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ORIGINAL PAPER

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Kinetic analyses of waterborne Ca and Cd transport and their interactions in the gills of rainbow trout (*Oncorhynchus mykiss*) and yellow perch (*Perca flavescens*), two species differing greatly in acute waterborne Cd sensitivity

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Abstract We evaluated the differential nature of interactions between waterborne Ca and Cd transport in the gills of yellow perch (*Perca flavescens*) and rainbow trout (*Oncorhynchus mykiss*), two species with a more than 400-fold difference in acute waterborne Cd tolerance. The J_{max} (maximum rate of uptake) and K_m (inverse of affinity) for Ca uptake, in the absence of Cd, were significantly lower in yellow perch (120.48 nM g^{-1} wet wt h^{-1} and 92.17 μM, respectively) relative to rainbow trout (188.68 nM g^{-1} wet wt h^{-1} and 243.90 μM, respectively). Similarly, the J_{max} for Cd uptake, at the lowest waterborne Ca level (100 μM) tested, was significantly lower in yellow perch (0.27 nM g^{-1} wet wt h^{-1}) relative to rainbow trout (0.40 nM g^{-1} wet wt h^{-1}), but no significant difference was observed in the K_m values between the two species (yellow perch: 32.47 nM; rainbow trout: 31.27 nM). Waterborne Cd (0–890 nM) as well as waterborne Ca (100–1,000 μM) competitively inhibited branchial uptake of each other in both species. However, analyses of inhibitor constants for branchial Ca uptake by waterborne Cd ($K_{i[Cd^{2+}]}$) revealed that the inhibition was about 1.8 times more potent in rainbow trout compared to yellow perch. In contrast, analyses of inhibitor constants for branchial Cd uptake by waterborne Ca ($K_{i[Cd^{2+}]}$) indicated that the inhibition was more than three fold more potent in yellow perch than in rainbow trout. Higher branchial Ca uptake and more potent inhibition by Cd as well as higher branchial Cd uptake and less potent inhibition by Ca were also reflected in whole-body measurements of Ca and Cd influx in trout relative to perch. Overall, whole-body effects were in accord with the branchial kinetic analyses. These results further strengthen the conclusion that branchial influxes of Ca and Cd occur through common pathways. Moreover, interspecific differences in acute waterborne Cd sensitivity can be explained, at least in part, by the differential nature of interactions between waterborne Ca and Cd transport in fish gills.

Keywords Ca · Cd · Gill · Yellow perch · Rainbow trout

Abbreviations *FAAS* flame atomic absorption spectrophotometer · *GFAAS* graphite furnace atomic absorption spectrophotometer · J_{max} maximum rate of uptake · K_i inhibitor constant · K_m substrate concentration at which the rate of uptake is half of the J_{max} · *96 h LC50* concentration at which 50% mortality occurs after 96 h

Introduction

According to the present concept of branchial uptake of essential ions in freshwater fish, Ca^{2+} transport occurs via the mitochondria-rich 'chloride' cells (Flik et al. 1985; Perry and Wood 1985; Perry and Flik 1988). Ca^{2+} passes through the apical membrane of the chloride cell via voltage-independent Ca channels, driven by its electrochemical gradient. After entering the chloride cell, Ca^{2+} binds to proteins, such as calmodulin, thus keeping the intracellular Ca activity low. Ca^{2+} is then transported through the basolateral membrane by a high-affinity Ca^{2+}-ATPase. Cd^{2+} and Ca^{2+} are believed to share a common uptake pathway, the voltage-independent Ca channels, in the apical membrane of chloride cells in fish gills (Verbost et al. 1987,1989; Wicklund-Glynn et al. 1994). The primary effect of an acute increase in waterborne Cd on freshwater fish is believed to be an impaired branchial Ca influx that, in turn, leads to hypocalcaemia and ultimately death. Micromolar (or lower) concentrations of Cd in the water inhibit branchial Ca uptake (Verbost et al. 1987; Reid and McDonald 1988) and induce hypocalcemia (Giles 1984; Pratap et al. 1989). At the gill surface, Cd^{2+}

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competes with Ca^{2+} for high-affinity Ca binding sites (Playle et al. 1993 a, 1993b; Playle 1998) and once it enters the chloride cell, it blocks Ca uptake through the competitive inhibition of the Ca pump in the basolateral membrane of the chloride cells (Verbost et al. 1988).

Unidirectional flux measurements (using radiotracers) and kinetic analysis have proven to be valuable tools in characterizing branchial ion-transport systems in fish (Wood 1992). These techniques can indicate the nature of both transport and inhibitory mechanisms. For example, linear kinetics (a proportional relationship between uptake and substrate concentration) suggests diffusive pathways, whereas saturable Michaelis–Menten kinetics suggests carrier or channel-mediated transport. When the latter processes apply, increases in K_m (inverse of affinity) in the presence of putative inhibitor are diagnostic of competitive inhibition, while decreases in J_{max} (maximum transport rate) are indicative of non-competitive inhibition. Such experiments have been used to investigate the effects of Al (McDonald and Milligan 1988; McDonald et al. 1991), Cu (Laurén and McDonald 1985; Reid and McDonald 1988), Zn (Spry and Wood 1985, 1988, 1989; Bentley 1992; Hogstrand et al. 1994, 1995, 1998), and Cd (Verbost et al. 1987, 1989; Wicklund-Glynn 2001) on branchial uptake and excretion processes. Kinetic analyses of the effects of Ca on Zn (Spry and Wood 1989) as well as Zn on Ca uptake (Hogstrand et al. 1994) have clearly shown that Ca competitively inhibits waterborne Zn uptake and vice versa, thereby suggesting Ca^{2+} and Zn^{2+} share the common branchial uptake pathway. Similar kinetic analysis using radiotracers have also indicated that branchial Cd uptake, like Zn, occurs through the Ca uptake pathway (Verbost et al. 1987). Recently Wicklund-Glynn (2001) has shown that waterborne Zn also competitively inhibits apical Cd uptake in zebrafish (*Danio rerio*) and suggested that Zn and Cd influx occurs through common pathways.

Different fish species have different sensitivity to acute waterborne Cd challenge. For example, some members of the fish family, Percidae, are believed to be highly tolerant to many toxic metals compared to most members of the Salmonidae, which are quite sensitive (Spear and Pierce 1979). Recently, we have found that the yellow perch (*Perca flavescens*), a representative of the Percidae family, is >400 times more tolerant to acute waterborne cadmium challenge than the rainbow trout (*Oncorhynchus mykiss*), a Salmonid, in terms of 96 h LC50 (concentration at which 50% mortality occurs after 96 h; Niyogi et al. 2004). Yellow perch thrive in many metal-impacted lakes of the Canadian Shield, while salmonids do not (Sherwood et al. 2000; Couture and Kumar 2003). One of the reasons for yellow perch being so tolerant relative to rainbow trout could be their better ability to maintain Ca homeostasis under acute waterborne Cd challenge. In this study we made an attempt to understand mechanistically the differences in tolerance between yellow perch and rainbow trout by examining the possible differential nature of interactions between waterborne Cd^{2+} and Ca^{2+} on branchial uptake processes. Because our interest was in gill uptake, yet significant Ca and Cd influx can also occur through the skin, fins, and head (Perry and Wood 1985; Wicklund-Glynn 2001), we measured the appearance rate of radiolabeled Ca and Cd exclusively in the gill tissue. However, to test whether the gill measurements could explain the consequences in the intact animal, we also measured whole-body Ca and Cd uptake rate at a range of different waterborne Cd and Ca levels, respectively. We compared the following aspects between the two species: (i) short-term (3 h) in vivo unidirectional branchial Ca uptake kinetics and the effects of waterborne Cd, (ii) short-term (3 h) in vivo unidirectional branchial Cd uptake kinetics and the effects of waterborne Ca, (iii) short-term (3 h) pattern of whole-body Ca influx at variable waterborne Cd concentrations, and (iv) short-term (3 h) pattern of whole-body Cd influx at variable waterborne Ca concentrations.

