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Binding of Trace Elements (Ag, Cd, Co, Cu, Ni, And Tl) to Cytosolic Biomolecules in Livers of Juvenile Yellow Perch (*Perca Flavescens*) Collected From Lakes Representing Metal Contamination Gradients

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Abstract: Biomolecules involved in handling cytosolic metals in the liver of the yellow perch (*Perca flavescens*) were characterized in juvenile fish collected from 4 lakes constituting metal contamination gradients. Using size-exclusion liquid chromatography coupled to an inductively coupled mass spectrometer, we determined metal distributions among ligands of different molecular weights in the cytosol, before and after a heat denaturation step designed to isolate metallothionein-like peptides and proteins. Silver, Cd, and Cu found in the heat-stable protein supernatants were indeed largely present as metallothionein-like peptides and protein complexes; but Co, Ni, and Tl, also present in the heat-stable protein supernatants, did not coelute with metallothionein-like peptides and proteins. This difference in metal partitioning is consistent with the known preference of "soft" metals such as Ag, Cd, and Cu(I) for thiolated ligands and the contrasting tendency of Co and Ni to bind to ligands with oxygen and nitrogen as donor atoms. Metal handling in the whole cytosol also reflected these differences in metal-binding behavior. For Cd and Cu, the importance of the molecular weight pool that includes metallothionein-like peptides and proteins increased relative to the other pools as the total cytosolic metal concentration ($[M]_{cytosol}$) increased, consistent with a concentration-dependent detoxification response. In contrast, for Ni and Tl the increase in $[M]_{cytosol}$ was accompanied by a marked increase in the high–molecular weight (670–33 kDa) pool, suggesting that hepatic Ni and Tl are not effectively detoxified. Overall, the results suggest that metal detoxification is less effective for Ni, Tl, and Co than for Ag, Cd, and Cu. *Environ Toxicol Chem* 2017;9999:1–11. © 2017 SETAC

Keywords: Metal; Native fish; Liver; Detoxification; Lake

INTRODUCTION

Concentrations of trace metals in aquatic animals—whole organisms or organs—are often used to assess their exposure to metals in aquatic systems [1]. Nevertheless, these measurements alone are not sufficient to reveal if the accumulated metal will cause toxic effects or not [2]; to link metal bioaccumulation to metal toxicity, complementary information about the localization of metals in animal cells in detoxified or biochemically sensitive fractions is needed [3,4]. Methods that involve differential centrifugation steps followed by heat denaturation are often used to discriminate between metals bound to heat-stable proteins and peptides, for example, metallothionein or metallothionein-like peptides and proteins and heat-denatured proteins (e.g., cytosolic enzymes) in aquatic animals [4,5]. In this context, a study on juvenile yellow perch (*Perca flavescens*) collected along a polymetallic concentration gradient showed that hepatic Cd and Cu were mainly found in the heat-stable protein fraction, whereas Ni and Zn burdens were associated with both the heat-stable protein and the heat-denatured protein fractions [6]. In addition, the authors found that Cd, Cu, and Ni increased in both cytosolic fractions along the spatial contamination gradient, a result that was consistent with a transplantation study [7] where temporal variations of hepatic Cd and Cu were followed in juvenile yellow perch that had been collected from an uncontaminated lake and placed in cages in a contaminated lake.

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However, although these techniques provide data about the partitioning of metals in the animal cells, they do not reveal detailed information about the different biomolecules targeted by the metals or about the affected metabolic pathways, this being essential information to discriminate between metal homeostasis/detoxification and metal-induced toxicity [8,9]. As an example, in the cell cytosol nonessential metals can bind to biomolecules of different molecular weights: at both extremes, to high– or low–molecular weight targets, such as enzymes and small peptides, respectively, and in the middle, to medium–molecular weight entities like metallothionein or metallothionein-like peptides and proteins [10,11]. Metal binding to ligands such as metallothionein or metallothionein-like peptides and proteins is normally seen as a sign of a detoxification response, whereas metal binding to enzymes or small peptides could be an indication of toxic effects [12].

In our recent work on *Chaoborus* larvae (Insecta, Diptera), we developed an approach to determine trace metal distributions among cytosolic biomolecules using size-exclusion chromatography coupled online to inductively coupled plasma mass spectrometry (SEC-ICP-MS) [13]. This method yields useful information about the properties of the metal–biomolecule complexes and furthers our understanding of metal interactions with subcellular biomolecules and of the metal-handling strategies used by aquatic animals. In the present study, we used a similar SEC-ICP-MS approach to determine the distribution of hepatic Ag, Cd, Co, Cu, Ni, and Tl in juvenile yellow perch (*P. flavescens*) collected from lakes contaminated to varying degrees by metals. Our objective was to compare the subcellular behavior of the different metals, with reference to their inherent binding preferences, and to evaluate to what extent the fish had mounted a successful detoxification response. The cytosolic family of metal-binding molecules in perch liver found to cover a wide range of molecular weights (700–0.1 kDa). Within this range, the biomolecules bound to "soft" metals such as Ag, Cd, and Cu(I) differed from those that bound to Co, Ni, and Tl. For some metals, notably Cd, Cu, Ni, and Tl, their distribution among the different cytosolic ligands varied as the total cytosolic bioaccumulated metal concentration increased, suggesting a concentration-dependent detoxification response.

MATERIALS AND METHODS

Sampling site and fish collection

Fish were collected in June 2013 in the Sudbury and Dorset (Ontario, Canada) areas. The Sudbury area was chosen on the basis of historical mining and smelting operations in the vicinity [14] and the available lake water metal concentrations (see Supplemental Data, Table S1), whereas the Dorset area is located approximately 250 km southeast from the Sudbury smelters, also on the Canadian Precambrian Shield, but can be considered to be much less affected by such activities. Because life stage and food quality can affect metal bioaccumulation in wild fish [15–17], juvenile perch at the planktivorous life stage (age 0+, 6–12 cm) were collected for the present study. They were captured using seine nets in 2 contaminated lakes of the Sudbury area (Hannah Lake, 46°26′N 81°02′W; Whitson Lake, 46°35′N 80°58′W) and in 2 reference lakes in the Dorset area (Dickie Lake, 45°08′N 79°05′W; Red Chalk Lake, 45°11′N 78°56′W) and dissected on site.

Following death by cervical dislocation, the liver from each individual was removed and frozen immediately in liquid nitrogen in cryogenic polypropylene containers. Liver samples were kept in liquid nitrogen until their transfer to a –80 °C freezer in our home laboratory. All procedures were carried out according to a protocol approved by our institutional animal care committee. Fish collection was authorized by a scientific collector's permit issued by the Ontario Ministry of Natural Resources.

