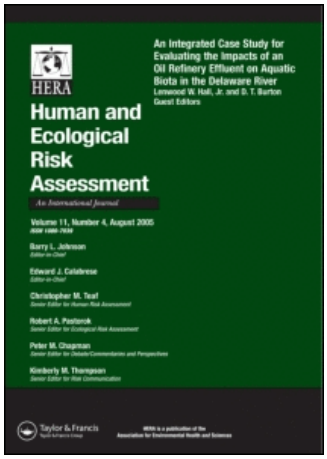

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Subcellular Distribution of Cadmium and Nickel in Chronically Exposed Wild Fish: Inferences Regarding Metal Detoxification Strategies and Implications for Setting Water Quality Guidelines for Dissolved Metals

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ABSTRACT

The objective of this study was to investigate metal detoxification in chronically exposed juvenile yellow perch (YP: *Perca flavescens*) and to field test the commonly assumed threshold toxicity model. Fish were collected from lakes located along a cadmium (Cd) and nickel (Ni) concentration gradient. Ambient dissolved metal concentrations were measured to evaluate exposure and total hepatic metal concentrations were determined as a measure of metal bioaccumulation. Hepatic metal partitioning among potentially metal-sensitive fractions (heat-denatured proteins, organelles) and detoxified metal fractions (metallothionein) was determined after differential centrifugation of YP liver homogenates. Major proportions of hepatic Cd were found in the heat-stable cytosolic peptides and proteins fraction (HSP; including metallothioneins), whereas Ni was mainly found in the potentially metal-sensitive heat-denaturable proteins fraction (HDP). For these chronically exposed fish there was no threshold exposure concentration below which binding of Cd or Ni to the heat-denaturable protein fraction or the organelle fraction did not occur. Metal detoxification was clearly incomplete and *P. flavescens* was subject to some metal-related stress, as evidenced notably by endocrine perturbations. Similar subcellular partitioning results were obtained when juvenile yellow perch were transferred from a reference lake to a Cd-contaminated lake and Cd accumulation was followed over time; there was no accumulation threshold below which Cd binding to the putative metal-sensitive fractions (HDP and organelles) did not occur. The presence of Cd and Ni in these fractions, even for low exposure concentrations and

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low hepatic accumulation, contradicts the threshold toxicity model that underpins metal toxicology theory and that is implicitly used in setting water quality guidelines for metals. Chronically exposed YP appear to have settled for a tradeoff between the cost of turning on their detoxification apparatus at full capacity, to completely suppress metal binding to metal-sensitive sites, and the alternative cost of allowing some binding of inappropriate metals to metal-sensitive sites.

Key Words: yellow perch, Cd, Ni, bioaccumulation, detoxification, threshold toxicity model, PNEC.

INTRODUCTION

Attempts to define the impacts of metals on aquatic ecosystems normally involve laboratory toxicity tests and, to a much lesser extent, field observations on impacted indigenous populations. Environmental Risk Assessment (ERA) approaches for metals have tended to rely on the former approach, comparing a "predicted no effect concentration" (PNEC) with either predicted or measured environmental concentrations. Virtually all PNECs for metals are currently derived from single species laboratory toxicity tests, the results of which are used to develop species sensitivity distributions with respect to metal M, assuming that this distribution represents the distribution of species sensitivities in a generic and diverse aquatic community (for an example of this approach, see Brix *et al.* (2001)). The PNEC may be chosen to protect a desired proportion of the (aquatic) species (e.g., 90, 95, or 99%), depending on the nature of the receiving water body and the desired degree of protection (Warne 2001). Until very recently, PNECs have been expressed in most jurisdictions in terms of dissolved metal (*i.e.*, no consideration of metal speciation in the water column; no consideration of diet-borne metal).[1]

The PNEC concept implicitly assumes that there is some threshold metal concentration below which the metal will not cause any deleterious effects. This assumption is consistent with classical metal toxicology concepts (Mason and Jenkins 1995). Many metals are biochemically essential, participating in reversible metal complexation reactions and playing key roles as co-enzymes, as electron acceptors or donors (oxidation-reduction reactions) and as allosteric promoters (affecting molecular conformations) (Finney and O'Halloran 2003). Nonessential metals that enter living cells are subject to the same complexation reactions, and metal toxicity is generally thought to involve the binding of "inappropriate" metals to physiologically important sites, leading to (i) blocked functional groups of biomolecules (often thiols); (ii) displacement of essential metals from their normal sites within biomolecules; (iii) changed conformation (and activity) of biomolecules. The overall response to increasing metal exposure is often presented as sigmoidal in shape, with a threshold below which the organism shows no signs of toxicity. It is generally assumed that

[1]Under the influence of the ongoing metals risk assessments in Europe (and with the increasing acceptance of the Biotic Ligand Model in regulatory agencies), there is widespread agreement that metal speciation in the water column (and water chemistry in general) must be taken into account (ICMM 2007).

below this threshold the organism has managed to prevent the binding of inappropriate metals to physiologically important sites, whereas above the threshold such binding occurs and leads to overt toxicity (Mason and Jenkins 1995).

In principle, the binding of inappropriate metals to physiologically important sites should be detectable analytically, by determining subcellular metal partitioning. Both chromatographic analyses (cytosol only) and differential centrifugation (cytosol + organelles) have been used for this purpose (Sanders and Jenkins 1984; Wallace *et al.* 2003; Giguère *et al.* 2006). The goal of such methods is to distinguish between "potentially metal-sensitive" fractions and "metal-detoxified fractions" within the cell. Conceptually, the metal-sensitive fraction should include physiologically sensitive molecules (small peptides such as glutathione; metalloenzymes; DNA or RNA) and organelles (mitochondria, endoplasmic reticulum and nuclei) (Wallace *et al.* 2003; Wang and Rainbow 2006). To protect these metal-sensitive targets, a variety of subcellular systems have evolved to permit the accumulation, regulation, and immobilization of trace metals (Mason and Jenkins 1995), including metal binding proteins (such as metallothionein), lysosomes, granules, and membrane-bound vesicles. Nonessential metals associated with these subcellular fractions are normally considered to have been detoxified (Wallace *et al.* 2003; Wang and Rainbow 2006).

Within the scientific framework outlined earlier, we set out to test the validity of the toxicity threshold model in the field, for the nonessential metals Cd and Ni. More specifically, we sought to identify relationships between the subcellular partitioning of these metals in a biomonitor species, yellow perch (YP: *Perca flavescens*), and the metal-exposure regime to which the fish had been exposed prior to collection. Two types of data were collected: (i) *spatial* data, from studies in which yellow perch were collected from lakes located along an existing metal gradient, downwind and downstream from past/current metal smelters; and (ii) *temporal* data, from field manipulations in which juvenile YP from a "clean" lake were subjected to a marked change in their metal exposure regime (either by transplantation to a metal-contaminated environment, or by changes to the metal content of their diet). In both cases, we determined how metal subcellular partitioning responded as a function of the metal exposure. The total metal concentration in the liver was taken as a measure of metal exposure, and we looked for evidence of a threshold response. We then tested for links between metal subcellular partitioning and the manifestation of deleterious effects at the organism level (physiology, endocrine and metabolic status, growth, reproductive status).

MATERIALS AND METHODS/DESCRIPTION OF EXPERIMENTAL APPROACH

Study Area

Our main study area was located in the Abitibi region, centered around the city of Rouyn-Noranda, approximately 600 km northwest of Montréal, Québec, Canada. A copper smelter has been in operation since 1927 in the city of Rouyn-Noranda, and although emissions are currently controlled, lakes located downwind from this smelter are contaminated with metals such as Cd, Cu, and Zn due to historical atmospheric deposition (Couillard *et al.* 2004). In addition to these direct inputs from

the smelter, some lakes in this region are also contaminated by runoff from mine tailings and mineralized outcrops. Additional complementary sampling was carried out in lakes in the Sudbury, Ontario, region, where historical inputs from nickel mining and smelting operations have affected ambient metal concentrations. Many Sudbury lakes present concentrations of Cu and Ni above background concentrations whereas many lakes in the Rouyn-Noranda area exhibit high concentrations of Cd, Cu and Zn. Within these study areas we chose lakes that were expected to have low, moderate or high ambient metal concentrations, based on their relative proximity to the local smelters and the prevailing wind direction.