Materials and methods

Experimental animals

Juvenile rainbow trout (*O. mykiss*, 8–12 g) and yellow perch (*P. flavescens*, 8–12 g) were obtained from Humber Springs Fish Hatchery (Orangeville, Ontario) and Kinmount Fish Farm (Kinmount, Ontario), respectively. Fish were maintained at least for 1 month in 500-l polyethylene tanks supplied with 2.0 l min^{-1} dechlorinated Hamilton tap water of moderate hardness (12–14 °C, pH 8.0, 0.6 mM Na, 0.7 mM Cl, 1.0 mM Ca, 120 mg l^{-1} as $CaCO_3$ hardness, 95 mg l^{-1} alkalinity, 3 mg l^{-1} dissolved organic carbon) prior to experiments. Fish were fed once a day at 2% of their body weight with commercial trout food (Granulated Hatchery Feed, Corey Feed Mills) and organic debris was siphoned out daily. No mortality was observed during this period.

Effect of waterborne Cd on short-term branchial Ca uptake

We characterized the differential effects of waterborne Cd on branchial Ca uptake in vivo between the two species following the method described in Zohouri et al. (2001). Sixteen polyethylene bags (four sets of four bags each, with each set representing one of three different Cd concentrations and a control) were filled with 3 l synthetic Ca-free water (0.7 mM Na and Cl added as NaCl, $KHCO_3$ = 1.9 mM, pH 8.0), fitted with an individual air line and placed in a water bath (temperature: 14 °C). Three bags of each set were spiked with Cd [$Cd(NO_3)_2$, 4 H_2O, Fisher Scientific, Canada] to achieve approximate total Cd concentrations of 89.2, 446.0, and 890.0 nM, respectively (control: 0 nM Cd). Each set was subsequently spiked with $Ca(NO_3)_2$, $4H_2O$ (Fisher Scientific, Canada) plus 7 μCi l^{-1} ^{45}Ca (as $CaCl_2$, specific activity = 12.26 mCi mg^{-1}, Perkin-Elmer, USA) to produce an exactly similar range of increasing total Ca concentrations (approximately 100, 250, 500, and 1000 μM, respectively) in all four sets. In total, 112 fish of each species (7 per bag) were transferred to 16 bags and exposed for 3 h. At the end, fish were killed by an overdose of anesthetic (tricaine methanesulfonate; Syndel, Canada; 1 g per flux bag), gill baskets were dissected out, rinsed separately in deionized water (Nanopure, Sybron Barnstead, Boston, USA) for 30 s, and stored at −20 °C. Water samples (5 ml) were taken in duplicate at the beginning and end of the exposure. One of each duplicate water sample was diluted in 10 ml scintillation fluor (aqueous counting scintillant; Amersham) and counted for ^{45}Ca in a beta counter (LKB Wallac

1217 Rackbeta Liquid Scintillation Counter). The remaining water samples were acidified with 50 µl concentrated trace metal grade HNO_3 and analyzed for total Cd and Ca. Total waterborne Cd was measured by using a graphite furnace atomic absorption spectrophotometer (GFAAS; Varian Australia, Model GTA 110) and a certified Cd standard for GFAAS (Fisher Scientific, Canada). Subsequently, total waterborne Ca was measured by using a flame atomic absorption spectrophotometer (FAAS; Varian Australia, Model 220FS) and a certified Ca standard for FAAS (Fisher Scientific, Canada). Later, following the procedure of Hogstrand et al. (1994), frozen gills were transferred to liquid nitrogen and ground to a fine powder with mortar and pestle. Powdered gills (~0.2 g, in duplicate) were placed in glass scintillation vials and digested in 2 ml liquid tissue solubilizer (NCS-II, Amersham) for 48 h at 50 °C. Samples were then neutralized with 20 µl glacial acetic acid, diluted in 10 ml scintillation fluor (organic counting scintillant; Amersham) and counted for ^{45}Ca on the scintillation counter. Counting efficiencies were determined by internal standardization, i.e., by addition/recovery tests of known amounts of ^{45}Ca to the samples.

The inward branchial flux (J_{in}) for Ca (in nM g^{-1} wet wt h^{-1}) into the gills was calculated according to Hogstrand et al. (1994):

$$J_{in} = cpm_g/(SA_w * CE * t) \quad (1)$$

where cpm_g is the average ^{45}Ca counts in gill samples (cpm g^{-1} wet gill tissue), SA_w is the measured mean specific activity of ^{45}Ca in the water [(cpm l^{-1} water)/total Ca in water (nM)], CE is the relative counting efficiency of the 'tissue-solubilizer-fluor system', compared to 'water-fluor system' and t is time.

Effect of waterborne Ca on short-term branchial Cd uptake

Inter-specific differences in branchial Cd uptake and the effect of waterborne Ca were also determined following the same methodology, and using the radiolabeled ^{109}Cd. Twelve polyethylene bags, (three sets of four bags each, with each set representing three different Ca concentrations) were filled with 3 l synthetic Ca-free water (0.7 mM Na and Cl added as NaCl, $KHCO_3 = 1.9$ mM, pH 8.0), fitted with an individual air line, and placed in a water bath (temperature: 14 °C). Four bags of each set were spiked with Ca [$Ca(NO_3)_2$, 4 H_2O, Fisher Scientific, Canada] to achieve approximate total Ca concentrations of 100, 500, and 1000 µM, respectively. Subsequently each set was then spiked with $Cd(NO_3)_2$, $4H_2O$ (Fisher Scientific, Canada) plus 3 μCi l^{-1} ^{109}Cd (as $CdCl_2$, specific activity = 3.45 mCi mg^{-1}, Perkin-Elmer, USA) to produce an identical range of increasing total Cd concentrations (approximately 44.6, 89.2, 223.0, and 446.0 nM, respectively) in all four sets. In total, 84 fish of each species (7 per bag) were transferred to 12 bags and exposed for 3 h. At the end fish were killed as described previously, gill baskets were dissected out, rinsed separately in deionized water for 30 s, and placed in 20-ml polyethylene vials. Duplicate water samples (5 ml) were taken at the beginning and end of the 3 h static exposure and acidified with 50 µl concentrated trace-metal grade HNO_3. Both water and gills were counted for radioactivity using a gamma counter (Packard Minaxi Auto-Gamma 5,000 Series). Water samples were further analyzed for total Cd by GFAAS and for total Ca by FAAS respectively as described earlier. The inward branchial flux of Cd (in nM g^{-1} wet wt h^{-1}) was calculated by an equation analogous to Eqn. 1, with CE = 1, because the counting efficiency of water and gill samples were identical in the gamma counter.