Preparation of subcellular fractions

Pools of 3 to 6 perch livers (~200 mg wet wt) were chopped manually using a surgical stainless steel razor blade and homogenized in 1.5 mL of 100 mM ammonium acetate (pH 7.4) with a motorized Potter-Elvehjem homogenizer equipped with a Teflon pestle (details in Supplemental Data). For each lake, all manipulations were conducted on 3 individual pools of perch livers ($n = 3$). The homogenate was then subjected to ultracentrifugation (100 000 *g* for 60 min at 4 °C) to separate the operationally defined cytosol from the pellet (nuclei + debris + granule-like fraction; Supplemental Data, Figure S1).

One 150-μL aliquot from each cytosol fraction was collected for measurement of total metal concentrations and another (also 150 μL) for SEC_{200}-ICP-MS analysis, as described in the next section. The remaining cytosol was subjected to heat denaturation at 80 °C for 10 min, placed on ice for 60 min, and then ultracentrifuged at 50 000 *g* for 60 min at 4 °C to separate the heat-stable protein fraction (as the supernatant; details in Supplemental Data) from the heat-denatured protein fraction (pellet). As was done for the whole cytosol, one 150-μL aliquot was removed from each heat-stable protein supernatant for total metal concentration measurements and a second 150-μL aliquot was used for SEC_{pep}-ICP-MS analyses (see next section).

SEC-ICP-MS analysis

Liquid chromatographic separations were conducted with a Thermo Scientific Spectra System high-performance liquid chromatography (HPLC) equipped with a P4000 quaternary gradient pump, an SCM1000 degassing pump, an AS3000 autosampler containing a 100-μL injection loop, and a UV2000 dual-wavelength detector. The sample flow-path of the chromatographic equipment contained metal-free components (mainly polyether ether ketone) to preserve the integrity of metal–biomolecule complexes and minimize unwanted surface interactions. An ICP-MS (Thermo Scientific XSeries 2) fitted with a nebulizer and platinum cones was used for metal detection. The chromatographic effluent was directly introduced via polyether ether ketone tubing into the HPLC detector for evaluation of protein absorbance at 280 and 254 nm and subsequently delivered to the ICP-MS instrument for ^{59}Co, ^{60}Ni, ^{65}Cu, ^{109}Ag, ^{111}Cd, and ^{205}Tl measurements. Note that we also

looked for Cr–biomolecule complexes in the samples analyzed, but because none were detected, we do not present any results for Cr. In addition, because absorbance measurements at 280 and 254 nm did not show any important overlap with the metal elution peaks, these results are not shown.

Separations of the whole cytosol (150 μL) and the heat-stable protein supernatants (150 μL) were carried out using Superdex 200 (SEC_{200}; 10–600 kDa linear mass separation range) and Superdex peptide (SEC_{pep}; 0.1–7 kDa linear mass separation range) size-exclusion columns (GE Healthcare), respectively. A solution of 100 mM ammonium acetate (pH 7.4) was used as the mobile phase at a flow rate of 0.7 mL min^{-1} (details in Supplemental Data). Biomolecules eluting before the highest–molecular weight marker (= column void volume) or after the lowest–molecular weight marker (as a result of interactions with the column stationary phase) were denoted as >670 kDa or <1.3 kDa for the SEC_{200} column and as >10 kDa or <0.1 kDa for the SEC_{pep} chromatograms.

Molecular masses for biomolecules eluting outside the column calibration ranges cannot be calculated from the calibration curve, which is not linear outside these limits, and cannot be compared between the 2 chromatographic columns. Because no biomolecules eluted before the void volume of the column, chromatograms are presented after 10 min of acquisition time to improve the visual presentation of the chromatographic data.

Metal quantification and controls

All laboratory-ware was soaked in 15% nitric acid solution and rinsed 5 times in ultrapure water before use, to minimize accidental metal contamination. The heat-stable protein and cytosol fractions were digested for 2 h at 80 °C with 400 μL of nitric acid (70%, v/v, Optima grade; Fisher Scientific). Once the solution had cooled, hydrogen peroxide was added (160 μL; 30%, v/v, Optima grade; Fisher Scientific). Digested samples were diluted with ultrapure water, and the total metal concentrations were determined in the whole cytosol and in the heat-stable protein supernatants by ICP-MS (^{59}Co, ^{60}Ni, ^{65}Cu, ^{109}Ag, ^{111}Cd, ^{205}Tl). These concentrations are expressed as the total metal burden in the whole cytosol and in the heat-stable protein fractions (nanomoles) divided by the total liver dry weight (grams, estimated as 20% of the wet wt). The 20% dry weight value is based on independent measures performed on yellow perch liver samples (A. Caron and Q2 Amyot, INRS-ETE, Quebec, QC, Canada, unpublished data) and is similar to the value found by Giguère et al. [6].

Recoveries (mean ± standard deviation [SD], $n = 5$) of the trace elements studied from the certified reference material lobster hepatopancreas (TORT-3; National Research Council of Canada) ranged from 83 to 100% for Cd (93 ± 5%), Co (100 ± 5%), Cu (93 ± 5%), and Ni (83 ± 4%). Recoveries from another certified reference material, dogfish liver (DOLT-4; National Research Council of Canada), varied from 88 to 91% for Ag (88 ± 2%), Cd (91 ± 2%), and Cu (93 ± 2%). The SEC_{200} column recoveries (means ± SD; $n = 6$; details in Supplemental Data) ranged from 83 to 112% for Ag (83 ± 21%), Cd (112 ± 2%), Co (92 ± 18%), Cu (92 ± 9%), Ni (104 ± 10%), and Tl (97 ± 16%).

Calculations and data treatment (statistics)

All numerical data are represented as means ± SD. To determine the distribution of each element studied among the different molecular weight pools (cytosol) or molecular weight fractions (heat-stable protein), each integrated peak area was normalized by total liver dry weight. Similarly, to identify the proportion of cytosolic metal in each SEC_{200} molecular weight pool (high-, medium-, and low-molecular weight) or in each SEC_{pep} molecular weight fraction (fractions 1, 2, 3), the ratio of the peak area of each molecular weight pool or fraction divided by the sum of the total chromatogram peak areas was calculated. When data did not respect the assumptions of normality (Shapiro-Wilk test) or homoscedasticity (Levene test), they were transformed. If these assumptions were still not met, a nonparametric test was applied. To compare total bioaccumulated metal concentrations in the cytosol or in the heat-stable protein fraction among fish livers collected from different lakes, one-way analysis of variance (ANOVA) followed by the Tukey test was used on log-transformed data. Differences in peak areas for each molecular weight pool or fraction, among fish collected from different lakes, were assessed using the nonparametric Kruskal-Wallis test, followed by Tukey's honest significant difference test on ranks. For peak area proportions in each molecular weight pool or fraction and for heat-stable protein contributions to the whole cytosol (percentage), one-way ANOVA was used on arcsine-transformed data to determine differences among fish collected from different lakes. Statistical analyses were performed with JMP Pro 12 Statistical Analysis Software (SAS Institute). For statistical significance, the threshold used were p levels of 0.05.