Biosentinel Selection

We chose yellow perch (*Perca flavescens*) as the sentinel organism for our studies, based on the following selection criteria: ubiquity, abundance, relative immobility, ease of sampling, metal tolerance, metal bioaccumulation capacity; available physiological and behavioral data, ecological role. Abundant and widely distributed across North America, this species does not travel over long distances and therefore its metal body burden tends to represent local sources (Aalto and Newsome 1990; Hontela *et al.* 1995).

Metals Studied

The chosen lakes represent a gradient in ambient concentrations of Cd, Cu, Ni, and Zn (Table 1), but in the present context (where we are seeking to detect a threshold response) we have chosen to emphasize those metals that are *non-essential* to fish (Cd, Ni) so as to be able to detect "inappropriate" metal binding without ambiguity. Cadmium and nickel are high profile metals under the Canadian Environmental Protection Act (CEPA). At the biochemical level, one metal (Cd) is recognized to be an excellent inducer of metallothionein (MT) biosynthesis (in the laboratory) and to bind to MT in the cytosol, whereas Ni is a "harder" cation than Cd in the Hard-Soft Acid-Base classification and is not recognized as a potent inducer of metallothionein synthesis (Frausto da Silva and Williams 2001).

Spatial Study—Sampling and Analysis

The lakes were characterized at a geochemical level, to measure the ambient exposure of the fish to metals. For each lake, we studied the response of the indigenous fish to the metal gradient at the cellular/biochemical level (metal concentrations in target organs, subcellular metal partitioning in the liver), and this was linked to effects at the physiological level (endocrine and metabolic status).

Water

Water samples were collected in the epilimnion of each of the studied lakes, using *in situ* diffusion samplers suspended 0.5 to 1 m above the lake bottom and at least 1.5 m under the surface in the epilimnion of each lake. After equilibration with the ambient water, they were retrieved and subsamples for trace metal, major cation, major anion, and organic carbon determinations were removed. The ambient pH was also measured in water collected at a depth of 0.5 m, near the diffusion samplers,

Table 1. Total dissolved metal concentrations, [M], free-metal ion concentrations, [M^{z+}], dissolved organic carbon, [DOC], and pH in the epilimnetic water of the studied lakes (Giguère *et al.* 2005).

Lake	[Cd] (nM)	[Cu] (nM)	[Ni] (nM)	[Zn] (nM)	[Cd^{2+}] (nM)	[Cu^{2+}] (nM)	[Ni^{2+}] (nM)	[Zn^{2+}] (nM)	[DOC] ($mg \cdot L^{-1}$)	[Ca] (μM)	pH
Rouyn-Noranda											
Dufault (DU)	6.7	180	10	1100	2.3	0.025	4.9	379	5.3	420	7.46
Osisko (OS)	1.6	140	20	110	0.25	<0.001	2.9	3.4	3.1	640	9.54
Vaudray (VA)	0.5	42	10	60	0.03	0.001	2.1	5.9	8.8	80	7.20
Opasatica (OP)	0.3	33	10	20	0.02	<0.001	3.1	1.7	7.7	220	7.75
Sudbury											
Hannah (HA)	2.4	370	2500	50	1.0	0.44	1603	24	3.7	270	7.02
Raft (RA)	1.7	130	1820	160	0.88	0.094	1284	80	2.6	90	6.68
Wavy (WA)	1.5	110	900	120	1.2	11	752	100	2.8	40	5.01
Laurentian (LA)	0.6	140	840	30	0.26	0.075	518	12	4.6	110	6.37
ratio max/min[a]	**22**	**11**	**250**	**55**	**115**	**$>1 \times 10^5$**	**763**	**223**	**3**	**16**	**3.4×10^4**

[a]Ratio of "maximum concentration : minimum concentration", as an expression of the water chemistry gradient. For pH, the ratio was calculated on the basis of the H^+-ion concentrations.

when they were retrieved. Sample collection from the diffusion samplers is described in detail in a previous paper (Giguère *et al.* 2004), as are the analytical methods used to determine the concentrations of the various analytes: major cations (Ca, Mg, Na, K); major anions (Cl, SO_4, NO_3); trace metals (Cd, Cu, Ni, Zn; Al, Fe, Mn); and dissolved organic and inorganic carbon.

Metal speciation at equilibrium was calculated for each lake with the Windermere Humic Aqueous Model (WHAM 6.0.1) (Centre for Hydrology and Ecology 2001). Details of the calculations can be found in Giguère *et al.* (2004).

Fish

Juvenile YP were collected from each lake using a seine net and kept alive in aerated coolers for <3 h. In the laboratory, the fish were killed with a blow to the head and their liver was removed for study. Livers were kept in acid-washed polypropylene containers in liquid nitrogen until analysis (described later).

The liver was chosen as the target organ on the basis of a previous study on indigenous YP in which this organ was shown to accumulate high Cd concentrations. The contribution of this organ to fish Cd and Cu burdens increased with increasing ambient metal concentrations (Giguère *et al.* 2004), a trend that is consistent with the important role of the liver in dealing with excess metals. To minimize possible variability related to fish age (Giguère *et al.* 2004), we collected juvenile fish of the same size class (7–10 g) for the spatial study. Average fish age, as evaluated on a subsample of 10 to 18 fish from each lake, varied from 1.2 to 2.3 years (Giguère *et al.* 2005). In the earlier study, differences in metal concentrations between 1-year and 2-year old perch were not significant.

For total metal analyses, livers were lyophilized, weighed, and then digested in an autoclave with 70% HNO_3. The digested samples were then analyzed for Cd, Cu, and Zn by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES). Analytical procedures and details of the quality assurance/quality control measurements can be found in Giguère *et al.* (2004, 2005, 2006).

Temporal Studies—Sampling and Analysis

In addition to the spatial study just described earlier, we also carried out caging experiments in which juvenile YP were transferred from an uncontaminated lake (Opasatica) to a heavily contaminated lake (Dufault). Uptake of Cd, Cu, and Zn and changes in metal subcellular partitioning were followed over 70 days (Kraemer *et al.* 2005). A second caging experiment was performed to determine the relative importance of waterborne and diet-borne Cd as sources of cadmium for juvenile YP (Kraemer *et al.* 2006b).

Water

Water samples were collected *in situ* with diffusion samplers, both within the cages and in the external environment. Subsamples were removed from the samplers and analyzed as described earlier for the spatial study. Details can be found in Kraemer *et al.* (2005, 2006b).

Fish

For the habitat-swap experiment, juvenile YP (less than 5 g) were collected from the reference lake (Opasatica) and kept within cages in the metal-contaminated lake (Dufault) for up to 70 d. Because this size class of perch feeds mainly on zooplankton and small aquatic insects (Gingras and Boisclair 2000), we designed enclosures to allow these prey to enter freely thereby exposing perch to both contaminated food and contaminated water. Fish were either transplanted from the reference lake to cages in the contaminated lake or were caged within the reference lake (caged control fish). Each cage initially contained 30–40 fish.

The fish in individual cages were sacrificed after 5, 10, 20, 30, 50, and 70 d of exposure. In addition to caged fish, indigenous perch were also collected from both lakes by seining at the beginning of the experiment and at 2–3 additional times over the summer (n = 7–11 fish at each sampling time). Fish were transported live back to a nearby laboratory (<2 h) where they were killed by a blow to the head after which they were immediately dissected and the liver was removed. Liver samples were combined in groups of five individual livers, stored under liquid nitrogen until thawing for subcellular analyses (described in the following section).

