

Cadmium uptake in isolated adrenocortical cells of rainbow trout and yellow perch

N.J. Raynal^a, A. Hontela^b, C. Jumarie^{a,*}

^aDépartement des Sciences Biologiques, Centre Toxén, Université du Québec à Montréal, C.P. 8888, Succ. Centre-Ville, Montréal, Québec, Canada H3C 3P8

^bDepartment of Biological Sciences, Water Institute for Semi-arid Ecosystems (WISE), University of Lethbridge, Lethbridge, Alberta, Canada T1K 3M4

Received 28 October 2004; received in revised form 23 March 2005; accepted 31 March 2005

Abstract

Cadmium uptake was studied in isolated adrenocortical cells of rainbow trout (*Oncorhynchus mykiss*) and yellow perch (*Perca flavescens*) to test the hypothesis that the greater sensitivity of trout cells to Cd-induced disruption of cortisol secretion observed in previous studies is correlated to higher level of metal accumulation. There was no evidence for interspecies differences in accumulation level, and a specific transport mechanism of similar affinity has been characterized in both fish species. However, inhibition of Cd uptake by calcium was observed in rainbow trout exclusively. The free metal ion Cd^{2+} and chlorocomplexes CdCl_n^{2-n} both contribute to Cd accumulation with different level of contribution between fish species. We conclude that interspecies differences in sensitivity to Cd endocrine disrupting effect are not necessarily related to different levels of metal accumulation but would rather be linked to transport pathways and metal speciation. Cadmium/calcium competition for uptake could be a determinant of the early Cd-induced impaired cortisol secretion in trout but not perch cells.

© 2005 Elsevier Inc. All rights reserved.

Keywords: Adrenocortical cells; Rainbow trout; Yellow perch; Cadmium uptake; Endocrine disruptor; Metal speciation; Calcium

1. Introduction

Environmental contamination by cadmium (Cd), a highly toxic metal released into the aquatic and terrestrial ecosystems by industrial activities poses significant health risks to both humans and wildlife. In 1993, the International Agency for Research on Cancer classified Cd and its compounds in Group 1 of carcinogenic substances to humans. In fish, there is also substantial evidence that growth, reproduction, respiratory functions and osmoregulation are affected by exposure to Cd (Pratap and Wendelaar Bonga, 1990; Sorensen, 1991; Hontela, 1997). Moreover, Cd causes apoptosis in skin epithelial cells of carp (Iger et al., 1994) and trout

(Lyons-Alcantara et al., 1998) as well as in rainbow trout hepatocytes (Risso-de Faverney et al., 2001). In addition, Cd has been identified to act as an endocrine disrupter exerting oestrogenic as well as androgenic effects in various species including fish (Kime, 1984; Garcia-Morales et al., 1994; Le Guével et al., 2000; Martin et al., 2002). Recently, it has been also reported that chronic exposure to Cd depresses the hypothalamic–pituitary–adrenocortical axis and impairs cortisol secretion (Hontela, 1997). Adrenocortical tissues of fish sampled in lakes contaminated by atmospheric depositions from mining activities contain high levels of Cd and exhibit an impaired cortisol secretion characterized by a blunted response to adrenocorticotrophic hormone (ACTH) or cAMP (Laflamme et al., 2000; Levesque et al., 2003). Cd has been shown to act directly on isolated steroidogenic cells responsible for cortisol secretion in rainbow trout, (*Oncorhynchus mykiss*) and yellow perch, *Perca flavescens* (Leblond and Hontela, 1999).

* Corresponding author. Tel.: +1 514 987 3000x7680; fax: +1 514 987 4647.

E-mail address: jumarie.catherine@uqam.ca (C. Jumarie).

Rainbow trout is one of the most sensitive fish to pollutants, particularly to metals including Cd (Hansen et al., 2002). The long term lethal value for trout exposed to Cd in water is 0.01 mg/l while it is 0.5 mg/l for perch (Alabaster and Lloyd, 1982). It has been suggested that differences of sensitivity to Cd between fish species is related to different rates of accumulation, in addition to various fates and patterns of distribution within tissues (Norey et al., 1990). A difference in sensitivity of specific target organs could however also explain the differences of vulnerability to metals. Recently, Lacroix and Hontela compared the sensitivity of steroidogenic cells isolated from the adrenocortical tissue of rainbow trout and yellow perch to Cd-induced disruption of cortisol secretion (Lacroix and Hontela, 2004). They reported that although slightly higher Cd concentrations were required to affect cell viability in rainbow trout compared to yellow perch, trout cells were more sensitive to the endocrine disrupting effect of Cd. Concentrations of Cd resulting in 50% inhibition of cortisol secretion (EC50) following a 1-h exposure in vitro were 0.09 mM and 0.26 mM in trout and perch cells, respectively. To explain the different sensitivities to Cd observed in vivo as well as in vitro between fish species, accumulation of Cd in specific target tissues needs to be characterized.

In this study, Cd uptake in the adrenocortical cells isolated from rainbow trout and yellow perch was investigated to: (i) increase our knowledge about transport mechanisms responsible for Cd uptake in fish cells, and (ii) determine whether or not species differences in sensitivity to Cd-induced endocrine disruption were related to different level of accumulation. Since Cd transport in various species and cell types involves specific mechanisms of transport (Blazka and Shaikh, 1992; Endo et al., 1999; Leffler et al., 2000; Jumarie et al., 2001), notably calcium (Ca) channels in fish gills (Verboost et al., 1989), the impact of Ca on Cd uptake has also been investigated. Uptake experiments have been conducted under well-controlled inorganic speciation conditions to estimate how much of Cd uptake is related to the free ion Cd^{2+} species.

2. Materials and methods

2.1. Chemicals

Minimum essential medium (MEM) was obtained from Sigma Chemical Co. (St-Louis, MO), whereas collagenase/dispase was from Boehringer Mannheim (Roche, Basel Switzerland). MS 222 was obtained from ICN Pharmaceuticals (Costa Mesa, CA). Culture 96-well microplates, multiscreen 96-well membranes (pore size: 1.2 μm) and punch tips (Millipore) were purchased from Fisher Scientific (Ottawa, ON). Labeled $^{109}\text{CdCl}_2$ (specific activity: 1.82–3.08 mCi/mg) was obtained from Perkin Elmer (Boston, MA).