Short-term whole-body Ca influx at variable waterborne Cd concentration

Possible inter-specific differences in whole-body Ca influx at different waterborne Cd concentrations in the acclimation water were also determined. Twenty-eight fish of each species (seven per bag) were exposed to 0 (control), 89.2, 223.0, and 446.0 nM, respectively, of total Cd [added as $Cd(NO_3)_2$, $4H_2O$, Fisher Scientific, Canada] in four polyethylene bags containing 3 l dechlorinated Hamilton tap water placed in a water bath at a temperature of 14 °C for 3 h. Five minutes before the introduction of fish, each bag was spiked with 7 μCi l^{-1} ^{45}Ca (as $CaCl_2$, specific activity = 12.26 mCi mg^{-1}, Perkin-Elmer, USA). At the end, fish were killed as described above, rinsed separately for 30 s in deionized water, blotted dry and stored at −20 °C. Later, the whole body of the fish was transferred to liquid nitrogen and ground to fine powder with a mortar and pestle (Hogstrand et al. 1994). Three aliquots of each fish and one set of water samples per treatment groups (5 ml, taken in duplicate at the beginning and end of the exposure) were processed and analyzed for ^{45}Ca as described previously. Total Ca and Cd were determined in the other set of water samples by FAAS and GFAAS, respectively. The whole-body Ca influx (in nM g^{-1} wet wt h^{-1}) was determined using an equation analogous to Eqn. 1, but replacing cpm_g with cpm_b, where cpm_b is the average ^{45}Ca count in the whole body (cpm g^{-1} whole body wet wt).

Short-term whole-body Cd influx at variable waterborne Ca concentration

The evaluation of possible inter-specific differences in whole-body Cd influx at different waterborne Ca concentrations were carried out following the same methodology as described above for the whole-body Ca influx experiment. Twenty-eight fish (seven per individual set) were exposed for 3 h to four different Ca concentrations [50, 150, 500, and 1000 µM, respectively; added as $Ca(NO_3)_2$, $4H_2O$, Fisher Scientific, Canada] in synthetic Ca-free water (0.7 mM Na and Cl added as NaCl, $KHCO_3 = 1.9$ mM, pH 8.0) containing approximately 446 nM Cd. The Cd concentration in the exposure water was achieved by spiking it with $Cd(NO_3)_2$, 4 H_2O (Fisher Scientific, Canada) and 3 μCi l^{-1} ^{109}Cd (as $CdCl_2$, specific activity = 3.45 mCi mg^{-1}, Perkin-Elmer, USA). At the end fish were killed as described previously, rinsed separately in deionized water for 30 s, blotted dry, and placed in 20-ml polyethylene vials. Duplicate water samples (5 ml) were taken at the beginning and end of the 3 h static exposure and acidified with 50 µl concentrated trace-metal grade HNO_3. Both water and whole body of the fish were counted for radioactivity using a gamma counter (Packard Minaxi Auto-Gamma 5,000 Series). Water samples were further analyzed for total Cd by GFAAS and for total Ca by FAAS. The whole-body Cd flux (nM g^{-1} wet wt h^{-1}) was calculated by an equation analogous to Eqn. 1, with CE = 1, because the counting efficiency of water and whole-body samples were identical in the gamma counter.

Calculations and statistical analyses

Free ionic waterborne Cd^{2+} and Ca^{2+} were estimated in exposure waters by using the MINEQL+ (version 4.5) aquatic chemistry program (Schecher and McAvoy 2001). Michaelis–Menten analyses of the relationships between free waterborne Ca^{2+} concentrations and branchial Ca uptake at different Cd concentrations as well as free waterborne Cd^{2+} concentrations and branchial Cd uptake at different waterborne Ca concentrations were performed using Lineweaver-Burke double reciprocal plots. Regressions were applied using Sigma Plot 2000 for Windows 98 (SPSS, Chicago, USA). All data were expressed as mean ± SEM (except in the double reciprocal plots). The parameter estimates for K_m and J_{max} of branchial Ca and Cd uptake, and rates of whole-body Ca and Cd influx in the two species were analyzed for statistical significance by using a two-way ANOVA followed by Bonferroni's multiple comparison. The level of significance was set to $p < 0.05$ in all comparisons. Inhibitor constants for branchial Ca uptake by waterborne Cd ($K_{i[Cd^{2+}]}$) and also for branchial Cd uptake by waterborne Ca ($K_{i[Cd^{2+}]}$) were determined from the regressions plots of apparent K_m/J_{max} vs. waterborne Cd and Ca, respectively (Segel 1976), where apparent K_m values were the values determined in the

presence of increasing concentrations of the respective inhibitors (i.e., waterborne Cd or Ca). Significance of differences in the respective inhibitor constant values between the two species was evaluated using a standard *t*-test ($p < 0.05$).

Results

Unidirectional branchial Ca uptake kinetics and the effect of waterborne Cd

In both species, short-term (3 h) unidirectional branchial Ca uptake showed saturation kinetics with increasing waterborne Ca concentrations both in the absence and the presence of waterborne Cd (Figs. 1a, 2a). Waterborne Cd inhibited branchial Ca uptake and this inhibitory effect gradually increased with increasing waterborne Cd concentrations in both species (Figs. 1a, 2a). Michaelis–Menten analyses (Figs. 1b, 2b) showed that J_{max} (J_{max} = inverse of the y intercept of the regression line) changed slightly (not statistically significant) with increasing waterborne Cd concentrations in both species (Table 1) except at the highest concentrations (at 890 nM). In contrast, K_m ($-K_m$ = inverse of the x intercept of the regression line) increased significantly ($p < 0.05$) with increasing waterborne Cd concentrations in both species (Table 1). For example, increasing the waterborne Cd concentrations from 0 nM to 890 nM produced approximately 827% and 632% increases of K_m and only 15% and 25% decrease of J_{max} in yellow perch and rainbow trout, respectively. These large increases of K_m compared to very small decreases in J_{max} in both species indicated a large competitive component and a small non-competitive component to the inhibition of unidirectional branchial Ca uptake by waterborne Cd.

Moreover, these analyses also showed that both in the absence and the presence of waterborne Cd, J_{max} and K_m values in rainbow trout were significantly ($p < 0.05$) higher than in yellow perch (Table 1). Since inhibition of branchial Ca uptake by waterborne Cd was predominantly competitive in nature in both species, it allowed us to determine an inhibitor constant ($K_{i[Cd^{2+}]}$) for each species from a regression plot of apparent K_m/J_{max} vs. free waterborne Cd^{2+} (Fig. 3; Segel 1976), where apparent K_m and J_{max} values were determined in the presence of increasing waterborne Cd concentrations. The $K_{i[Cd^{2+}]}$ (x intercept of the regression line = $-K_i$) was found to be approximately 1.8-fold higher (significant, $p < 0.5$) in yellow perch (276.43 ± 30.06 nM free waterborne Cd^{2+}) than in rainbow trout (155.25 ± 24.04 nM free waterborne Cd^{2+}), indicating that waterborne Cd was a more potent inhibitor of branchial Ca uptake in the trout.