For the diet manipulation study, we used a different caging approach to examine the relative importance of the diet and water as routes of Cd exposure for YP. For this study, juvenile YP (~1.5 g) were collected from the reference lake (Opasatica) and placed in cages located in either Lake Opasatica (2 cages per treatment group; 2 treatments) or Lake Dufault (2 cages per treatment group; 2 treatments). Each cylindrical cage was made of Nitex netting (64 μm mesh-aperture), which prevented the entry of macro-zooplankton; details of cage construction and deployment can be found in Kraemer *et al.* (2006b). Each cage initially contained 10 fish.

The cages were filled by passive entry of lake water through the Nitex netting. Yellow perch were allowed to acclimate to their cages for 24 h and were not fed during this time period. The caged fish in both lakes were then fed daily with macrozooplankton collected from either the reference lake or the Cd-contaminated lake, creating the four desired treatment regimes: reference food and reference water; Cd-contaminated food and reference water; reference food and Cd-contaminated water; and Cd-contaminated food and Cd-contaminated water. Fish were sampled after 15 and 30 d of exposure and the liver was removed and stored in liquid nitrogen for subcellular analyses. For these experiments, liver samples were pooled into groups of 2–5 livers.

Metal Subcellular Partitioning

Differential centrifugation

A differential centrifugation procedure adapted from that of Wallace *et al.* (2003) was used to separate various subcellular compartments: (1) a fraction comprising nuclei, cell membranes, intact cells and connective tissue, termed "nuclei/debris" hereafter; (2) a granule-like or resistant fraction; (3) mitochondria; (4) a fraction combining microsomes and lysosomes; (5) cytosolic heat-stable proteins, including metallothioneins, termed "HSP" hereafter; and (6) cytosolic heat-denatured

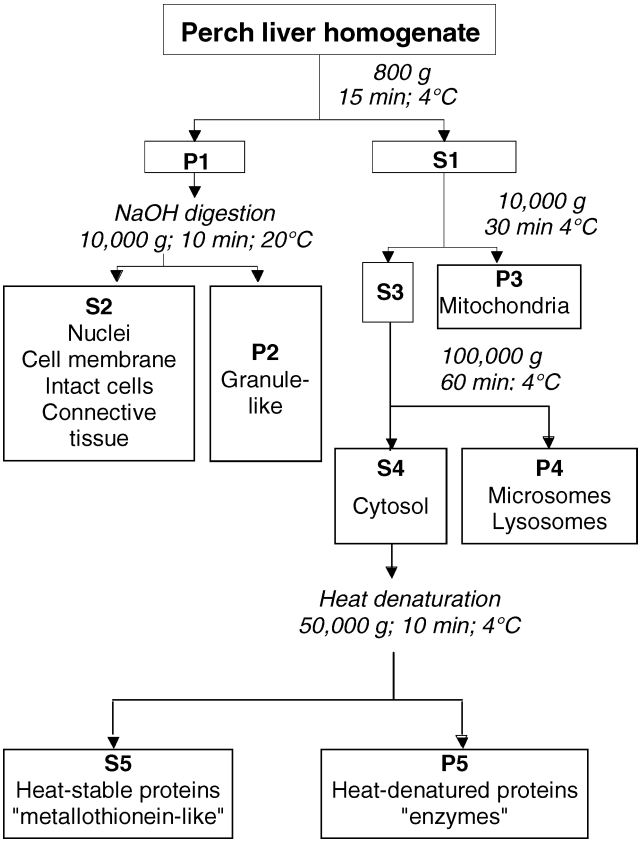

Figure 1. Differential centrifugation method used to separate the various subcellular fractions. Modified from Giguère *et al.* (2006) with permission from Elsevier. P = pellet; S = supernatant.

proteins, termed "HDP" hereafter (Figure 1). Details of the centrifugation approach, a critical evaluation of its strengths and weaknesses, and metal mass balances can be found in Giguère *et al.* (2006). In some cases we employed a less elaborate fractionation scheme, wherein fractions (1) and (2) were combined (as "cellular debris"), as were fractions (3) and (4) (as "organelles").

Metal concentrations in the differential centrifugation fractions

Aliquots taken from each whole homogenate and all centrifugation pellets were digested with nitric acid. Concentrations of Cd and Ni in the digested samples and in the supernatants were measured by ICP-AES. For both the supernatants and the pellets, metal concentrations in a given fraction are expressed on a total organ dry weight basis, for example, total Cd burden (nmol) measured in a particular fraction ÷ weight of the organ (g dry weight). The digestion procedures are described in detail in Giguère *et al.* (2006).

Fish Physiology

Oxidative stress

To evaluate the importance of oxidative stress in juvenile yellow perch captured in lakes located along the metal gradient, we investigated the status of their hepatic antioxidant defense system and looked for evidence of lipid peroxidation (Giguère *et al.* 2005). Glutathione-dependent oxido-reductase enzymes were analyzed on the basis of kinetic enzymatic reactions as monitored by spectrophotometric methods. The activity of the selenium-independent glutathione peroxidase was measured according to the method of Lawrence and Burk (1976). The activity of glutathione-reductase was determined according to the method described by Carlberg and Mannervick (1985), optimized for perch liver. Reduced and oxidized glutathione were measured by reversed phase liquid chromatography followed by a post-column derivatization with *ortho*-phthalaldehyde according to Cossu *et al.* (2000). Lipid peroxidation was quantified by measuring malondialdehyde concentrations according to the colorimetric method of Sunderman *et al.* (1985). Proteins were measured according to the method of Bradford (1976) using bovine albumin as the standard.

Endocrine and metabolic status

Complementary biochemical measurements were carried out on adult (4+), juvenile (1+), and young of the year (YOY) yellow perch (Levesque *et al.* 2002, 2003; Gravel *et al.* 2005). Blood, liver, and muscle samples were collected and analyzed for hormones (cortisol, triiodothyronine T3, thyroxine T4) and various biochemical parameters (glucose, glycogen, lipids). The functional integrity of the interrenal organs secreting cortisol was assessed *in vitro* using the adrenocorticotropic hormone (ACTH) challenge test (Laflamme *et al.* 2000) and a histopathological examination of vital organs such as gills, liver, and kidney was performed (Levesque *et al.* 2003). In addition, reproductive status was estimated by gonad size (gonado-somatic indices or GSI, gonad weight as % of body weight). These measures enabled us to evaluate the endocrine and metabolic status of the indigenous fish living along the metal concentration gradient.

Statistics

In the analysis and interpretation phase of this project, we dealt with various types of data: limnological (habitat quality); geochemical (ambient metal levels); cellular (effectiveness of detoxification at the biochemical level); and whole organism (physiology, endocrine, and metabolic status). Appropriate statistical techniques were used to treat the results within each data type, and to explore the anticipated links among the different hierarchical levels.

Metal concentration differences among lakes were compared using a non-parametric test (Kruskal-Wallis) and a Tukey honestly significant difference (HSD) test was performed on ranks to discriminate among lakes. Our original hypothesis was that non-essential metals would be effectively detoxified in fish collected from lakes at the low end of the metal exposure gradient, but that above a certain threshold metal concentration these metals would "spill over" into potentially metal-sensitive subcellular pools. We were thus looking for a threshold response, particularly for

relationships between metal exposure and accumulation in potentially metal-sensitive subcellular fractions. To test this hypothesis, relationships between variables were initially examined in bivariate scatter plots. When bivariate plots indicated a possible linear (non-threshold) relationship, simple regression models were tested using Statistica software. For each of the significant relationships presented, we verified if the residuals were normally distributed and if some outliers had biased the regression coefficients. For all statistical analyses, p-levels of 0.05 were used as the threshold for statistical significance.