2.2. Experimental animals

Rainbow trout, *Oncorhynchus mykiss* (body weight 80–110 g) were bought from a local commercial supplier: la Pisciculture Laurentienne. Yellow perch, *Perca flavescens* (body weight 50–110 g) were captured by seine in Lake Memphremagog (Québec, Canada) which is a well characterized pristine lake (Girard et al., 1998). Fish were maintained in a 600 l freshwater tank at 15 ± 1 °C with a constant flow rate of 3.8 l/min of filtered and oxygen-saturated water (hardness 70 mg/l CaCO_3). At the arrival in the laboratory, yellow perch were treated one week with antibiotic (oxytetracycline, Quick cure, sulfamethazin) before the period of acclimation. Fish were fed daily (ad libitum): rainbow trout with a commercial trout food at the recommended rate (10 g/kg of fish) and yellow perch with pieces of beef meat. The experiments began after 2 weeks of acclimation for both fish species.

2.3. Preparation of cell suspension

The adrenocortical cell suspension method has been described in details by Leblond and Hontela (1999) for rainbow trout and was also applied to yellow perch. Fish anaesthetized with MS 222 were bled from the caudal vasculature and perfused with saline solution (0.7% NaCl) to remove as much blood as possible. Adrenocortical tissue (head kidney) was then dissected and washed twice with complete medium (MEM supplemented with 5 g/l BSA and 2.2 g/l NaHCO_3 , pH 7.4) before digestion with 2.0 mg/ml collagenase/dispase for 1 h at room temperature. The cell suspension was filtered with a 30 μm mesh cloth and the filtrate was washed 3 times (centrifugation 300 $\times g$ for 1 min at 15 °C and resuspension) with a Ringer solution containing (in mM): 111 NaCl, 1.8 CaCl_2 , 2.7 KCl, 2.4 NaHCO_3 , 5.5 dextrose, 25 Hepes adjusted to pH 7.4 with 1 mM NaOH. The total cell suspension was kept in a conical tube (50 mL Sarsted tube) and was incubated in the Ringer solution for 2 h with gentle agitation before the beginning of the transport measurements.

2.4. Cadmium transport and efflux measurements

Cadmium uptake experiments were performed at 15 °C in serum-free transport media referred to as chloride (Cl^-) and nitrate (NO_3^-) media and containing, respectively, (in mM): 111 NaCl/ NaNO_3 , 2.7 KCl/ KNO_3 , 1.8 $\text{CaCl}_2/\text{Ca}(\text{NO}_3)_2$, 2.8 NaHCO_3 , 5.5 Dextrose and 25 Hepes buffered to pH 7.4 with NaOH 1 N. Some experiments were conducted in the presence of an excess of 100 μM unlabeled Cd used as a specific inhibitor of ^{109}Cd uptake, or in the absence of $\text{CaCl}_2/(\text{NO}_3)_2$ (in the latter case, additional 2.7 mM NaCl was used to keep the ionic strength of the exposure medium constant). Transport media were always prepared in advance and allowed to equilibrate in plasticware overnight at room temperature.

^{109}Cd was added to the cell suspension from stock concentration to get a final ^{109}Cd concentration of $0.5\ \mu\text{M}$ ($0.09\ \text{ppm}$) which does not affect adrenocortical cell viability ($\text{LC}_{50}=10.8\ \text{mM}$, Leblond and Hontela (1999)). After specific selected time of incubation, 5 replicates each of $200\ \mu\text{l}$ were sampled from the cell suspension: $50\ \mu\text{l}$ were kept for protein determination according to Bradford (1976), and the remaining $150\ \mu\text{l}$ were rapidly rinsed 3 times with ice-cold ^{109}Cd -free chloride medium containing $2\ \text{mM}$ EDTA by ultrafiltration using a 96-well multiscreen vacuum system to remove the excess radioactivity according to the procedure previously established (Jumarie et al., 2001). Filters were then cut off from the multiscreen 96-well microplates using punchtips and radioactivity of cells on filters was determined using a gamma counter (Cobra II, Canberra Packard Canada).

Cadmium efflux measurements were performed at $15\ ^\circ\text{C}$. The cells were first exposed to $0.5\ \mu\text{M}$ ^{109}Cd in a chloride medium for 1 h, at which time accumulation process was stopped by a 3-min centrifugation ($300\times g$). Cells were then resuspended in a ^{109}Cd -free efflux chloride medium and sampled at specific times and processed as previously described for uptake experiments. Cellular uptake and efflux are both expressed as $\text{pmol } ^{109}\text{Cd}$ per mg protein.

2.5. Cadmium speciation and experimental conditions

Cadmium speciation in the transport media was calculated as previously (Jumarie et al., 2001) using the MINEQL⁺ chemical equilibrium program (Schecher and McAvoy, 1994) and the NIST stability constants database (Martell et al., 2001). Cd transport was studied in a chloride medium where Cd is mainly present as chlorocomplexes (CdCl_n^{2-n}) (Table 1). However, to assess the impact of metal speciation on Cd transport, the Cl^- ligand (of high affinity for Cd) was changed for NO_3^- (of much lower affinity for Cd) to increase Cd^{2+} levels over chlorocomplexes formation ($\log K$ values for CdCl^+ and CdNO_3^+ are 1.98 and 0.50, respectively: K is the formation constant for Cd complexation). Indeed, as shown in Table 1, the total dissolved metal in the nitrate medium was mainly recovered as the free ion Cd^{2+} compared to chloride exposure

Table 1
Cadmium speciation in the chloride and nitrate exposure media

Chloride		Nitrate	
Species	% Total	Species	% Total
Cd^{2+}	17	Cd^{2+}	84
CdCl^+	63	CdNO_3^+	11
CdCl_2	18	CdHCO_3^+	4
CdCl_3^-	1.3	–	–

The composition of the transport media is described in Materials and methods. Cadmium speciation was calculated using the MINEQL⁺ chemical equilibrium program. Species listed are those representing more than 1% of the total dissolved metal.

conditions. Therefore, to evaluate the relative contribution of the free metal ion Cd^{2+} and chlorocomplexes CdCl_n^{2-n} to the total uptake of Cd, experimental conditions were designed as described previously (Jumarie et al., 2001). Briefly, NO_3^- (which does not bind Cd as much as Cl^-) concentration was successively changed for Cl^- according to speciation calculations, so that only total CdCl_n^{2-n} levels increased from 0 to $0.8\ \mu\text{M}$ whereas that of the free metal cation Cd^{2+} was maintained constant at $170\ \text{nM}$ (Table 2).

2.6. Cell viability

Cell viability was assessed using iodide propidium exclusion and FAXSCAN analyses. No more than 2% loss in cell viability was recorded regardless of the exposure medium (chloride or nitrate) used (data not shown).

2.7. Data analysis

Accumulation (uptake) time-course data were analyzed according to the linear Eq. (1)

$$A = A_0 + v.t \quad (1)$$

in which the initial rate v of accumulation is the slope of the regression line and A_0 represents the zero-time accumulation value.