Unidirectional branchial Cd uptake kinetics and the effect of waterborne Ca

The short-term (3 h) unidirectional branchial Cd uptake also exhibited saturation kinetics with increasing waterborne Cd concentrations at all waterborne Ca levels (100, 500, and 1,000 μM, added as inhibitor) in both species (Figs. 4a, 5a). Waterborne Ca inhibited branchial Cd uptake, and this inhibitory effect gradually increased with increasing waterborne Ca concentrations in both species (Figs. 4a, 5a). Michaelis–Menten analyses (Figs. 4b, 5b) showed that J_{max} (calculated as described before) of branchial Cd uptake did not show

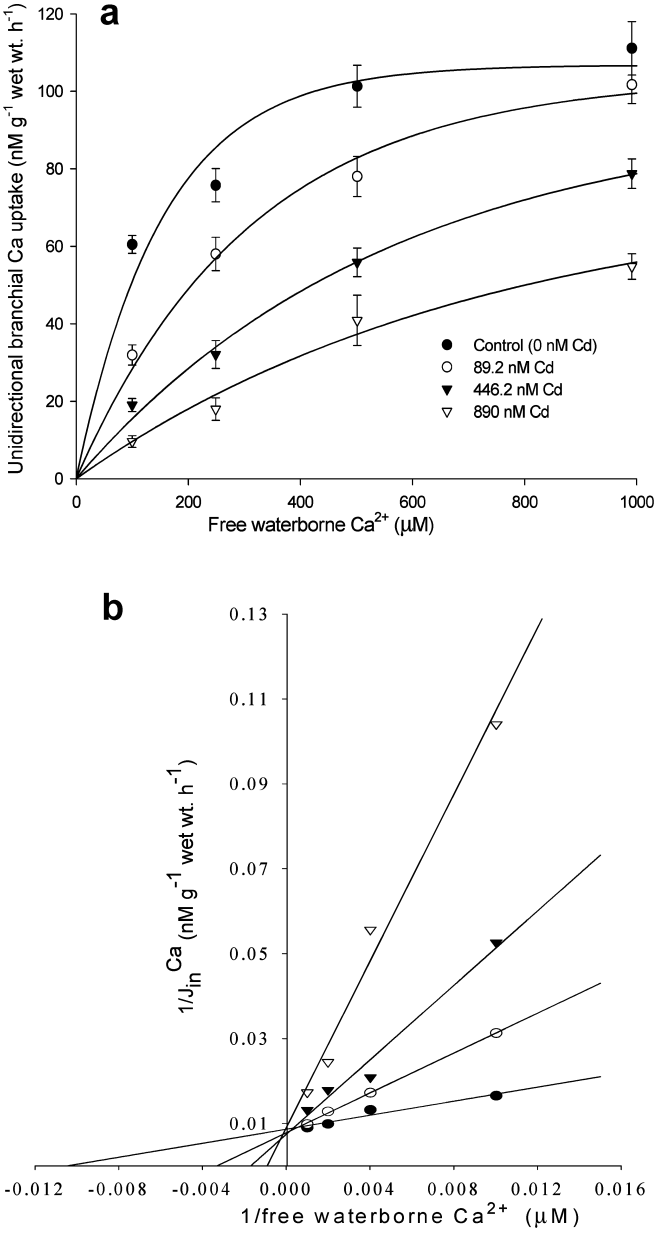
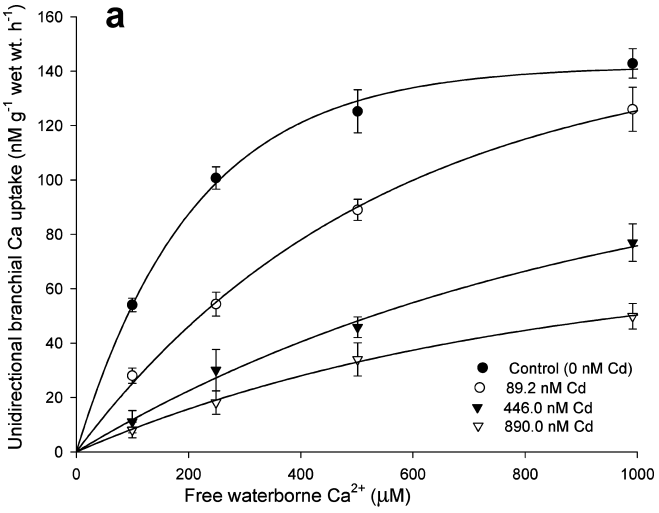


Fig. 1 **a** Unidirectional branchial Ca uptake at various waterborne Ca concentrations in synthetic water (0.7 mM Na and Cl, $KHCO_3$: 1.9 mM, pH 8.0, temperature: 14 °C) and the effect of four different waterborne Cd concentrations (includes control or 0 nM Cd) in yellow perch. The curves were fitted to the respective data sets by using Sigma Plot 2000 for Windows 98 (SPSS, Chicago, USA). Data presented as mean ± SEM ($N = 7$). **b** Michaelis–Menten analyses by Lineweaver–Burke regression (double reciprocal) plot of the data from **a**, showing the predominant competitive nature of inhibition of branchial Ca uptake by waterborne Cd



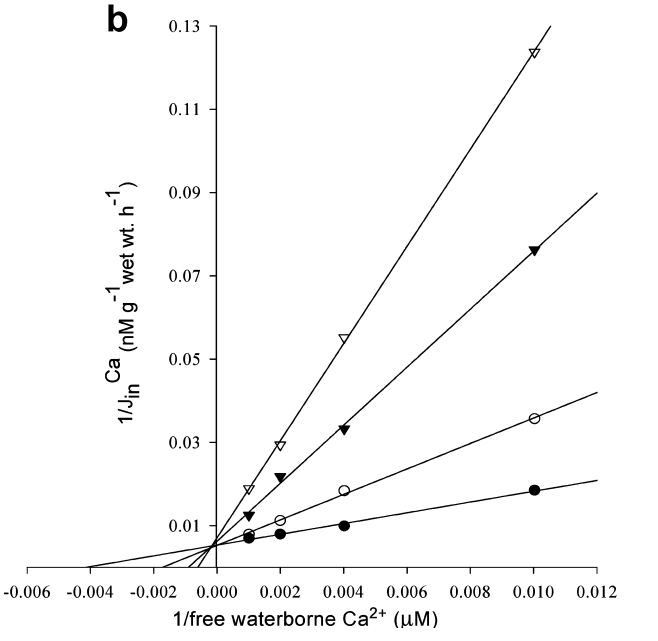


Fig. 2 **a** Unidirectional branchial Ca uptake at various waterborne Ca concentrations in synthetic water (0.7 mM Na and Cl, $KHCO_3$: 1.9 mM, pH 8.0, temperature: 14 °C) and the effect of four different Cd concentrations (includes control or 0 nM Cd) in rainbow trout. The curves were fitted to the respective data sets by using Sigma Plot 2000 for Windows 98 (SPSS, Chicago, USA). Data presented as mean ± SEM ($N = 7$). **b** Michaelis–Menten analyses by Lineweaver–Burke regression (double reciprocal) plot of the data from **a**, showing the predominant competitive nature of inhibition of branchial Ca uptake by waterborne Cd

any notable change at any waterborne Ca concentrations in either species (Table 2). Contrastingly, K_m increased significantly ($p < 0.05$) with increasing waterborne Ca concentrations in both species (Table 2). For example, increasing the waterborne Ca concentrations from 100 μM to 1000 μM produced approximately 600% and 320% increases of K_m in yellow perch and rainbow trout, respectively. These large increases of K_m compared to very small changes (< 10%, not statistically significant) in J_{max} in both species suggested that the inhibition of unidirectional branchial Cd uptake by waterborne Ca was also competitive in nature. Note also the large difference in magnitude between Ca transport (Figs. 1, 2) and Cd transport (Figs. 4, 5). K_m values for Cd transport were approximately four orders of magnitude lower, and J_{max} values for Cd transport were approximately three orders of magnitude lower than the corresponding values for Ca transport (Tables 1, 2).