RESULTS

Exposure Gradient

Aqueous metal concentrations varied markedly from lake to lake. The dissolved metal gradient (defined as the ratio of the highest observed metal concentration/lowest metal concentration for the entire set of lakes) followed the order [Ni] (250) > [Zn] (55) > [Cd] (22) > [Cu] (11) (Table 1). The two lakes used in the transplantation and caging studies, lakes Dufault and Opasatica located in the Rouyn-Noranda area, represented the largest Cd gradient, dissolved Cd concentrations in Lake Dufault being ~22 times higher than in Lake Opasatica.

Spatial Gradient Study

Metal accumulation (Cd, Ni, Cu, Zn)

In lakes at the high end of our exposure gradient, Cd, Cu, and Ni (but not Zn) accumulated in yellow perch liver to concentrations well above background tissue values (Table 2). The $[M]_{max}/[M]_{min}$ ratio for hepatic Cu was similar to that for the lake waters (~13), whereas the ratios for Cd (14), Ni (36), and especially Zn (1.9) were lower than those for the lake waters (compare the last rows in Tables 1

Table 2. Hepatic metal concentrations in indigenous yellow perch collected from the study lakes (Giguère *et al.* 2005).

Lake	[Cd] ($nmol \cdot g^{-1}$)	[Cu] ($nmol \cdot g^{-1}$)	[Ni] ($nmol \cdot g^{-1}$)	[Zn] ($\mu mol \cdot g^{-1}$)
Rouyn-Noranda				
Dufault (DU)	421 ± 34	476 ± 75	5.6 ± 4.4	2.18 ± 0.07
Osisko (OS)	282 ± 19	1360 ± 537	6.8 ± 3.2	2.07 ± 0.02
Vaudray (VA)	320 ± 62	335 ± 35	4.1 ± 1.4	2.18 ± 0.17
Opasatica (OP)	36 ± 1	146 ± 10	2.3 ± 0.9	1.58 ± 0.05
Sudbury				
Hannah (HA)	92 ± 16	1380 ± 355	26.6 ± 4.1	1.97 ± 0.18
Raft (RA)	509 ± 82	1920 ± 721	82.8 ± 11.7	2.63 ± 0.21
Wavy (WA)	118 ± 6	399 ± 29	20.9 ± 2.4	1.80 ± 0.06
Laurentian (LA)	42 ± 9	265 ± 63	16.1 ± 3.3	1.39 ± 0.05
ratio max/min	**14**	**13**	**36**	**1.9**

and 2). Clearly yellow perch handle zinc differently from the three other metals, maintaining hepatic concentrations of this essential metal within very narrow limits.

Metal subcellular partitioning (Cd, Ni)

To evaluate to what extent the accumulated cadmium had been detoxified, we plotted Cd concentrations in the HDP, organelle, and HSP fractions as a function of total hepatic Cd for yellow perch collected from the Rouyn-Noranda lakes. The spatial study was carried out in the summers of 2001 (open and solid circles; Figure 2; Giguère *et al.* 2006) and 2003 (open triangles; Figure 2; Kraemer *et al.* 2006a). Similar trends were observed for both studies; Cd concentrations in each of the subcellular fractions (HDP, organelles and HSP) increased linearly ($p \leq .001$) with increasing total hepatic Cd and no threshold was observed.

Similarly, a plot of Ni concentrations in the HDP, organelle and HSP fractions as a function of total hepatic Ni for yellow perch collected from the Sudbury and Rouyn-Noranda lakes showed linear trends with no hint of a threshold response (Figure 3).

Fish health status

Direct effects of metals on yellow perch endocrine systems were detected in a pattern linked to liver metal concentrations in lakes located along the metal exposure gradient, in both the Rouyn-Noranda and Sudbury areas. Adult and 1+ perch collected from the more contaminated lakes exhibited an attenuated cortisol stress response *in vivo* (Figure 4), whereas young of the year (YOY) did not (Gagnon 2004; Gravel *et al.* 2005; Rasmussen *et al.* 2008). The ability of adult and 1+ perch to mount a stress response (as indicated by their ability to raise their cortisol levels following a standardized confinement stress) decreased as a function of the accumulated Cd concentration. In addition, lower levels of the thyroid hormones T3 and T4, key hormones for regulation of intermediary metabolism and osmoregulation, were measured in adult YP from contaminated lakes but not in 1+ fish (Levesque *et al.* 2003, Gravel *et al.* 2005), suggesting that an exposure of at least one year is necessary to induce this specific endocrine impairment.

In addition to the endocrine response, lower condition factor and anomalies in intermediary metabolism and use of energy reserves were observed in adult YP from contaminated lakes sampled in both study regions. The seasonal summer to fall build-up of liver glycogen and triglycerides that occurs in fish from the reference lakes was not observed in contaminated lakes (Levesque *et al.* 2002). Moreover, enzymatic activities mediating lipid, carbohydrate and protein metabolism were altered. Results for the 1+ yellow perch suggested an impaired capacity to mobilize liver glycogen reserves. Overall, the physiological effects tended to increase in the sequence YOY < 1+ < adult perch. Morphological alterations indicative of pathology were observed in gills, thyroid, and interrenal tissues of fish from contaminated lakes (Levesque *et al.* 2003), whereas the gonads appeared healthy but at an earlier maturation stage compared to fish from reference lakes.

In contrast to the clear evidence for metal-induced perturbations of the yellow perch endocrine systems, the indications for oxidative stress in perch livers are less persuasive. Glutathione concentrations and glutathione reductase activities did

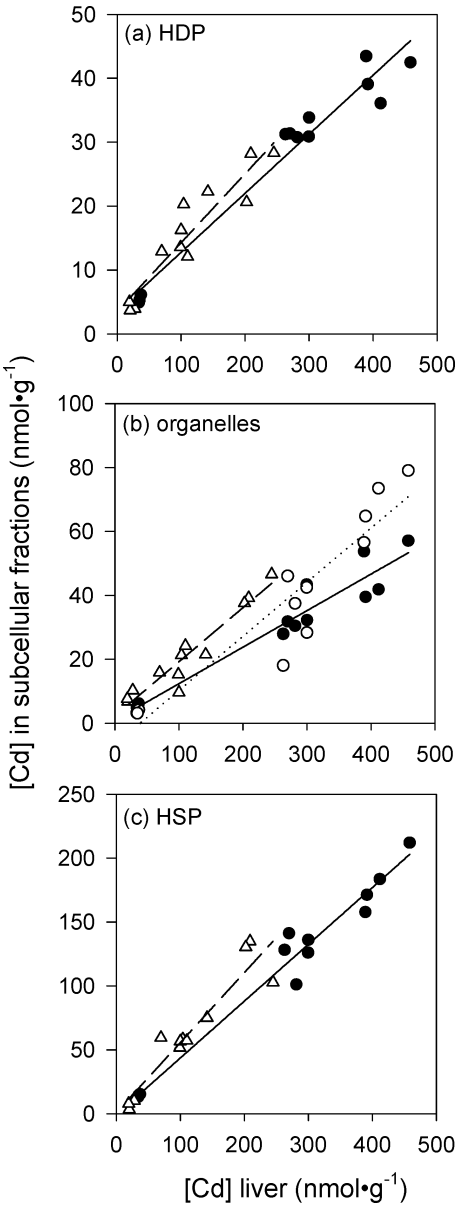

Figure 2. Cadmium concentrations in (a) the heat-denaturable protein fraction (HDP), (b) the organelle fraction, and (c) the heat-stable protein fraction (HSP) as a function of total hepatic Cd in juvenile perch sampled from the gradient of Rouyn-Noranda lakes. Data from the 2001 spatial study are represented by circles; in panel b solid circles represent lysosomes and microsomes, open circles represent mitochondria (Giguère *et al.* 2006). Data from the 2003 spatial study are represented by open triangles (Kraemer *et al.* 2006a). Each point represents data for individual fish collected at a single sampling station.