Kinetic parameters of Cd uptake in adrenocortical cells were determined by one-time point measurements at 2 min (rainbow trout) and 5 min (yellow perch), using $0.5\ \mu\text{M}$ ^{109}Cd and increasing concentrations of unlabeled Cd ranging from 0 to maximally $50\ \mu\text{M}$. Kinetic data, normalized to 1 min, were analyzed in term of total Cd ($^{109}\text{Cd} + \text{Cd}$) according to the Michaelis–Menten Eq. (2)

$$v = \frac{V_{\max}[\text{Cd}_{\text{total}}]}{K_m + [\text{Cd}_{\text{total}}]} + k_D[\text{Cd}_{\text{total}}] \quad (2)$$

in which K_m and V_{\max} have their usual meaning (apparent affinity and maximal velocity of uptake, respectively), and k_D stands for all nonspecific contributions to total uptake.

The 5-min accumulation data measured as a function of chlorocomplexes formation were analyzed according to the linear Eq. (3)

$$A_5 = A_0 + k_{D5}[S] \quad (3)$$

in which A_5 is the initial 5-min accumulation, $[S]$ is the concentration of chlorocomplexes ($[^{109}\text{CdCl}_n^{2-n}]$), and k_{D5} is the proportionality constant for the accumulation process (measured at 5 min).

All linear and nonlinear regression analyses of the uptake data were performed using Prism 3.0 software© 2001 (GraphPad Software). The errors associated with kinetic parameter values given in the text represent the standard errors of linear/nonlinear regression (SER). Statistical analyses on efflux data were performed with the two-tailed Student's t test for unpaired data on small samples with

Table 2

Cd speciation as a function of increasing chloride concentration

$[^{109}\text{Cd}]_{\text{total}}$ (nM)	$[\text{Cl}^-]$ (mM)	$[\text{NO}_3^-]$ (mM)	$[^{109}\text{Cd}^{2+}]$ (nM)	$[^{109}\text{CdCl}^+]$ (nM)	$[^{109}\text{CdCl}_2]$ (nM)	$[^{109}\text{CdCl}_3^-]$ (nM)	$[^{109}\text{CdNO}_3^+]$ (nM)
200	0	117	170	–	–	–	29.1
269	13	104	170	71	2.1	–	26.1
344	26	91	170	142	8.6	–	22.8
422	39	78	170	213	19.3	–	19.5
506	52	65	170	284	34.3	1	16.3
594	65	52	170	355	53.6	2	13
686	78	39	170	426	77.2	3.4	9.8
784	91	26	170	497	105	5.4	6.5
886	103	13	170	568	137	8.1	3.2
994	117	0	170	639	174	11.5	–

Cadmium speciation was calculated using MINEQL⁺ chemical equilibrium program, as described in Materials and methods. Components listed are those for which the concentrations were modified to ensure that $[\text{Cd}^{2+}]$ remained constant as the chlorocomplex levels were modified. Species listed are those representing more than 1% of the total dissolved metal (in addition to CdHCO_3^+ representing less than 3.5% of total Cd for all experimental conditions).

Welch's correction using InStat software© 2001 (GraphPad Software). Statistical significance was assessed at the $p < 0.05$ level.

3. Results

3.1. Time course of ^{109}Cd uptake

As shown in Fig. 1, the accumulation of $0.5 \mu\text{M}$ ^{109}Cd in rainbow trout (A) and yellow perch (B) adrenocortical cells increased linearly as a function of time. Quite large inter-individual variations in ^{109}Cd accumulation levels were observed among fish of the same species, but no inter-species differences could be noted. Indeed, in both fish species, the uptake time course data could be well analyzed according to Eq. (1) with similar uptake rate value ($v = 1.18 \pm 0.09$ and 1.11 ± 0.03 pmol $^{109}\text{Cd}/\text{min}/\text{mg}$ protein for trout and perch cells, respectively) showing that ^{109}Cd (filled circles) is highly and rapidly accumulated in these fish endocrine cells. Fig. 1 also shows that there is no evidence for an equilibrium of uptake following a 1 h-exposure to $0.5 \mu\text{M}$ Cd, suggesting high levels of cellular accumulation (binding) capacity. A 95% reduction of the uptake rate v for ^{109}Cd was obtained in the presence of an excess of $100 \mu\text{M}$ unlabeled Cd (open circles) in both fish species. In this study uptake data are expressed relative to tracer exclusively whereas unlabeled Cd is used as a potent competitive inhibitor of ^{109}Cd accumulation. These results therefore clearly demonstrate the involvement of: (1) a specific transport system(s) in metal uptake in adrenocortical cells from both fish species; (2) similar initial uptake rate with no evidence for any significant differences in the 1 h-level of Cd accumulation in rainbow trout and yellow perch cells.

3.2. Cd/Ca interaction in Cd uptake

Fig. 2 shows the results representative of 3 different experiments designed to assess whether or not ^{109}Cd uptake

may be affected by the presence of Ca in the exposure medium. The uptake time course of $0.5 \mu\text{M}$ ^{109}Cd has been studied in the absence (open circles) and in the presence (filled circles) of 1.8 mM Ca. A strong inhibitory effect on ^{109}Cd transport in trout cells (Fig. 2A) was clearly observed

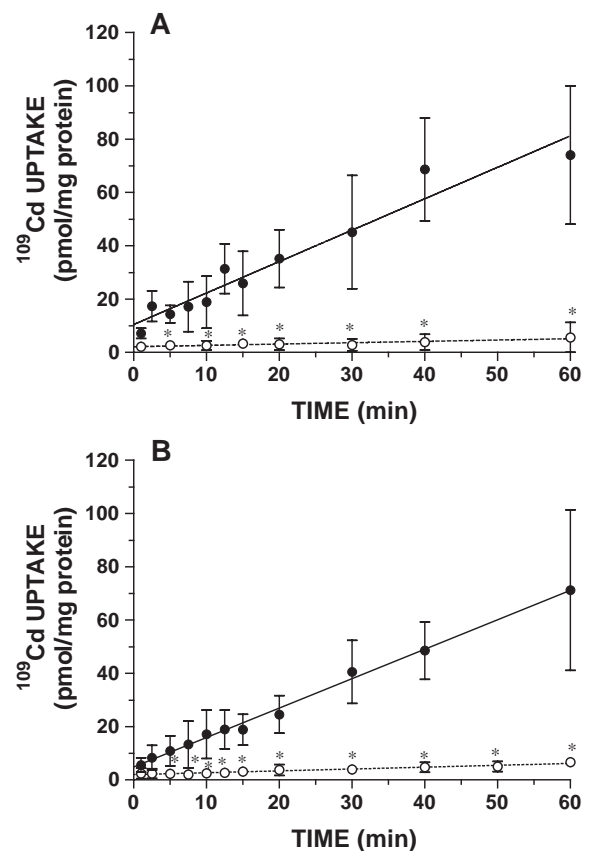


Fig. 1. Time-course of $0.5 \mu\text{M}$ ^{109}Cd uptake in adrenocortical cells of Rainbow trout (A) and Yellow perch (B) in a chloride medium in absence (filled circles) or presence (open circles) of $100 \mu\text{M}$ unlabeled Cd. The lines shown are the best-fit curves over data points obtained according to Eq. (1). Points are means \pm SD evaluated on five to seven different cell preparations (5–7 fishes). *Significant differences ($p < 0.05$) compared with control values measured in the absence of unlabeled Cd.