RESULTS AND DISCUSSION

Total metal concentrations (cytosol and heat-stable protein fraction)

For metals known to have been emitted from the Sudbury smelters (e.g., Cd, Cu, and Ni), liver concentrations for perch collected from the different lakes followed a similar "gradient" (i.e., defined in the present study as a progressive increase when comparing mean metal concentrations) for both the cytosols and the heat-stable protein fractions. This progressive increase was greater for Ni and Cd, as indicated by their respective $[M]_{Max}$ to $[M]_{Min}$ ratios (Table 1), than for Cu, this latter metal being subject to reasonable homeostatic control in yellow Q3 perch. For Ag and Tl, metals for which there are no known point sources in the Sudbury area, hepatic concentrations were low and only a modest concentration gradient (low $[M]_{Max}$ to $[M]_{Min}$ ratios; Table 1) existed among values obtained for fish from the different lakes.

Interestingly, although there are also no known point sources of Co in the study area, a modest interlake concentration gradient was seen for hepatic Co concentrations. This gradient differed from that observed for the other metals, but, again, the $[M]_{Max}$ to $[M]_{Min}$ ratios were quite low both in the cytosol and in the heat-stable protein fraction. Ultimately, these considerations

TABLE 1: Hepatic metal (Ag, Cd, Co, Cu, Ni, and Tl) concentrations (± standard deviation; $n = 3$) in the cytosol and in the heat-stable protein supernatants from livers of juvenile *Perca flavescens* collected from 4 lakes[a]

	Trace metals (nmol g^{-1} dry wt)					
	Ag	Cd	Cu	Co	Ni	Tl
Cytosol						
Dickie Lake	0.086 (0.014)A	7.4 (0.23)C	92 (12)B	5.4 (1.7)B	1.3 (0.40)C	0.71 (0.11)A
Red Chalk Lake	0.11 (0.033)A	17 (2.0)B	143 (42)A,B	14 (0.10)A	1.4 (0.26)C	0.64 (0.029)A
Hannah Lake	0.052 (0.004)B	27 (2.2)A	132 (6.8)A,B	5.7 (1.5)B	8.2 (1.2)B	0.72 (0.073)A
Whitson Lake	0.090 (0.0096)A	32 (2.6)A	200 (32)A	10 (1.0)A	18 (3.3)A	0.33 (0.050)B
$[M]_{Max}$ to $[M]_{Min}$	2.2	4.4	2.2	2.6	14	2.2
Heat-stable protein and peptide fraction						
Dickie Lake	0.044 (0.0057)B,C	4.4 (0.42)C	49 (6.1)B	2.9 (0.78)B	0.27 (0.089)D	0.46 (0.085)A
Red Chalk Lake	0.071 (0.022)A	11 (1.5)B	84 (29)A,B	7.6 (0.17)A	0.61 (0.16)C	0.44 (0.026)A
Hannah Lake	0.029 (0.0043)C	19 (1.8)A	81 (9.2)A,B	3.4 (0.76)B	3.1 (0.65)B	0.53 (0.060)A
Whitson Lake	0.059 (0.0026)A,B	23 (2.9)A	131 (31)A	5.9 (0.25)A	6.3 (0.65)A	0.24 (0.047)B
$[M]_{Max}$ to $[M]_{Min}$	2.5	5.2	2.7	2.6	23	2.2
Heat-stable protein contribution (%)						
Dickie Lake	51 (6.4)B	58 (3.9)B	53 (6.3)A	55 (3.5)A	22 (12)B	64 (4.0)B
Red Chalk Lake	65 (2.2)A,B	64 (5.0)A,B	58 (3.1)A	54 (1.5)A	44 (4.6)A	69 (1.1)A,B
Hannah Lake	55 (4.9)A,B	72 (4.2)A	61 (3.9)A	59 (4.7)A	38 (4.4)A,B	74 (2.6)A
Whitson Lake	65 (6.4)A	71 (3.6)A	65 (5.1)A	58 (3.5)A	36 (3.1)A,B	75 (2.7)A

[a]Within the table, for each metal, values with the same letter are not significantly different, whereas different letters indicate significant differences (analysis of variance followed by Tukey test, $p < 0.05$).
$[M]_{Max}$ to $[M]_{Min}$ = maximum metal concentration divided by minimum metal concentration (i.e., metal gradient ratio).

about the total hepatic metal concentrations allowed us to compare the contamination levels in the lakes: in general, fish collected from Whitson Lake showed the highest hepatic metal concentrations in the cytosol and heat-stable protein fractions (with the exception of Ag, Co, and Tl), whereas yellow perch from Dickie Lake generally had the lowest metal concentrations (Table 1). This observation is consistent both with a previous study reported by Drevet [18], where similar contrasting metal concentrations of Cd and Ni were measured in fish collected from these lakes, and with the long-term monitoring data on aqueous metal concentrations in these lakes (Supplemental Data, Table S1).

In their study of hepatic metal concentrations in perch collected in the Sudbury area, Giguère et al. [6] also collected juvenile yellow perch from Hannah Lake. Mean hepatic concentrations were quite different for the 2 sampling campaigns: for fish captured in June 2001 in Hannah Lake, total hepatic concentrations were approximately 2.3 times higher for Cd (~ 92 nmol g^{-1} dry wt), 6.2 times higher for Cu (~ 1400 nmol g^{-1} dry wt), and 1.4 times higher for Ni (~ 27 nmol g^{-1} dry wt) than in the fish captured in the present study. These differences suggest that a major decrease in the degree of metal contamination of Hannah Lake occurred in the 2001 to 2013 period. This trend is also evident in the long-term monitoring data on aqueous metal concentrations in Hannah Lake (Supplemental Data, Table S1).

Comparisons between the mean metal concentrations in the whole cytosol and in the heat-stable protein fraction (Table 1) indicate that for most metals the heat denaturation step removed less than half of the total metal originally present in the cytosol. The contribution of the thermostable heat-stable protein fraction was consistently above 50% (51–65% Ag, 58–71% Cd, 54–59% Co, 53–65% Cu, 64–75% Tl); only for Ni (22–44%) was this proportion appreciably less than 50%.