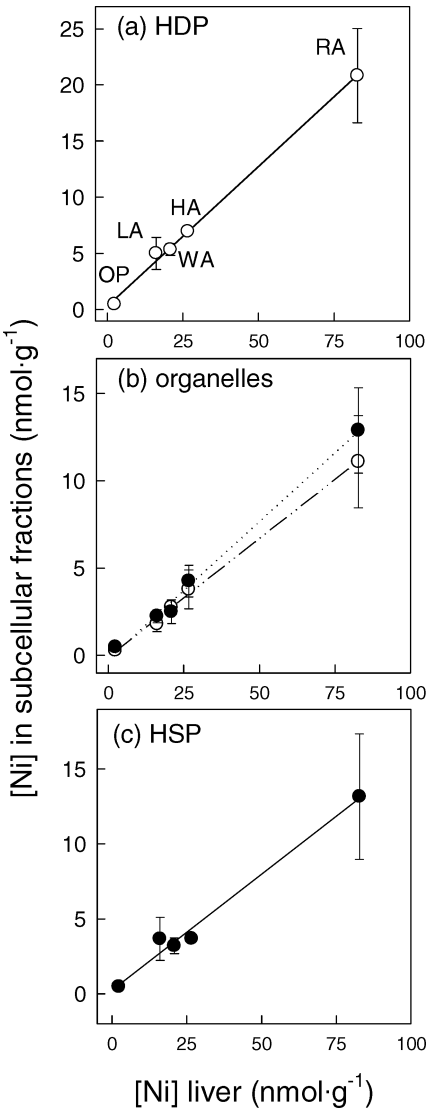

Figure 3. Nickel concentrations in (a) the heat-denaturable protein fraction (HDP), (b) the organelle fraction, and (c) the heat-stable protein fraction (HSP) as a function of total hepatic Ni in juvenile perch sampled from the gradient of Rouyn-Noranda and Sudbury lakes. Data are from the 2001 spatial study (Giguère *et al.* 2006); in panel b solid circles represent lysosomes and microsomes, open circles represent mitochondria. Each point represents data for fish collected at a single sampling station (mean ± SD; N = 3). See Table 1 for lake abbreviations.

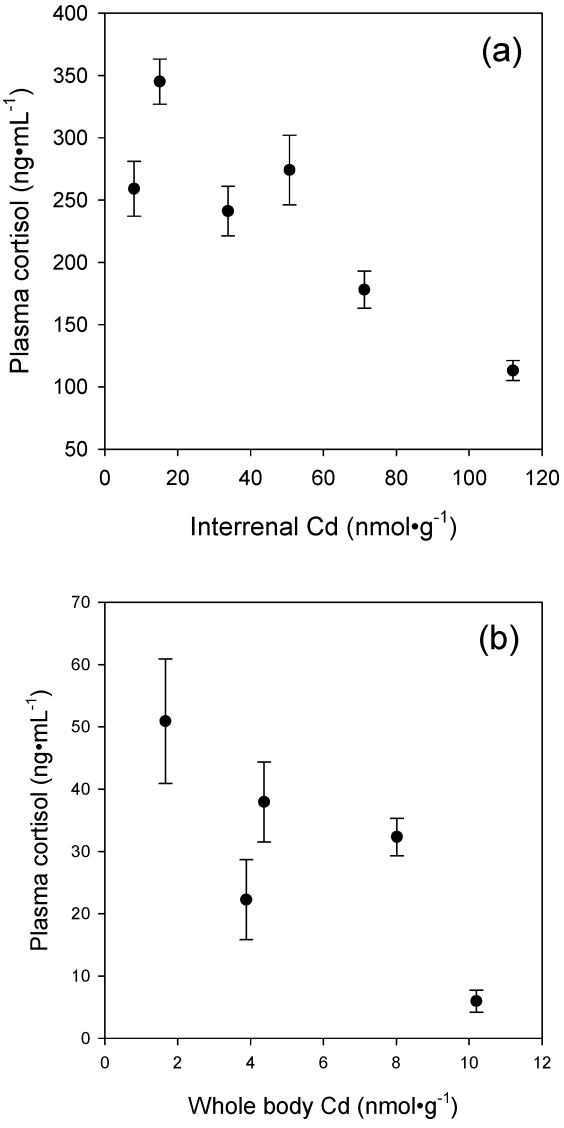

Figure 4. Cortisol levels (mean ± SE) in perch after a standardized stress test, as a function of bioaccumulated Cd. (a) Plasma cortisol levels in adult perch; (b) whole body cortisol levels in juvenile 1+ perch. Data taken from Laflamme *et al.* (2000) and Gravel *et al.* (2005). Each point represents data for fish collected at a single sampling station (N = 10–25).

decrease along the exposure gradient (Giguère *et al.* 2005), but in contrast increased hepatic Cu concentrations were associated with reduced lipid peroxidation, a response opposite to that predicted from basic principles. Overall the results suggest that oxidative stress will not have direct repercussions on the health of the perch at the individual level. We speculate that the observed increase in metallothionein concentrations with increasing accumulated metals (Laflamme *et al.* 2000, Giguère *et al.* 2006) might afford protection against reactive oxygen species.

Transplantation Study

Hepatic Cd concentrations in YP transplanted from Lake Opasatica to Lake Dufault increased markedly over the 70-day experiment, rising from 23 to ~350 nmol $Cd \cdot g^{-1}$ (Kraemer *et al.* 2005). As we were interested in how subcellular Cd concentrations changed as a function of exposure, we plotted the Cd concentrations in the various subcellular fractions as a function of total hepatic Cd concentrations. Cadmium concentrations in the HDP ($r^2 = 0.49$, $p \leq .01$), organelle ($r^2 = 0.98$, $p \leq .001$) and HSP ($r^2 = 0.94$, $p \leq .001$) fractions all increased linearly as a function of increasing total hepatic concentrations, with no threshold concentration below which Cd was absent from the putative metal-sensitive fractions, HDP and organelles (Figure 5).

Diet Manipulation Study

In the experiment examining the relative importance of food versus water as sources of Cd for YP, fish were exposed to Cd in food only, water only or a combination of these two routes, and Cd concentrations were measured in the liver on days 0, 15, and 30. We again expressed changes in Cd concentrations in the subcellular fractions as a function of exposure (*i.e.*, total hepatic Cd concentrations) for all three times (Figure 6). Here we see that unlike the response observed in the transplantation study, a nonlinear response ($r^2 = 0.70$, $p \leq .001$) was observed for the HDP fraction, suggesting a threshold of approximately 80 $nmol \cdot g^{-1}$ total hepatic Cd (Figure 6a). Below this threshold, Cd concentrations in this fraction remained relatively constant, but for concentrations above ~80 $nmol \cdot g^{-1}$, Cd began to increase in the HDP fraction. This threshold response was not observed in the organelle fraction, where Cd concentrations increased in this fraction in a linear manner with increasing total hepatic Cd concentrations ($r^2 = 0.37$, $p \leq .01$; Figure 6b). Similarly, Cd concentrations in the HSP fraction increased linearly with increasing total hepatic Cd ($r^2 = 0.33$, $p \leq .01$; Figure 6c).