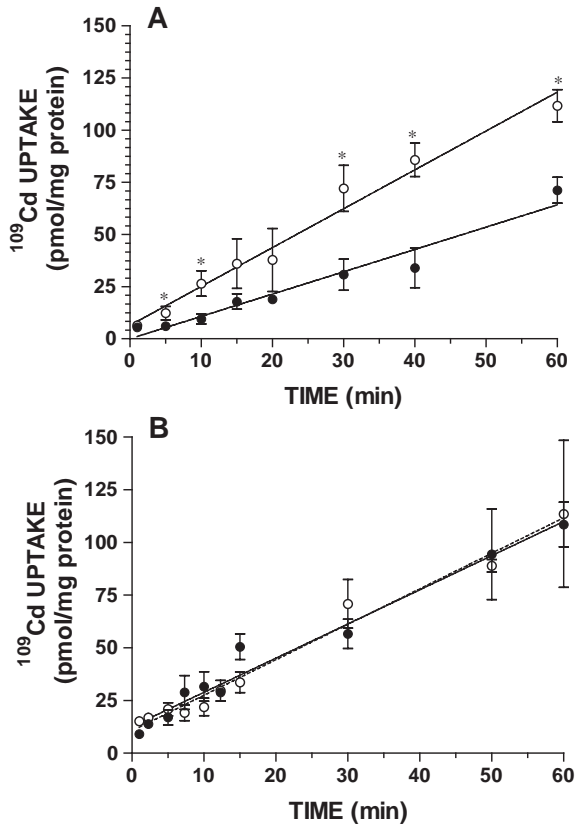


Fig. 2. Time-course of $0.5 \mu\text{M}$ ^{109}Cd uptake in adrenocortical cells of Rainbow trout (A) and Yellow perch (B) in a chloride medium in presence (filled circles) or absence (open circles) of 1.8 mM Ca. The lines shown are the best-fit curves over data points as obtained according to Eq. (1). Points are means \pm SD evaluated on five determinations of the same cell preparation (1 fish). Data shown are representative of three different experiments. *Significant differences ($p < 0.05$) compared with values measured in the presence of Ca.

in the presence of Ca: the initial uptake rate fell from 1.86 ± 0.11 to 1.07 ± 0.09 pmol $^{109}\text{Cd}/\text{min}/\text{mg}$ protein and a 36% decrease in ^{109}Cd accumulation was observed at 1 h, compared to Ca-free exposure chloride conditions. In contrast, Cd uptake levels in perch cells were similar whether Ca was present or absent (Fig. 2B).

3.3. Kinetic parameters of Cd accumulation

To further characterize the specific component of ^{109}Cd uptake we have determined the kinetic parameters by one-time point analysis, measuring the initial accumulation of total Cd ($0.5 \mu\text{M}$ ^{109}Cd + increasing levels of unlabeled Cd) in rainbow trout (Fig. 3) and yellow perch (Fig. 4). The data points obtained in a standard (1.8 mM Ca) chloride medium (filled circles) could be analyzed according to Eq. (2) with the following parameter values (normalized to 1 min): $V_{\text{max}} = 4.1 \pm 1.0$ pmol/min/mg protein, $K_m = 1.2 \pm 0.9 \mu\text{M}$ and $k_D = 0.21 \pm 0.04$ pmol/ $\mu\text{M}/\text{min}/\text{mg}$ protein for trout cells; $V_{\text{max}} = 21.4 \pm 2.5$ pmol/min/mg protein, $K_m = 3.4 \pm 0.8 \mu\text{M}$ and $k_D = 1.2 \pm 0.1$ pmol/ $\mu\text{M}/\text{min}/\text{mg}$ protein for perch cells.

The much higher k_D value for Cd uptake was obtained for perch cells, showing a more important contribution of the nonspecific component of uptake which leads to higher levels of accumulation as a function of increasing concentration of total Cd (compare Figs. 3A and 4A). Correction of the uptake data for the nonspecific contribution ($k_D \times [\text{Cd}]$) allows a better appreciation of the Michaelis–Menten kinetics: the 5-fold higher V_{max} value estimated for Cd uptake in the perch cells also contribute to increase the level of accumulation at high metal concentrations whereas comparable K_m values are obtained in both fish species (Figs. 3B and 4B).

Since results shown in Fig. 2 suggest Cd/Ca interaction in trout cells exclusively, kinetic parameters were also determined in the absence of Ca in trout cells only. It is

