Tipping, Ambleside, UK) was used to estimate free metal ion concentrations, with the assumptions discussed in Croteau et al. [3].

RESULTS AND DISCUSSION

Metallothionein in Chaoborus

The three species of *Chaoborus* present in our study lakes all possess a metal-binding protein that has features consistent with those of MT, i.e., a strong affinity to form complexes with Hg (stronger than those with Cd, Cu, or Zn), thermostability, and acid resistance. The concentrations of MT-like protein ranged from 100 to 270 nmol binding sites/g (Table 2), which is slightly lower than those found for the freshwater bivalve *Pyganodon grandis* (163–414 nmol binding sites/g dry wt; [11]) but higher than those found in the mayfly *Hexagenia limbata* (18–106 nmol binding sites/g dry wt; Y. Couillard, unpublished data). The presence of a metal-binding protein in *Chaoborus* larvae could explain in part their accumulation of and tolerance to trace metals. Increased metal resistance has been associated with MT induction in other aquatic (mayflies [6], chironomids [13]) and terrestrial (collembolans [14]) insects.

Because all of the *Chaoborus* species that we studied appear to have a MT-like protein (Table 2), the presence or absence of such a protein cannot explain differences in Cd accumulation among them [8]. In contrast, Aoki et al. [6] reported marked species-to-species differences for the mayfly *Baetis*; a species present in metal-contaminated streams possessed a MT-like protein, whereas two others that did not were restricted to uncontaminated rivers.

We compared the concentrations of Cd and MT-like protein in *Chaoborus* species from the same lake. In the two lakes in which they coexisted (Lakes Tilton, ON, and Bousquet, QC, Canada; Table 2), larvae of *Chaoborus punctipennis* had higher concentrations of a MT-like protein and the same or higher concentrations of Cd than did larvae of *Chaoborus flavicans*. In contrast, the concentrations of MT-like protein in *C. punctipennis* were not significantly different from those in *Chaoborus americanus* (Lake Turcotte, *t* test, $p = 0.34$), in spite of the fact that Cd concentrations in the former species were three times lower than those of the latter species (*t* test, $p = 0.004$). This apparent inconsistency could result from the small number of samples that we were able to collect for analysis. The large number of larvae (300–1,000) that had to be sorted to prepare a single sample limited the number of samples that we could prepare. More data are clearly required to explain species-specific differences in the relationship between Cd contamination levels in *Chaoborus* and their MT-like protein levels.

Concentrations of larval Cd and MT-like protein

The concentrations of MT-like protein in *Chaoborus* were directly related to larval Cd concentrations (Fig. 1a, $p < 0.01$). Several previous studies have reported a linear relationship between the concentrations of MT and those of Cd in aquatic animals [11,15,16]. However, a curvilinear relationship also fits our data well ($p < 0.001$), i.e., there appears to be a plateau in concentrations of the MT-like protein at approximately 200

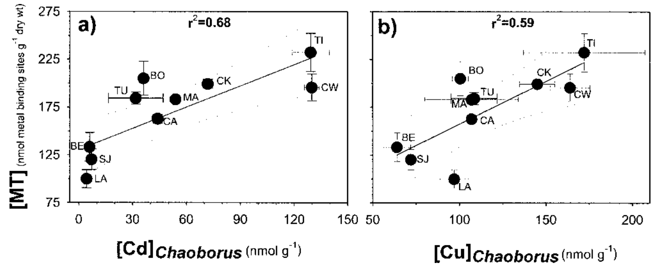

Fig. 1. Metallothionein-like protein concentrations (MT ± standard error) in larvae of the genus *Chaoborus* as a function of their (**a**) Cd concentrations (± standard deviation) and (**b**) Cu concentrations (± standard deviation). Data for the manipulated Lake Turcotte, Quebec, Canada (TU), were excluded from statistical analyses. Each point corresponds to a particular lake (see Table 1 for lake codes).

nmol binding sites/g dry weight (Fig. 1a). We would require data from larger number of lakes to choose credibly between these alternatives.