Moreover, J_{max} of branchial Cd uptake was significantly greater in rainbow trout than in yellow perch at all three waterborne Ca concentrations (Table 2). In contrast, significant differences in K_m values between the two species were observed at higher waterborne Ca concentrations only (500 μM and 1000 μM; Table 2). As inhibition of branchial Cd uptake by waterborne Ca also appeared to be competitive in nature in both species, an inhibitor constant ($K_{i[Cd^{2+}]}$) was determined as before for each species from a regression plot of apparent K_m/J_{max} vs. free waterborne Ca^{2+} (Fig. 6; Segel 1976), where apparent K_m and J_{max} values were determined in the presence of increasing waterborne Ca concentrations. The $K_{i[Cd^{2+}]}$ (x intercept of the regression line $= -K_i$) was found to be more than three fold higher (significant, $p < 0.05$) in rainbow trout (176.43 ± 23.98 μM free waterborne Ca^{2+}) than in yellow perch (49.91 ± 10.17 μM free waterborne Ca^{2+}), indicating that waterborne Ca was a more potent inhibitor of branchial Cd uptake in the perch.

Table 1 Maximum rate of uptake (J_{max}) and substrate concentration at which uptake is half of J_{max}(K_m) values for unidirectional branchial Ca uptake in rainbow trout and yellow perch at various waterborne Cd concentrations (derived from Figs. 1b, 2b, respectively). Significant differences ($p < 0.05$) of $J_{max}$and K_m values between the two species for identical treatments are indicated by an *asterisk*. Significant differences ($p < 0.05$) of J_{max} and K_m values within the same species relative to control (0 nM waterborne Cd) are indicated by a*dagger*

Cd in water	J_{max} (nM g^{-1} wet wt h^{-1})		K_m (μM)	
(nM)	Yellow perch	Rainbow trout	Yellow perch	Rainbow trout
0	120.48 ± 10.51	188.68 ± 23.62*	92.17 ± 13.45	243.90 ± 22.63*
89.2	128.21 ± 14.83	185.19 ± 20.44*	312.50 ± 22.92†	526.32 ± 34.27*†
446.0	129.87 ± 12.19	158.73 ± 10.92*	649.35 ± 36.37†	1184.13 ± 55.51*†
890.0	102.04 ± 5.38†	140.84 ± 8.70*†	854.70 ± 46.73†	1785.71 ± 63.10*†

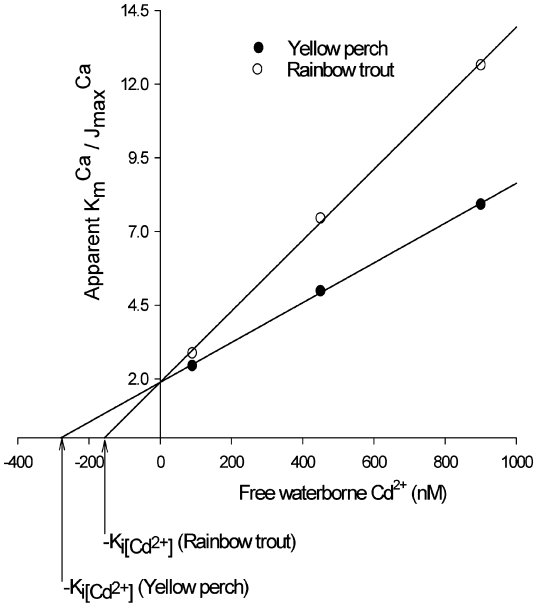


Fig. 3 Inhibitor constants ($K_{i[Cd^{2+}]}$) for unidirectional branchial Ca uptake by waterborne Cd. Apparent K_m/J_{max}, derived from the values presented in Table 1, were plotted against free waterborne Cd^{2+} concentrations. The regression equations for yellow perch and rainbow trout were $y = 0.007x + 1.935$ ($R^2 = 0.98$) and $y = 0.012x + 1.863$ ($R^2 = 0.98$) respectively, where y and x represent apparent K_m/J_{max}, and inhibitor (waterborne Cd) concentrations respectively. The inhibitor constants ($-K_{i[Cd^{2+}]}$ = x intercept of the regression line) for yellow perch and rainbow trout were 276.43 ± 30.06 nM and 155.25 ± 24.04 nM free waterborne Cd^{2+}, respectively. Regressions were significantly different ($p < 0.05$)

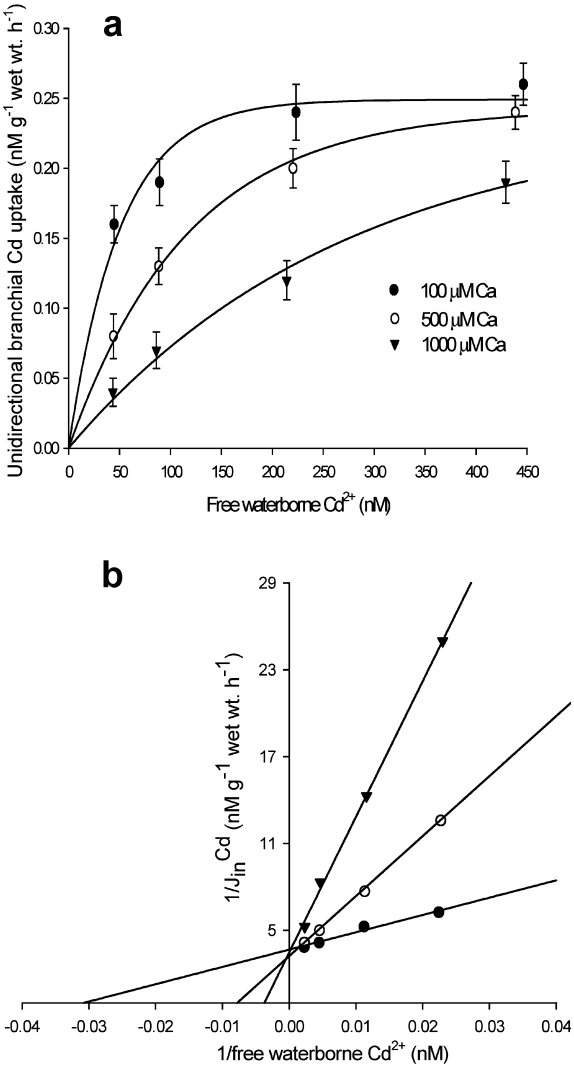


Fig. 4 **a** Unidirectional branchial Cd uptake at various waterborne Cd concentrations in synthetic water (0.7 mM Na and Cl, $KHCO_3$: 1.9 mM, pH 8.0, temperature: 14 °C) and the effect of three different waterborne Ca concentrations in yellow perch. The curves were fitted to the respective data sets by using Sigma Plot 2000 for Windows 98 (SPSS, Chicago, USA). Data presented as mean ± SEM ($N = 7$). **b** Michaelis–Menten analyses by Lineweaver–Burke regression (double reciprocal) plot of data from **a**, showing the competitive nature of inhibition of branchial Cd uptake by waterborne Ca

Effect of waterborne Cd on whole-body Ca influx

In order to explore the consequences of these relationships for the intact animal, we measured whole-body Ca influx in the two species in their moderately hard acclimation water (with a Ca concentration of about 1000 μM) at a range of different Cd concentrations. The rate of whole-body Ca influx in the acclimation water was significantly lower in the yellow perch than in the rainbow trout in the absence of Cd (Fig. 7), as could be predicted from the differences in J_{max} values of branchial Ca uptake (Table 1; Figs. 1, 2). Waterborne Cd produced significant inhibition of whole-body Ca influx in both species, however this inhibition tended to increase at a much greater rate in rainbow trout than in yellow perch with increasing waterborne Cd concentrations, in accord with the 1.8-fold higher $K_{i[Cd^{2+}]}$ in the yellow perch (Fig. 3). At 446 nM of total waterborne Cd (approximately 284 nM of free Cd^{2+}), which is acutely toxic to rainbow trout but not to yellow perch under the existing water chemistry (Niyogi et al. 2004), the whole-body Ca influx was significantly higher in yellow perch compared to that in rainbow trout, which was completely the reverse of the pattern observed under control conditions (i.e., in the absence of waterborne Cd).