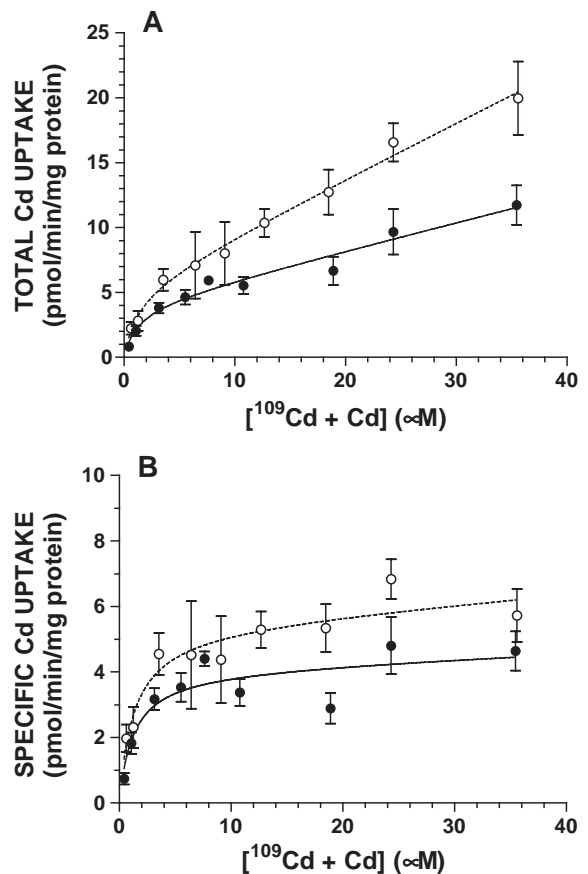


Fig. 3. Determination of kinetic parameters for Cd uptake in adrenocortical cells of Rainbow trout. Initial accumulation values were estimated by one-time point analysis as described in the text using a tracer ^{109}Cd concentration of $0.5 \mu\text{M}$ and unlabeled Cd ranging from 0 to $35 \mu\text{M}$ in a chloride medium. The lines are the best-fit curves to Eq. (2). (A) Total uptake with the following kinetic parameter values: $V_{\text{max}} = 4.1 \pm 1.0$ or 5.4 ± 0.9 pmol/min/mg protein; $K_m = 1.2 \pm 0.9$ or $1.3 \pm 0.7 \mu\text{M}$; $k_D = 0.21 \pm 0.04$ or 0.43 ± 0.03 pmol/min/ $\mu\text{M}/\text{mg}$ protein in presence (filled circles) or absence of 1.8 mM Ca (open circles). (B) Specific component of uptake as obtained after subtraction of the diffusional uptake ($k_D \times [\text{Cd}]$) from total uptake values. Points are means \pm SD evaluated on five determinations of the same cell preparation (1 fish). Data shown are representative of three different experiments.

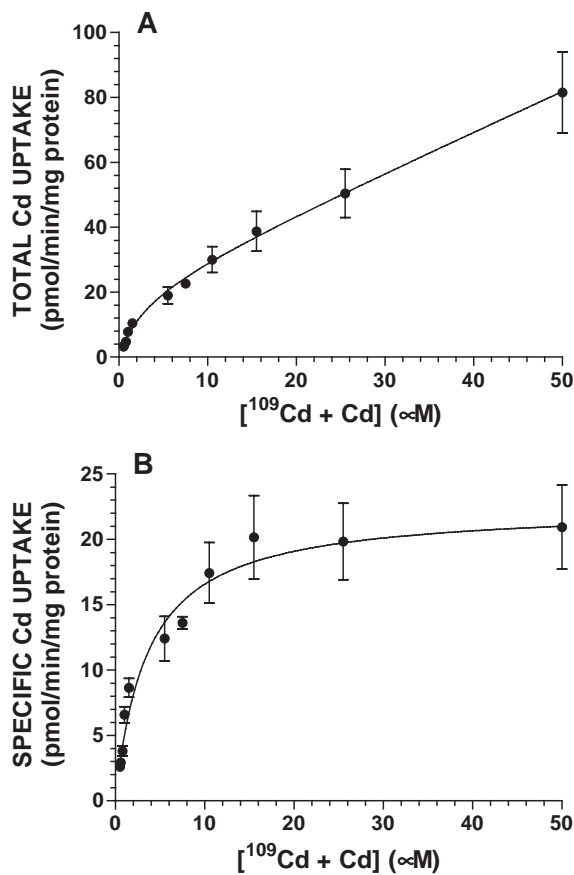


Fig. 4. Determination of kinetic parameters for Cd uptake in adrenocortical cells of Yellow perch. Initial accumulation values were estimated by one-time point analysis as described in the text using a tracer ^{109}Cd concentration of $0.5 \mu\text{M}$ and unlabeled Cd ranging from 0 to $50 \mu\text{M}$ in a chloride medium. The lines are the best-fit curves to Eq. (2). (A) Total uptake with the following kinetic parameter values: $V_{\max} = 21.4 \pm 2.5$ pmol/min/mg protein; $K_m = 3.4 \pm 0.8 \mu\text{M}$; $k_D = 1.2 \pm 0.1$ pmol/min/ μM /mg protein. (B) Specific component of uptake as obtained after subtraction of the diffusional uptake ($k_D \times [\text{Cd}]$) from total uptake values. Points are means \pm SD evaluated on five determinations of the same cell preparation (1 fish). Data shown are representative of three different experiments.

noteworthy that a 2-fold higher k_D value (0.43 ± 0.03 pmol/ μM /min/mg protein) was obtained for Cd uptake in the absence of Ca (Fig. 3A, open circles) whereas no significant differences were noted for the V_{\max} (5.4 ± 0.9 pmol/min/mg protein) or the K_m ($1.3 \pm 0.7 \mu\text{M}$) value (Fig. 3B).

3.4. Cadmium uptake as a function of inorganic speciation

Since Cd/Ca interaction for uptake is assumed to involve the free metal ion Cd^{2+} , rather than chlorocomplexes, we have designed an experimental procedure allowing us to estimate the relative contribution of Cd^{2+} and CdCl_n^{2-n} in the total uptake of Cd. As explained in Materials and Methods, NO_3^- in the transport medium was successively changed for Cl^- and the total concentration of Cd was adjusted in such a way that only $[\text{CdCl}_n^{2-n}]$ increased whereas $[\text{Cd}^{2+}]$ was systematically equal to 170 nM (Table

2). Results of this study are shown in Fig. 5 where the data points obtained for total $[\text{CdCl}_n^{2-n}]$ ranging from 0 to $0.8 \mu\text{M}$ correspond to the experimental conditions described in Table 2. Although $[\text{Cd}^{2+}]$ was unchanged, a linear increase in the 5-min accumulation of ^{109}Cd was clearly observed as a function of increasing chlorocomplexes formation; the resulting data could be fitted successfully to Eq. (3) with a proportionality constant (k_{DS}) of 25.8 ± 3.0 and 27.6 ± 3.4 pmol/ μM /5 min/mg protein for rainbow trout (Fig. 6A) and yellow perch (Fig. 6B) cells, respectively. Using the nonzero intercept uptake value (at zero CdCl_n^{2-n}) which can be attributed to 170 nM Cd^{2+} (12.2 ± 1.4 and 22.1 ± 1.3 pmol/5 min/mg protein, for trout and perch, respectively), it was estimated that under conditions where membrane transport systems were not saturated, the 5-min uptake of Cd^{2+} increased linearly with a proportionality constant of about 71 and 130 pmol/ μM /mg protein in trout