In contrast, there was no relationship between the concentrations of larval Zn and the MT-like protein ($p = 0.53$), a result that is consistent with the reported ineffectiveness of ambient Zn in inducing MT-like proteins both in the laboratory [17] and in the field [11]. However, there was a significant relationship between larval Cu and MT-like protein concentrations (Fig. 1b, $p = 0.01$). Since we do not see a correlation between MT and external ambient Cu, we do not think that Cu is acting to induce MT biosynthesis. Rather, we suggest that MT concentrations increase as a result of Cd exposure and that this induced MT then acts as a competitive ligand within the cytosol and sequesters part of the available Cu [18]. Since copper is an essential micronutrient, the organism will tend to compensate for this lowering of the internal free Cu^{2+} concentration by increasing total copper levels in the cytosol. The overall result is an increase in cytosolic MT, Cd, and Cu and the observation of a correlation between Cd and Cu concentrations in larvae ($r^2 = 0.95$, $p < 0.01$).

If the rate of metal influx exceeds the net rate of metal-binding protein biosynthesis, incoming excess metal could bind nonspecifically to other intracellular ligands. This phenomenon, often termed spillover [19], would correspond to the onset of potential adverse effects of the metal on the animal. We evaluated spillover in a simplistic way by calculating the ratio of tissue [Cd] to [MT-like protein] (Table 2). The MT-like protein concentrations in *Chaoborus* larvae were more than sufficient to bind all of the Cd in the insect, suggesting that no Cd spillover occurred. Although this result is consistent with the presence of this animal in metal-contaminated lakes, our simplistic scenario ignores the presence of Cu that is also likely to be bound to the MT-like protein and the fact that an unknown proportion of the Cd is not in the cytosol and thus not bound to MT.

Relationships between MT, Cd in the insect, and Cd in water

Despite an extremely wide range of Cu and Zn concentrations in our study lakes (Table 1), *Chaoborus* Cu and Zn varied little among lakes ($p > 0.05$; Table 2), suggesting that *Chaoborus* larvae are able to regulate the concentrations of these essential metals [3,20]. Ignoring highly manipulated Lake Turcotte, larval Cd concentrations were directly related to those in water (Fig. 2a, $p < 0.01$). Lake Turcotte remains an outlier even if we take into account the competitive influence of hydrogen ions and other cations on Cd bioaccumulation using the approach described by Hare and Tessier [3,21]. Thus, we chose to exclude Lake Turcotte from our statistical analyses but to retain it for comparative purposes in the figures. Metallothionein-like protein concentrations in *Chaoborus* were directly related to concentrations of the free Cd^{2+} ion (Fig. 2b, $p < 0.001$). A direct relationship has also been reported for a bivalve mollusk [11], but there have been few other comparable studies in nature. If all of the Cd in *Chaoborus* were in the cytosol, then we might expect to observe a stronger relationship between MT and Cd in *Chaoborus* ($r^2 = 0.68$) than between MT in *Chaoborus* and Cd in water ($r^2 = 0.89$). Based on the r^2 values for these relationships, the reverse was the case, suggesting that part of the Cd in *Chaoborus* is bound to cell fractions other than MT that are less bioreactive (granules, membranes, etc.). The corollary to this observation is that the concentrations of MT-like protein in larvae of the insect *Chaoborus* could thus be used to monitor the concentrations of bioreactive Cd, i.e., the concentration of internal Cd that was able to initiate a biochemical response.

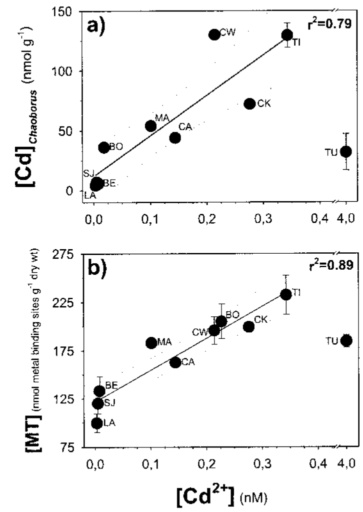

Fig. 2. Relationships between (**a**) Cd concentrations in larvae of the genus *Chaoborus* (± standard deviation) and free Cd ion concentrations and (**b**) metallothionein-like protein concentrations (MT ± standard error) in larvae of the genus *Chaoborus* and free Cd ion concentrations. Data for the manipulated Lake Turcotte, Quebec, Canada (TU), were excluded from statistical analyses. Each point corresponds to a particular lake (see Table 1 for lake codes).