Metal binding to cytosolic ligands (whole cytosol)

For the SEC_{200}-ICP-MS analyses of the whole cytosol, 3 molecular weight pools were defined as high-molecular weight (>670–33 kDa, elution time 10–22 min), medium-molecular weight (33–1.3 kDa, elution time 22–32 min), and low-molecular weight (<1.3 kDa, elution time >32 min). The partitioning of the trace metal elements studied (Ag, Cd, Cu, Co, Ni, and Tl) among these pools was examined for fish collected along the metal contamination gradient (Figure 1). Note that the chromatograms for the Red Chalk Lake and Hannah Lake samples are not presented in Figure 1 because they show similar trends to what was observed for the samples collected from Dickie and Whitson Lakes, respectively; the results for Red Chalk and Hannah Lakes can, however, be found in Supplemental Data, Figure S2.

The peak areas of Cd and Cu in the medium-molecular weight pools significantly increased as total cytosol metal concentrations increased (Figures 1 and 2). For Cd a single medium-molecular weight peak was detected at 25 min, but for Cu 2 medium-molecular weight peaks were observed (22.5 and 25 min); these 2 peaks were of similar areas in fish collected from Dickie Lake, but in fish from Whitson Lake (more contaminated) the peak at 25 min had greater intensity than the peak at 22.5 min, suggesting a change in Cu handling when the ambient Cu (or Cd) concentration is high. Indeed, in fish from Whitson Lake the concentration of Cu in the high-molecular weight pool was much lower than that in fish from the other lakes (Figure 2). In a similar pattern, Cd concentrations in the high-molecular weight pool stayed quite low, even in fish from the most contaminated lakes, whereas increasing concentrations of

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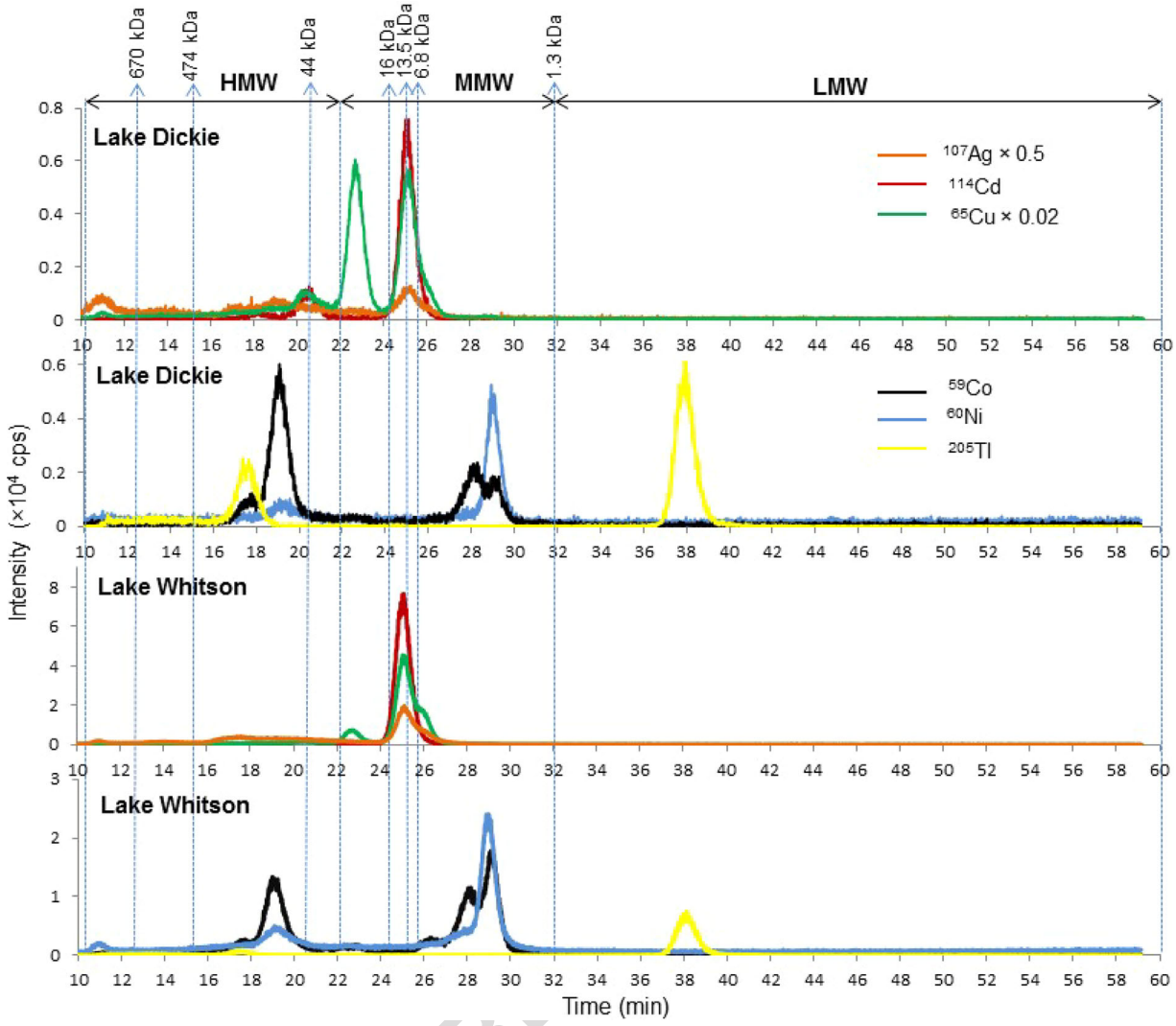

FIGURE 1: Size-exclusion chromatography coupled online to inductively coupled plasma mass spectrometry (Superdex 200) chromatograms of metal (Ag, Cd, Co, Cu, Ni, and Tl)–binding biomolecules from hepatic cytosols of *Perca flavescens* collected from Dickie Lake (low metal-contaminated lake; 2 upper panels) and Whitson Lake (high metal-contaminated lake; 2 lower panels). Vertical dotted lines represent elution times of the molecular weight standards. The 3 operationally defined molecular weight pools comprise: high molecular weight (>670–33 kDa, elution time 10–22 min), medium molecular weight (33–1.3 kDa, elution time 22–32 min), and low molecular weight (<1.3 kDa, elution time >32 min). HMW = high molecular weight; LMW = low molecular weight; MMW = medium molecular weight.

this metal were detected in the medium-molecular weight fraction along the contamination gradient. The proportions of cytosolic Cd and Cu that were bound to the medium-molecular weight ligands significantly increased ($p < 0.05$) with increasing cytosolic metal concentrations, from 81 to 94% and from 71 to 98%, respectively (Supplemental Data, Figure S3). The predominance of cytosolic partitioning of Cd and Cu in the medium-molecular weight pool was also reported for *Anguilla anguilla* [19,20], *Chaoborus* larvae [13], and *Carassius auratus gibelio* [21], where concentrations of both elements in the medium-molecular weight pool significantly increased as the level of metal contamination increased.