As we were interested in why an apparent threshold was observed in the HDP fraction during this study and not in the field transplantation study, we graphed changes in Cd concentrations in the HDP fraction for perch receiving each of the three Cd treatments used in the Cd source manipulation study: Cd via food only, Cd via water only, and Cd via food + water (Figure 7). There were no significant increases in the Cd concentrations in the HDP fraction in the liver of perch receiving Cd via food only (Figure 7a; $p > .05$). However, an apparent threshold of 80 $nmol \cdot g^{-1}$ total hepatic Cd was observed in perch receiving Cd via water only (Figure 7b; $r^2 = 0.94$, $0 \leq .001$). Similar to the transplantation study, Cd concentrations increased

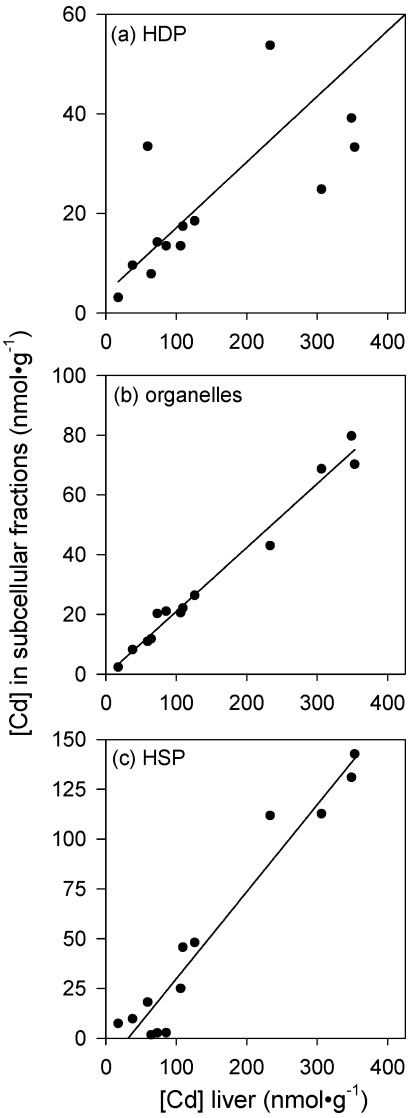

Figure 5. Cadmium concentrations in (a) the heat-denaturable protein fraction (HDP), (b) the organelle fraction, and (c) the heat-stable protein fraction (HSP) as a function of total hepatic Cd in juvenile perch transplanted from Lake Opasatica (low Cd exposure) to Lake Dufault (high Cd exposure). Data are from the 2002 habitat swap experiment (Kraemer *et al.* 2005). Each point represents data for individual fish collected at 0, 5, 10, 20, 30, 50, or 70 d during the experiment; these data were then plotted as a function of hepatic Cd.

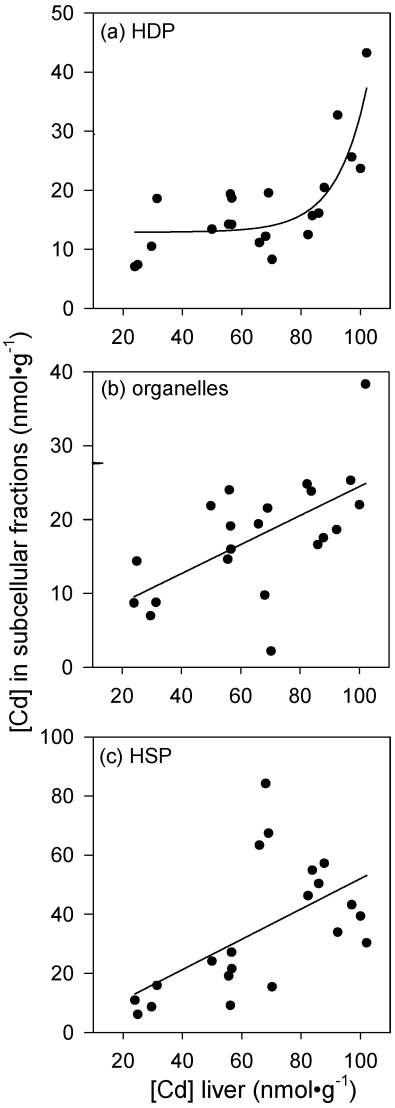

Figure 6. Cadmium concentrations in (a) the heat-denaturable protein fraction (HDP), (b) the organelle fraction, and (c) the heat-stable protein fraction (HSP) as a function of total hepatic Cd in juvenile perch that were exposed to four separate Cd exposure regimes: reference food and reference water; Cd-contaminated food and reference water; reference food and Cd-contaminated water; and Cd-contaminated food and Cd-contaminated water. Reference food and water were from Lake Opasatica; contaminated food and water were from Lake Dufault. Each point represents data for individual fish collected from one of the exposure regimes, at one of three times (0, 15, or 30 d).

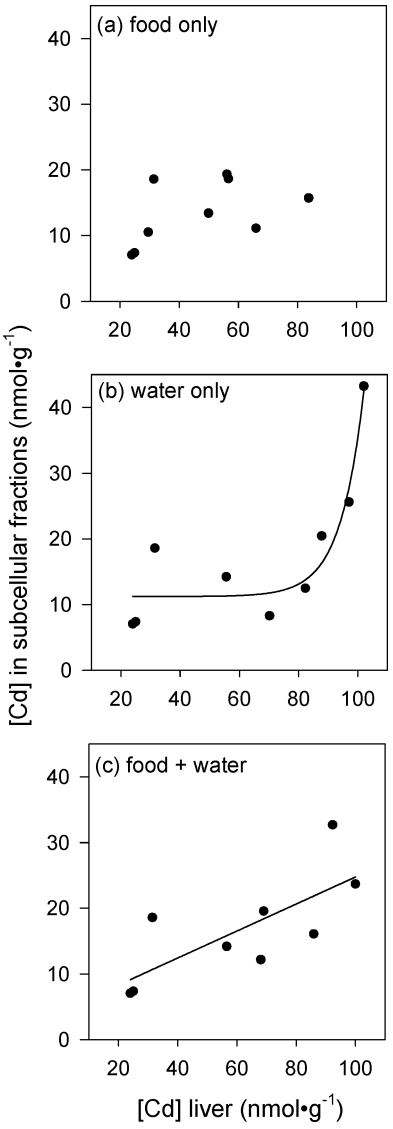

Figure 7. Cadmium concentrations in the heat-denaturable protein fraction (HDP) as a function of total hepatic Cd in juvenile perch that were exposed to: (a) Cd-contaminated food and reference water; (b) reference food and Cd-contaminated water; and (c) Cd-contaminated food and Cd-contaminated water. Reference food and water refer to Lake Opasatica; contaminated food and water refer to Lake Dufault. Each point represents data for individual fish collected from one of the exposure regimes, at one of three times (0, 15, or 30 d).

in the HDP fraction in a linear manner with increasing total hepatic Cd in perch receiving Cd via food + water sources (Figure 7c; $r^2 = 0.70$, $p \leq .01$).

DISCUSSION

The overall objective of this study was to test the validity of the toxicity threshold model in the field, for the nonessential metals Cd and Ni. We sought answers to the following questions: Are indigenous aquatic organisms that are chronically exposed to low to moderate concentrations of nonessential metals able to detoxify the metals successfully? Is there a threshold exposure concentration below which metal detoxification is complete, and above which inappropriate metals bind to metal-sensitive sites?

To answer these questions we adopted two complementary approaches: (i) spatial studies, in which we collected wild YP from lakes representing a spatial gradient in aqueous Cd and Ni concentrations; and (ii) temporal studies, in which metal-naïve YP collected from a reference lake were exposed in the field to high Cd concentrations and followed over time. We used hepatic metal concentrations in the YP as an integrative measure of their past metal exposure (Giguère *et al.* 2004).

Spatial (Inter-Lake) Studies

In the spatial (inter-lake) studies, Cd and Ni accumulated to higher levels in lakes at the high end of our exposure gradient than in the control lakes. The liver and the kidney (including the interrenal tissue) are the organs in which the internal Cd and Ni concentrations are highest. Accumulation of Cd in perch liver is accompanied by the induction of metallothionein-like peptides (Laflamme *et al.* 2000), but the results for the subcellular partitioning of Cd indicate that its detoxification by metallothionein is *incomplete* (*i.e.*, even in the moderately contaminated lakes, some of the Cd is present in subcellular fractions other than metallothionein). There is no indication of a threshold level below which all the Cd is completely bound by the metallothionein (Figure 2). Similarly, detoxification of hepatic Ni is incomplete, with Ni being found in the organelles fraction and in the "enzyme" or heat-denatured proteins fraction. Again, there is no threshold exposure concentration below which Ni does not appear in these potentially metal-sensitive fractions (Figure 3).