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REFERENCES

1. Saether OA. 1972. Chaoboridae. In Elster HJ, Ohle W, eds, *Das Zooplankton der Binnengewässer*. E. Schweizerbart'sche, Stuttgart, Germany, pp 257–304.
2. Hare L, Carter JCH. 1986. The benthos of a natural West African lake, with emphasis on the diel migrations and lunar and seasonal periodicities of the *Chaoborus* populations (Diptera, Chaoboridae). *Freshw Biol* 16:759–780.
3. Croteau M-N, Hare L, Tessier A. 1998. Refining and testing a trace metal biomonitor (*Chaoborus*) in highly acidic lakes. *Environ Sci Technol* 32:1348–1353.
4. Roesijadi G. 1992. Metallothioneins in metal regulation and toxicity in aquatic animals. *Aquat Toxicol* 22:81–114.
5. Yamamura M, Suzuki KT, Hatakeyama S, Kubota K. 1983. Tolerance to cadmium and cadmium-binding proteins induced in the midge larva, *Chironomus yoshimatsui* (Diptera, Chironomidae). *Comp Biochem Physiol C* 75:21–24.
6. Aoki Y, Hatakeyama S, Kobayashi N, Sumi Y, Suzuki T, Suzuki KT. 1989. Comparison of cadmium-binding protein induction among mayfly larvae of heavy metal resistant (*Baetis thermicus*) and susceptible species (*B. yoshinensis* and *B. sahoensis*). *Comp Biochem Physiol C* 93:345–347.
7. Everard L, Swain R. 1983. Isolation, characterization and induction of metallothionein in the stonefly *Eusthenia spectabilis* following exposure to cadmium. *Comp Biochem Physiol C* 75:275–280.
8. Croteau M-N, Hare L, Tessier A. 2001. Differences in Cd accumulation among species of the lake-dwelling biomonitor *Chaoborus*. *Can J Fish Aquat Sci* 58:1737–1746.
9. Carignan R, Rapin R, Tessier A. 1985. Sediment pore-water sam-

pling for metal analysis: A comparison of techniques. *Geochim Cosmochim Acta* 49:2493–2497.
10. Dutton MD, Stephenson M, Klaverkamp JF. 1993. A mercury saturation assay for measuring metallothionein in fish. *Environ Toxicol Chem* 12:1193–1202.
11. Couillard Y, Campbell PGC, Tessier A. 1993. Response of metallothionein concentrations in a freshwater bivalve (*Anodonta grandis*) along an environmental cadmium gradient. *Limnol Oceanogr* 38:299–313.
12. Tipping E. 1994. WHAM—A chemical equilibrium model and computer code for waters, sediments, and soils incorporating a discrete site/electrostatic model of ion-binding by humic substances. *Comput Geosci* 20:973–1023.
13. Postma JF, Kyed M, Admiraal W. 1995. Site specific differentiation in metal tolerance in the midge *Chironomus riparius* (Diptera, Chironomidae). *Hydrobiologia* 315:159–165.
14. Hensbergen PJ. 1999. Metallothionein in *Orchesella cincta*. PhD thesis. Vrije Universiteit, Amsterdam, The Netherlands.
15. Bebianno MJ, Langston WJ. 1991. Metallothionein induction in *Mytilis edulis* exposed to cadmium. *Mar Biol* 108:91–96.
16. Wang D, Couillard Y, Campbell PGC, Jolicoeur P. 1999. Changes in subcellular metal partitioning in the gills of freshwater bivalves (*Pyganodon grandis*) living along an environmental cadmium gradient. *Can J Fish Aquat Sci* 56:774–784.
17. Roesijadi G, Unger ME, Morris JE. 1988. Immunochemical quantification of metallothioneins of a marine mollusc. *Can J Fish Aquat Sci* 45:1257–1263.
18. Gully JR, Mason AZ. 1993. Cytosolic redistribution and enhanced accumulation of Cu in gill tissue of *Littorina littorea* as a result of Cd exposure. *Mar Environ Res* 35:53–57.
19. Mason AZ, Jenkins KD. 1995. Metal detoxification in aquatic organisms. In Tessier A, Turner DR, eds, *Metal Speciation and Bioavailability in Aquatic Systems.* Wiley, New York, NY, USA, pp 479–608.
20. Hare L, Tessier A. 1998. The aquatic insect *Chaoborus* as a biomonitor of trace metals in lakes. *Limnol Oceanogr* 43:1850–1859.
21. Hare L, Tessier A. 1996. Predicting animal cadmium concentrations in lakes. *Nature* 380:430–432.