Effect of waterborne Ca on whole-body Cd influx

The whole-body Cd influx in the two species at 446 nM waterborne Cd and variable waterborne Ca levels in the synthetic water (Fig. 8) exhibited similar profiles as observed in the case of whole-body Ca influx at variable waterborne Cd concentrations (Fig. 7). The rates of whole-body Cd influx were significantly lower in yellow perch relative to rainbow trout at all four concentrations of waterborne Ca tested. Once again these findings were in accord with our previous observation of the lower J_{max} values of branchial Cd uptake in the

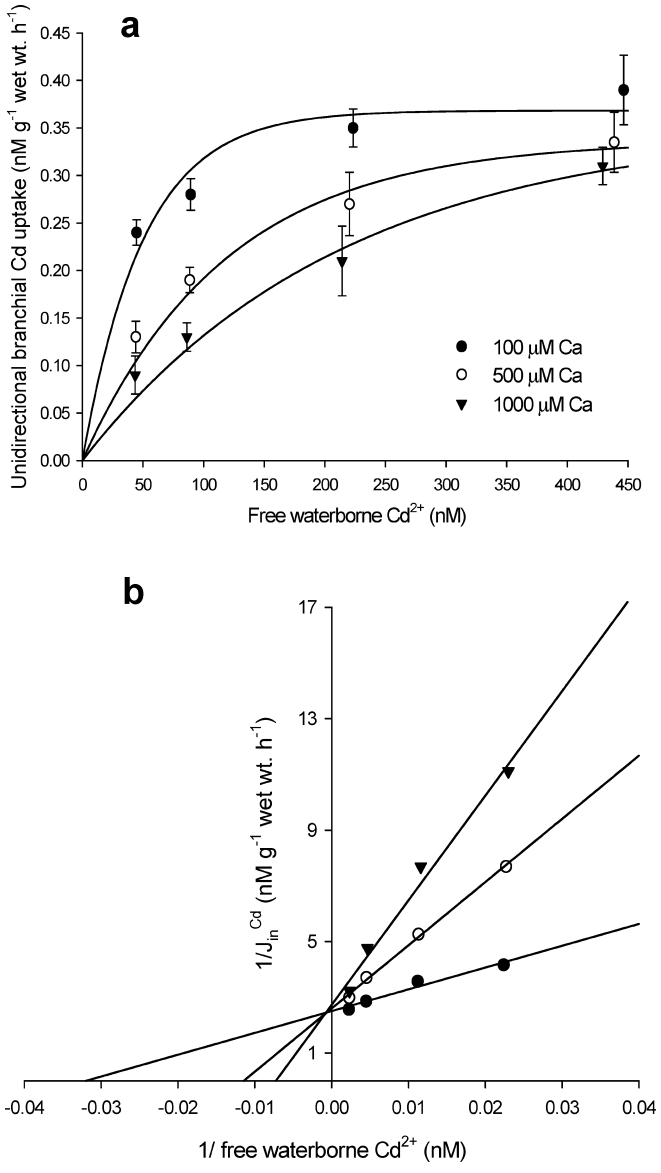


Fig. 5 **a** Unidirectional branchial Cd uptake at various waterborne Cd concentrations in synthetic water (0.7 mM Na and Cl, $KHCO_3$: 1.9 mM, pH 8.0, temperature: 14 °C) and the effect of three different waterborne Ca concentrations in rainbow trout. The curves were fitted to the respective data sets by using Sigma Plot 2000 for Windows 98 (SPSS, Chicago, USA). Data presented as mean ± SEM ($N = 7$). **b** Michaelis–Menten analyses by Lineweaver–Burke regression (double reciprocal) plot of data from **a**, showing the competitive nature of inhibition of branchial Cd uptake by waterborne Ca

yellow perch (Table 2; Figs. 3, 4). Waterborne Ca produced significant inhibition of whole-body Cd influx in both species, however this inhibition seemed to increase at a much greater rate in the yellow perch than in rainbow trout with increasing waterborne Ca concentrations. For example, increasing the total waterborne Ca levels from 50 μM to 1,000 μM yielded 71% inhibition of whole-body Cd influx in the yellow perch, whereas the same increase in Ca level produced only 43% inhibition in the rainbow trout, which is in reasonable agreement with the result of a greater than three-fold higher $K_{i[Cd^{2+}]}$ in the rainbow trout relative to the yellow perch (Fig. 6).

Discussion

The major objective of this study was to evaluate whether the interactions between waterborne Ca^{2+} and Cd^{2+} in gills between the two species were differential in nature. If yes, then how could it possibly influence the tolerance to acute waterborne Cd?

The results showed that waterborne Cd competitively inhibited the branchial uptake of Ca in both species, and waterborne Ca also competitively inhibited branchial Cd uptake in both species. In addition, the small but significant decrease in J_{max} of branchial Ca uptake in both species at a high waterborne Cd concentration (890 nM) indicated the possibility of a minor non-competitive component to this inhibition. In comparison, only minor and insignificant changes in J_{max} of branchial Cd uptake were observed in both species at the highest waterborne Ca concentration tested (1,000 μM), indicating the truly competitive nature of inhibition of waterborne Ca upon branchial Cd uptake under the existing conditions. The K_m of branchial Ca uptake in the absence of any inhibitor (Cd) (i.e., the true K_m) reported in this study (243.9 ± 22.6 μM; Table 1) for juvenile rainbow trout (8–12 g) was in reasonable agreement with the values reported in the previous experiments of a similar kind. Perry and Wood (1985) reported a K_m of 280.0 ± 70.0 μM for waterborne Ca uptake in rainbow trout (average fish weight: 244 g) acclimated in almost identical water quality. Hogstrand et al. (1994, 1998) reported the K_m values of 92.0 ± 8.0 μM and 34.0 ± 7.0 μM for the same in rainbow trout of average weight of 21 g and 2.5 g, respectively, also acclimated under similar conditions. In contrast, J_{max} values of waterborne Ca uptake reported by them were several fold lower [12.2 ± 2.0 nM g^{-1} wet wt h^{-1} (Perry and Wood 1985), 53.0 ± 3.0 nM g^{-1} wet wt h^{-1} (Hogstrand et al. 1994) and 44.0 ± 5.0 nM g^{-1} wet wt h^{-1} (Hogstrand et al. 1998)] compared to the value (188.7 ± 23.6 nM g^{-1} wet wt h^{-1}) determined in our study. Considering the fact that our J_{max} measurements of waterborne Ca uptake were based on unit wet weight of the gills (which constitute only a few percent of body weight) compared to theirs which were determined as unit wet weight of the whole-body basis, the higher values observed in our study were not unexpected as gill is the primary uptake site of all essential metal ions (Marshall 2002). However, our data related to J_{max} and K_m of waterborne Cd uptake could not be compared due to the absence of any previously published reports in this aspect for rainbow trout, either branchial or whole body. Similarly, our data on J_{max} and K_m of both branchial Ca and Cd uptake in yellow perch could not be compared as well since we evaluated the branchial Ca and Cd uptake kinetics in this species for the first time.