In contrast to the observations for Cd and Cu, the partitioning of Ag between the high- and medium-molecular weight pools did not change with increasing Ag concentrations (Figure 2; Supplemental Data, Figure S3); but this may be attributable to the low cytosolic concentrations and to the modest metal concentration gradient (low $[M]_{Max}$ to $[M]_{Min}$ ratios observed; Table 1). However, within the medium-molecular weight pool Ag did coelute (with Cd and Cu) under the peak at 25 min, which is consistent with the known affinity of these 3 "soft" class B trace metals for thiolated ligands. A similar coelution pattern for Ag was reported for field-collected samples of the phantom midge [13] and for hepatic cells of *A. anguilla* [19,20].

Both Co and Ni were mainly found in the same high-molecular weight pool (peak at 19 min) and in the medium-molecular weight pool associated with similar but different medium-molecular weight ligands (peak at 28 min for Co and peaks eluting ~ 29 min for both metals; see Figure 1), these medium-molecular weight ligands being of lower molecular weight than those observed for Ag, Cd, and Cu. The relative importance of the high- and medium-molecular weight pools

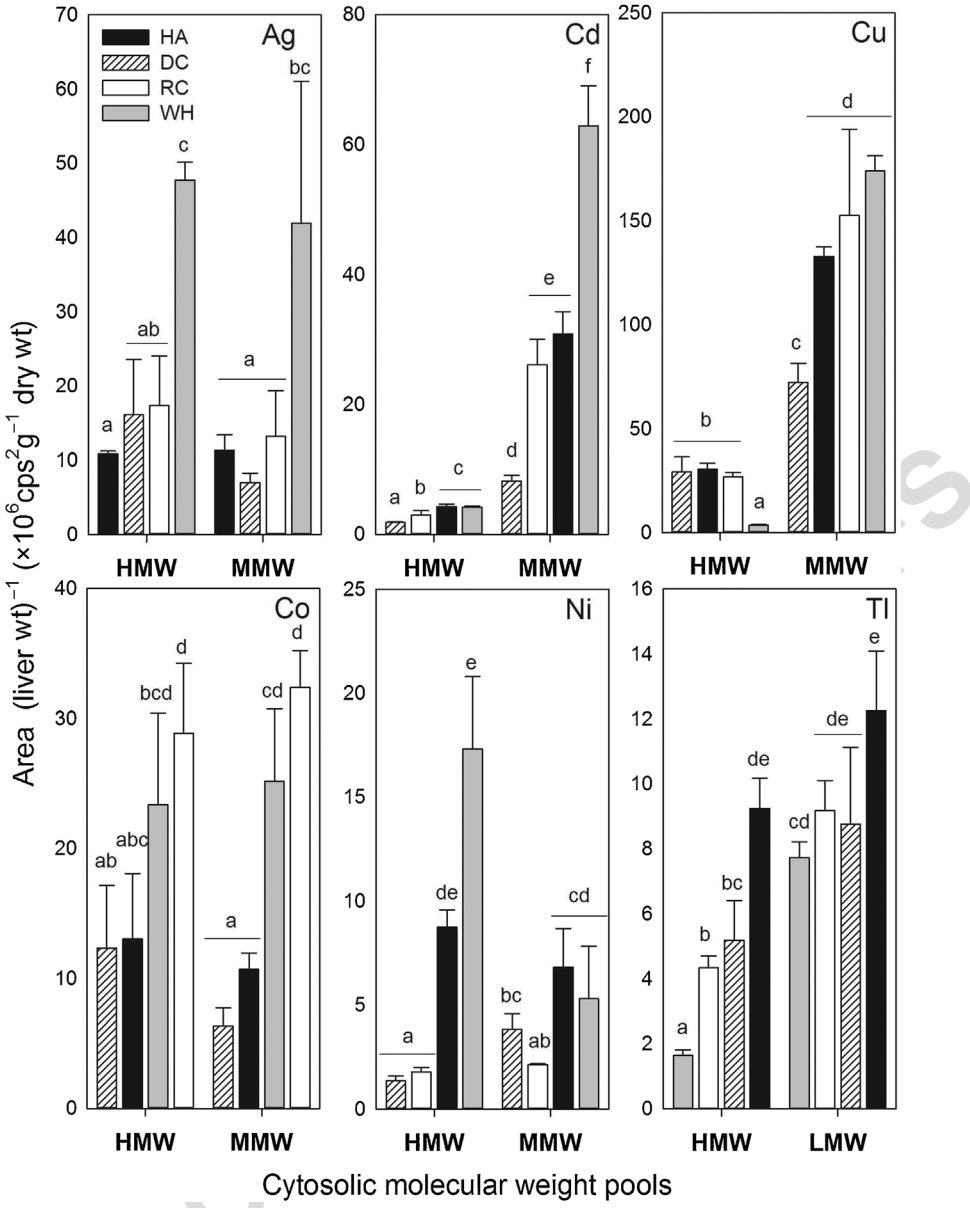

FIGURE 2: Peak areas (mean ± standard deviation, $n = 3$) normalized by liver weight for Ag, Cd, Co, Cu, Ni, and Tl in the high molecular weight (>670–33 kDa, elution time 10–22 min), medium molecular weight (33–1.3 kDa, elution time 22–31 min), and low molecular weight (<1.3 kDa, elution time >31 min) size-exclusion chromatography (Superdex 200) pools in the hepatic cytosols of *Perca flavescens* collected from Hannah Lake, Dickie Lake, Whitson Lake, and Red Chalk Lake. Bars with the same letters indicate nonsignificant differences, whereas different letters indicate that the differences are significant (analysis of variance followed by Tukey test, $p < 0.05$). DC = Dickie Lake; HA = Hannah Lake; HMW = high molecular weight; LMW = low molecular weight; MMW = medium molecular weight; RC = Red Chalk Lake; WH = Whitson Lake.

changed along the metal contamination gradient (Figure 2; Supplemental Data, Figure S3) but with opposite trends; for Co the relative contribution of the medium-molecular weight pool increased slightly in importance as the total cytosolic concentration increased, whereas for Ni the contribution of the medium-molecular weight pool decreased markedly as the cytosolic Ni concentration increased (Supplemental Data, Figure S3). This observation is of interest because metallothioneins and metallothionein-like peptides and proteins are found in the medium-molecular weight fraction with a molecular weight ranging from 9 to 20 kDa [22,23] and therefore do not seem, in the present study, to be involved in Ni detoxification. This aspect will be discussed in the next section *Metal binding to metallothionein-like peptides and protein and other thermostable ligands*.