Given this evidence for incomplete Cd and Ni detoxification, one might expect to observe toxicity at the cellular and physiological levels. Sub-lethal toxicity is indeed detected in the indigenous fish, the severity of the symptoms (*e.g.*, endocrine impairment) being linked to metal exposure along the contamination gradient (Figure 4). In an earlier paper (Campbell *et al.* 2003), we pointed out that effects of metal contamination on indigenous fish may be direct (physiological) or indirect (foodweb mediated). In the present study we have focused on 1+ perch, a life stage that feeds on plankton in all the lakes (reference and metal-contaminated). We chose to study this life stage in order to minimize any foodweb mediated effects, reasoning that 1+ perch should be relatively unaffected by the changes in the benthic foodweb that have been shown to affect adult perch (Sherwood *et al.* 2002, Kovecses *et al.* 2005). The diagnosis of cortisol impairment in 1+ perch thus provides strong evidence

that the perturbation of the YP endocrine system is caused by direct effects of metals rather than effects mediated through changes in foodwebs.

Temporal (Transplantation) Studies

The caging experiments added a temporal component to the preceding studies. Indeed, we anticipated that the results from the habitat swap and diet manipulation studies would perhaps be more comparable to the results from the earlier laboratory studies, reviewed by Mason and Jenkins (1995), in that in both cases "naïve" organisms were subjected to a step-change increase in metal exposure. In the transplant experiments, Cd accumulated in the four organs studied (gill, gut, liver, kidney) (Kraemer *et al.* 2005). In the liver (the only organ where subcellular partitioning was followed over time), Cd concentrations increased in *all* subcellular fractions; there was no clear threshold accumulation below which the putative metal-sensitive fractions were protected (Figure 5). If the results from the spatial studies and those from the transplant experiment are plotted with the same X-axis (total hepatic Cd), the distributions of subcellular Cd are indistinguishable (data not shown), suggesting that the metal-naïve organisms were behaving in a similar manner to the metal-adapted indigenous fish.

Only in the case of the diet manipulation study did we see some evidence of a threshold response (*i.e.*, Figures 6a and 7b), for the HDP fraction (but not for the organelles). When we teased apart the data for the diet manipulation study, looking at the results for the fish exposed primarily to waterborne Cd or to diet-borne Cd (Figure 7), a clear threshold response was only seen for the water-only exposure. At hepatic Cd concentrations less than ~80 nmol·g^{-1}, Cd concentrations in the HDP (enzyme) fraction did not increase, suggesting that Cd detoxification was reasonably effective (although Cd did increase in the organelle fraction in these fish). In fish from the food-only treatment, hepatic Cd concentrations never exceeded the 80 nmol·g^{-1} threshold, so we cannot evaluate whether the 1+ yellow perch were better able to handle waterborne Cd or diet-borne Cd.

Why did we see an apparent threshold for movement of Cd into the HDP fraction in the diet manipulation experiment (Figure 6a) but not in the habitat swap experiment (Figure 5a)? In the latter experiment total hepatic Cd concentrations after 30 days (~120 nmol·g^{-1}) were somewhat higher than those achieved at the end of the diet manipulation experiment (day 30: 90–95 nmol·g^{-1}). We speculate that the greater Cd influx in the 2002 habitat swap experiment may have exceeded the capacity of the juvenile YP to keep Cd out of the HDP intracellular pool. Note that ambient Cd concentrations were somewhat higher in summer 2002 than in summer 2003, when the diet manipulation experiment was performed.

Somewhat analogous results, relating metal influx rates to metal-induced effects, were reported by Baudrimont *et al.* (1999) for a field experiment in which bivalves (*Corbicula fluminea*) were transplanted from an uncontaminated site to different metal-contaminated sites in the River Lot in southeastern France. At the most contaminated site, metal influx rapidly exceeded the detoxification capacities of the transplanted bivalves, metallothionein synthesis ceased, and complete mortality ensued. However, at the moderately contaminated site metal influx was lower, metal detoxification processes were maintained, and the bivalves survived.

Implications for Metal Ecotoxicology

There appears to be a fundamental disconnect between the traditional metal toxicology paradigm, which presupposes that for a given metal there exists a threshold concentration below which the organism can effectively detoxify the metal, and our results for aquatic organisms chronically exposed to metals. For YP collected along a metal concentration gradient, we have shown that there is no threshold exposure below which YP succeed in keeping accumulated Cd and Ni from binding to inappropriate sites within their liver cells. This result represents a failure of the classical metal toxicology model for the nonessential metals Cd and Ni in YP.[2]

In analyzing the results of our study, we have interpreted the presence of Cd and Ni in cellular organelles (mitochondria, microsomes, lysosomes) and in the heat-denaturable protein (HDP) fraction as evidence for incomplete metal detoxification. Such an interpretation is consistent with earlier work on subcellular metal partitioning (*e.g.*, see Vijver *et al.* (2004) and Wang and Rainbow (2006) for reviews), but the operational nature of the distinction between detoxified and non-detoxified metal must be recognized. For example, although the presence of Cd or Ni in the HDP fraction demonstrates that the metal has bound to the protein, it does not necessarily indicate that the functioning of the protein has been impaired. If binding occurs outside the active site of enzymes and/or does not change the protein's conformation, enzymatic activities or protein functions will presumably not be impaired. For Ni, a metal without strong affinity for thiol groups, this scenario of innocuous binding is perhaps more plausible than for Cd, which would normally be expected to seek out the type of functional groups found in enzyme active sites.

We can ask the question, why do yellow perch from the less contaminated lakes "choose" not to synthesize the extra MT and ensure complete Cd sequestration? Clearly, they have the metabolic *capacity* to do this because the steady-state concentrations of metallothionein in fish from the lakes with high ambient Cd concentrations are much greater than those in the fish from the less contaminated lakes. Is there a significant metabolic cost involved in achieving complete metal detoxification? This question of the energetic cost of metal detoxification is somewhat controversial (Mason and Jenkins 1995) but at the simplest (anthropomorphic) level it does indeed appear as though chronically exposed yellow perch have settled for a tradeoff between the cost of turning on the detoxification apparatus at full capacity, to completely suppress metal binding to metal-sensitive sites, and the alternative cost of allowing some binding of inappropriate metals to metal-sensitive sites.

We speculate that this capacity to tolerate incomplete metal detoxification may be an acquired trait, linked to long-term genetic adaptation. Indeed, Bourret et al. (2007) have recently assessed the potential role of metal contamination in driving evolutionary changes by documenting patterns of genetic diversity at 9 microsatellite loci among 20 populations of wild yellow perch in the Rouyn-Noranda and Sudbury areas. Their results suggest that the selective response to contamination has been sufficiently large to substantially lower the effective population sizes and thus reduce within-population genetic diversity. The 50+ years of metal contamination have

[2]Analogous results have been reported for a freshwater mollusc living in lakes in the same Rouyn-Noranda study area (Campbell *et al.* 2005; Bonneris *et al.* 2005a,b).

significantly impacted patterns of genetic diversity observed among the populations of wild yellow perch and as such may have affected their capacity to respond to metal-induced stress.

On a positive note, it should be mentioned that although metal detoxification is imperfect, there was nevertheless no clear threshold metal concentration *above which* the perch could not at least partially detoxify incoming metals. The fish were still able to handle the incoming metals reasonably successfully even in the lakes at the high end of the metal exposure gradient.