Table 2 J_{max} and apparent K_m for unidirectional branchial Cd uptake in rainbow trout and yellow perch at various waterborne Ca concentrations (derived from Figs. 4b, 5b, respectively). Significant differences ($p < 0.05$) of $J_{max}$and K_m values between the two species for identical treatments are indicated by an *asterisk*. Significant differences ($p < 0.05$) of J_{max} and K_m values within the same species relative to 100 μM waterborne Ca treatment are indicated by a *dagger*

Ca in water	J_{max} (nM g^{-1} wet wt h^{-1})		Apparent K_m (nM)	
(μM)	Yellow perch	Rainbow trout	Yellow perch	Rainbow trout
100	0.27 ± 0.04	0.40 ± 0.03*	32.47 ± 4.80	31.27 ± 5.76
500	0.29 ± 0.04	0.39 ± 0.03*	125.04 ± 17.62†	86.66 ± 10.17*†
1000	0.26 ± 0.03	0.37 ± 0.04*	227.28 ± 23.07†	131.58 ± 11.13*†

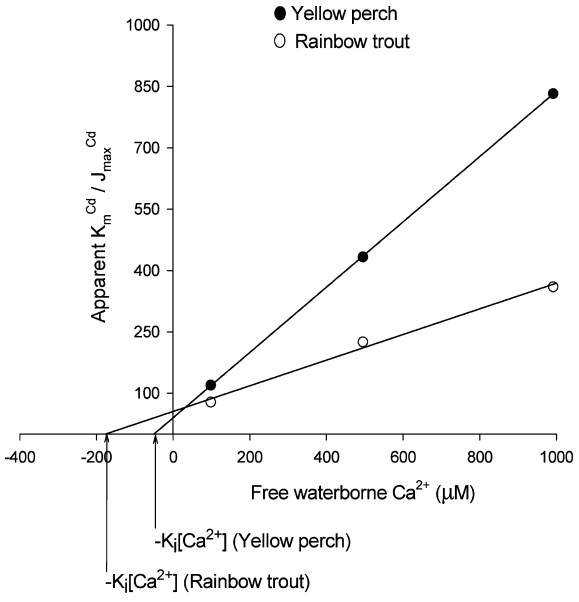


Fig. 6 Inhibitor constants ($K_{i[Cd^{2+}]}$) for unidirectional branchial Cd uptake by waterborne Ca. Apparent K_m/J_{max}, derived from the values presented in Table 2, were plotted against free waterborne Ca^{2+} concentrations. The regression equations for yellow perch and rainbow trout were $y = 0.798x + 39.826$ ($R^2 = 0.98$) and $y = 0.313x + 55.294$ ($R^2 = 0.99$), respectively, where y and x represent apparent K_m/J_{max}, and inhibitor (waterborne Ca) concentrations respectively. The inhibitor constants ($-K_{i[Cd^{2+}]} = x$ intercept of the regression line) for yellow perch and rainbow trout were 49.91 ± 10.17 μM and 176.43 ± 23.98 μM free waterborne Ca^{2+}, respectively. Regressions were significantly different ($p < 0.05$)

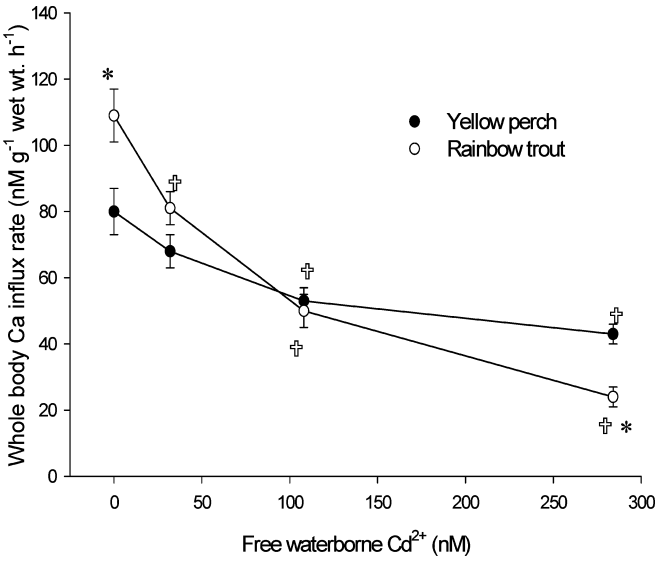


Fig. 7 Effect of variable waterborne Cd in moderately hard dechlorinated Hamilton tap water (1 mM Ca, 0.6 mM Na, 0.7 mM Cl, 120 mg l^{-1} $CaCO_3$ hardness, 95 mg l^{-1} alkalinity, 3 mg l^{-1} DOM, pH 8.0, temperature: 14 °C) on whole body Ca influx in yellow perch and rainbow trout. All values were expressed as mean ± SEM ($N = 7$). Significant differences ($p < 0.05$) between the two species for identical treatments are indicated by an *asterisk*. Significant differences ($p < 0.05$) within the same species relative to control (0 nM waterborne Cd) are indicated by a *dagger*

McWilliams (1983) postulated two types of Ca^{2+} uptake sites in the gills of brown trout. The higher-affinity site was suggested to be the Ca^{2+} transport site, and the lower-affinity site was believed to control permeability. Reduced availability of waterborne Ca^{2+} would thus have two probable effects: (i) competitive replacement of Ca^{2+} from some transporting component in the gill by Cd^{2+}, and (ii) an increase in the general permeability of the gill allowing entry of Cd^{2+} into the gill by a nonspecific and unsaturable route, perhaps simple diffusion through paracellular channels. The results of our experiments strongly supported the occurrence of the first proposed effect. We did not observe any increase in J_{max} of branchial Cd uptake with decreasing waterborne Ca; therefore, the occurrence of the other factor is unlikely.

Waterborne Ca^{2+} and Cd^{2+} are believed to enter the gill via the same La^{3+}-sensitive apical voltage-independent Ca^{2+} channels of the chloride cells (Verbost et al. 1987, 1989). Cd^{2+} competes with Ca^{2+} for binding sites at the apical gill surface (Playle et al. 1993a, 1993b; Hollis et al. 1997), and once internalized, it competitively inhibits Ca^{2+} transport in the basolateral membrane by occupying the Ca^{2+} transport site of the Ca^{2+}-ATPase (Verbost et al. 1988). The results of the present study were likely a reflection of the combined effects of these two processes in the gill. Chang et al. (1997) also reported that waterborne Ca uptake was competitively inhibited in tilapia larvae (*Oreochromis mossambicus*) exposed for 4 h to water containing 0.18 μM Cd and 0.2 mM Ca relative to control (0 μM Cd). The K_m increased significantly ($p < 0.05$, approximately 20 fold) whereas J_{max} increased only slightly (1.2 fold, not significant). Waterborne Zn^{2+}, another