With regard to Tl, this metal was mainly bound to high-molecular weight (17.5 min) and low-molecular weight (38 min) ligands, and the high-molecular weight ligand was of a different molecular weight from that of the high-molecular weight ligands responsible for binding Co and Ni. Thallium concentrations increased in both the high- and low-molecular weight pools as total cytosolic Tl concentrations increased. However, as was the case for Ni, the partitioning of Tl between the high- and low-molecular weight ligands changed with increasing metal

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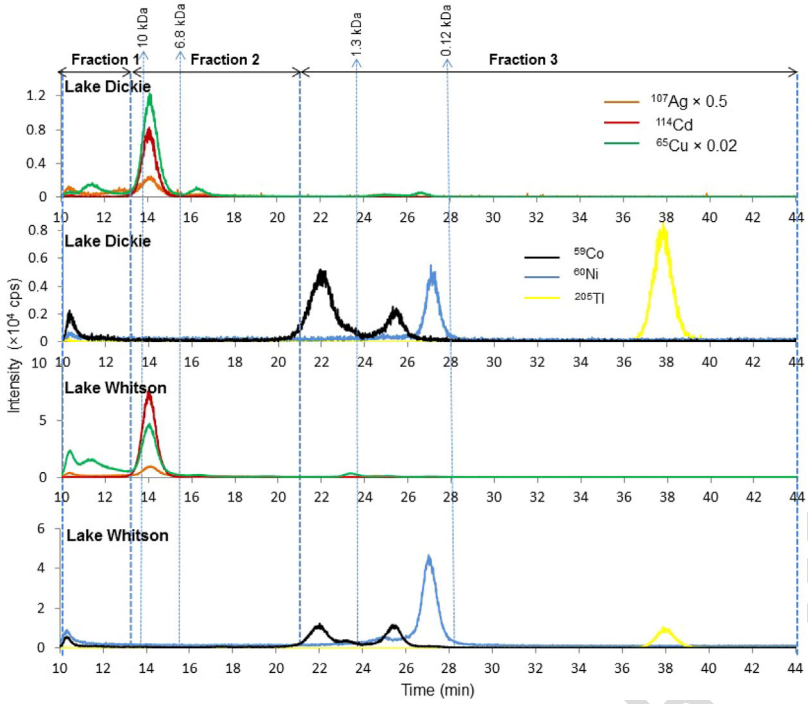

FIGURE 3: Size-exclusion chromatography coupled online to inductively coupled plasma mass spectrometry (Superdex peptide) chromatograms of metal (Ag, Cd, Co, Cu, Ni, and Tl)–binding biomolecules of hepatic heat-stable protein fractions of *Perca flavescens* collected from Dickie Lake (2 upper panels) and Whitson Lake (2 lower panels). Vertical dotted lines represent elution times of the molecular weight standards. The 3 operationally defined molecular weight fractions comprise fraction 1 (>10 kDa, elution time 10–13 min), fraction 2 (10–1.1 kDa, elution time 13–21 min), and fraction 3 (<1.1 kDa, elution time >21 min).

concentrations. Considering Dickie Lake fish as more contaminated with Tl than those from Lake Whitson (Table 1), the peak at 17.5 min was almost nondetectable for fish from Whitson Lake, but it increased markedly for fish from Dickie Lake (Figure 1). This observation is confirmed by peak area measurements, which showed that as the total cytosolic Tl concentration increased from Whitson Lake to Hannah Lake, the relative importance of the high-molecular weight fraction increased (Supplemental Data, Figure S3). These results are surprising, given the contamination gradient of only approximately 2 (Table 1); and they suggest that Tl-binding ligands are able to respond to small Tl concentration variations in the cytosolic fraction. In our previous work on *Chaoborus* larvae, Tl was only found in the low-molecular weight fraction and a $[M]_{Max}$ to $[M]_{Min}$ ratio of 4.7 was observed among the studied lakes [13]. In yellow perch liver, it appears that Tl can bind to at least 2 major ligands, both in putative metal-sensitive molecular weight pools.

Metal binding to metallothionein-like peptides and protein and other thermostable ligands

For the SEC_{pep}-ICP-MS analyses of the heat-denatured cytosol, 3 molecular weight fractions were defined as fraction 1 (>10 kDa, elution time 10–13 min), fraction 2 (10–1.1 kDa, elution time 13–21 min), and fraction 3 (<1.1 kDa, elution time >21 min). For Ag, Cd, and Cu, a dominant metal–biomolecule complex elutes at 14 min (corresponding to 9.3 kDa; molecular weight estimated using data from the molecular weight calibration column) for fish collected from all 4 lakes (Dickie and Whitson, Figure 3; Red Chalk and Hannah, Supplemental Data, Figure S4). As was observed in the cytosolic medium-molecular weight pool, in the heat-stable protein samples we also see a marked increase in Cd and Cu concentrations bound to fraction 2 ligands along the concentration gradients (Figure 4). In contrast, concentrations of Cd and Cu in fraction 1 ligands do not respond to the contamination gradient, suggesting that ligands found in fraction 2 (probably metallothionein and metallothionein-like peptides and proteins) play an important role in handling these 2 metals (Supplemental Data, Figure S5). This is consistent with the results reported by Van Campenhout et al. [20], where Cd and Cu were largely associated with metallothionein (~10 kDa) in liver cells of European eels. A slightly different pattern was seen for Ag, where fraction 2 dominated the partitioning of this metal in the heat-stable protein samples (Figures 3 and 4), as was the case for Cd and Cu; but the Ag concentration in fraction 1 also increased along the concentration gradient, and the relative proportions of the 2 fractions did not change (Supplemental Data, Figure S5).

Cobalt present in the heat-stable protein samples was distributed between fractions 1 and 3 but was absent from fraction 2 where Ag, Cd, and Cu were found. Elution of Co in fraction 3 was consistently greater than that observed in fraction 1 (Figures 3 and 4; Supplemental Data, Figure S5). Cobalt concentrations in fraction 1 increased slightly along the metal concentration gradient, but the incoming Co was bound preferentially to fraction 3 ligands (Figure 4). Nickel behavior