Comparison with Current Water Quality Guidelines

Current water quality guidelines for cadmium and nickel vary considerably from one jurisdiction to another (Table 3), at least in part because regulatory frameworks differ among jurisdictions (*e.g.*, "criteria," "guidelines," "trigger values"). In the past, the protocols used to derive these values also differed among regulatory agencies, but in recent years there has been a general movement toward risk-based approaches and the use of species sensitivity distributions (Warne 2001; ICMM 2007).

Increased water hardness protects against the toxicity of both Cd and Ni; for Cd the effect seems to be largely related to competition between Cd^{2+} and Ca^{2+} for epithelial uptake sites (Wood 2001), whereas for Ni the Mg^{2+} ion plays a dominant role (Pane *et al.* 2003). In principle, the Biotic Ligand Model (BLM) approach could be used to take these interactions into account and adjust the water quality guideline as a function of water hardness, but in neither case has this yet been implemented. Instead, most jurisdictions continue to use empirical algorithms to correct for the hardness effect. The values in Table 3 were calculated in this manner for a water hardness range of 5 to 65 mg $CaCO_3 \cdot L^{-1}$, which corresponds to the approximate range found in our two study areas. However, it should be pointed out that the hardness correction equations were derived from experiments run in the hardness range from 40 to 210 mg$\cdot L^{-1}$ $CaCO_3$ (USEPA 2001) and the extrapolation to soft waters with hardness values below 40 is done with some trepidation.

Comparing the dissolved Cd levels in our lakes (Table 1: 0.3–6.7 nM) with the guideline values in Table 3 for very soft waters (0.11, 0.15, 0.26 nM), we note that all our lakes exceed this lower limit; three of the lakes (Raft, Hannah, and Dufault) also reach or exceed the current draft European Union $PNEC_{water}$ for Cd (1.7 nM). The observation that yellow perch are unable to completely detoxify Cd, even in our cleanest lakes, is thus consistent with these guideline values, even though they were extrapolated below the tested hardness correction range.

The situation for dissolved Ni (Table 1: 8–2500 nM) is somewhat less clear, because there is a major gap in our concentration gradient (no lakes in the 20 to 800 nM range). The Rouyn-Noranda lakes (8–17 nM) are all well below the guideline values in Table 3 for very soft waters (30, 56, 70 nM), whereas all the Sudbury lakes are well above the guidelines (840–2500 nM). The evidence for incomplete detoxification of Ni is limited to the Sudbury lakes (Figure 3).

CONCLUSIONS

The results of this field study raise important questions about current models of metal toxicology. These models assume that indigenous aquatic organisms can

Table 3. Some current regulatory limits for aqueous Cd and Ni.

	Study lakes concentration range		Regulatory limits							
			Canadian WQG		USEPA		EU risk assessment		Australia/ New Zealand WQG	
	$ng \cdot L^{-1}$	nM	$ng \cdot L^{-1}$	nM	$ng \cdot L^{-1}$	nM	$ng \cdot L^{-1}$	nM	$ng \cdot L^{-1}$	nM
Cd	34–750	0.3– 6.7	17[a]	0.15	29– 170[b]	0.26– 1.5	190[c]	1.7	12– 120[d]	0.11–1.1
Ni	470– 147000	8– 2500	3300[e]	56	4000– 36000[f]	70– 620	5000– 28700[g]	85– 490	1700– 15400[h]	30– 265

[a]Value taken from the CCME website (http://www.ccme.ca/sourcetotap/cadmium.html), for an unspecified water hardness (site visited 2007–03-06).

[b]Value taken from USEPA (2006) "Criterion Continuous Concentration" or CCC, an estimate of the highest concentration of a material in surface water to which an aquatic community can be exposed indefinitely without resulting in an unacceptable effect. The value was corrected for a hardness of 5 or 65 $mg \cdot L^{-1}$ $CaCO_3$, giving a range from 29 $ng \cdot L^{-1}$ at the low hardness to 170 $ng \cdot L^{-1}$ at the higher hardness (see USEPA 2006, Appendices A and B).

[c]Predicted No Effect Concentration (PNEC) taken from the draft European Community risk assessment for Cd, for an unspecified hardness (EU 2003).

[d]Trigger value taken from the Australia/New Zealand Water Quality Guidelines (A-NZ 2000), for protection of 99% of indigenous species in freshwater ecosystems. The value was corrected for a hardness of 5 or 65 $mg \cdot L^{-1}$ $CaCO_3$, giving a range from 12 $ng \cdot L^{-1}$ at the low hardness to 120 $ng \cdot L^{-1}$ at the higher hardness (see A-NZ 2000, Tables 3.4.1 and 3.4.3).

[e]Value taken from Demers *et al.* (2006), who used Ni as an example of the new Canadian protocol for developing Water Quality Guidelines, for an unspecified hardness.

[f]CCC value taken from USEPA (2006). The value was corrected for a hardness of 5 or 65 $mg \cdot L^{-1}$ $CaCO_3$, giving a range from 4 $\mu g \cdot L^{-1}$ at the low hardness to 36 $\mu g \cdot L^{-1}$ at the higher hardness (see USEPA 2006, Appendices A and B).

[g]Tentative PNEC values pending the finalization of the EU Existing Substances Risk Assessment of Nickel (Schlekat C, NiPERA, pers comm, 2007–03-07). PNECs were derived by normalizing a chronic nickel aquatic toxicity database (27 species) with chronic Biotic Ligand Models (BLMs). Water quality parameters (pH, hardness, and dissolved organic carbon [DOC]) from seven freshwater scenarios representing typical EU surface waters were used to parameterize the BLMs. The HC5 from a log-normal distribution of species-mean EC10/NOEC values was divided by an Assessment Factor of 2 to yield PNEC values. The lowest PNEC came from a water with pH = 7.7, hardness = 48 mg/L, and DOC = 2.8 mg /L. The highest PNEC came from a water with pH = 6.9, hardness = 260 mg/L, and DOC = 12 mg/L.

[h]Trigger value taken from the A-NZ (2000), guidelines for protection of 99% of indigenous species in freshwater ecosystems. The value was corrected for a hardness of 5 or 65 $mg \cdot L^{-1}$ $CaCO_3$, giving a range from 1.7 $\mu g \cdot L^{-1}$ at the low hardness to 15.4 $\mu g \cdot L^{-1}$ at the higher hardness (see A-NZ 2000, Tables 3.4.1 and 3.4.3).

tolerate exposure to low concentrations of nonessential metals, and that toxicity only occurs above a certain metal exposure threshold, where metal detoxification is incomplete and where the nonessential metals bind inappropriately to metal-sensitive intracellular sites. In our spatial studies, where we examined fish that had been subject to chronic/life-long metal exposures, we could find no threshold exposure concentrations below which binding of "inappropriate" metals to potentially metal-sensitive sites did not occur. In other words, regardless of their ambient concentration, metals taken up by yellow perch from the environment seem to exist in both bioreactive and immobilized pools. Similarly, in our transplantation study where metal-naïve fish were subjected to a step-change in metal exposure, there was no threshold in accumulated cadmium below which the fish were able to keep Cd from binding to potentially metal-sensitive sites, even though their maximum capacity for synthesizing metallothionein had not been reached. We conclude that tolerance to nonessential metals like Cd and Ni must involve both (incomplete) detoxification *and* some metabolic compensation for any deleterious effects caused by the binding of the metals to inappropriate cellular sites.

The implications of our findings for Environmental Risk Assessment are mixed. Taking cadmium as an example, it is reassuring to note that where we observed incomplete Cd detoxification in yellow perch, the ambient Cd concentrations were above water quality guideline values for dissolved Cd. This would not have been the case 10 years ago, when the Canadian guideline value for Cd (200 ng $Cd \cdot L^{-1}$, 1.8 nM) was more than 10 times higher than the current value. The downward revision of the Cd guidelines clearly was appropriate. On the other hand, the absence of an exposure threshold below which Cd was completely detoxified leaves open the possibility that the true NOEC (No Observed Effect Concentration) will turn out to be even lower than current estimates (Table 3).

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