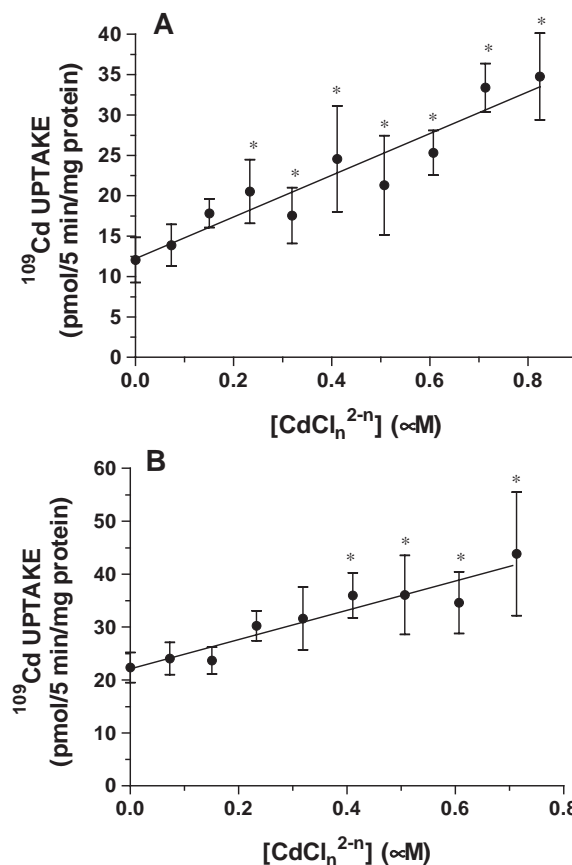


Fig. 5. Short term (5-min) uptake of ^{109}Cd in adrenocortical cells of Rainbow trout (A) and Yellow perch (B) measured as a function of increasing concentrations of $^{109}\text{CdCl}_n^{2-n}$. Experimental conditions corresponding to each data point are described in Table 2. In all cases the free Cd^{2+} ion concentration was maintained at 170 nM . The lines shown are the best-fit curves over data points as obtained according to Eq. (3). Data points are means \pm SD evaluated on five determinations of the same cell preparation (1 fish). Data shown are representative of three different experiments. *Significant differences ($p < 0.05$) compared with values measured in the absence of chloride using a nitrate medium (control value at $[\text{CdCl}_n^{2-n}] = 0$).

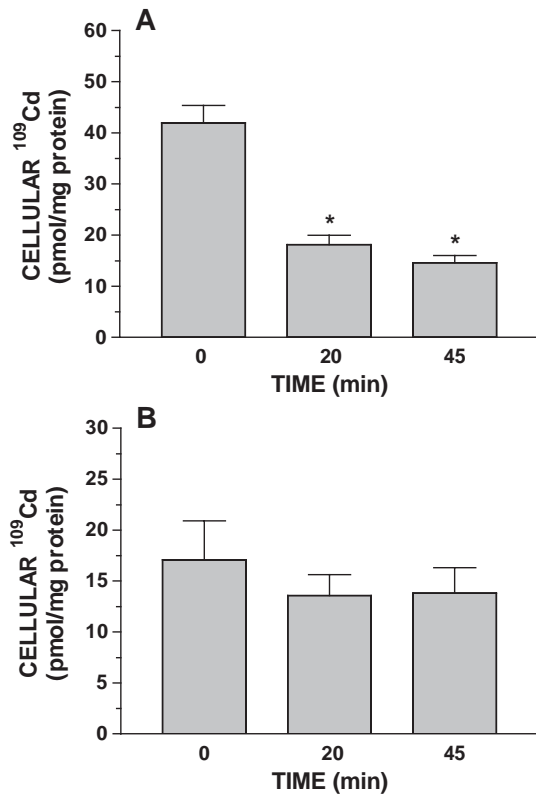


Fig. 6. ¹⁰⁹Cd efflux from adrenocortical cells of Rainbow trout (A) and Yellow perch (B) cells pre-exposed for 1 h to 0.5 μM ¹⁰⁹Cd (chloride medium). Cellular ¹⁰⁹Cd release was recorded as described in the text. After pre-incubation, cells were suspended in a chloride medium in absence of ¹⁰⁹Cd. The levels of ¹⁰⁹Cd remaining in cells were evaluated after a 45-min period of incubation in a Cd-free medium. Data points are means ± SD evaluated on five determinations of the same cell preparation (1 fish). Data shown are representative of three different experiments. *Indicates significant differences ($p < 0.05$) compared to no efflux conditions (total initial cellular metal).

and perch cells, respectively. Comparison of these values with that of 26 and 28 pmol/μM/mg protein obtained for CdCl_n^{2-n} uptake suggests that chlorocomplexes were transported about 0.4 and 0.2 times as rapidly as the free metal ion in trout and perch cells, respectively. Also, from the accumulation value related to 170 μM ¹⁰⁹Cd²⁺, it can be estimated that the free metal ion Cd²⁺ contributes 35% and 50% of total accumulation measured under standard conditions (experiment 10 in Table 2, where total ¹⁰⁹Cd uptake were 34.8 ± 5.4 and 43.9 ± 11.7 pmol/5 min/mg protein in trout and perch cells, respectively).

3.5. Cellular Cd efflux

Cadmium accumulation but also Cd cellular retention is an important determinant of cytotoxicity. Therefore we have studied cellular Cd efflux at 15 °C in cells pre-exposed to 0.5 μM ¹⁰⁹Cd (chloride exposure conditions) for 1 h. As shown in Fig. 6, a 20-min period of incubation in Cd-free efflux chloride medium resulted in a 56% loss of Cd content

in rainbow trout cells (Fig. 6A); at 45 min of efflux, 65% of the metal initially accumulated was lost. In contrast, no significant cellular efflux could be detected in perch cells during the 45-min period studied (Fig. 6B). Thus, Cd accumulation is partially reversible in rainbow trout but not in yellow perch adrenocortical cells; these results suggest a higher proportion of cellular metal tightly bound to intracellular components in perch cells compared to trout.

4. Discussion

The present study demonstrates, for the first time, that Cd is highly and rapidly transported in fish adrenocortical cells. Moreover, and contrary to the general hypothesis, our results show that the difference in sensitivity to a metal between fish species is not necessarily related to different levels of accumulation in target organs. Although isolated adrenocortical cells of rainbow trout have been shown to be more sensitive to Cd-induced disruption of cortisol secretion compared to yellow perch (Lacroix and Hontela, 2004), we found similar uptake rate and level of Cd accumulation in both fish species (Fig. 1). Also, in both trout and perch cells, ¹⁰⁹Cd uptake was highly inhibited by the presence of an excess of unlabeled Cd, demonstrating that Cd uptake occurs via a specific and displaceable mechanism of transport. Additional kinetic characterization revealed the involvement of a system of very high affinity but low capacity in cells of both fish species (Figs. 3 and 4). The similar K_m values of 1–3 μM ensure that uptake experiments performed with 0.5 μM ¹⁰⁹Cd were indeed carried under conditions far from transport saturation. Note that, although our kinetic data did not reveal any heterogeneity, more than one of the Cd species listed in Table 1 may conceivably participate in the transport process. Accordingly, the K_m values estimated in this study should be considered as operational parameters only.