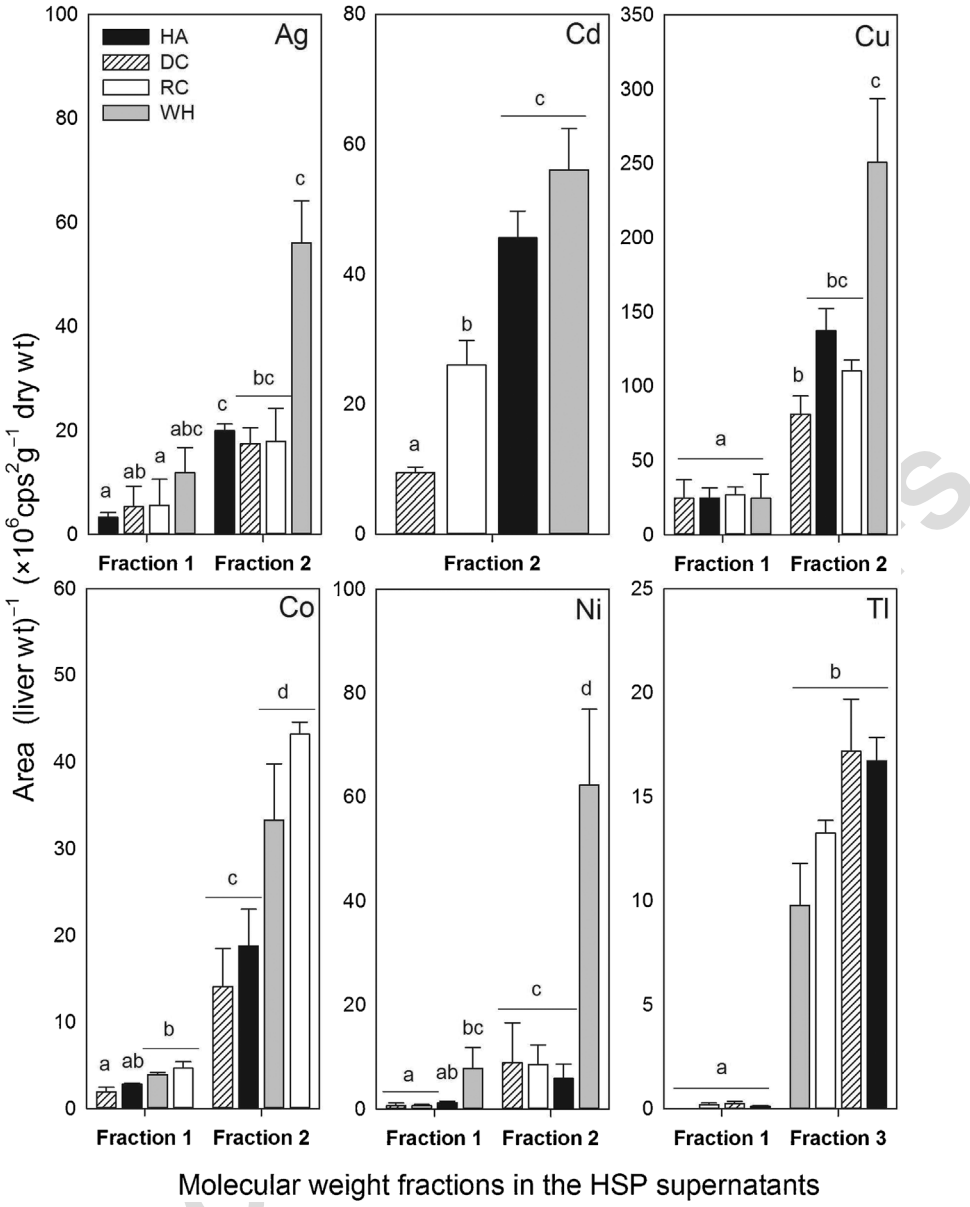

FIGURE 4: Peak area (mean ± standard deviation, $n = 3$) normalized by liver weight for Ag, Cd, Co, Cu, Ni, and Tl for the 3 molecular weight size-exclusion chromatography (Superdex peptide) fractions including fraction 1 (>10 kDa, elution time 10–13 min), fraction 2 (10–1.1 kDa, elution time 13–21 min), and fraction 3 (<1.1 kDa, elution time >21 min) in the hepatic heat-stable protein fractions of *Perca flavescens* collected from Hannah Lake, Dickie Lake, Whitson Lake, and Red Chalk Lake. Bars with the same letters indicate nonsignificant differences, whereas different letters indicate that the differences are significant (analysis of variance followed by Tukey test, $p < 0.05$). DC = Dickie Lake; HA = Hannah Lake; HSP = heat-stable protein; RC = Red Chalk Lake; WH = Whitson Lake.

resembled that of Co, with Ni also appearing in fractions 1 and 3 but not in fraction 2; but there was no evidence of coelution of the 2 metals. Note that the gap in the heat-stable protein chromatogram observed between the Ni peak at 27 min and the Co peak at 25.5 min suggests that the apparent coelution of these 2 metals in the cytosol (broad peak ~ 29 min) was in fact hiding 2 different ligands. Finally, concentrations of Tl in fraction 1 stayed low along the contamination gradient, as observed for the other metals; and as the total heat-stable protein concentration increased, Tl partitioning was dominated by a single peak of very low molecular weight (elution at 38 min) in fraction 3 (Figures 3 and 4).

As mentioned briefly in the previous section *Metal binding to cytosolic ligands (whole cytosol)*, it is important to note that the respective retention times for the Co, Ni, and Tl peaks indicate that these metals are not linked to metallothionein or metallothionein-like peptides and proteins and that the major heat-stable protein peaks observed are found in fraction 3. Our previous work on *Chaoborus* larvae also showed that Tl and Ni were mainly bound to low-molecular weight thermostable ligands, which were suggested to be amino acids or organic acids such as malate, citrate, histidine, and cysteine [13]. In their analysis of the roots, xylem, shoots, and protoplasts of the metal hyperaccumulator plant *Thlaspi caerulescens*, Ouerdane

et al. [24] found that Ni was complexed with low-molecular weight ligands such as malate, citrate, histidine, ethylenediaminetetraacetic acid, and nicotianamine, that is, ligands at least 20 times smaller in molecular weight than metallothioneins. In addition, the work of Amari et al. [25] indicated a particular involvement of malate and citrate in Ni translocation and accumulation in the different organs of the plants *Mesembryanthemum crystallinum* and *Brassica juncea* and a possible link between the high citrate and histidine concentration in the roots of *M. crystallinum* and its high tolerance to toxic levels of Ni.