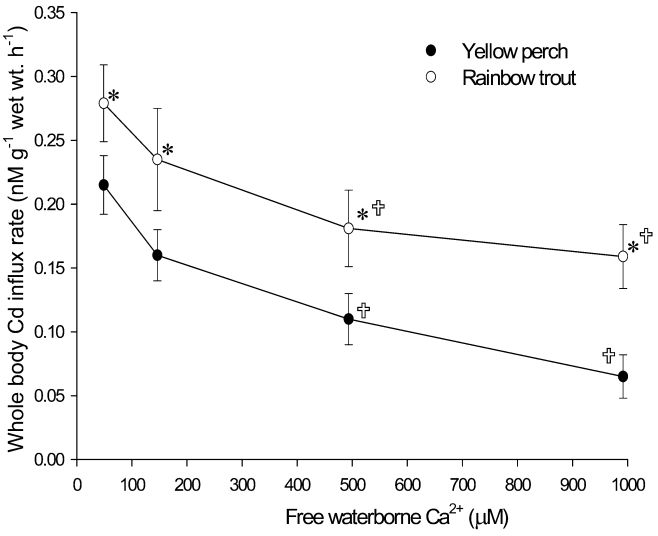


Fig. 8 Effect of variable waterborne Ca on whole-body Cd influx in yellow perch and rainbow trout exposed to 446.0 nM Cd in synthetic water (0.7 mM Na and Cl, $KHCO_3$: 1.9 mM, pH 8.0, temperature: 14 °C). All values were expressed as mean ± SEM ($N = 7$). Significant differences ($p < 0.05$) between the two species for identical treatments are indicated by an *asterisk*. Significant differences ($p < 0.05$) within the same species relative to 50 μM waterborne Ca treatment are indicated by a *dagger*

element known to share the same uptake sites and route with Ca^{2+} (Perry and Wood 1985; Spry and Wood 1989), has also been reported to competitively inhibit Ca uptake and vice versa (Spry and Wood 1989; Hogstrand et al. 1994, 1995, 1998). Again it has yet to be established whether this competition takes place at the apical side or at the basolateral side or at both. Recently, Wicklund-Glynn (2001) has also shown that waterborne Zn competitively inhibits Cd uptake in the gills of zebra fish, *D. rerio*, after 24 h exposure. Therefore, our results agree quite well with previous studies and further corroborate the fact that Ca, Cd, and Zn share the common uptake pathway in fish gill.

Waterborne Cd produced inhibition of branchial Ca uptake in both species; however, this inhibitory effect was differential in nature between the two species. Kinetic analysis of the inhibition of branchial Ca uptake by Cd indicated that the inhibitor constant ($K_{i[Cd^{2+}]}$) for Ca uptake in yellow perch was approximately 1.8-fold higher than rainbow trout, i.e., the inhibition was approximately 1.8-fold less potent in the perch relative to the trout. Similarly, the inhibitor constant for branchial Cd uptake ($K_{i[Cd^{2+}]}$) was found to be more than three fold higher in rainbow trout compared to yellow perch, i.e., the inhibition was more than three fold less potent in the trout relative to the perch. The whole-body Ca influx at different waterborne Cd concentrations, which gradually changed from significantly higher to significantly lower in rainbow trout in comparison to yellow perch with increasing waterborne Cd, further substantiated the differential nature of the toxic effects of waterborne Cd on Ca homeostasis between the two species. Interestingly, 50% inhibition of whole-body Ca influx occurred in rainbow trout at approximately 100 nM free waterborne Cd^{2+}, yet for yellow perch, the same percentage inhibition of Ca influx was observed at a concentration of ≥280 nM waterborne Cd^{2+} (Fig. 7), observations which were in reasonable agreement with 1.8-fold higher $K_{i[Cd^{2+}]}$ values of branchial Ca uptake in perch relative to trout (Fig. 3). Likewise, an approximate 20-fold increase in free waterborne Ca^{2+} produced about 1.7-fold greater inhibition of whole-body Cd influx in yellow perch relative to rainbow trout (Fig. 8), as could be expected from the greater-than three-fold higher $K_{i[Cd^{2+}]}$ value of branchial Cd uptake in trout compared to perch (Fig. 6).

Yellow perch are >400-fold more tolerant (in terms of 96 h LC50) to acute waterborne Cd relative to rainbow trout. The 96 h LC50 values in moderately hard, dechlorinated Hamilton tap water (hardness: 120 mg l^{-1} as $CaCO_3$) were 8,140 μg l^{-1} (72,420 nM) in yellow perch and 19 μg l^{-1} (169 nM) in rainbow trout (Niyogi et al. 2004). Considering the fact that acute Cd toxicity occurs primarily due to the disruption of Ca homeostasis in fish, there can be three probable ways through which greater Cd tolerance may exist in different fish: (i) through the prevention of inhibition of Ca uptake (i.e., inhibition occurs but at much higher Cd concentrations), (ii) by reducing the Ca loss or Ca efflux, and (iii) a combination of both mechanisms. Based on the findings of the present study, it appears that the first option applies to yellow perch as it showed better ability to resist the inhibition of branchial and whole-body Ca uptake by waterborne Cd exposure. Yellow perch exhibited significantly lower J_{max} of branchial Ca and Cd uptake relative to rainbow trout in all exposure conditions, which indicated that yellow perch accumulate Cd more slowly than rainbow trout in their gills and other tissues since both ions share the common uptake pathway, which was also evident from the whole-body Cd influx rates in the two species. Verbost et al. (1989) reported that inhibition of Ca influx by waterborne Cd is not instantaneous, it happens after substantial buildup of Cd burden in the gill epithelial cells. Therefore, the slower buildup of Cd burden in yellow perch gills was likely a factor responsible for the lower Cd-induced inhibition of branchial Ca uptake in this species compared to rainbow trout.

However, it appears that the magnitude of difference in this inhibitory effect of waterborne Cd on Ca uptake was relatively small compared to the magnitude of difference in the 96 h LC50 values between the two species, and therefore probably not the only factor responsible for the massive difference in their acute waterborne Cd sensitivity. Freda and McDonald (1988) and later McDonald et al. (1991) studied the permeability differences between yellow perch and rainbow trout against low pH, a condition which is known for its ability to increase the ionic permeability of the gill, and concluded that tolerance in yellow perch was mainly attributable to the ability of their gills to resist net ion loss and to limit

the increase in branchial permeability (by reduced chloride cell proliferation and the larger depth of tight junctions). Therefore, the suggested second option of tolerance may also play a role since it is likely that yellow perch benefit from the difference in chloride cells and tight junctions to resist or prevent Ca loss due to acute waterborne Cd exposure. In addition, more effective detoxification, storage and elimination capacities of Cd in yellow perch relative to rainbow trout could also be other contributing factors to greater Cd tolerance. Clearly, further research is needed to evaluate these hypotheses.

The water in most of the metal-contaminated lakes in Canadian Shield (e.g., Rouyn-Noranda and Sudbury regions), where yellow perch is the most abundant species (Sherwood et al. 2000; Couture and Kumar 2003), are extremely soft in nature (Ca: 0.05–0.2 mM) (Giguére et al. 2003; Niyogi et al. 2004). The waterborne Cd levels in the same metal-contaminated lakes have been reported to be 1–3 nM (Couture and Kumar 2003; Giguére et al. 2003; Niyogi et al. 2004). In comparison, in our study, the range of waterborne Ca used (0.1–1mM) was moderately higher, and Cd levels (89–890 nM) used were many folds higher. Since this study was designed primarily to understand the mechanisms of inter-specific difference in waterborne Cd tolerance, it required using acute levels of Cd, i.e., concentrations that are well above the environmentally realistic levels. Nevertheless, our study has demonstrated that interactions between branchial Ca and Cd transport are differential in nature between yellow perch and rainbow trout, benefiting the former to mitigate waterborne Cd toxicity more efficiently relative to the latter. Clearly, this could be one of the many probable reasons for yellow perch being able to thrive in the metal-contaminated lakes where sensitive salmonids like rainbow trout do not survive.

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