A clear difference between Cd uptake in adrenocortical cells of both fish species was the inhibitory effect of Ca occurring in rainbow trout cells exclusively. The initial uptake rate and the 1-h accumulation level of 0.5 μM ¹⁰⁹Cd in trout cells both decreased in the presence of 1.8 mM Ca (Fig. 2). However, the presence of Ca did not result in modification of the K_m nor the V_{max} value but rather lowered by half the proportional constant k_D for Cd uptake (Fig. 3). This result is unexpected since k_D represents the nonspecific contribution of Cd accumulation, i.e. passive diffusion process which, conceptually, should not be inhibited. However, a Michaelis–Menten kinetic does reduce to a linear kinetic when $[\text{Cd}] \ll K_m$, and uptake increases linearly as a function of $[\text{Cd}]$ with a proportionality constant equal to the ratio V_{max}/K_m . The part of Cd transport for which saturation is observed under our experimental conditions (and then the kinetic parameters determined in this study) is not necessarily related to transport mechanism subjected to Ca inhibition (thus would

not be affected whether Ca is present or absent). Transport systems in which Cd may compete with Ca may have much lower affinity (for Cd) thus Cd/Ca interactions would appear as a decrease in the proportionality constant for Cd accumulation.

Competition between Cd and Ca has already been described, notably in fish gills (Verbost et al., 1989). Competition for Ca^{2+} influx in adrenocortical cells may be responsible, at least in part, for impairment of cortisol secretion observed in vitro (Leblond and Hontela, 1999; Lacroix and Hontela, 2004) since it has been shown, in mammalian cells, that cortisol secretion following ACTH stimulation requires Ca^{2+} influx (Davies et al., 1985; Laird et al., 1991; Enyeart et al., 1993). Assuming that Ca influx is also involved in cortisol secretion in steroidogenic fish cells, it could be hypothesized that this step in the signal transduction pathways may be more sensitive to Cd in rainbow trout cells compared to yellow perch. It is noteworthy that our speciation studies show that chlorocomplexes species and the free metal ion both contribute to the initial 5-min accumulation of Cd, but also suggest a higher contribution of Cd^{2+} uptake (the metal species expected to compete with Ca^{2+}) in perch cells (Fig. 5). Clearly, the exact role Ca plays in cortisol secretion in the adrenocortical tissue of rainbow trout and yellow perch should be addressed in future studies. For the present time, our results show that about 50% of Cd uptake is inhibited by Ca under inorganic conditions in rainbow trout cells only.

Other notable differences demonstrated between trout and perch adrenocortical cells concern the reversibility of Cd accumulation. A significant cellular Cd efflux has been measured in rainbow trout whereas Cd release was nonsignificant in perch cells (Fig. 6). The reversibility of Cd accumulation may favor metal excretion but it also reveals the presence of a significant proportion of intracellular Cd which is free or loosely bound. In the trout cells, the “labile” pool of metal may be available for interaction with target molecules within the cells to exert toxic effects. Indeed, the free metal ion Cd^{2+} is generally considered to be the toxic metal species when Cd^{2+} levels exceed metallothionein (MT) binding capacity, and it has been suggested that Cd is less extensively sequestered by MT in rainbow trout (Thomas et al., 1983). Resistance to toxicants may depend on the balance between the “labile” and the “tightly bound” pool of intracellular metal rather than simply total cellular metal content. The exact role MT plays in intracellular Cd handling in fish adrenocortical cells deserves further investigation. Here we show that intracellular Cd retention may vary among fish species.

Generally, the concentrations of xenobiotics used for exposure during in vitro studies are in the same order of magnitude as environmental levels. However, concentrations do not necessarily reflect the level of accumulation within the target tissues. In vitro exposure conditions may well differ from in vivo exposure, and the use of small

concentrations of xenobiotics does not ensure their environmental relevance. Therefore, comparison between levels of uptake, rather than levels of exposure, provides a better estimation of how in vitro data are relevant to the field situation. In the present laboratory study, we can compare the in vitro accumulation data with field data. Laflamme et al. (2000) measured metal concentrations, including Cd, in the adrenocortical tissue of yellow perch from contaminated and reference lakes. Total metal tissue content specifically due to environmental contamination above background has been estimated as 122.5 μg metal/g dry tissue (from a metal mixture content of 11 μg Cd, 3.5 μg Cu and 108 μg Zn/g dry tissue leading to impaired cortisol secretion). As 1 μg metal/g dry tissue is equivalent to 0.29 μg metal/g wet tissue (Levesque et al., 2003), the total metal tissue content of 122.5 μg metal/g dry tissue corresponds to 35.5 μg metal/g wet tissue, i.e. about 35 ppm. In vitro, we measured a mean accumulation of 150 pmol Cd/mg protein over a 10-h exposure to 0.5 μM ^{109}Cd in adrenocortical perch cells (data not shown). This uptake value can be converted to 16.8 ng Cd/mg protein, i.e. about 17 ppm which compares quite well with metal contents in the adrenocortical tissue of yellow perch measured by Laflamme et al. (2000) in their field study. Thus, the Cd exposure used in the present study is a close replicate of the environmental exposure in a mining area in northern Quebec.

In conclusion, we have studied for the first time Cd accumulation in isolated adrenocortical cells of rainbow trout and yellow perch, two fish species that have been extensively used in toxicological studies both in the laboratory and the field (Hontela, 1997). We have demonstrated that interspecies differences in sensitivity to endocrine disrupting effects of Cd cannot be directly correlated to different levels of accumulation. Our study underlines the role of membrane transport pathways, rather than simple accumulation, play as determinants mechanisms of metal-induced endocrine disruption.

Acknowledgments

The authors thank J. Dorval, B. Goulet, A. Gravel, A. Lacroix, G. Sherwood for help in the field and V.S. Leblond for assistance in the laboratory. This research was supported by the Discovery grants from the Natural Sciences and Engineering Research Council of Canada (C.J. and A.H.), and the Canadian Network of Toxicology Centres (C.J. and A.H.).

References

- Alabaster, J.S., Lloyd, R., 1982. Water Quality Criteria for Freshwater Fish. Butterworths, London.
- Blazka, M.E., Shaikh, Z.A., 1992. Cadmium and mercury accumulation in rat hepatocytes: interactions with other metal ions. Toxicol. Appl. Pharmacol. 113, 118–125.

- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of micrograms quantities of protein utilizing the principle of protein dye-binding. *Anal. Biochem.* 72, 248–254.
- Davies, E., Kenyon, C.J., Fraser, R., 1985. The role of calcium ions in the mechanism of ACTH stimulation of cortisol synthesis. *Steroids* 45, 551–560.
- Endo, T., Kimura, O., Sakata, M., 1999. Further analysis of cadmium uptake from apical membrane of LLC-PK1 cells via inorganic anion exchanger. *Pharmacol. Toxicol.* 84, 187–192.
- Enyeart, J.J., Mlinar, B., Enyeart, J.A., 1993. T-type Ca^{2+} channel are required for adrenocorticotropin-stimulated cortisol production by bovine adrenal zona fasciculata cells. *Mol. Endocrinol.* 7, 1031–1040.
- Garcia-Morales, P., Saceda, M., Kenney, N., Kim, N., Salomon, D.S., Gottardis, M.M., Solomon, H.B., Sholler, P.F., Jordan, V.C., Martin, M.B., 1994. Effect of cadmium on estrogen receptor levels and estrogen-induced responses in human breast cancer cells. *J. Biol. Chem.* 269, 16896–16901.
- Girard, C., Brodeur, J.C., Hontela, A., 1998. Responsiveness of the interrenal tissue of yellow perch (*Perca flavescens*) from contaminated sites to an ACTH challenge test in vivo. *Can. J. Fish. Aquat. Sci.* 55, 438–450.
- Hansen, J.A., Welsh, P.G., Lipton, J., Cacula, D., Dailey, A.D., 2002. Relative sensitivity of bull trout (*Salvelinus confluentus*) and rainbow trout (*Oncorhynchus mykiss*) to acute exposures of cadmium and zinc. *Environ. Toxicol. Chem.* 21, 67–75.
- Hontela, A., 1997. Endocrine and physiological responses of fish to xenobiotics: role of glucocorticosteroid hormones. *Rev. Toxicol.* 1, 1–46.
- Iger, Y., Lock, R.A.C., Van der Meij, J.C.A., Wendelaar Bonga, S.E., 1994. Effects of water-borne cadmium on the skin of the common carp (*Cyprinus carpio*). *Arch. Environ. Contam. Toxicol.* 26, 342–350.
- Jumarie, C., Fortin, C., Houde, M., Campbell, P.G.C., Denizeau, F., 2001. Cadmium uptake by Caco-2 cells: effects of Cd complexation by chloride, glutathione and phytochelatins. *Toxicol. Appl. Pharmacol.* 170, 29–38.
- Kime, D.E., 1984. The effect of cadmium on steroidogenesis by testes of the rainbow trout, *Salmo gairdneri*. *Toxicol. Lett.* 22, 83–88.
- Lacroix, A., Hontela, A., 2004. A comparative assessment of the adrenotoxic effects of cadmium in two teleost species, rainbow trout, *Oncorhynchus mykiss*, and yellow perch, *Perca flavescens*. *Aquat. Toxicol.* 67, 13–21.
- Laflamme, J.S., Couillard, Y., Campbell, P.G.C., Hontela, A., 2000. Interrenal metallothionein and cortisol secretion in relation to Cd, Cu, and Zn exposure in yellow perch, *Perca flavescens*, from Abitibi lakes. *Can. J. Fish. Aquat. Sci.* 57, 1692–1700.
- Laird, S.M., Hinson, J.P., Vinson, G.P., Mallick, N., Kapas, S., Teja, R., 1991. Control of steroidogenesis by the calcium messenger system in human adrenocortical cells. *J. Mol. Endocrinol.* 6, 45–51.
- Le Guével, R., Petit, F.G., Le Goff, P., Métivier, R., Valotaire, Y., Pakdel, F., 2000. Inhibition of rainbow trout (*Oncorhynchus mykiss*) estrogen receptor activity by cadmium. *Biol. Reprod.* 63, 259–266.
- Leblond, V.S., Hontela, A., 1999. Effects of in vitro exposures to cadmium, mercury, zinc, and 1-(2-Chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane on steroidogenesis by dispersed interrenal cells of rainbow trout (*Oncorhynchus mykiss*). *Toxicol. Appl. Pharmacol.* 157, 16–22.
- Leffler, P.E., Jin, T., Nordberg, G.F., 2000. Differential calcium transport disturbances in renal membrane vesicles after cadmium-metallothionein injection in rats. *Toxicology* 143, 227–234.
- Levesque, H.M., Dorval, J., Van Der Kraak, G.J., Campbell, P.G.C., Hontela, A., 2003. Hormonal, morphological, and physiological responses of yellow perch (*Perca flavescens*) to chronic environmental metal exposures. *J. Toxicol. Environ. Health, Part A* 66, 657–676.
- Lyons-Alcantara, M., Mooney, R., Lyng, F., Cottell, D., Mothershill, C., 1998. The effect of cadmium exposure on the cytology and function of primary cultures from rainbow trout. *Cell Biochem. Funct.* 16, 1–13.
- Martell, A.E., Smith, R.M., Motekaitis, R.J., 2001. NIST Standard Reference Database 46. Critically Selected Stability Constants of Metal Complexes. U.S. Department of Commerce, Gaithersburg, MD. Version 6.0.
- Martin, M.B., Voeller, H.J., Gelmann, E.P., Lu, J., Stoica, E.G., Herbert, E.J., Reiter, E., Singh, B., Danielsen, M., Pentecost, E., Stoica, A., 2002. Role of cadmium in the regulation of the AR gene expression and activity. *Endocrinology* 143, 263–275.
- Norey, C.G., Brown, M.W., Cryer, A., Kay, J., 1990. A comparison of the accumulation, tissue distribution and secretion of cadmium in different species of freshwater fish. *Comp. Biochem. Physiol., C* 96, 181–184.
- Pratap, H.B., Wendelaar Bonga, S.E., 1990. Effect of water-borne cadmium on plasma cortisol and glucose in the cichlid fish, *Oreochromis mossambicus*. *Comp. Biochem. Physiol., C* 95, 313–317.
- Risso-de Faverney, C., Devaux, A., Lafaurie, M., Girard, J.P., Bailly, B., Rahamani, R., 2001. Cadmium induces apoptosis and genotoxicity in rainbow trout hepatocytes through generation of reactive oxygen species. *Aquat. Toxicol.* 53, 65–76.
- Schecher, W.D., McAvoy, D., 1994. MINEQL⁺: A Chemical Equilibrium Program for Personal Computers. Version 3.01. Environ. Research Software, Hallowell, ME.
- Sorensen, E.M., 1991. Cadmium. Metal Poisoning in Fish. CRC Press, Boca Raton, FL, pp. 175–234.
- Thomas, D.G., Solbe, J.F., Kay, J., Cryer, A., 1983. Environmental cadmium is not sequestered by metallothionein in rainbow trout. *Biochem. Biophys. Res. Commun.* 110, 584–592.
- Verbost, P.M., Van Rooji, J., Flick, G., Lock, R.A.C., Wendelaar Bonga, S.E., 1989. The movement of cadmium through freshwater trout branchial epithelium and its interference with calcium transport. *J. Exp. Biol.* 145, 185–197.