Effects of heat denaturation: Comparison of metal partitioning before and after heat denaturation of the hepatic cytosol samples

Comparisons between the whole cytosol and the heat-stable protein fraction are not straightforward because different size-exclusion columns were used for the HPLC separations and retention times are necessarily different. In addition, any metal–ligand complex that was originally present in the cytosolic high-molecular weight pool and that survived the heat denaturation step would be too large to be separated on the SEC_{pep} column (because the upper molecular wt cutoff for SEC_{pep} is 10 kDa). Nevertheless, some reasonable deductions can be made by focusing on the medium- and low-molecular weight pools and noting how they are affected by heat treatment. For example, in the medium-molecular weight pool it is clear that the ligand that eluted at 25 min in the whole cytosol separations corresponds to the ligand eluting at 14 min in the heat-stable protein chromatograms because the Ag, Cd, and Cu peaks were exactly superimposed in both cases (compare Figures 1 [25 min] and 3 [14 min]). However, the Cu peak that was observed at 22.5 min in the cytosol seems to be absent in the heat-stable protein samples (the small peak at 11.5 min in fraction 1 of the Dickie Lake chromatogram [Figure 3] corresponds better to a similar peak at 20.5 min in the cytosol); the apparent absence of this metal–biomolecule complex suggests that it is thermosensitive and proteic. This result is consistent with the idea that the fish response to increased concentrations of "soft" metals such as Ag, Cd, and Cu involves a "shift" to detoxified fractions, in the present study being heat-stable protein ligands, presumably metallothionein or metallothionein-like peptides and proteins.

The 2 Co peaks that were observed at 28 and 29 min in the cytosolic medium-molecular weight pool appear to be preserved in the heat-stable protein chromatograms with better separation (22 and 25.5 min in fraction 3) and with a slight shift in the estimated molecular weights attributable to the imprecision below 10 kDa for the SEC_{200} column (see *Materials and Methods*). On the other hand, the large Co peak observed at 19 min in the whole cytosol did not appear in the void volume of the heat-stable protein chromatograms, a result that is consistent with a considerable proportion of Co being present in the heat-denatured protein fraction (i.e., the pellet resulting after heat treatment of the whole cytosol; details in Supplemental Data). For Ni and Tl, a similar pattern was observed because the major peaks for both metals in the cytosol (medium- and low-molecular wt pools, respectively) were also observed in fraction 3 of the heat-stable protein chromatograms (compare Figures 1 and 3), but the Tl peak observed at 17.5 min in the cytosol high-molecular weight pool was not present in the void volume of the heat-stable protein chromatograms. As suggested previously for Cu and Co, the disappearance of this ligand suggests that it is a protein subject to heat denaturation.

Overall, these results suggest that when changes among the relative proportions of the high-, medium-, and low-molecular weight pools are observed along the contamination gradient (Supplemental Data, Figure S3) but we do not see any such changes in the relative proportions of fractions 1, 2, and 3 in the heat-stable protein chromatograms (Supplemental Data, Figure S5), we can conclude that the changes in the whole cytosol involved biomolecules (proteins) that were denatured by the heat treatment. For example, for Ni (and to a lesser extent for Tl) we observe an increase in the proportion of metal bound in the cytosolic high-molecular weight pool with increasing concentration of metal (Supplemental Data, Figure S3). Because we did not detect any Ni in the void volume of the heat-stable protein chromatogram (Supplemental Data, Figure S2), we can conclude that the "shift" seen in the cytosol involves a binding of Ni to heat-denatured protein ligands in the high-molecular weight pool and, therefore, implies a potential risk of toxicity at high concentrations (until the molecular nature of these thermolabile high-molecular wt ligands has been determined, this risk of toxicity is best qualified as "potential" because the heat-denatured protein ligands may include Ni chaperones, involved in intracellular metal trafficking [26], in which case the presence of Ni in the high-molecular weight pool would not be a harbinger of toxicity). This reasoning is in agreement with that of Giguère et al. [6], who concluded that most metals found in the heat-stable protein fraction (Cd, Cu, and Zn) are largely bound to metallothionein-like peptides and proteins and that the poor affinity of Ni for thiolated cytosolic proteins explains why this metal is mostly associated with ligands other than metallothionein or metallothionein-like peptides and proteins.

CONCLUSION

Previous studies on the intracellular fate of metals in aquatic organisms have largely relied on differential centrifugation and heat denaturation to determine the partitioning of metals in the subcellular environment. For metals in the cytosol, this approach has typically involved a gentle homogenization step followed first by ultracentrifugation to obtain an operationally defined cytosol and then by heat denaturation to distinguish between thermostable and thermolabile metal–biomolecule complexes. Researchers in this area (including ourselves) have tended to interpret the presence of metals in the thermostable metal fraction (heat-stable protein) as an indication that the metals found in this fraction were bound to metallothionein or metallothionein-like peptides and proteins; in the case of nonessential metals such as Ag and Cd, this putative binding to metallothionein is normally interpreted as evidence for successful detoxification.

The present study supports the assumption that Ag, Cd, and Cu found in the heat-stable protein supernatants are largely

present as metallothionein or metallothionein-like peptides and protein complexes. However, this is not the case for Co, Ni, and Tl, which are also present in the heat-stable protein supernatants but do not elute in the molecular weight range corresponding to metallothionein and metallothionein-like peptides and proteins. This difference in metal partitioning is consistent with the known preference of soft metals such as Ag, Cd, and Cu(I) for thiolated ligands and the contrasting tendency of Co and Ni to bind to ligands with oxygen and nitrogen as donor atoms. Future work should focus on identifying these latter ligands, present in fractions 1 and 3 of the heat-stable protein supernatants, because we do not know whether they correspond to metal-sensitive ligands (in which case they could potentially be used as biomarkers of metal-induced effects) or rather to low-molecular weight metal chaperones that serve to buffer the internal concentrations of nonchalcophile elements [27]. This ambiguity extends to Tl, which is also present in the heat-stable protein supernatants and is normally classified as a chalcophile but does not elute with the metallothionein and metallothionein-like peptides and protein peaks.

Finally, looking at metal handling in the whole cytosol from a toxicological perspective (and limiting our conclusions to those metals for which there was an appreciable metal concentration gradient), we note that for Cd and Cu the relative importance of the medium-molecular weight pool (which includes metallothionein and metallothionein-like peptides and proteins) tends to increase relative to the other pools as the total cytosolic metal concentration increases, an observation that is consistent with a concentration-dependent detoxification response. On the other hand, for Ni and Tl the increase in total cytosolic metal concentration is accompanied by a marked increase in the high-molecular weight fraction, a trend that suggests that hepatic Ni and Tl are not being effectively detoxified.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3998.

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Data availability—Data are available in the Supplementary Data files and from the corresponding author (peter.campbell@ete.inrs.ca).

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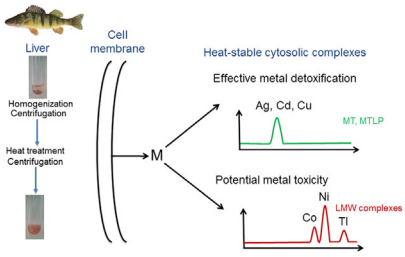

Binding of Trace Elements (Ag, Cd, Co, Cu, Ni, And Tl) To Cytosolic Biomolecules in Livers of Juvenile Yellow Perch (Perca Flavescens) Collected From Lakes Representing Metal Contamination Gradients